

REPORT

of the

Session of the Research Group of the Standing Technical Committee

of the

**EUROPEAN COMMISSION FOR THE CONTROL OF FOOT-AND-MOUTH
DISEASE (EUFMD)**

held at

Chania, Crete (Greece)

**11 October 2004 (*Closed Session*)
12-15 October 2004 (*Open Session*)**

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INTRODUCTION

A Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease (EUFMD) was held in Chania, Crete (Greece), from 11 to 15 October 2004. The Session was composed of two parts, one for Closed business, open only to elected members of the Standing Technical Committee, the Secretariat and seven invited Observers, and an Open part from 12 to 15 October for open discussion of significant technical items with wide participation (over 120 persons) of experts in technical and regulatory affairs relating to FMD control from 48 countries. Observers came from most regions including the Americas (10 persons), Australasia (2), Asia (5), North Africa (4), Near East (7), and Southern and Eastern Africa (4).

The meeting was chaired by Dr Kris De Clercq (Belgium). Members of the Group present were: Drs. Aldo Dekker (Netherlands); Franco De Simone (Italy); Matthias Greiner (Denmark); Bernd Haas (Germany); François Moutou (France); David Paton (UK); José Sanchez-Vizcaíno (Spain); Ms Nilay Ünal (Turkey) and Hagai Yadin (Israel). Apologies were received from Vilmos Pálfi (Hungary) who was unable to attend.

The EUFMD Secretariat was represented by Dr Keith Sumption (Secretary), Dr Dónal Sammin (Associate Professional Officer) and Ms Maria Solari, (secretarial assistance).

Introduction to the Closed Session

The Chairman of the Research Group, Dr Kris De Clercq, welcomed the members of the Group and the observers and passed the floor to Professor Preben Willeberg. The latter expressed his great interest in the work of the Group, and explained that he was present because the Executive had nominated him as the liaison person with the Group, to ensure that there is a two way dialogue between the Group and the Executive. In particular he indicated the importance of passing the concerns of the Executive to the Group to enable the key issues to be addressed and to better identify the type of information that they require. He suggested that his attendance be considered "a pilot project" to identify means of better liaison between the Group and Executive.

Dr De Clercq welcomed the observers present, including Professor Soren Alexandersen, Dr Naci Bulut and Dr Emiliana Brocchi, each of whom had contributed to papers for items to be discussed. Apologies were received from Vilmos Palfi. Dr Schudel of the OIE and Dr Füssel of DG-SANCO were able to join the follow-up meeting on the 13th.

Note: as several observers could not join the Session on the 11th, a follow-up session was held on Wednesday, 13th. The report below has brought together discussions held on both days.

Introduction to the Open Session

Professor Christos Avgoulas, General Secretary of the Ministry of Rural Development and Food opened the Session and gave a speech of welcome to the participants. The Government of Greece was pleased to host the Session because of the importance of FMD for agriculture in Greece, which continues to the present moment. He emphasized that FMD can have devastating consequences both to stock-breeding and to the economy of the affected countries. The last epidemic of Foot-and-Mouth disease that struck Europe in 2001 reminded Governments in a really dramatic way that we must always be alert to the seriousness of FMD and pointed out the urgency and importance of immediate diagnosis. The work of the research teams is to achieve the targets which will help in the realization of this goal. Although the Foot-and-Mouth disease has been in our agenda for a long period of time, it is evident that constant research is of utmost importance. The level of FMD research in Europe should assist in reaching the rapid lab diagnosis that is required. The importance of the Session can be seen from the number of participants and the breadth of countries represented. He expressed gratitude to FAO for support to FMD control in the region and to Greece, and indicated the strong support of the Greek Ministry for this type of co-operative activity.

In response, on behalf of the Executive Committee of the EUFMD, Professor Preben Willeberg thanked the Government of Greece for the welcome and for arranging the Session in such a beautiful location. He re-iterated his remarks made to the Closed Session that the issues being discussed and the work of the Group, were of vital importance to those charged with decision making in disease control policy. The Executive Committee was therefore concerned to ensure the Group were encouraged in their activities and to focus on the most significant issues for decision makers. He thanked the scientific groups represented for their contributions to the debate and hoped the success of the Session would be noted for years to come.

Dr Sumption, Secretary of the EUFMD Commission, thanked the previous speakers for their encouraging words. A record number of observers had requested to attend the current Session from a

wider range of countries than ever before. The presence of so many persons from FMD endemic countries was welcomed, as this ensured that attention would be given to the problems for laboratories and epidemiologists working in the endemic areas; all should benefit from co-operation to reduce the threat of FMD in these regions. He recalled that type Asia-1 had swept across Iran, Caucasus, Turkey and into Greece as recently as 2000, and therefore the importance of maintaining close working relations between technical staff should assist to reduce the risk of this occurring again. Since the outbreak of SAT 2 in Libya in 2003, it is also clear that Europe, the Near East and North Africa must encourage FMD surveillance in eastern and sub-Saharan Africa as a form of early warning, and therefore he hoped the participants from these regions would be encouraged through contacts with other FMD scientists at the Session.

Representing the FMD Institute of Athens, Dr Helen Hondrokouki gave a short presentation on the history of the FMD Institute. In the early years, the threat of exotic FMD infections ensured that the Institute had carried out significant research leading to rapid production of vaccines to counter the threats. Recent work had focussed on early detection of FMD and other infections, through surveillance in high risk parts. FAO support had been very significant, especially in the early days.

Mr. Gregorios Archondakis, Vice-Mayor of Chania, welcomed the participants and wished everyone a pleasant stay in the historic region of Chania.

Following the opening speeches, Dr De Clercq, Chairman of the Research Group, reviewed the Agenda items and requested the Secretary to make the proposal for the reporting groups. Each item, as proposed, would have a reporting group, to be comprised of a member of the Research Group, speakers who had presented papers, and a rapporteur. He proposed the names for each reporting group, and these were accepted. In addition, feedback was encouraged from those representing the State veterinary services in Europe, and from those representing non-European countries not free of FMD.

REPORT ON DISCUSSIONS HELD IN THE CLOSED SESSION

11 October 2004

Item 1 - Adoption of the Agenda

The Provisional Agenda (Appendix 1) was adopted, with the exception that the order of discussion was altered to postpone discussion of some items until the 13th.

Item 2 - Papers for adoption

2.1 Minimum requirements for FMD serology laboratories

Dr Haas stated that this paper was initially developed by the biosecurity working Group following the Gerzensee Session, reviewed and revised following consultations with additional laboratory experts at the EUFMD workshop in Cordoba, and was then further revised after receipt of comments upon circulation to additional European experts. The document had been circulated in final draft form ("version 6") to RG members prior to the Crete Session.

After discussion of a point regarding the location of laboratories in relation to control zones, the paper (Appendix 2) was adopted by RG members without further amendment.

The Chairman congratulated the Group on this important milestone for sero-diagnosis of FMD infection in Europe.

Note – follow up on 13th October

The position reached by the Research Group was summarized for those who were not present on the 11th. Dr Schudel considered that the paper could be used as an input into the deliberations of the Biological standards Commission of the OIE. He informed the Group that an ad hoc Group on laboratory biosecurity will be established by the OIE and in the next two years will develop texts for revision of 2 Chapters. He expressed the wish that the EUFMD Research Group work closely with this ad hoc Group, with possible representation at their meetings.

2.2 Diagnostic reagent bank

Dr Haas gave the background to the paper. It was agreed the paper be revised to change the title to a position paper of the Group, and to place the final sentence as a recommendation, and with these amendments **be adopted as the position** of the Group on the issue of establishment of a diagnostic reserve. The final version incorporating these changes is given in Appendix 3.

The Chairman described the risk that if national banks are created only in major countries or within the EU block there could become significant gaps in access to diagnostic kits in crisis situations. The suggestion to use the EUFMD/EC Trust Fund to fund the bank in the short term was raised.

Note – follow up, 13th October

The position reached by the Research Group was summarized for those not present on the 11th. In response Dr Füssel thanked the Group for their work and indicated a legal base for financing of the FMD reagent bank by the EC now exists. Decision on the form of funding mechanism had not been finalized and that the position paper would be useful in this respect. He indicated that tenders would take into consideration the technical recommendations of the Session.

Following discussion, the Group agreed that:

- There are benefits to recommending tests on the basis of their performance characteristics rather than by name.
- The Group should come to a recommendation on the minimum performance standards for test kits.
- Decision on test selection for the time being is based on the outputs of the Brescia workshop.
- The updating or replacement of the Brescia workshop data for the selection of tests should be placed on the Agenda for future Group Sessions.

2.3 Sample transport

The Group agreed the title of the paper (Appendix 4) should be revised to read "Summary of the Current Regulations....." since the aim was to guide laboratory staff to the relevant international regulations, and practices of the WRL, in one document. The Secretary drew attention to the recommendation of the Cordoba workshop that the "summary" be updated yearly or sooner if relevant changes occur.

The Session agreed:

1. To further review the document and provide comments to the Secretariat (*Action: Dr Paton*).
2. To designate a contact person on the RG for updating the transport regulations (*Current designate: Dr Palfi*).
3. To discuss the issue of changes in the international regulations concerning shipment of diagnostic specimens with the representative of the OIE.

Note -on follow up on the 13th

The Secretary summarized the work of the Group. Given the primary importance of UN regulations relating to carriage of dangerous goods by air, and the difficulties currently faced by laboratories to transport diagnostic specimens for FMD confirmation, he proposed that Dr Schudel be given the floor to outline the OIE actions being taken with a view to reducing the adverse consequences of current regulations for the work of disease surveillance.

Dr Schudel outlined the position of the OIE taken by the OIE in suppositions to the UN SubCommittee of experts on transport of dangerous goods (UNSCETDG). The elements of the OIE proposal made in July 2004 approved by UNSCETDG, but which required final ratification in December 2004, enabled differentiation between lower risk diagnostic specimens and cultures of infectious agents. The entry into force of these changes is not expected until 1st January 2007, and therefore OIE has approached the International Civil Aviation Organisation (ICAO) for an addendum to the 2005-2006 Technical Instructions to be published, which would if agreed, bring forward the implementation to 2005.

The Session:

1. Supported the OIE position; and
2. Recommended that the national delegates on UNSCETDG and ICAO be requested to support the position.

Item 3 - Progress reports of the Working Groups established after the 2003 Gerzensee Session

3.1 Assisted delivery of samples from third countries

The Secretary gave an update on activities aimed at supporting delivery of virus isolates for characterization from the Horn of Africa/East Africa region, following the agreement made at Gerzensee to focus attention on this region because of potential for introduction to EUFMD member countries, via the Near East and North Africa. He also updated the Group on recently initiated FAO projects in West Africa and Central Asia which should improve delivery of samples from these regions for FMDV characterisation.

Conclusion

1. Despite the relatively slow progress made in establishing agreements, the Group strongly recommended continuation of the efforts.

3.2 Vaccine selection for the European banks

3.2.1 Vaccine selection and related issues

Dr Paton provided a paper on this subject (Appendix 5). In summary, he was pleased to provide re-assurance that the characterisation of isolates received in the last year did not indicate the Gerzensee recommendations should be updated (on the antigens in the European vaccine banks).

In discussion, Dr Paton voiced concern that there is an emerging issue relating to strengthening of regional reference labs around the world to the potential detriment of the role of the WRL as a global reference facility. While strengthened regional surveillance was clearly desirable if there were further reduction in timeliness of reporting of virus characterisation results this would negatively affect early identification of trends and events in FMD risk. The EUFMD General Session was suggested as an

occasion to discuss this issue, since it is the appropriate event at which to make decisions upon the level of support for the WRL to be provided from the EUFMD Commission.

Recommendation

1. The global function of the WRL and support given by international bodies should be included in the Agenda of the EUFMD General Session.

3.2.2 Development of models to improve risk assessment/communication of FMDV circulation

The EUFMD Secretariat had agreed to take this item forward. Ideas for developing predictive tools had been discussed with Dr Perez of the University of California Group, whose activities in temporal and spatial mapping of FMD have significantly progressed since Gerzensee, to the point that two papers would be presented at the Open Session. The Secretary considered that prediction of FMD circulation may provide a basis for better risk visualisation, and would assist in identifying areas where the level of risk may be high but surveillance information low, which would help identify targets for improved surveillance. In addition the models may assist in risk analysis calculations if shown to be significantly better than the limited and patchy information from most endemic regions.

It was agreed that:

1. The Group be involved and should consider contributing to design of proposals for international collaborative projects on FMD risk mapping. (*Follow-up: Dr P Willeberg, Dr Paton, Dr Sumption*).
2. The development of global risk analysis tools should take into consideration requirements for prevention of agro-terrorism.
3. A focal point in the Group for information on agro-terrorism issues should be designated. (*Dr Sanchez-Vizcaíno agreed to act as focal point*).

Dr Sanchez-Vizcaíno and Dr Sumption agreed to liaise with EC (DG-Research), and to identify the outcomes of NATO Sessions relating to prevention of agro-terrorism.

3.3 Comparative evaluation of candidate DIVA tests

Dr de Simone presented the work of the WG (Appendix 6: report of Brescia WS) which had been entrusted with the task at the Gerzensee meeting; the most significant event to report was the Brescia workshop in May, the organisation of which had required very significant efforts by many parties and could be considered a major success in organisation and output. The WS was supported by EC (through the Improcon project) and through FAO/EUFMD, and the diagnostic companies concerned provided kits at reduced costs. Over three thousand sera were received after heat inactivation, from 9 countries. Eleven people worked in the first 10 days, and very rapidly produced the final preliminary report. Some repeat testing occurred during and following the WS, and final agreement between authors was required before the final report could be produced. One of the key findings is that there were not found to be significant differences in diagnostic specificity (D_{Sp}) between vaccinated and non-vaccinated populations. Regarding the results for discrimination of the most critical animal infection status Group, most tests approached 90% (and after re-testing approach 95%) sensitivity. Discrepancy analysis suggested that differences in test result could be exploited, to improve D_{Sn} or D_{Sp}, for example through parallel testing or re-testing for confirmation of test status. Quality control of kit batches will be essential to maintain confidence in expected test performance.

Principal gaps were in the number of suitable sera available to be tested from sheep and pigs. In the latter there is limited sera available in Europe even if present elsewhere; it is seen as very important that European laboratories can make their own assessment of suitability of diagnostic tests for use in pigs and to have sufficient sera for control purposes available as these would be necessary to support any decision that would result in large scale testing.

The Chairman thanked Dr de Simone and all participants and supporters of this action for the immense effort. He indicated that the final report of the workshop will be agreed between participants and published shortly.

Discussion

The Secretary indicated that in response to the report of the WS, the Secretariat had worked to establish collaboration with Groups working principally on diagnostics for pigs in Asia and as a result

Dr Dyrting from Hong Kong had been partially funded to attend the Crete Session to discuss a programme for collection and supply of suitable sera.

The need to determine suitable test regimens for practical situations, based on an analysis of the workshop data, was mentioned by several members. Dr Willeberg stressed the importance of comprehensive review of test performance in order to develop the guidelines for post-vaccination surveillance (PVS). Dr Bulut indicated that guidelines for interpretation of data could be useful to address the needs of Turkey, using data from problem areas and case-studies. As an example he mentioned that in recent experience, only 11 of 58 positives tested positive on re-bleeding 6 weeks later.

Dr Dekker proposed that further statistical analysis such include maximum likelihood analysis of ELISA results be undertaken, and offered to undertake this.

Regarding performance of alternative tests to the OIE index method, the Chairman considered that results indicate that one or more tests behave sufficiently similarly to the Panaftosa test to enable it to act as equivalent to the OIE index test. Dr Dekker supported this, on the basis that there is no significant difference between most test systems for sensitivity and specificity, thereby providing a measure of confidence that laboratories can select between several of the available tests.

On the issue of recommending test systems, Dr Haas proposed that it was not necessary to make recommendations, rather the publication of diagnostic performance should assist countries to make their own selection of tests.

Dr Willeberg stressed the exceptional importance of the WS was appreciated by the Executive Committee and thanked those responsible for the organization.

Conclusions

1. The Group has identified tests that perform very similarly to the OIE index test, for use in cattle.
2. Provisional figures for test performance have been identified, but which will not be released until final quality checks have been completed, unless required in an emergency situation; the provisional figures can assist the modeling on test use and on impact in surveillance.
3. The Group does not need to recommend a particular test, rather laboratories should be given sufficient information on which to base their own decisions in consultation with their state veterinary services. Guidance on strategies for test application and analysis would be helpful to the latter.

Recommendations

1. The working Group is strongly encouraged to continue to finalise and publish the comparative analysis.
2. The RG should at an early stage identify strategies for test application and analysis to give guidance on the use of single or multiple tests for detection of vaccinated and putatively infected animals.
3. A proposal for activities (to be conducted under Letter of Agreement) should be developed to address the requirements of the European laboratories for sera from pigs for NSP validation purposes. (*Action: Dr De Clercq and Donal Sammin to develop, to be funded via the EUFMD/EC Trust Fund*).

3.4 Working Group on post-vaccination surveillance

3.4.1 Gerzensee issue: Progress on parameter estimations

Matthias Greiner provided an overview (Appendix 7) of some recent developments relating to parameter estimation on a) performance of diagnostic tests, and b) on design prevalence.

On the first, he illustrated the system intended to provide a transparent and recognized level of test validation ("fit for purpose"), under development by the OIE Biological Standards Commission (OIE-BSC). He suggested that no-gold standard methods be applied to analysis of the data outputs of the Brescia workshop, and to compare these results to other quasi-gold standards such as the current OIE index test for NSP serology.

Regarding the other parameters required in the design of surveillance using NSP tests, he stated that absence of infection/disease is technically impossible to prove, and therefore the target (detection

limits) need to be defined, for example 0.2% between herds and 5% within herds. However defining a scientifically justifiable lower limit for prevalence which requires to be detected is more controversial; here the question is –“at what lower level would there be no virus circulation?”

He recommended that field data should be used to better define the design level prevalence. He indicated that goals need to be defined for test validation; fixed values for required DSe and Dsp are not useful; and that consensus is needed about the levels of “design prevalence”.

Discussion

The use of concept of circulating infection was discussed. A move to the use of “circulation” was seen by some as a simplistic attempt to avoid the problem of carrier animals and detection of low prevalences. However since sustainable, continuous transmission sufficient to maintain an epidemic (“circulation”) is likely to leave far more evidence in the form of recovered, antibody positive animals, the relative ease of testing for circulation has some potential advantages.

There was general agreement that:

1. The disadvantages and advantages of a move from the current surveillance objectives, to adopting the “absence of circulation” objective after emergency vaccination in normally unvaccinated populations, needs to be clearly set out.
2. The issue of low target prevalence is stumbling block to progress on defining acceptable levels of PVS, and therefore the EUFMD Commission should explore alternatives.

3.4.2 Post-vaccination serosurveillance (PVS) for presence of FMD infected animals

Dr Paton outlined the paper prepared with Dr De Clercq and Dr Dekker (Appendix 8), which would be presented in full at the Open Session. The Closed Session was an opportunity to air some of the issues for which the Group would need to propose solutions if doubts about the feasibility of PVS were to be assuaged. Following the Brescia workshop, the DSn and DSp of NSP tests are now far better known and can be applied to model the application to detection of infected farms. The paper raised several significant concerns, and proposed several possible solutions. One issue is that of testing small herds for absence of infection. One solution considered would be to not vaccinate small herds, but this could operationally objectionable to some stakeholders.

Discussion

The effort of the authors to define the problems and possible solutions was widely appreciated. However, some issues raised were not seen as major problems by all members. Dr Haas considered the risk posed by false negative animals in small herds would in principle not be different for such animals between small and large herds. Dr Greiner suggested there would be statistical methods applicable to defining small herds, and grouping of small herds.

Conclusions

1. The small herd problem requires further study to determine if there are guidelines that can be useful to decision makers in the application of vaccination.
2. The problems which require value judgments by policy makers should be clearly set out.
3. The existing Brescia WS data should be sufficient to undertake case studies and scenario modelling to better define the impact and feasibility of surveillance options.
4. The level of complexity in the scenario modeling needs to be defined and may require a new dedicated project to undertake.

Recommendations

1. The Group should identify the problems that may be addressed by technical means, that may be addressed by redefinition of existing standards, and those where higher level policy decisions are needed.
2. A working Group is needed to address the small Group problem (*Action: Matthias Greiner, Aldo Dekker, Kris De Clercq and David Paton*; Dr Greiner kindly developed a paper relating to this for the Session report – Appendix 9).
3. Attention to the issues for FMD free countries in regaining FMD free status when emergency vaccination is applied must be addressed when changes to the OIE Code and surveillance guidelines are under development or considered for adoption.
4. The OIE Code and surveillance guidelines require re-examination and a WG continues to be required to identify possible Code or guideline changes.

Note on the follow-up discussions with EC and OIE representatives

The issues raised in the Closed Session were reviewed and briefly discussed with the OIE and EC representatives. Dr Schudel indicated that a proposal prepared by the Group should be received by January 2005. This could then be considered by the OIE ad hoc Group, or possibly directly reviewed by the Scientific Commission.

It was agreed it was necessary to:

1. Include and as far as possible, address the issues raised by the Group relating to 3.8.7.
2. Base the proposed revisions on the draft guidelines, as currently exist in the OIE Code.
3. Establish a working Group (David Paton, Matthias Greiner and Keith Sumption) with the aim of producing an agreed text by end of November, for circulation and comment by the Group and EC representative before submission to the OIE.

3.5 FAO Phase XVIII progress and plan

Dr Paton provided a paper (Appendix 10) detailing activities and progress of Phase XVIII. The Committee warmly supported continuation of the Phase and congratulated the WRL team on the progress made. It was agreed that the conclusions and recommendations of the discussion held in Open Session should be considered by a meeting of the subGroup concerned with Phase XVIII and its follow-up, on the 15th November, and their report integrated into the Session report (Appendix 11).

3.6 Proficiency panel for virus detection; progress report (pilot study)

Dr Paton provided a position paper on "Progress and future prospects for standardisation of FMD tests" (Appendix 12). This included the issue of proficiency panels for virus detection tests.

The Chairman thanked Dr Paton for the report which provided a timely reminder and suggestion of the need for further action.

The Committee agreed that:

1. The action needs to be continued, there being currently no system for external evaluation of laboratory competence in virus detection.
2. Additional funding would be required for some elements, for example for cost of transportation of the panels.

The Secretary agreed to consider a request for funding and that an estimate of additional costs should be prepared.

3.7 Working Group on penside tests

The Working Group had made some progress in two areas: 1) a preliminary set of guidelines had been developed at the Cordoba workshop, restricted mainly to general principles applicable to selection and use of portable, rapid tests; and 2) a rapid antigen and antibody detection test (chromatographic strip tests) were utilised in a study in Anatolia, Turkey (Appendix 13), and results compared to conventional laboratory procedures.

Donal Sammin gave the background to the work undertaken in Turkey; the evaluation of tests was undertaken as a secondary objective to the evaluation of FMD outbreak investigation procedures in an endemic region. The potential of rapid tests to provide an immediate result which would support the identification of current or recent infection in a group of animals was attractive. It was assumed before the field study that signs of clinical infection may be transient and not easily found in backwards and forwards tracing, thereby creating a greater need for confirmation off recent infection in animals with recovering lesions.

Regarding performance of chromatographic strip-tests for antigen:

- Dr Bulut stressed that intact vesicles are not commonly found during investigations and therefore to be useful tests should give positive results with the commonly observed stages of the lesions. The antigen test used was found to have a low sensitivity, except with fluid from intact vesicles, whereas with OP fluids from animals with lesions, only 3/38 were positive. Only 16 could be considered conclusive with OP fluids, which may be related to viscosity of samples.

Regarding performance of chromatographic strip-tests for antibody:

- With the NSP strip tests, 6 (strong positive result) to 35% (trace positive) of animals with lesions considered to be between 3 and 10 days old gave a test positive, and when samples 4 to 7 days later, between 11 to 48% (strong to trace, respectively) were positive. When tested by laboratory ELISA methods, 73-100% were positive in 4 Groups and 97-100% when re-tested. The Rapid Test (RT-Ab) may have use in some investigations despite the lower diagnostic sensitivity, as flock or herd test where a positive result is of significance. However, in an endemic area, positives may result from previous waves of infection and thereby complicate detection of recent exposure. In the study, NSP antibodies were detected very early after the lesions were present and this was considered the result of exposure several months earlier, presumably to a different antigenic type.

Dr De Clercq thanked the Working Group for their efforts to evaluate tests in real-time. The experience should be useful in considering how to use existing tests, and the level of sensitivity and specificity required in portable tests.

Conclusions

1. The chromatographic tests evaluated have some potential as an adjunct to thorough veterinary clinical examination and epidemiological investigation.
2. The low sensitivity of both the antigen and antibody test strips indicates that negative results must be treated with a great deal of caution; suspicions gained through surveillance investigations will require confirmation through validated laboratory tests.
3. Rapid availability of SP and NSP serology results can be extremely useful in the investigation of FMD in endemic areas where vaccination is practiced.
4. Interpretation of SP and NSP serology results may be difficult in endemic areas, particularly where vaccination is used. Guidance is needed on the interpretation of herd profiles.

Recommendations

1. It is strongly recommended to continue with similar, or extended, surveillance studies in endemic areas of eastern Turkey.
2. Greater involvement of the RG in the surveillance activities in countries not free of FMD should be encouraged by the EUFMD Commission, which should provide a high level of mutual benefit.
3. The RG should assist in the analysis and interpretation of herd/village level serology data from eastern Turkey.

3.8 Laboratory contingency planning

The EUFMD Cordoba workshop in April 2004, organised by Dr Sanchez-Vizcaíno, Dr De Clercq together with the Secretariat, and with financial support from DG-SANCO, had been very successful and the key points in the report (Appendix 14) were highlighted.

Following discussion, it was agreed that there is a need to consider the revised LCP of the WRL as potential replacement of the model plan developed and discussed at Cordoba. Action: Dr Paton to forward the updated WRL model laboratory contingency plan (LCP) to the Secretariat for circulation to the working Group.

3.9 Working Group on FMD Virus inactivation kinetics

Prof. Alexandersen presented a short proposal (Appendix 15) to establish a working Group, to take forward the recommendations of the Gerzensee Session to develop study plans and carry forward with potential funding sources.

In resolution, there are two items of agreement:

1. A Working Group should develop a study plan, including definition of the level of infectivity in pork products.
2. The Executive Committee should consider if the RG should develop a draft Section for the OIE Code, on treatment of pork from areas not free of FMD, or other sections of the Code relating to conditions for trade in pigs/pig meat from areas not free of FMD.

3.10 Laboratory sero-diagnostic capacity

The Secretary drew attention to the discussion and recommendations of the WS in Cordoba. He indicated there remains a need to provide guidelines on what constitutes adequate capacity for sero-

diagnosis to meet post-outbreak requirements. To take this forward the participation of Dr Hammond from the Geelong Laboratory was invited to present the Australian approach to determining laboratory sero-diagnostic requirements post-outbreaks.

Recommendation

A paper on "adequate sero-diagnostic capacity" should be developed by the Group, in advance of the EUFMD General Session in 2005.

3.11 Bio-security standards for FMD laboratories

The need to continue the WG on bio-security/bio-containment to revise the EUFMD Minimum Standards for FMD laboratories was discussed. This text has had an importance since the 1980's and the paper adopted in 1993¹ is a reference text in the EC Directive. The Gerzensee Session agreed that revision of the 1993 paper should be undertaken in late 2004 following first development of the guidelines for serology laboratories. The Group reviewed this requirement and considered that the need to revise the 1993 Standards was not clear as these had worked without recognized problem for at least 10 years, and any change would have financial implications for laboratories which may even lead to closure of facilities.

Recommendation

Comments should be solicited, from Group members, on the 1993 Standards, in order to assist the Chairman, and also the OIE, in a decision upon development of new texts on biocontainment for FMD laboratories. *Action: Secretariat to circulate 1993 Standards.*

Item 4 - Short report of EUFMD/EC supported studies relating to validation of DIVA tests

4.1 Prevalence in vaccinated herds exposed to infection – report of study undertaken in Israel

The Secretary reported that during the year Dr Yadin made a proposal to use the opportunity of outbreaks occurring in Israel to undertake a study on the spread of infection in vaccinated populations. The EC (DG-SANCO) had agreed to support the action in order to better establish parameters for design of post-vaccinal surveillance.

Dr Yadin reported that the studies were designed to gain information on the level of prevalence in herds where FMD exposure had occurred through contact with infected animals. The study was possible since in Israel such exposed herds are not culled, and highlighted some aspects of the epidemiology of FMDV in vaccinated populations which may be of major significance for control of infection both in vaccinating countries, and in countries considering using emergency vaccination. The experience highlighted that spread in well vaccinated herds may be minimal even where direct or indirect transmission from a clinical case within the herd occurs, and that in well vaccinated groups of animals on same management unit, a few animals may sero-convert without clinical signs being seen, indicating exposure and infection had occurred.

The data provides two estimates of intra-herd prevalence that may be very useful in modeling NSP test use in PVS, and in the absence of other data, used in defining guidelines for PVS.

From the epidemiological investigations, he concluded (Appendix 16) that:

- Sheep are high risk factor as source of infection for cattle herds.
- Feedlot fattening systems, particularly those with vaccination problems, are at high risk of developing clinical FMD.
- Vaccination programmes in feedlot systems need attention to ensure adequate re-vaccination is applied.

Discussion

The study was found to be extremely useful and provide very interesting data to support further work on design of surveillance. The figures of within herd prevalence, of 0 to 5% in 6 groups (2/119, 1/58, 1/61, 6/120, 0/49, 6/71), in two vaccinated dairy herds with no clinical signs, on farms where FMD

¹ Appendix 6 (ii), Report of the 30th Session of the European Commission for the Control of Foot-and-Mouth Disease, Rome, Italy, 27-30th April, 1993.

Online version: <http://www.fao.org/ag/againfo/commissions/docs/SecurityStandards.pdf>

had occurred in associated feedlots, could provide useful indicators of the prevalence to expect in vaccinated herds in Europe exposed to infection but where no clinical signs are observed. However, since only two farms were studied where no FMD signs had been observed, the number of observations were seen as inadequate.

Recommendation

Further data on intra-herd prevalence in vaccinated herds exposed to infection is needed, and therefore it was recommended that similar studies should be conducted if further opportunities arise, especially in the context of emergency vaccination.

4.2 Collection of sera/specimens for validation of DIVA tests for detection of animals received from SAT virus infections

The context of the study (Reported in Appendix 17) was provided by Donal Sammin; following the Gerzensee (2003) and Çesme (2002) Sessions, to address the lack of suitable sera for validation of NSP tests for detection of antibodies to SAT viruses, the opportunity had arisen to utilize field exposure in Zimbabwe and a contract had been developed with the Zimbabwean authorities. Funding was agreed with the EC (DG-SANCO) through the EUFMD/EC Trust Fund. Together with David Paton, a report was presented on the activities and results, which had been successful in meeting almost all of the objectives; sera had been collected from a high number of animals subsequently shown to be persistently infected with SAT1 or SAT2 virus, and therefore this enabled evaluation of indirect tests including NSP tests, as markers of infection. The results demonstrated that NSP tests have the expected sensitivity for SAT1 and SAT2 and give confidence to the use of NSP tests for these types and therefore do not support inferences from some earlier field studies that NSP tests for SAT infections have a lower sensitivity. In addition, a unique dataset of test results has been obtained on performance of tests which will assist selection of diagnostic tests for detection of carriers.

Dr Paton highlighted the value of the study for collection of samples for validation purposes and cautioned that the herd vaccination/infection status could not be considered similar to vaccination and infection status expected under disease management scenarios in Europe.

He concluded:

1. The study provided useful data on the prevalence of SAT 1 and 2 virus carriers in cattle herds 1-5 months after FMDV infection and on their ease of detection by different virological and serological methods.
2. Virological tests on nasopharyngeal brush swabs scored very few cattle as infected compared to the conventional approach of testing samples obtained with a probang sampling cup.
3. Routine RT-PCR was equivalent to, and optimised RT-PCR more sensitive than, virus isolation for the detection of SAT 1 and SAT 2 FMDV in probang cup samples.
4. SPCE and NSPE tests readily detected animals that had been infected with SAT 1 and SAT 2 FMDV viruses.
5. Sensitivity estimates of NSPE for detection of FMDV carriers (75-90%) were very similar to those obtained with experimental sera during the NSPE workshop in Brescia in May 2004. By comparison, the VNT could detect all carriers.
6. Since none of the herds from which virological data were available had been optimally vaccinated and since clinical disease was obvious, the study provides limited insight into the prevalence of carriers likely following subclinical infection in well vaccinated herds.

He recommended that:

1. Final conclusions should wait until the results of all tests, such as antibody detection tests on saliva samples, use of RT-PCR internal standards and completion of data analysis.
2. It would be useful to conduct similar exercises involving herds with a more certain vaccination status and following use of emergency vaccination in a previously disease-free region, and also in areas where disease has occurred in vaccinated pigs and sheep.

Dr De Clercq congratulated those involved and expressed gratitude on behalf of the RG for the valuable samples collected and the results obtained.

Recommendations

1. A further presentation to the group be made after all tests have been completed, which should also provide guidance on use of single or parallel tests for detection of carriers.

2. The distribution of aliquots of the sera should be restricted to RG members participating in the comparative evaluation of DIVA tests, because of the scarcity of sera from animals whose status has been assessed by other tests for evidence of virus infection.

Item 5 - Items arising from the Executive Committee 69th and 70th Sessions

5.1 Performance of the new oil adjuvanted vaccine and conventional vaccines produced by the SAP Institute in 2004

Dr Ünal presented information (Appendix 18) on vaccine quality assessments conducted in 2004.

Responsibility for vaccine control has still not been transferred to Bornova Institute; FMD Institute continued to control the sterility, safety and potency of the vaccines as usual. BVCRI inspected the safety and potency tests to be conducted in FMD Institute, and FMD Institute sent a dossier for each production batch to BVCRI. She stated that in addition to the regular controls, BVCRI will, at random, inspect control tests being performed.

Conventional, aqueous vaccines (AI-sap) vaccines continue to be the principal type produced and applied in the field. A pilot study in 2002 with trivalent, Montanide ISO 206 (Seppic) oil adjuvanted vaccine (W/O/W) had indicated prolonged and satisfactory levels of antibody. Ninety-four cattle and 90 sheep were tested at 28 dpv and 194 dpv, with protective levels in around 60% cattle at 194 days. A 1 ml sheep dose gave 85% immunity. They had therefore proposed to produce oil adjuvant vaccine for national campaigns in 2004 but could not because of delays in procurement of antigen concentration equipment. However oil vaccine was produced in quantities (5 million doses) that allowed some regional use within Turkey, and also export to Georgia (575,000 bivalent and 500,000 oil adjuvanted trivalent vaccine).

She gave results of 5 serological tests for vaccine potency conducted in 2004 in naive cattle in field locations. For Asia-1, O and A types, the % with protective titres was 93-100%, 90-98% and 87-98% immunity. The regime in the country was been changed in 2004, with move to bivalent vaccine in the autumn campaigns, except in the eastern/south-eastern border area.

Conclusion

1. The detailed results of the challenge tests and serological assessments on each batch are of importance to the EUFMD Commission in light of the results obtained of EQA in 2003 and the mission report of 2003.

Recommendations

1. The detailed potency test results (laboratory challenge and full results of field serological tests) should be made available to the Commission, until the time that recommendations of the 2003 mission have been seen to be implemented satisfactorily.
2. Further attention should be given to undertaking the recommendations of the 2003 expert mission to the SAP Institute. Expertise in the Group (Aldo Dekker) can assist in technical advice on fulfillment of the recommendations.

5.2 Guidelines for monitoring performance of FMD vaccines and vaccination in the field

The Secretary presented the background to this item. In recent years vaccination zones (buffer zones) had been supported in Europe for FMD control, in Thrace region and in the Trans-Caucasus countries. The paper had been commissioned to bring guidance to the Executive on sero-monitoring of these campaigns; in Thrace region the sero-monitoring was based on recommendations of the Research Group, while in the Trans-Caucasus, sero-monitoring in 2003 had been by the FGI-ARRIAH who had applied a lower test cut-offs for assessment of immune status, and thereby obtained results which might be interpreted as overestimation of protection. In addition there is a need to optimise timing of sample collection.

Dr Yadin presented the draft guidelines (Appendix 19) which he had prepared together with Dr Barteling and Dr Suttmoller. The authors emphasized the need to test (or inspect test results) of vaccine prior to and following purchase. They did not recommend a system of only testing after delivery. In Israel, since 1992, vaccine quality has been monitored with a system of testing batches of vaccine before use, and vaccination performance monitored by assessment of herd immunity rates every year. The system currently involves sampling in 4 age groups on 6 farms, located in different

areas. The system had enabled early detection of vaccine performance problems. The paper was presented for discussion.

The Secretary thanked the authors for their efforts. He noted the authors had elected to extend the scope of the paper beyond that requested by the Secretariat, since they considered that post-import testing was inadequate to ensure the quality of purchased vaccines would meet the requirements, and in addition, it is important to win the confidence of stakeholders by full and early publication of information on vaccine quality and potency.

Discussion

Dr Haas supported the use of serological assessment of potency, but considered that it must be on the basis of a well established relationship between titres and protection, obtained with multiple batches. He considered that, depending on the laboratory and the virus strain, the necessary titre to ensure that a vaccine batch will pass a challenge test will often be much higher than the LPBE titre of 1:100, which is frequently cited as a generally applicable cut-off. He therefore warned against the use of test cut-off figures based on invalid assessments, because this could lead to an overestimation of protection.

Paul Sutmoller stressed that field monitoring is not an alternative to quality checks on the purchased vaccine. Both components are required to ensure the eventual herd immunity meets expectation.

The necessity of obtaining panels of reference post-vaccination sera for each strain which is present in the vaccine bank was emphasised in order to ensure that titres recorded for a vaccine batch can be compared to titres of homologous reference batches.

Conclusion

1. Sero-monitoring vaccination in the field provides a useful measure of application of vaccines, but in addition vaccine potency must be monitored by challenge tests or by serological tests where a well established relationship has been described.

Recommendation

1. The RG should finalise, as soon as possible, the sections relating to testing of the vaccine after arrival and in the monitoring of campaigns. The revision of the draft paper should be scrutinised by the Group before a decision to adopt it is made.

5.3 Terms of reference/vision for the Research Group of the Standing Technical Committee

The Secretary presented the Terms of Reference (ToR) for the Standing Technical Committee, which had been agreed at the 70th Executive Committee Session:

1. To provide technical guidance to the Executive Committee of the EUFMD Commission, and thereby to the member states and wider international community.
2. To identify technical gaps relating to FMD control that should be brought to the attention of the Executive Committee, and/or the member states.
3. To assist in the maintenance of expertise on all aspects of FMD control.

He explained that the ToR are not defined in the EUFMD Constitution or Rules of Procedure, and appear to date to the foundation of the Committee in 1957. The ToR did not greatly differ from those of 1957 except in the aspect of maintenance of expertise in Europe, where the present situation is far different from that occurring when FMD was endemic in mainland Europe. Dr Willeberg added that that the Executive were concerned that the Committee maintained sufficient expertise and activities relating to control of the disease to ensure continuation of support to decision makers. The Chairman asked for position of each member on the ToR and the Terms of Reference were unanimously upheld.

Item 6 - Items arising from EUFMD implemented actions in FMD Control in TransCaucasus under EC support

6.1 Plan for assessment of potency, and induction of NSP antibodies by FMD vaccines produced in Armenia and Georgia

The Secretary requested guidance from the RG on the protocol to be recommended to test FMD vaccines for induction of antibodies to NSP tests. This request follows problems suspected to result

from unpurified vaccines in the above countries, but which is also suspected to be a problem in other countries in the region.

Recommendation

Testing of FMD vaccines for induction of antibodies to NSP antigens should follow the protocol given in the position paper of the European Medicines Agency (EMA) of June 2004 on requirements for vaccines against FMD, prepared by the ad hoc Group of the Committee for Veterinary Medical Products (CVMP), at which the Research Group had been represented by the Chairman. The part of the position paper relating to testing for NSP induction, is given in Appendix 20.

Item 7 - Items raised by the Committee members

National responses to new Directive: expert groups/simulation exercises

The Chairman introduced the subject and that requested the members to outline the activities in their countries on simulation exercises to test contingency plans (CPs), and the role of experts in their national expert Groups. From the response it is clear most countries plan to undertake simulation exercises in the next 1-2 years, and there is a developing body of knowledge and expertise from which to develop "best practice". The subject of laboratory CPs was raised and the issue of use of European expertise in the national expert Groups.

Recommendation

It was agreed that it is necessary for the Executive Committee to address the issue of expertise and competence of the national expert Groups, including the use of international experts, and therefore is recommended as an Agenda item for the 2005 General Session.

Item 8 - Upcoming issues and items for consideration in new workplan

The discussion on the new workplan (ie October 2005 - onwards) was deferred for discussion at a later time, ahead of the EUFMD General Session.

Item 9 - Workplan of the EUFMD Research Group to mid-2005

The Session agreed that the 2004 plan should continue into the second year, as envisaged at Gerzensee, with incorporation of the recommendations of the current Session.

Item 10 - EUFMD Research Group Sessions in 2005 and 2006

The locations and provisional dates of the 2005 and 2006 Sessions are:

Insel Riems, Germany	Dates: 20-23 September 2005 (OIE and David Paton/Coordination Action to approve)
Eilat, Israel	Dates: 17-20 October 2006 (H Yadin to confirm dates)

REPORT ON DISCUSSIONS HELD IN THE OPEN SESSION

12-15 October 2004

Item 1 – Recent findings in molecular epidemiology of FMDV

Dr Jean-Francois Valarcher provided an overview of the occurrence of FMD worldwide since January 2003 (Appendix 21), highlighting the work of the FAO World Reference Laboratory for FMD, the genetic diversity found within the different serotypes and the available evidence on antigenic matching to vaccine strains. Dr Wilna Vosloo provided a summary (Appendix 22) of genetic and antigenic information on the extremely diverse FMD viruses circulating in sub-Saharan Africa. She discussed the epidemiology and the role of wildlife in the persistence and spread of some serotypes in different regions and outlined some of the difficulties posed for disease control.

Dr Kirsten Tjørnehøj described the antigenic and genetic characterisation of a new lineage of type O from Uganda that has been associated with a syndrome of chronic photophobia in convalescent cattle (Appendix 23). Nick Knowles also described laboratory sequencing studies of FMDV isolates from several East African countries which had identified two previously unrecognised lineages of type O FMDV with differing geographic locations and a possible role for recombination in the generation of new strains (Appendix 24).

Dr Laurids Christensen outlined ambitions for the development of high capacity sequencing at Lindholm and discussed the potential for fine tracing of FMD outbreaks by genetic comparison of isolates from different herds (Appendix 25). He presented sequence data for resolution of the spread of FMDV between herds in the Danish outbreak of 1982-3. Jose Ignacio Nunez analysed sequences from three genomic regions and antigenic profiles with monoclonal antibodies to determine the likely origin and the substitution rate for FMDV type O isolates from the 1993 outbreak in Italy (Appendix 26).

Dr Dan Haydon discussed the prospects for further study of the microevolution of FMDV (Appendix 27), pointing out that there was great potential to use genetic variation to trace outbreaks more accurately, to identify the role of recombination in the emergence of new strains and to predict antigenically significant changes in the amino acid sequences of isolates.

Conclusions

1. FMDV is still active in many parts of the world and there are significant gaps in our knowledge of the global diversity of the virus and of the likelihood for different viruses to spread.
2. Two new type O lineages have been recognised in East Africa, as well as cases of a heat intolerance/photophobia syndrome thought to be a chronic sequel to FMD. The origins and mechanism of emergence of new variants are unresolved, although RNA recombination between FMD viral genomes may play a role and is being increasingly demonstrated.
3. However, recent reports of serotype C in East Africa, Asia and South America are cause for some concern and the origin of these outbreaks is not yet clarified.
4. There is a shortage of standardised antigenic information to aid vaccine selection, but available evidence suggests that current recommendations remain appropriate. The antigenic diversity of SAT strains cause difficulty for vaccine selection and SAT 2 is of the most concern due to its high degree of antigenic diversity, widespread distribution, and frequent historical association with FMD outbreaks in livestock.
5. High resolution analyses of epidemiological dynamics are powerful tools that remain to be fully exploited.

Recommendations

1. A better coordination between reference laboratories will improve the global surveillance of FMD.
2. More research is needed to better define the extent of genetic and antigenic diversity amongst FMD viruses circulating in sub-Saharan Africa.
3. A more comprehensive system of applying vaccine matching is needed as well as the harmonisation of existing techniques and the development and validation of improved methods. More linkages between antigenic and genetic comparisons are needed to improve our ability to predict vaccine coverage.

4. That the laboratory and techniques used for confirmation of an outbreak is recorded in the information system of the OIE (Handistatus II).
5. The presence of serotype C and the occurrence of serotype SAT 3 in some Central, West and East African countries must be confirmed by a reference laboratory and their origin determined.
6. Information on genetic diversity of FMD viruses should be linked to more studies of the epidemiology of the infection in endemic regions to improve predictions on the risk of the spread of FMD viruses.
7. To gain a better understanding of the evolution and spread of FMD more complete genomic sequence data should be generated to identify recombination and mechanisms involved in the emergence of new variants.
8. High volume sequencing capacity should be established in several European laboratories and the latest methods of sequence analysis should be applied to improve our understanding of the evolution of FMD virus and to develop methods for the fine resolution of virus spread.

Item 2 - Surveillance: for what purpose and how much is enough?

Dr Jordi Serratosa described the new European Food Standards Agency (EFSA) and its anticipated role in animal disease risk assessment and its aim to give independent scientific advice on matters related to food and feed safety (Appendix 28). Discussions highlighted the need for this agency to work with existing bodies and that it should not become a competitor for the EUFMD to avoid conflicting advice to stakeholders.

Prof. Prem Kumar Uppal highlighted the potentially underestimated role and importance of small ruminants, particularly in the Middle East and Asia, in FMDV outbreaks and persistence at the population level (Appendix 29). He proposed epidemiological studies to improve our knowledge in this area and the need to include sheep in any surveillance programme.

Dr. Marius Gilbert also highlighted the role of small ruminants and illegal movements across borders in the Middle East region and demonstrated the use of GIS systems for combining spatial data to assess risk factors for outbreaks (Appendix 30).

Dr. Andres Perez continued with the demonstration of the use of spatial data and the development of new Bayesian techniques for mapping the global risk for FMDV (Appendix 31). Issues of data reliability and ground referencing the data were discussed along with the need for collaboration with national veterinary organisations to improve the data quality.

Prof. Mark Thurmond gave an overview of the need and prospect for a global surveillance system and presented the prototype web based portal through which information could be accessed in a real-time global surveillance network (Appendix 32). The need for collaboration was again highlighted.

Dr Hassan Wishte² summarized the implementation of a new geo-referenced database in Iran, and reported that it is now operational for 22 of 28 provinces in Iran (Appendix 33). This system records and reports not only disease outbreaks but also other epidemiologically relevant data.

Conclusions

1. Cooperation among national and international bodies on global FMD control and surveillance activities is essential.
2. Methods to validate, summarize, visualize and distribute global FMD surveillance information should be further developed and refined. These approaches, which include modelling and statistical expressions and relations, improve our ability to interpret large amounts of data and to draw clear and reasonably confident conclusions from complex information.
3. The GISVET system facilitates national surveillance of transboundary animal diseases (TADs) in Iran, and should assist understanding of spatial and temporal trends in FMD in this country which may provide insights for wider application.

Recommendations

1. The Executive Committee should consider the proposals from EFSA and the UC Davis-FMD Surveillance and Modeling Laboratory about cooperation or partnership in the proposed joint FMD activities.

² Presentation made later in the programme, but because of relevance is summarised in this section

2. Governments and international organizations should facilitate and support activities in the following R&D areas:
 - a. Improved livestock census data for specific regions of importance for FMD risk.
In particular sub-national data on livestock density, animal movements, people movements, product movements.
 - b. Improved understanding of the specific epidemiologies of different serotypes and their interactions with the different host species.
Raised awareness and appreciation and design studies to address the role of small ruminants and domestic buffalo and wildlife species in the persistence of FMDV in domestic populations.
 - c. Raised awareness of geospatial data and ways of combining data from different sources to provide summary statistics, analysis and predictions.
Quantitative and analytic methods and approaches should be strongly encouraged in presenting scientific data in order to succinctly and clearly interpret data and results in ways that offer confidence assessments and that are compatible with decision and policy making.
 - d. The processes for sharing information, data and reagents that will maximise the utility of the available information while minimising delays related to legal requirements and competition.
3. Training in spatial epidemiology should enable the wider application and significance of the GISVET surveillance system to be realised in Iran. Adequacy of information at regional level should be strengthened by the transfer of rapid, validated diagnostic test methods to regional laboratories.

Item 3 - Transmission and its control

All presentations in this Session aimed at the quantification of transmission of FMDV. Prof. Soren Alexandersen showed that the number of infectious animals could influence the speed and intensity of the infection in contact pigs and sheep (Appendix 34). Dr Isabel Esteves described that an estimated dose of 300 TCID₅₀ was sufficient to infect contact sheep by the airborne route (Appendix 35). John Gloster showed with ample data that current airborne spread models, although very well validated for spread over long distances, are far less accurate in predicting airborne spread over short distances (Appendix 36). These airborne spread models should be used with caution during an outbreak. Both Dr Phaedra Eblé (Appendix 37) and Dr Karin Orsel (Appendix 38) showed estimates of the reproduction ratio in pigs and cows respectively. They both showed that vaccination 7-14 days before infection reduced the reproduction ratio significantly below 1. Dr Phaedra Eblé also showed that additional data from the experiments can be used to estimate other transmission parameters. Dr Sarah Cox showed that increasing the antigen payload in the vaccine might reduce the local replication and therefore the development of carrier animals (Appendix 39). Prof Ulrich Wernery showed results that camelids (Tylopoda) are less susceptible to FMDV than ruminants and swine (Appendix 40). He suggested that this difference should be further studied and reflected in the OIE code.

Conclusions

1. The number of pigs and sheep kept together in direct contact influence incubation period and the efficiency of spread.
2. Even at low concentration, airborne transmission occurred after longer term exposure of sheep to an FMDV containing aerosol. Surprisingly levels of airborne virus excreted by lambs were as high as levels excreted by adult sheep.
3. FMD airborne prediction models can currently provide useful advice. Further research on the models and their input parameters is still necessary.
4. Several vaccination strategies before infection significantly reduced transmission of foot-and-mouth disease virus in co-mingled calves and pigs; $R_v < 1$ and/or $R_v < R_c$.
5. Estimates (transmission rate β , infectious period T and reproduction ratio R) from transmission experiments can be of importance to model FMDV transmission in order to optimise control strategies during future outbreaks.
6. High potency emergency vaccines are capable of reducing local virus replication in the all important early post exposure period following severe direct contact challenge.
7. Increasing antigen payload results in an improved immune response which has an effect on local virus replication and persistence.
8. Camelids possess a low susceptibility to FMD, and do not appear to be long-term carriers of the FMDV.

Recommendations

1. More studies should be done using varying conditions (housing, species etc.) and different strains of virus to provide a better understanding of the epidemiology of FMD. Such studies should also provide the necessary data for modelling disease and airborne spread and calculations of R.
2. Meteorologists should include local flows in their transport and dispersion models at the earliest time possible.
3. Results of airborne spread models should be evaluated in a multidisciplinary manner. Assessment of emergency vaccines and antigen payload should also address subclinical infection.
4. More studies are needed in camelids to quantify their susceptibility.
5. More attention is needed to identify factors that accurately predict between herd transmission.

Item 4 - Managing diagnostic demands

Dr Jef Hammond gave an overview on the steps being taken to increase Australia's preparedness for an outbreak of FMD (Appendix 41). As a result of FMD simulation exercises a number of changes have been made to the way in which Australia will deal with an outbreak. AAHL has undergone major changes and improvements in its platform capabilities to support FMD diagnosis and surveillance. These include the establishment of an emergency response plan, the introduction of a Laboratory Information Management System (LIMS) and robotic sample handling along with a variety of testing options including high-throughput PCR screening.

Dr Nigel Ferris outlined the prospects for improved laboratory diagnosis of FMD using real-time RT-PCR (Appendix 42). The real-time PCR was of superior sensitivity compared to virus isolation. However, modified probes were necessary to detect a group of isolates from Wales.

Scott Reid described the use of automated real-time RT-PCR to detect FMDV in milk. Again RT-PCR matched closely the results of virus isolation but also detected FMDV after mild (72°C) heat-treatment (Appendix 43). However, no positive results could be obtained by PCR or VI in milk before the onset of clinical signs.

Dr Emiliana Brocchi presented a paper on the mapping of neutralizing sites on FMDV type Asia 1 and relationships with sites in other serotypes (Appendix 44). She produced 24 new Mabs of which 10 were neutralizing and described a new independent site on the C-terminus of VP3. In a second presentation (Appendix 45) she described the validation of a new solid phase competition ELISA (SPCE) based on the use of a single neutralizing monoclonal antibody for the measurement of antibodies to FMDV type Asia 1, which appears to reduce the variability of the SPCE and which can be more easily mass-produced.

Dr Wesley O. Johnson gave a talk on the application of the Bayesian probability diagnostic assignment method to predict FMDV infection from serological results (Appendix 46). He suggested development of improved analytical, quantitative and statistical methods to evaluate distribution of laboratory readings from various groups of animals as an alternative to the determination of optimal cut off values for tests. The objective would be to estimate the probability of infection in individual animals and at the herd level.

Conclusions

1. The real time RT-PCR currently in use at the FAO World Reference Laboratory for FMD provides an extremely sensitive and rapid additional procedure for improved diagnosis.
2. The Mab-based SPCE has a very high specificity and sensitivity and has some advantages over polyclonal-based SPCEs.
3. Current analytical methods provide valuable tools for assessing diagnostic protocols.

Recommendations

1. Lab Contingency plans should be constantly reviewed, tested and updated.
2. Molecular diagnostic development should be closely linked to molecular epidemiology.
3. The feasibility of PCR-testing of milk to detect FMD infection should be further investigated.
4. The development and funding of a mAb-bank should be investigated.
5. The development of SPCE using Mabs for all serotypes and the conversion of in-house SPCEs to complete kits is encouraged.
6. The standardization of terminology for neutralizing sites of FMDV should be considered.

- Analytical methods for evaluating and determining protocols to quantify the presence or absence of FMD in animals/herds/regions/countries should be explored.
- NCPs should include guidance to the lab on the capacity to be established for the situation in post-outbreak surveillance.

Item 5 - Pathobiology & Diagnostics

Dr Melvyn Quan started this session by describing a model of FMDV dynamics in pigs infected by FMDV, which related virus concentrations in the circulation, interstitial space and cells of the epithelium (Appendix 47). Dr Eoin Ryan then described the early pathogenesis of FMD in contact infected lambs (Appendix 48).

FMDV infection of the host cell is assumed to follow attachment to a surface integrin, and both Dr Don King and Dr Nigel Ferris described experiments using the integrin $\alpha\beta 6$. Dr King had transfected an MDBK cell line with $\alpha\beta 6$ in an attempt to increase its susceptibility to FMDV (Appendix 49), and Dr Ferris used $\alpha\beta 6$ to trap FMDV in an antigen ELISA (Appendix 50), generating type-specific reactions with monoclonal antibody detectors in contrast to high levels of heterotypic reactivity with polyclonal detectors.

Questions from the audience resulted in discussion of the use of integrins for other diagnostic tests (Dr Sammin) and the advantage of *in situ* hybridisation for studying the pathogenesis of FMDV (Dr Goris).

Conclusions

- A linear model of the early FMDV dynamics *in vivo* did not adequately describe experimental data but this discrepancy could be explained by a limited infection rate of epithelial cells at low FMDV concentrations, and the model adapted to better fit the observed data.
- FMDV in lambs is initially dermatotropic but thereafter infected lambs either clear the virus or sustain high-level viraemia with consequent myocardial involvement and death.
- Neither the expression of integrin $\beta 6$ on the cell surface nor the expression of SV5-v increased the sensitivity of transfected MDBK cell-lines to field isolates of FMDV.
- Recombinant $\alpha\beta 6$ protein binds FMDV and this property has potential to be exploited in diagnostic assays such as FMDV antigen detection.

Recommendations

- Further studies on mathematical models of early FMDV infection, comparing the outputs of the model with the outcome of infecting epithelial cells, are required to demonstrate the validity of the model.
- The pathogenesis of FMD in lambs should be further investigated using *in situ* methods to identify patterns of infection and to correlate this with the progression of disease.
- Efforts to develop alternative cell-lines, sensitive to field isolates of FMDV, should continue; additional bovine/porcine cell lines should be transfected with $\beta 6$ and/or other integrin subunits.
- Diagnostic applications of the selective binding of FMDV by integrins should be further explored, in particular the potential use of this format in rapid "penside" tests for virus should be considered.

Item 6 – Sero-diagnosis – improvements and standardisation

Dr David Paton presented the aims and preliminary results for the FAO Phase XVIII serological standardisation exercise which incorporated the distribution to 22 participating laboratories of reference sera, reagents for solid phase competition ELISA and a proficiency panel of unknown sera (Appendix 10). A preliminary analysis was summarised for the inter-laboratory comparative testing conducted in 2004. Recommendations were made concerning future direction of serological standardisation exercises.

Dr Nesya Goris described the preparation of secondary reference sera for day-to-day control of assay performance and presented data on the use of different software for the visualisation of trend analysis (Appendix 51).

Conclusions

1. The Phase XVIII comparative testing exercise revealed an overall, high level of consistency in results between laboratories for both reference sera and proficiency panel using both NSP and SP tests.
2. Antigenic variability of type A strains can affect sensitivity of "structural protein" tests that use "heterologous" virus/antigens.
3. SPCE specificity data for serotypes A, Asia 1 and O appeared to be similar.
4. Control charts are an essential part of internal quality control and for maintenance of quality accreditation and the mutual recognition of results.

Recommendations

1. An annual round of inter-laboratory proficiency testing is essential for quality accreditation. This should be the core activity of future Phase exercises. An improved proficiency panel is needed for NSPE.
2. The issue of establishing reference sera should be separated from that of proficiency testing and further steps are urgently needed to realise the objective of their production.
3. The purpose and use of reference sera in FMD serodiagnosis needs to be clarified and the development and distribution of reference sera could be simplified by distribution of strong positive and negative sera only. Different dilutions could be specified for the local generation of weak positive standards applicable for different tests and test purposes.
4. National laboratories wishing to obtain strong positive sera for the generation of secondary standards are encouraged to make arrangements to obtain such sera from laboratories undertaking animal experiments.
5. More work needs to be done on the development and validation of tests for the detection of antibodies to SAT serotypes.
6. Different cut-offs need to be identified for the SPCE tests taking account of the purpose of testing as well as the specificity and the sensitivity of the tests.
7. The OIE should consider recommending the use of BEI inactivation or gamma irradiation to render sera for inter-laboratory transfer free of infectious FMD virus.
8. Future inter-laboratory comparative trials should give priority to the establishment of best practices in quality control issues, such as trend analysis.
9. A working group should be convened to establish the plan for future activities on inter-laboratory standardisation exercises.

Item 7 – Optimisation of conventional vaccines

Three papers were presented in this session, one dealt with improvement of vaccine and two with monitoring of vaccine quality for field application.

Dr. Bernd Haas presented a paper (Appendix 52) on the prediction of vaccine potency (PD50 values according to the European Pharmacopoeia) of FMDV type A and Asia₁ vaccines on the basis of liquid-phase-blocking ELISA (LPBE) results. The correlation between group mean LPBE titers and PD50 values was in the range 0.8 to 0.9. However, correlation may be strain dependant.

A discussion paper on guidelines for control of Foot-and-Mouth Disease (FMD) vaccine quality and performance in the field was presented by Dr. Simon Barteling (Appendix 19). The elements needed for the implementation of FMD control by vaccination were evaluated. Emphasis was put on quality control of the vaccine and monitoring of performance in the field.

Dr. Eliana Smitsaart reported results of experiments for enhancing specific antibody response by the addition of saponin to double or single oil emulsion vaccines (Appendix 53).

Conclusions

1. Serology could be one of the methods used in batch release testing, as given in the "Position Paper on Requirements for Vaccines against Foot-and-Mouth Disease" issued by EMEA. However, tests have to be calibrated to make data from different laboratories comparable and it has to be checked whether data on additional strains within a serotype fit into an established correlation.
2. The added saponin to double oil emulsion vaccine based on Montanide ISA 206 enhanced significantly the immune response in pigs and cattle. Saponin in single oil emulsion vaccine

induced higher immune response in pigs than saponin- DOE vaccine. No adverse side effects were associated with the addition of saponin to vaccine oil formulations.

Recommendations

1. Laboratories and producers are encouraged to make their data and sera available to groups working on correlations between serology and protection.
2. Buyers should check quality of vaccines by audit or testing.
3. Vaccine performance should be monitored by serological screening of different age groups on selected farms at different locations in the country on an annual basis.
4. Laboratories using serology for evaluation of herd protection should validate the test in their own laboratory. 80 % of the animals should reach titres considered indicative of protection.
5. Evaluation and reporting of results should be carried out annually as well in order to inform and involve stakeholders.
6. Further studies are encouraged to investigate the protective capacity of the addition of saponin to the vaccine formulation and to characterize the specific immune response associated to the adjuvant effect of saponin.

Item 8 – Regulatory issues affecting FMD vaccine selection and use

Dr David Mackay summarised the current regulatory requirements for FMD vaccines within the EU (Appendix 54). The Committee for Veterinary Medicinal Products has recently adopted a Position Paper on Requirements for Vaccine against FMD (EMA/CVMP/775/02). This paper proposes practical means whereby manufacturers can overcome the regulatory 'hurdles' that currently act as deterrents to authorisation in the EU. Following the recent review of EU pharmaceutical legislation, there is currently an opportunity to amend the annexes to directive 2001/82/EC to make specific provision for the unique requirements of FMD vaccines. The Commission was encouraged to make use of this opportunity to promote the authorisation of FMD vaccines in the interests of animal health and consumer protection.

Dr Tim Doel reviewed how virus strains are selected for use in FMD vaccines (Appendix 55). Well established vaccine strains are suitable in the great majority of cases. New vaccine strains are required when outbreaks occur due to field strains against which existing vaccine strains do not provide adequate protection. Existing vaccine strains of serotypes O, C and Asia 1 generally provide a sufficient spectrum of antigenic coverage that the possible development of new vaccine strains is rarely necessary, although some strains may be developed as a result of a specific customer request. In contrast, new variants of type A repeatedly emerge requiring constant surveillance and the possible development of appropriate, new vaccine strains. The wide genetic and antigenic diversity of the SAT serotypes makes vaccine strain selection more difficult and further work to characterise the antigenic coverage of existing, and newly developed, SAT vaccine strains was encouraged.

In the discussion that followed an aspiration was expressed that a system of surveillance, and selection and distribution of vaccine strains, would be set up for FMD that would operate in a similar way to the network of WHO human influenza reference laboratories.

Conclusions

1. That authorisation of FMD vaccines is strongly desirable in the interests of animal health and consumer protection.
2. That sufficient general guidance on the requirements for authorisation already exists in the European Pharmacopoeia, the OIE Manual and in EU legislation and guidelines.
3. That the recently adopted Position Paper EMA/CVMP/775/02 on 'Requirements for Vaccines against Foot-and-Mouth Disease' provides additional, specific guidance on the requirements for authorisation of FMD vaccines within the EU.
4. That the position paper may serve as a useful model for regulatory agencies in other regions.
5. That surveillance and the development of new vaccine strains continues to be essential, particularly for serotypes A and the SAT serotypes.
6. That submission of samples from countries worldwide is essential for this surveillance to be worthwhile.

Recommendations

1. Member Countries of the EU FMD Commission should use vaccines for which marketing authorization has been gained, wherever possible.
2. Manufacturers should obtain appropriate licences for their FMD vaccines in any country or region where they might be used.
3. The European Commission should amend the annexes to Directive 2001/82, as amended, and Commission Regulation 1084/2003 to make specific provision for the exceptional requirements of FMD vaccines.
4. Active surveillance by national, regional and the FAO World Reference Laboratories should remain a priority to detect new antigenic variants and supply potential new vaccine strains to manufacturers for adaptation.
5. Submission of samples to national reference laboratories, and interchange of samples between reference laboratories, is strongly encouraged, and the EUFMD Commission should facilitate this process where required for risk assessment purposes for Europe.

Item 9 – Novel vaccines

The first presentation by Dr Artur Summerfield presented novel immunological approaches for emergency Foot-and-Mouth disease (FMD) targeting the innate immune defence (Appendix 56). In addition, the immunological basis for breaking the barrier between the non-mucosal and mucosal immunological compartments was presented. The knowledge is being used towards improving adjuvants to induct mucosal immunity.

The second presentation by Dr Zhidong Zhang presented a study (Appendix 57) on cytokine and Toll-like receptor mRNAs in the nasal-associated lymphoid tissues (NALT) in cattle during FMD virus (FMDV) infection in order to explore host factors which are involved in controlling FMDV infectivity at the mucosal surface (pharyngeal regions). The level of cytokine IL-1alpha, TNF-alpha, IFN-alpha, beta and gamma and Toll-like receptor 3 and 4 mRNA was investigated by real-time RT-PCR. Data showed that IFN- α mRNA was significantly up-regulated during the acute stage of disease and TNF- α mRNA was significantly up-regulated during persistence.

The third presentation given by Dr Shugene Lynn showed full protection against FMDV challenge following single dose synthetic emergency vaccine (Appendix 58). The UBITH-VP1 O synthetic peptide vaccine for swine was evaluated in a 1-shot emergency vaccine protocol, and full protection was observed at 28 dpv. Numerous earlier studies proved the peptide vaccine to be safe and effective in challenge and in field trials when used as a 2-shot schedule. Swine at 28dpv lacked neutralizing antibodies. Neutralizing antibodies were induced by a boost at 28dpv. INF-gamma is a possible correlate of immunity that may account for the protection at 28dpv. Correlates of immunity need further investigation. The peptide vaccine is a chemically defined vaccine for easy quality control and for use as a marker vaccine.

The fourth presentation by Dr Luis Rodriguez presented early development of adenovirus-vectored FMD vaccine and antiviral pINF-alpha (Appendix 59). A novel approach combining vaccination with adenovirus-vectored FMD empty capsid (VLP) and adenovirus-vectored porcine interferon was evaluated for rapidly controlling and minimizing the impact of FMD outbreaks.

Dr Paul Barnett presented a strategy for DNA vaccination involving a protein antigen boost (Appendix 60). Advantages and limitations of a DNA vaccine were followed by an overview of the plasmid construction and previous experimental results. A pig experiment aimed at further optimising this was detailed involving single, double and triple DNA injection and a final protein boost. Specific and neutralizing antibody response following protein boost was significantly enhanced compared to conventional vaccinates. Results from a DTH test suggested that multiple DNA plasmid administration can de sensitise against the antigen in this system.

The final presentation by Dr Belén Borrego presented a study on immunogenicity and protection conferred by DNA vaccines based on FMDV minigenes in a mouse model (Appendix 61). Different DNA constructs based on the FMDV antigens called –"BTT", fused to different cell – targeting signals, have been tested in a mouse model in order to analyse their immunogenicity and protection capacity. The plasmid coding for BTT fused to a signal peptide was able to induce neutralizing protective antibodies. However, the best construction seemed to be the one expressing the BTT epitope alone.

Conclusion

1. Targeting the innate immune defences, particularly, induction of interferon- α production by natural interferon producing cells (NIPC), has the potential to induce protection against FMD.
2. Mucosal cellular immune response may have a highly significant role in controlling FMDV, and improving current adjuvants for conventional FMD vaccine to induce mucosal immunity may reduce or prevent infection and the development of carrier animals.
3. A synthetic peptide vaccine for FMDV O in swine was reported to be safe and efficient in 2-shot protocol, and as a 1-shot emergency protocol.
4. Use of the peptide vaccine is fully compatible with requirements for sero-surveillance with NSP antibody tests.
5. Human adenovirus 5-vectored FMD vaccines seem as effective as current commercial vaccines in inducing early protection against FMD.
6. Human adenovirus 5-vectored pINF- α conferred an antiviral state and complete protection against FMD challenge in swine as early as 1 dpv and for up to 3dpv.
7. The immediate and long-term protection resulting from use of a combination of Ad5-A24 vaccine and Ad5-pINF- α inoculations may provide an important tool to control FMD for emergency situations.
8. FMDV DNA (P1) vaccination in swine followed by an inactivated FMDV antigen and protein 3D boost resulted in higher antibody responses, and may be a more efficient vaccination strategy than single shots of DNA vaccine or conventional vaccines.
9. Specific immune responses to FMDV were significantly enhanced in pigs receiving two P1 DNA vaccinations and a protein antigen boost than a single DNA vaccination followed by a protein antigen boost.
10. A DNA vaccine based on FMDV minigenes can protect against a viral challenge in the mouse model. Protection can occur in the absence of neutralizing antibodies.

Recommendations

1. Research be continued to improve adjuvants for FMD vaccines through novel immunological understanding of effector mechanisms active in control of FMD.
2. Further studies to fully investigate local (mucosal) interaction between virus and host during infection to define the associations of FMDV-induced changes with viral persistence/clearance. Improving our understanding of this will provide fundamental knowledge to help develop improved strategies for FMD control as well as improved vaccines which are able to prevent the development of carriers.
3. The work on Ad5-FMD vaccine and vectored delivery of antivirals is encouraged to progress to a stage where it can be evaluated in the control of FMD outbreaks in the field.
4. Further work is encouraged on use of DNA vaccination strategies and regimes that incorporate primer/boost regimes. Further work must be done to elucidate the mechanisms involved in the protection observed in pig and mouse systems.
5. OIE guidelines should be developed or revised relating to importation of fresh meat products from vaccinated pigs, from countries that are FMD-free with vaccination.
6. The relevant texts for European countries relating to marketing authorisation of FMD vaccines should be reconsidered to allow authorisation of peptide-based vaccines.
7. An assessment of advantages, disadvantages, and regulatory steps and timetable required in the realisation of the various novel vaccine technologies as emergency tools should be made to guide further investment.

Item 10 - International Issues

Dr Keith Sumption informed the meeting of a paper that was adopted by the EUFMD Research Group setting the 'minimum requirements for FMD serology laboratories' (Appendix 2). The Research Group also produced a position paper on the establishment of a 'diagnostic reagent bank' (Appendix 3) and a paper summarizing the information on the regulations concerning sample transport (Appendix 4).

During the Session on International Issues a presentation from the World Organization for Animal Health was made by Dr Alejandro Schudel, informing on the latest standards and guidelines related to FMD (Appendix 62). The new criteria for OIE listed diseases and notification, the procedures for validation and certification of diagnostic assays, the changes in Chapter 2.2.10 on FMD introducing the concept of virus circulation, the standards for diagnostics and vaccines and the advances made in the validation process for the NSP test for bovines were described as well as the actions implemented by the OIE on the United Nations Sub-Committee of Experts on the Transport of Diagnosis Goods (UNSCETDG). OIE has proposed to amend the model regulations on the transport of dangerous goods with regard to diagnostic materials from animal origin to be included in Category B and for the

consideration of "substances from animals for which there is a low probability that infectious substances are present, or where the concentration is at the level naturally encountered, not to be subject to these Regulations (2.6.3.2.3.2)" since these samples do not represent a risk to transport workers or to the environment.

Conclusion

1. The meeting endorsed and supported the actions taken by EUFMD /FAO and OIE on these subjects.

Recommendations

1. The actions taken by OIE to guarantee a realistic possibility for transport of samples should be supported.
2. Laboratories should improve their collaboration and information exchange, which will support global FMD control.
3. Close collaboration between the EUFMD Research Group and the OIE experts is essential for a coordinated improvement of the international standards.

Item 11 - Persistent and subclinical infections – Diagnostic and surveillance issues

Drs. Paul Suttmoller and John Bashiruddin discussed vaccinated carriers and recovered carriers, respectively. An account of the actual risks compared to the perceived risks of FMD carriage by vaccinated carriers was discussed by Dr Suttmoller and co-authors, who considered that based on historical data, the risk of transmission of FMDV from carriers after emergency vaccination is smaller than the risk of introduction of FMDV by illegally imported meat (Appendix 63). Further it was suggested that the risk of transmission of FMDV from carriers might be of the same magnitude as the risk of import of meat from animal populations in countries using vaccination against FMD. Dr Bashiruddin from experimental evidence concluded that there were differences in the rate of carriage and clinical presentation dependent on the age of the cattle at the time of infection (Appendix 64).

Drs. Franco De Simone and Kris De Clercq reported on the results of the validation exercise, undertaken as part of the EU ImproCon project, for various NSP-ELISA kit tests on sera from naïve, FMDV vaccinated, infected and vaccinated plus infected populations of cattle, sheep and swine (Appendix 6). Extensive analyses were shown and the workshop results were considered to have produced sufficient information to enable test comparison. However, they considered further analysis is needed before publication of the final report. Dr. Kitman Dyrting reported on the NSP-ELISA evaluation that is part of the Coordination Action of the IAEA/FAO, on pig sera from Hong Kong (Appendix 65). The sensitivity of NSP ELISA systems applied to the detection of exposed animals after outbreaks in pigs was reported to decrease with time subsequent to outbreaks.

Dr. Nesya Goris considered the validation and batch-to-batch consistency of commercial diagnostic kits and their testing to ensure consistency of test results (Appendix 66).

Conclusions

1. Experimental infection with the FMDV type O virus responsible for the outbreak in 2001 in the UK resulted in more carrier cattle in older than in younger cattle.
2. High specificities (CI lowest test, highest test system-prelim) with the current NSP ELISA systems were obtained in both unvaccinated and vaccinated, non-infected cattle.
3. The finding that samples from naive animals that scored false positive in one NSP tests often scored correctly in the other NSP tests may provide a basis for use of confirmatory tests to increase specificity.
4. Where there is no relevant national system, batch-to-batch testing is necessary when using diagnostic kits to ensure consistency of results; this could be organised internationally.

Recommendations

1. Science-based risk assessments should be made to compare the risk associated with persistent infections, and risks associated with different eradication methods of FMD and trade in animals and animal products.
2. The ability and likelihood of FMD carrier bulls to transmit disease by the sexual route should be investigated.

3. Efforts should be made to corroborate the finding of higher susceptibility to virus carriage in older animals, and to investigate the basis for the observation.
4. Extensive collaborations to evaluate and validate diagnostic tests are very valuable and should be encouraged for all types of diagnostic tests.
5. Control of vaccines, and monitoring of vaccination programmes and more epidemiological research is necessary in regions where FMD is endemic in pig populations.
6. NRL should use International Standard Sera to control diagnostic tests including those commercially produced. Batch testing should always be included as part of the IQC.
7. More samples from vaccinated and infected pigs from different time points after infection should be obtained for the validation of NSP diagnostics in pig sera.

Item 12 – Test development and standardisation

Dr Liesbeth Jacobs described the use of the CEDI test for detecting cattle that became carriers of FMD virus following challenge in a vaccine potency test over a two year period (Appendix 67). The use of the Chekit 3ABC ELISA in experimentally infected sheep was reported by Dr Laila El-Shehawy to be valuable in differentiating between infected and vaccinated sheep (Appendix 68).

A competition 3ABC ELISA using biotinylated 3ABC antigen was shown by Dr Kitching to be a potentially useful screening test for evidence of FMD virus infection in different species (Appendix 69). Positive samples could be confirmed using a multiplex luminex-based assay, which was very specific and measured antibodies to 3A, 3B, 3ABC and 3D proteins. Further work is required to define the characteristics of these two tests.

A correlation between the production of interferon – gamma and virological protection in vaccinated and challenged cattle was reported by Dr Satya Parida (Appendix 70). This could also potentially be used to confirm infection in vaccinated cattle.

Dr Paton described the measurement of secretory IgA in saliva by a capture ELISA (Appendix 71). This assay may be an indicator of oropharyngeal replication of FMD virus and was promising for detection of persistently infected cattle in vaccinated groups.

Conclusion

1. Additional assays to differentiate infection from vaccination such as a multiplex luminex-based assay, IgA and gamma interferon assays are being developed, which have potential for use as confirmatory tests.

Recommendations

1. Fitness for purpose should be considered when selecting a test for NSP antibody detection.
2. There is a need of confirmatory tests, with equal or better sensitivity and specificity as screening tests. The development of novel technology and the detection of alternative infection indicators is encouraged.
3. Panels of sera should be evaluated to validate new NSP tests, and provision should be made by FAO or other international organizations to support laboratories preparing these panels.

Item 13 - Surveillance using DIVA tests

Six papers were presented in which the field application of NSP antibody tests was described.

Drs Donal Sammin and David Paton reported on the rationale and laboratory test results of a field study in Zimbabwe that evaluated the performance of diagnostic methods for detecting SAT-type FMDV infection in cattle (Appendix 17). Dr Hagai Yadin reported on the FMD outbreaks in Israel in January 2004 and described the findings of post-outbreak serosurveillance with DIVA tests (Appendix 16). Dr Georgi Georgiev reported on the use and interpretation of NSP serosurveillance in Bulgaria in the years 2002 and 2003, which was mainly targeted at sentinel herds located at the Bulgarian-Turkish border (Appendix 72). Dr Naci Bulut presented the results of the serosurveillance conducted in the Thrace region of Turkey after the Spring 2004 FMD vaccination campaign which included the evaluation of both post vaccinal immunity and of tests for subclinical infection (Appendix 73). Follow-up investigations of NSP seropositive results have provided confidence that at the time of the surveillance there was no evidence of virus circulation in the Thrace region.

Dr Helen Hondrokouki presented preliminary results of serosurveillance conducted in the Evros prefecture of North-West Greece for both FMD and Peste des petits ruminants (PPR) (Appendix 74). Dr Ingrid Bergmann gave an overview of the occurrence of FMD in South America, presented data on the genetic and antigenic characterisation of recent isolates including a type C virus from Brazil and discussed the approach of the PANAFTOSA laboratory to diagnosis and serosurveillance (Appendix 75).

Conclusions

1. The ability of NSP tests to detect FMDV infection in vaccinated cattle under field conditions allows prevalence rates in vaccinated populations to be estimated.
2. NSP tests can be used in the serodiagnosis of SAT 1 and SAT 2-type FMD infections, such that they can be considered as serotype-independent serodiagnostic tests; SPCE tests also readily detected antibodies to these viruses.
3. The sensitivity of NSP tests for detection of SAT serotype FMDV carriers (75-90%) was very similar to estimates obtained with experimental sera during the NSP workshop in Brescia in May 2004.
4. Optimised RT-PCR was found to be more sensitive than virus isolation for detecting virus in probang samples; virus was detected rarely in nasopharyngeal swabs.
5. Serotype O and A FMD viruses isolated in South America between 2000 and 2004 represent strains that are indigenous to the region. Strains circulating in the Andean region are clearly separate from those that have occurred in the Southern Cone region.

Recommendations

1. Age stratification should be used as part of the assessment of potential virus circulation in a population following FMD outbreaks or in the determination of the absence of virus circulation.
2. To further evaluate the use of NSP tests for DIVA purposes they should be applied in different epidemiological situations and in different susceptible species.
3. Follow-up epidemiological investigations and additional laboratory tests are indicated where NSP seropositive animals are identified.
4. Sampling strategies which require the use of appropriate validated tests, should be developed that would assist countries in regaining the disease free status after an FMD outbreak and where vaccination has been used.
5. NSP serosurveillance (and follow-up investigation) should be conducted at least on an annual basis, and more frequently where risk indicates, in the Thrace region of Turkey and in neighbouring regions of Greece and Bulgaria.
6. Careful consideration should be given to the statistical validity of the sampling regime for the surveillance purpose intended and to subsequent interpretation of the data.
7. The work presented should be continued, with further analysis and sequencing of strains from epidemiologically important regions of S. America.

Item 14 - Regulatory compliance

Dr David Paton reviewed progress on the development of serosurveillance strategies that could be used by European countries adopting a vaccinate-to-live policy for controlling future FMD outbreaks (Appendix 76). Information is now available on the sensitivity and specificity of diagnostic tests for use in cattle. However, uncertainty remains over: (i) the level of certainty with which freedom from infection must be demonstrated; (ii) how to interpret results from herd-based tests when herds comprise small numbers of animals and (iii) details of how to resolve test specificity problems by retesting and resampling.

Conclusions

1. Many of the problems associated with false positive results may be overcome by retesting and resampling/retesting.
2. It is impossible to prove complete absence of infection.
3. The issues of confidence in the status of a group of animals is greatest in small herds.

Recommendations

1. Further work is needed to define sensitivity and specificity parameters for use of DIVA tests with susceptible species other than cattle.

2. LCPs should include decision trees to indicate the follow-up tests to be conducted.
3. Laboratories should make quantitative estimates of follow-up testing and resampling to confirm the seropositive test results obtained with NSP screening tests.
4. A consensus needs to be developed on the appropriate threshold level for detection of carriers in vaccinated animals following the adoption of a vaccinate-to-live outbreak-control option in EU Member States. Once this has been achieved, it should be possible to refine guidelines for post-outbreak serosurveillance.
5. It is recommended that the EUFMD provide further input to the development of the OIE Guidelines on FMD serosurveillance.

Item 15 - Managing the decision making process in control of FMD and in the priority setting of research and development

Professor Julian Hilton gave a paper on the importance of communications systems that enable cross-talking between the emergency services (*interoperability*) (Appendix 77). He illustrated how interoperability may be applied to planning for emergency response to FMD and other epizootic diseases, at national and European level. He indicated that communications systems are rapidly developing which have applicability for the needs he observed in the decision makers for disease control. With moderate investment this could greatly reduce problems within and between veterinary services and avoid some of the communications problems observed in 2001.

Mary Marshall made a case for engagement with stakeholders in decision making on investment in research, development and implementation of research findings relating to prevention and control of FMD (Appendix 78). She considered that stakeholders can be supportive to decision making process in various ways, as well as acting to keep the issues to the front of the agenda for funding agencies. She suggested that the process of two way communication between stakeholders and the scientific community could have advantages for each party, and outlined how this could be organised. She emphasised the importance of the competence of members of expert groups in the range of disciplines required for integrated control of FMD, and suggested European expertise could be effectively used to balance of panels where expertise is restricted.

Conclusions

1. Recent developments in information systems are relevant to the decision making in risk management process, and to communication of risk management and scientific opinions.
2. Emergency management requires an effective suite of communications tools that will enable laboratory and epidemiology experts to focus on essential tasks while maintaining or enhancing the public understanding of risk and risk management decisions.
3. Stakeholders could provide a positive contribution to the priority setting process of researchable questions on FMD prevention, surveillance and control.
4. The mechanisms for collection and review of stakeholder opinion require careful development to ensure technical questions can be presented in a format that enables an effective and rapid response by bodies that fund research or are required to provide a response.

Recommendations

1. That the EUFMD Commission develops a working group to identify user requirements for information management.
2. That this working group prepare a paper outlining options for information management and communication relevant to the needs of end-users in decision making at central or field level.
3. That this group address the options for improving knowledge transfer and training of national experts on FMD control, to meet current and future anticipated demand for FMD expertise.
4. That member countries of the EUFMD Commission ensure that national experts on FMD control are familiar with the published positions of the EUFMD Standing Technical Committee, and of the contact points for request for additional expertise provided by the STC.
5. Stakeholder groups are encouraged to develop a mechanism for presenting of priorities for research or opinion on FMD to those responsible for co-ordination of research on FMD.
6. Two way communication between stakeholders and policy makers should be improved.
7. Stakeholders in the livestock sector should be encouraged to make their own contribution to disease control, particularly through application of biosecurity measures at all relevant points of animal management and marketing.
8. The role of the EUFMD Research Group be further considered and developed to help meet the needs of the European member states for a range of competences in their national FMD expert groups.

**Research group of the Standing Technical Committee of the European Commission for
the Control of Foot-and-Mouth Disease**

Closed Session, 11 October 2004

Provisional Agenda

			Original Paper ref.
1	Opening of the Session and Adoption of the Agenda		
2	Papers for adoption - post-Gerzensee working groups		
	2.1 Biosecurity working group	<i>Haas/Sanchez-Viscaino</i>	2a
	i. Paper for adoption: Minimum requirements for FMD serology laboratories		
	2.2 Diagnostic reagent bank		
	ii. For adoption: EUFMD Position paper	Bernd Haas	2b
	2.3 Sample transport		
	2.3.1 For adoption: Guidelines paper	Vilmos Palfi	2c
3	<i>Progress reports</i> - post-Gerzensee working groups		
	3.1 Assisted delivery of samples from third countries	<i>Secret.</i>	
	3.2 Vaccine selection for the European banks	<i>David Paton</i>	
	3.2.1 Related issues - the OIE ad hoc group on vaccine and antigen banks		
	3.2.2 Global FMD mapping/modelling - surveillance for circulating strains	<i>Secret.</i>	
	3.3 Comparative evaluation of candidate DIVA tests		
	3.3.1 Findings and recommendations of the Improcon/EUFMD/EC Brescia workshop	Kris de Clercq	
	3.4 Post-vaccination surveillance		
	3.4.1 Gerzensee issues: within herd prevalence estimates, NSP test performance	Matthias Greiner	
	3.4.2 New EC Directive: is compliance with PVS requirements technically feasible?	<i>Paton/De Clercq/Dekker</i>	3f
	3.5 FAO Phase XVIII progress and plan	David Paton	3h
	3.6 Proficiency panel for virus detection; progress report (pilot study)	<i>David Paton</i>	3i
	3.7 Pen-side tests –evaluation/guidelines on use	Sammin, Unal, Bulut	
	3.8 Laboratory contingency planning	<i>Secret.</i>	
	3.9 Virus inactivation kinetics	<i>Alexandersen</i>	3l
	3.10 Laboratory sero-diagnostic capacity		
	3.11 Bio-security working group		
4	Short report of EUFMD/EC supported studies relating to validation of DIVA tests		
	4.1 Prevalence in vaccinated herds exposed to infection – Israel	<i>Hagai Yadin</i>	4n
	4.2 Collection of sera/specimens for DIVA test validation – SAT viruses	Donal Sammin	
5	Items arising from the Executive Committee 69 th and 70 th Sessions		
	5.1 Performance of new oil adjuvanted vaccine and conventional vaccines produced by the SAP Institute in 2004	<i>Nilay Unal</i>	
	5.2 Guidelines for monitoring performance of vaccines and vaccination in the field	Hagai Yadin	5q

	5.3 Terms of Reference/Vision for the Research group of the Standing Technical Committee	Secret.	5r
6	Items arising from EUFMD implemented actions in FMD Control in TransCaucasus under EC support		
	6.1 Plan for assessment of potency, and induction of NSP antibodies by FMD vaccines produced in Armenia and Georgia	Haas, Secret.	
7	Items raised by the Committee members		
	7.1 National responses to new Directive: expert groups/simulation exercises		
8	Upcoming issues and items for consideration in new workplan		
9	Workplan of the EUFMD Research Group to mid-2005		
10	EUFMD Research Group Sessions in 2005 and 2006		

**OPEN SESSION, 12-15 October 2004
PROVISIONAL TIMETABLE**

DAY/TIME	PRESENTER/ FIRST AUTHOR	Pg no. of abstract book	ABBREVIATED TITLE
Tuesday 12th			
8.30	Opening Ceremony		Representatives of the Government of Greece, and the EUFMD Executive Committee
8.50	Sumption		The Open Session; purpose and procedures
9.00	De Clercq		Progress report <i>Recent findings in molecular epidemiology of FMDV</i>
9.10	Valarcher	1	Global FMD situation in 2003 and 2004
9.40	Vosloo	2	FMD in sub-Saharan Africa
10.10	Coffee		
10.40	Christensen	3	Novel type O lineage detected
10.55	Knowles	4	Identification of a ninth foot-and-mouth disease virus type O toptotype
11.10	Christensen	5	High-resolution molecular analysis of the 1982-3 FMD epidemic in Denmark
11.30	Nunez	6	Genetic and antigenic analysis of Italian 1993 FMDV isolates
11.45	Haydon	7	Outstanding but tractable questions regarding the micro-evolution of FMDV <i>Surveillance – for what purpose and how much is enough?</i>
12.15	Serratos		EFSA – statement
12.30	Lunch		
14.00	Uppal	8	FMD in small ruminants -cause for concern
14.10	Gilbert		FMD in Turkey and Iran - trends and relationships
14.40	Perez	9	Epidemiological models for global surveillance of foot-and-mouth disease
15.00	Thurmond	11	Concepts and Considerations for Global FMD Surveillance
15.20	Coffee		
			<i>Transmission -and its control</i>
15.50	Alexanderson	12	Exposure intensity and FMD transmission
16.20	Esteves	13	Natural aerosol transmission of FMD in sheep
16.35	Gloster	14	Understanding airborne transmission of FMD
16.55	Orsel	15	Effect of vaccination on transmission
17.15	Eblé	16	Quantification of experimental transmission of FMDV O Taiwan in pigs
17.35	Cox	17	Emergency FMD vaccine: effect of antigen payload
17.55	Discussion		
18.10	Close		Depart for Tour of old Chania and dinner
Wednesday 13th			
8.00	Wernery	18	FMD and camelids: international relevance of current research <i>Managing Diagnostic demand</i>
8.20	Hammond	19	Laboratory surge capacity - Australian approach
8.40	Ferris	20	Prospects for improved lab diagnosis using real time RT-PCR
8.55	Reid	21	Use of automated RT-PCR to detect FMDV in milk
9.15			Discussion point
9.20	Brocchi	22	Validation of a SPCE, for antibodies to FMDV type Asia 1.
9.40	Grazioli	23	Mapping of neutralising sites on FMD virus type Asia 1
9.55	Johnson	24	Bayesian methods and predicting FMDV infection from serologic results
10.10	Coffee		
			<i>Sero-diagnosis – improvements and standardisation</i>
10.30	Paton	25	Phase XVIII serology exercise
11.00	Goris Nesya	26	Development of secondary standards for SPCE
11.20	Discussion		
			<i>Regulatory issues affecting FMD vaccine selection and use</i>
11.30	Mackay	27	Progress and regulatory requirements for FMD vaccines within the European Union

DAY/TIME	PRESENTER/ FIRST AUTHOR	Pg no. of abstract book	ABBREVIATED TITLE
12.00	TBC		Issues and approaches - selection of vaccine seed viruses
12.20	Discussion		
12.30	Lunch		
			<i>Optimisation of conventional vaccines</i>
14.00	Haas		Prediction of protection by FMD vaccines on the basis of in-vitro results
14.20	Smitsaart (Biogenesis)	28	Addition of saponin to double oil emulsion FMD vaccines
14.40	Barteling/ Yadin	29	Guidelines for field monitoring of vaccines & vaccination
15.10	Coffee		
			<i>Novel vaccines</i>
15.40	Summerfield (IVI)	30	Novel immunological approaches for emergency FMD vaccines
16.00	Zhang	31	Immune responses in nasal associated lymphoid tissues
16.15	Rodriguez		Progress on vectored vaccines expressing anti-virals for early protection
16.30	Lynn_UBI	32	Full protection in pigs against FMDV challenge following synthetic emergency FMDV vaccines
16.50	Barnett/ Yanmin Li	33	Enhancing immune responses to DNA vaccines against FMD
17.05	Borrego	34	DNA vaccines based on FMDV mini-genes in a mouse model
17.25	Close		
19.00	Poster Session		
21.00-*	10 mins each		*Programme subcommittee may opt to bring some of these into main sessions-authors will be informed on 12 th
			<i>Pathobiology and diagnostics</i>
	Durand	35	Identification of FMDV replication in epithelial cells
	Ahmed, Raza	36	Laser microdissection studies of FMDV in pigs
	Quan	37	Modelling early FMD virus dynamics in vivo
	Ryan	38	Pathogenesis of FMD in young lambs
	King	39	Towards engineered cell lines for diagnosis
	Ferris	40	Recombinant integrin $\alpha v \beta 6$ as a capture reagent
Thursday 14th			<i>International issues including access to reference laboratory services</i>
8.15	TBC		Transport of Specimens
8.35	Schudel	41	Update from the OIE
			<i>Persistent and subclinical infections - diagnostic and surveillance issues</i>
8.55	Sutmoller	42	Discussion Paper on the risks posed by FMD carriers among vaccinated cattle
9.15	Bashiruddin	43	Age and the likelihood of persistence in FMDV infected cattle
9.30	De Clercq	44	Comparative evaluation of NSP tests -Brescia workshop
9.50	Dyrting	45	Swine and NSP tests
10.05	Luyten	46	Need for control before release of FMD ELISA kits
10.25	Discussion		
10.30	Coffee		
			<i>Surveillance using DIVA tests</i>
11.00	Sammin	47	Serological responses in relation to vaccination and natural infection
11.30	Yadin	48	Post-outbreak serosurveillance in Israel, 2004
12.00	Discussion		
12.15	Georgiev	49	Use of NSP ELISA as a tool for FMDV serosurveillance in Bulgaria
12.30	Lunch		
14.00	Bulut	50	Serosurveillance following FMD vaccination campaign in Turkish Thrace

DAY/TIME	PRESENTER/ FIRST AUTHOR	Pg no. of abstract book	ABBREVIATED TITLE
14.20	Jacobs_Cedi	51	<i>Test development and standardisation</i> The FMD-NS ELISA, to detect FMDV infected animals in a vaccinated population
14.35	El-Shehawy	52	3ABC ELISA for the diagnosis of FMD in Egyptian sheep.
14.40	Clavijo	54	New strategies for DIVA
15.00	Parida	56	In vitro production of Interferon- γ from whole blood of FMD vaccinated and infected cattle
15.15	Parida 2/Paton	57	Secretory IgA as an indicator of oropharyngeal FMDV replication
15.30	Coffee		
16.00	Paton/De Clercq	58	<i>Regulatory compliance</i> Discussion on Postvaccination surveillance requirements
16.30	End		
16.30- 18.30	Report prep		Reporting groups prepare conclusions and recommendations
Evening	Gala dinner		
Friday 15th			
8.30	Hilton (Avis)		Knowledge management in FMD prevention and control
9.00	Marshall	59	Policy and science of FMD control: the stakeholders contribution to decision-making
9.30	Feedback session		Feedback groups present – and discussion
10.00	Break		Hand-in reports to Secretariat
10.30			Excursion or free time
15.00	Circulation of report		
17.00	Close of Session		

ABSTRACTS PROPOSED / SELECTED FOR POSTER PRESENTATIONS

PRESENTER/ FIRST AUTHOR	Page no. of abstract book	ABBREVIATED TITLE
Avalos	60	FMDV RNA detection in pig samples by conventional and Real Time RT-PCR methods
Avalos	61	Immunogenicity and protection conferred by synthetic peptides
Doel, Claudia	62	Evaluation of real time RT-PCR for the detection of carrier animals
Dukes	63	A novel but simple molecular method for the detection of FMDV
Fowler	64	Toward the construction of an O1 Manisa chimeric vaccine
Grazioli	65	Production of monoclonal antibodies against the three FMDV SAT serotypes helpful as standardised reagents in diagnostic assays
Horsington	66	Potential sequence variations between viral genomes from various FMDV infected tissues
King	67	A ring test for the lab detection of FMDV by VI and RT-PCR
Sakamoto	68	Purification of RNA dependant RNA polymerase and its predicted three-dimensional structure
van Borm	69	Analytical validation of One-Step real-time RT-PCR for FMDV RNA
Kim	70	Production of a single-chain variable fragment antibody against FMDV
Persson	71	Development of an indirect ELISA for the detection of antibodies to the non-structural protein 3ABC of the Foot-and-Mouth Disease virus; the use of a polyclonal conjugate that allows a multi-species detection of antibodies
Sangula	73	Complexity of Foot-and-Mouth disease outbreaks in Kenya
Bergmann	74	Diagnostic tools for epidemiological surveillance in South America
Staes	75	Rolling circle amplification for detection of FMDV

Chairpersons and Reporting Groups for Open Session items

<i>Date</i>	<i>Subject</i>	<i>Time</i>	<i>Chair</i>	<i>Rapporteur/Supporting members for reporting</i>
Tuesday	FMD trends/Mol epid	9.00	David Paton	Vosloo, Christensen, Haydon, <i>Valarcher</i>
Tuesday	Global Surveillance	12.15	Preben Willeberg	Thurmond, Gilbert, <i>Bronsvoort</i>
Tuesday	Transmission and refined control	15.50	Aldo Dekker	Orsel, Cox, <i>Alexandersen</i>
Wedns	Camel FMD	8.00	Keith Sumption	
Wedns	Managing diagnostic demand	8.20	Bernd Haas	Hammond, Johnson, <i>Ferris</i>
Wedns	Stnd ⁿ of serodiagnosis	9.20	Kris De Clercq	Paton, <i>Brocchi</i>
Wedns	Regulatory issues –FMD vaccines	11.30	Kris De Clercq	<i>MacKay</i>
Wedns	Optimising conventional vaccines	14.00	Hagai Yadin	Barteling, Smitsaart, <i>Rweyemamu</i>
Wedns	Novel vaccines	15.40	Jose Sanchez-Viscaino	Summerfield, Chang, <i>Barnett</i>
Wedns	Poster Session	19.00	Stephan Zientara	Georgiev, Unal, <i>Zientara</i>
Thurs	Pathobiology/diagnosis	21.00	Donal Sammin	Sammin, King, <i>Kitching</i>
Thurs	Int ^l issues/transport	8.15	Kris De Clercq	De Clercq, <i>Schudel</i>
Thurs	Persistent infections	8.55	Aldo Dekker	Sutmoller, Dyrting, <i>Bashiruddin</i>
Thurs	Surveillance using DIVA	11.00	Matthias Greiner	Moutou, Sammin, Paton, <i>Fuessel</i>
Thurs	DIVA test development	14.20	Franco de Simone/ Emiliana Brocchi	Parida, Jacobs, Kitching, <i>Merza</i>
Thurs	Compliance with Post vaccination surveillance	16.00	Francois Moutou	Moutou, Sammin, Paton, <i>Füssel</i>
Fri	Various	8.30	Keith Sumption	
	Report	15.00	Kris De Clercq	Sumption

Minimum standards for bio-security for laboratories undertaking serology with blood samples from areas not considered free from foot-and-mouth disease

The following is a supplement to "Security Standards for FMD laboratories" adapted by the EUFMD General Session in 1993¹

Introduction

The following Minimum standards for bio-security for laboratories undertaking serology including for foot-and-mouth disease during an FMD emergency or during FMD surveillance after an outbreak only apply to

the testing of blood samples from holdings without clinical signs by

laboratory tests based on FMDV antigens where virus infectivity has been inactivated by documented procedures², or those produced by techniques which do not require live FMD virus

Blood sampling is often combined with surveillance and staff taking samples may also examine the mouth of possibly infected animals, which may increase the risk of a surface contamination of packing material. This risk, as well as the risk of leakage during transport has to be mitigated by appropriate provisions. The risk of FMD occurring as a result of sero-diagnostic activities within laboratories is associated with escape of virus following receipt of blood samples from viraemic animals. While the likelihood of virus being present in samples originating from holdings without clinical signs during an FMD epidemic generally is moderate to low, it is almost impossible to predict due to the dynamic nature of an epidemic. However, the maximum virus titres in blood of viraemic animals is about 10 000 to 100 000 fold lower than in vesicular material.

Risk categories for blood samples

Blood samples coming from holdings with clinical signs indicating the possible presence of FMD are considered **FMD sample risk category 3**. They must be examined in laboratories meeting the "Security Standards for FMD laboratories" adapted by the EUFMD General Session in 1993.

Blood samples originating in an area that is not considered free of FMD, but coming from holdings without clinical signs indicating the possible presence of FMD are considered **FMD sample risk category 2**. They must be examined in laboratories meeting the "Minimum standards for bio-security for laboratories undertaking serology for foot-and-mouth disease".

Blood samples originating in an area considered free of FMD coming from holdings without clinical signs indicating the possible presence of FMD are considered **FMD sample risk category 1**. They can be examined in any laboratory.

Minimum Requirements

Personnel

1. A disease security officer (DSO) and deputy (DDSO) must be designated, and one or both present on-site at all periods in which samples are being received, and contactable at all periods when sero-diagnostic activities are ongoing.
2. The DSO/DDSO must have sufficient experience and technical training to enable assessment of FMD risk and risk management procedures.
3. There must be a designated restricted area or areas with controls in place to limit human access
4. Personnel must be authorised to enter the restricted area by the DSO/DDSO
5. Authorised personnel working in the restricted area must be trained in disease security and evidence of the training recorded. Where facilities for the inactivation of waste from the restricted area are located outside of this area, also staff working with such waste must be

¹ Appendix 6 (ii), Report of the 30th Session of the European Commission for the Control of Foot-and-Mouth Disease, Rome, Italy, 27-30th April, 1993.

Online version: <http://www.fao.org/ag/againfo/commissions/docs/SecurityStandards.pdf>

- trained in disease security and evidence of the training recorded.
6. Authorised personnel must change clothing before entering the restricted area and take a shower when leaving the restricted area. Authorised personnel must not have any contact to animals of susceptible species, must not enter buildings or enclosed fields where animals of susceptible species are kept, and must not handle items used in the care of susceptible species, for at least 3 days after leaving the restricted area. The agreement of the authorised personnel to these conditions must be recorded and a reminder notice of these conditions placed in a visible location at the exit point of the restricted area.
 7. Entry and exit of personnel to the restricted area should be recorded.
 8. Entry and exit points to the restricted area will be kept to the minimum– preferably a single point of entry/exit.
 9. A step-over line, or other clearly demarcated boundary shall indicate the exit point.
 10. In case the shower facilities are not placed at the border of the restricted area, outer protective garments, including shoes or shoes coverings, shall be removed before exit from the restricted area. All clothing worn in the restricted area must be stored in a secure way, e.g. in designated lockers, until treatment.
 11. An incident recording system, SOPs for risk identification and notification procedures and target response time, must be in place to ensure early notification of the authorities responsible for FMD surveillance in the event that
 - Samples have been received which are considered FMD sample risk category 3
 - Samples have been received in unsatisfactory state of packaging

Buildings

12. Susceptible livestock must not be kept on the premise where the restricted area is located.
13. Access doors to the restricted area should display a warning sign that access is restricted to authorised personnel only
14. Changing facilities and lockers are required to enable staff to deposit unessential items outside the restricted area
15. Entering of the laboratory premise by farmers or staff working on farms should be avoided. If possible, it should be attempted to separate vehicles bringing samples from vehicles entering the premise for other purposes.
16. Shower facilities must be available onsite, preferably at the border of the restricted area.
17. Sample reception area
 - a. The restricted area must contain a specified area for reception of packages.
 - b. This area must:
 - i. Be easily disinfectable in the event that leakage of samples occurs into packing materials or following opening of the packages;
 - ii. Be equipped to enable packages considered to potentially contain FMD risk category 3 to be re-packaged into appropriate transport containers for dispatch to laboratories licensed for handling FMD virus,
 - iii. have suitable facilities for waste disposal and have hand-washing facilities at exit points
18. Serum separation area
 - a. The restricted area must contain a specified area for serum separation
 - b. This area must have suitable facilities for surface disinfection and waste disposal and have hand-washing facilities at exit points
19. Serum testing area
 - a. The restricted area must contain a specified area for testing
 - b. This area must have suitable facilities for surface disinfection and waste disposal and have hand-washing facilities at exit points
20. Serum storage area
 - a. The restricted area must contain a specified area for the storage of samples
 - b. This area must have suitable facilities for surface disinfection
21. Communications and reporting office space
 - a. The laboratory housing the FMD serology facility must demonstrate an adequate provision of office space, computing and communications facilities and organisation, sufficient to reduce the need to a minimum for staff, papers and physical records to exit the restricted area on a daily basis.
 - b. Facilities should be in place (for example, electronic communications, facsimile) to prevent any need for untreated papers to exit the restricted area
22. Rest rooms

The restricted area should have sufficient rest rooms and lavatory facilities in relation to the staff number expected at peak periods of activity.
23. Location of autoclave

Facilities for wet heat treatment must be present on the site, preferably with sufficient

capacity for throughput at the maximum operating capacity of the serology laboratory. (see also 26. Solid waste)

Waste

24. Liquid waste

- a. Heat or chemical treatment of all waste water is the PREFERRED treatment, in compliance with the prescribed standards specified for FMD laboratories.
- b. Alternatively, or additionally, the laboratory may demonstrate that it has put in place a system for inactivation of virus if present in liquid waste that has contacted risk materials. If treatment of all liquid waste from the restricted area (including waste water from the showers) is not possible, at least the ELISA buffers and washing fluids must be collected and treated.

25. Solid waste

- a. For biological, solid waste, and all solid, disposable materials that have been in contact with specimens, treatment by wet-heat, in accordance with the level of heat effect specified for security standards for FMD laboratories, in an autoclave within or at an entrance point to the restricted area is the preferred option.
- b. If such a treatment of all solid waste is not possible, it may be packed into suitable watertight containers and, after spraying of the containers with disinfectant, removed for treatment at a different site.

26. Removal of equipment, materials and clothing from the restricted area

- a. Removal of any equipment from the restricted area shall be subject to authorisation by the disease security officer
- b. The reason for removal, date, and destination will be recorded, precautions taken to reduce potential virus contamination
- c. The DSO will ensure that equipment which has been in contact with risk materials (specimens) will not be removed from the restricted area without treatment in accordance with the procedures given in the Security Standards of FMD Laboratories

27. Declassification of the restricted area

- a. A decontamination plan must be agreed with the competent authorities, before restrictions can be lifted.
- b. If heat treatment or scanning of all paper from the restricted area is not possible, it should be packed into suitable containers, which should be disinfected and kept under lock for at least two years. If the containers have to be opened before, this has to be done in a restricted area meeting the standards described above.

Biosecurity Requirements for Testing of Blood Samples (for Any Disease)

Sample Risk Category	Sample Risk	Laboratory Requirements
Cat. 3 = FMD signs	Material considered infective	FMD Lab ¹ ("3+")
Cat. 2 = no clinical FMD in holding (dynamic)	status unclear, epidemiological reason for suspicion	FMD Serology Lab ("2+")
Cat. 1 = FMD free area	No obvious risk	No specific requirements

All properly produced FMD kits are considered Risk Category 1.

Movements of Blood Samples

Sample Cat. 3	only to	FMD Lab of "3+" status
Sample Cat. 2	only to	FMD Lab ("2+ or 3+")
Sample Cat. 1	To	any lab including FMD Lab ("2+ or 3+")

Packaging of samples of risk category 2

Samples must be put into watertight primary containers (e.g. plastic tubes) and the primary containers must be packed in watertight secondary packaging, which should be a strong crushproof and leak-proof container, with absorbent material that can absorb the entire contents of all the primary containers. The packaging process must include a disinfection of the secondary packaging. Preferably, the packaging should comply with the European agreement concerning the international carriage of dangerous goods by road (ADR).

Diagnostic reagent banks for FMD

Position paper of the EUFMD Research Group – October 2004

Introduction

The traditional main role of laboratories specialised on foreign animal diseases has been the rapid confirmation of new outbreaks. In future, testing for proving freedom of disease and testing as a basis for movement, slaughter and trade certificates will become more and more important. New tests allow the detection of infected animals in a vaccinated population. However, in “peace times” few samples are tested for FMD. In order to meet the sudden demand for rapid mass testing in an emergency, reagent banks are required in addition to the already existing vaccine banks.

The Council Directive on Community measures for the control of foot-and-mouth disease requires serological tests in all cases, where a diagnosis based on clinical signs would not be sufficient to detect the disease fast enough and with the necessary degree of confidence. As a general rule, when the presence of foot-and-mouth disease is suspected in the absence of clinical signs, a sampling protocol suitable to detect 5% prevalence with at least 95% level of confidence must be used. This means about 60 samples per stable and, in areas of high animal densities, ten or even hundred thousands of samples in total. Many of these tests will have to be performed under an enormous time pressure. If vaccination is used, testing for infection with FMDV, either by an assay for antibodies against non-structural proteins of the FMDV, or by another approved method, shall meet the statistical condition mentioned above. However, it might even be decided to test samples taken from all vaccinated animals of susceptible species and their non-vaccinated offspring in all herds in the vaccination zone. Since a vaccine bank typically contains between one and five million cattle doses, this could mean as many tests.

A look at the 2001 FMD epidemic confirms that we have to be prepared to test hundred thousands or even millions of samples. In the UK, in order to prove freedom of FMD, 3 million samples had to be tested with a throughput of 200 000 samples a week. This became possible after the solid-phase-competition ELISA (SPCE) had been established in five additional laboratories. Because there was and still is no validated commercial test kit available, a considerable lead-in time was required until the full serological capacity was reached.

In the Netherlands, the laboratory diagnosis of animal diseases is centralized at ID Lelystad, (now CIDC-Lelystad). Thus, in contrast to many other FMD institutes, ID Lelystad had the experience, equipment and staff for large scale serological screening. About 200 000 sera were tested by a mab-based competition-ELISA. Its specificity was not fully satisfactory, but the necessary retesting by VNT could be done on the site.

In retrospect, Europe was lucky that the countries that had to do most of the testing also had large, well staffed and experienced laboratories. In many other countries, there would have been a much longer delay until the necessary throughput could have been achieved.

The strategic problems of FMD serology

The first strategic problem with FMD serology is, that in “peace times” there is almost no market for FMD test kits in Europe because the number of samples to be tested is small and these testing is done by a few specialized laboratories using in-house methods. This situation is complicated by the fact that there are 7 serotypes which all require their own test, if “anti-structural” antibodies have to be determined. In addition, even some of the many subtypes may require their own special tests. As a consequence, there is limited incentive for the industry to develop and keep ready test kits that could be used in laboratories not specialized on FMD.

The second strategic problem is, that because of the security concerns, the complication caused by the many different subtypes and the necessity to check doubtful results by VNT using infectious virus, the small number of samples regularly examined are mostly tested in relatively small, specialized laboratories, which often concentrate on research, not on high throughput serology. In a crisis, however, FMD laboratories would be expected to quickly scale up their serological screening capacity to levels reaching or exceeding the whole serological capacity of all the countries veterinary laboratories combined. This is a problem especially for countries with a decentralized laboratory structure like Germany, where almost all the routine work is performed by regional (state) diagnostic laboratories, while the national reference lab belongs to the Federal Research Center. Since the regional laboratories often already have the staff, the experience, the rooms and the sample handling robots for mass serology, enabling them to perform mass serology also for FMD is much more

economic than to duplicate their capacity in a research laboratory. This however, would require a long lead-in time, unless ready to use tests kits are available in sufficient number and quality. It is sometimes argued, whether laboratories which do not meet the criteria for laboratories working with FMD Virus should be allowed to run serological tests for FMD antibodies. However, as long as these tests do not require working with infectious virus, the only risk would be spread of the minute amounts of virus, which might be present in blood samples from viremic animals. Using proper laboratory procedures and prohibiting contact of staff handling such samples with susceptible animals would reduce the risk that this virus causes further outbreaks to a level that is absolutely negligible in a situation in which the disease is already present in the field.

Requirements for tests kept in FMD diagnostic reagents banks

The formal requirements are laid down in ANNEX XIII of Council Directive 2003/85 EC. Tests either must be described in the OIE "Manual of Standards for Diagnostic Tests and Vaccines" (Prescribed Tests) for international trade, or they must have been shown to match or exceed the sensitivity and specificity parameters laid down in the OIE Manual or in the annexes to Community legislation, whichever is the more stringent. The Commission may, in accordance with the procedure referred to in Article 89 (Decision by the Standing Committee on the Food Chain and Animal Health) decide to adopt more stringent testing procedures or approve additional tests. The Commission may consider the scientific advice produced by the meetings of the National Laboratories to be organised by the Community Reference Laboratory.

While cattle are the most important species, preferably, the tests should also be suitable for use with small ruminants and pigs. Originally, NSP tests were developed mainly to detect infected animals in a vaccinated population. In the absence of complete type specific kits for all seven serotypes, the serotype-independent NPS tests kits could also be used for large scale testing of sera from non-vaccinated cattle and pigs. However, since NSPs are generally less immunogenic than structural proteins, seroconversion to NSP in non-vaccinated animals will be slightly delayed compared to conventional, type specific tests.

All the usual requirements for tests for broad scale serology apply. The tests kept in a FMD diagnostic reagents bank must be

- sensitive
- specific
- robust (high reproducibility in different labs)
- easy to use (complete, no special equipment or experience required)
- economic

The EUFMD-Workshop in May 2004 in Brescia has produced valuable data on the properties of commercial test kits as well as some in-house tests.

There is an urgent requirement for procedures to ensure international acceptance of NSP test results. In some countries there are legal provisions for marketing authorization and batch release of diagnostic tests. In any case, accredited laboratories have to check the performance of tests and batches of kits they are using. Whereas such procedures for FMD NSP-test have to be implemented on the laboratory and national level, international harmonization of these procedures is crucial. While the final objective is an agreement on the level of the OIE, an EU-wide agreement can probably be achieved much easier.

Quality Control Issues

While the main concern of the trading partners is sensitivity – necessitating international harmonization - specificity is mainly in the interest of the countries applying the test and different national panels to determine specificity are acceptable and may even be necessary because of different "background reactivities" and different control strategies. However, specificity requirements may to some extent depend on the phase of eradication.

It is suggested to create a panel of internationally recognized reference sera to ensure a minimum sensitivity for the purpose of screening of areas. Since creating a definitive panel will take considerable time, it should be attempted to create a provisional panel for the needs of the EU using already existing sera. Possibly a provisional panel of 10 – 30 sera, containing "early" and "late" post infection sera would be sufficient and the results could be analysed by a scoring system. A serum scored consistently positive in one test may be consistently negative in another test of equal diagnostic sensitivity. National panels of negative sera, in which "problem sera" may be overrepresented, could be used to check specificity.

While producers should run their own tests and provide documents on the results, it is recommended that national labs perform batch testing before batches are used in disease control.

Numbers and timelines

The main economical problem for a FMD diagnostic reagents bank is the limited storage time of complete test kits. Even if only the coated plates would have to be discarded, the annual costs per sample, for which reagents are stored, could exceed one €. Therefore it is recommended to use a model analogous to the vaccine bank: Antigen, antibodies and other reagents should be produced in advance and stored in a deep freezer. Pilot batches of complete kit should be prepared and tested and a limited number of complete kits should be kept ready for dispatch.

What is the number of kits required for a FMD contingency? Since the scale of epidemics can't be predicted, the throughput of the veterinary laboratory system of a major country and the number of doses in vaccine banks should serve as guideline:

The first test kits should reach the customers within 24 – 48 hours. The numbers of ready to use tests should be sufficient to cover the time until newly produced kits are available. With an appropriate stand-by contract in place, this time could be as short as 2-3 weeks. Considering plausible initial throughput figures for laboratory systems of major countries, tests for at least 50000 samples would be required in order to avoid a situation in which kits become the "bottleneck" of disease control.

Two to three weeks after the order, the bank holder should be able to reach a weekly production of kits for at least 200000 samples.

The reagents kept in storage should allow the production of kits for at least 2 million samples in total.

For the whole EU, these estimates for a major country should be multiplied by the factor 2.5, so that a total capacity of 5 million samples is reached – identical to the number of doses per strain in the European vaccine bank.

Recommendation:

A working group of SANCO, EUFMD and WRL should prepare a tender for a European reagent bank, which could also serve as a guideline for national reagent banks. For international reagent banks, labels and manuals in should be in English, and additional national manuals should be prepared with support of the national laboratories.

Summary of Current Regulations for the Safe Transport of Materials Containing infectious FMD Virus by Air

Update Version: October 2004

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DISCLAIMER:

The information provided is for guidance purposes only. Since the regulatory situation changes over time and from country to country, the reader is referred to the information sources listed below for updates and to verify technical information. FAO does not accept responsibility for completeness or accuracy of the text and in relation to any claims arising out of the application of these guidelines.

1. Introduction

These instructions relate to the transport of Foot and Mouth Disease Virus (FMDV) containing materials, and stress the importance of a working relationship between the groups involved – the Sender, the Carrier and the Receiver – in order to provide for the safe transport.

The packaging of FMD virus specimens must be designed to minimize the potential risk for damage during transport. Any un-controlled escape of the infectious material may be the source of a new epidemic.

The international regulations for the transport of infectious materials by any mode of transport are based upon the Recommendations of the United Nations Committee of Experts on the Transport of Dangerous Goods (UN). The International Air Transport association (IATA) has also incorporated the UN Recommendations.

This document provides practical guidance to facilitate compliance with the 44th Edition of IATA Dangerous Goods regulations. These regulations are updated on a regular basis and the reader is referred to: http://www.iata.org/whatwedo/dangerous_goods for further information.

Furthermore, the instructions prepared by IAH Pirbright Laboratory Experts for collection and transportation of specimens for vesicular virus investigation was also considered.

The following references were used to prepare these Instructions:

1. R.P. Kitching and A.I. Donaldson: Collection and transportation of specimens for vesicular virus investigation. Rev. sci. tech. Off. Int. Epiz, 1987, 6 (1), 263-272
2. Dangerous Goods. Guidelines for the transport of infectious substances and diagnostic/clinical specimens by air. 44th edition of the IATA Dangerous Goods Regulation. EBSA Sixth Annual Conference, Lyon, France, 15-16 May 2003
3. Guidelines for the safe transport of Infectious Substances and Diagnostic Specimens. WHO/EMC/97.3
4. OIE, Manual of Standards for diagnostic tests and vaccines 2004.
5. OIE, Terrestrial Animal Health Code 2004.

6. The FMD homepage of the FAO World Reference Laboratory for FMD:
http://www.iah.bbsrc.ac.uk/primary_index/current_research/virus/Picornaviridae/Aphthovirus/index.html

2. Transport Planning

It is the responsibility of the sender to ensure the correct designation, packaging, labeling and documentation of all infectious substances and diagnostic specimens.

The efficient transport and transfer of infectious materials requires good coordination between the sender, the carrier and the receiver (receiving laboratory), to ensure that the material is transported safely and arrives on time and in good condition.

Such coordination depends upon well-established communication and a partner relationship between the three parties.

All have specific responsibilities to carry out in the transport effort.

The sender

1. Makes advance arrangements with the receiver of the specimens including investigating the need for an import permit;
2. Makes advance arrangements with the carrier to ensure:
 - that the shipment will be accepted for appropriate transport
 - that the shipment (direct transport if possible) is undertaken by the most direct routing, avoiding arrival at weekends;
3. Prepares necessary documentation including permits, dispatch and shipping documents;
4. Notifies the receiver of transportation arrangements once these have been made, well in advance of expected arrival time.

The carrier

1. Provides the sender with the necessary shipping documents and instructions for their completion;
2. Provides advice to the sender about correct packaging
3. Assists the sender in arranging the most direct routing and then confirms the routing;
4. Maintains and archives the documentation for shipment and transport;
5. Monitors required holding conditions of the shipment while in transit;
6. Notifies the sender of any anticipated (or actual) delays in transit.

The receiver

1. Obtains the necessary authorization(s) from national authorities for the importation of the material;
2. Provides the sender with the required import permit(s), letter(s) of authorization, or other document(s) required by the national authorities;
3. Arranges for the most timely and efficient collection on arrival;
4. Immediately acknowledges receipt to the sender.
Shipments should not be dispatched until:
 - advance arrangements have been made between the sender, carrier and receiver
 - the receiver has confirmed with the national authorities that the material may be legally imported
 - the receiver has confirmed that there will be no delay incurred in the delivery of the package to its destination.

3. Requirements for laboratories handling and possessing FMD virus

There is a recognized risk that disease may occur as a result of accidental release of FMDV from laboratories handling infectious virus for diagnostic, research and vaccine production purposes. It is therefore necessary to have in place strictly applied measures to prevent the accidental release of the virus from laboratories, and therefore few laboratories in Europe are able to accept samples known to contain or suspected to contain FMDV, for diagnostic purposes.

Regulatory measures are therefore in place to license laboratories for handling FMDV and the licensing authority has the responsibility to check that the risk of escape from laboratories is addressed through an adequate bio-containment system.

IN European Union member states, only a few laboratories are licensed to undertake work with live virus, such as confirmation of FMDV infection by virus isolation. These are stated in Annexes to EC Council Directive 2003/85/EC¹.

Annex 11 of this Directive lists the national laboratories licensed to handle live FMD virus. In Annex XII reference to the biosecurity systems for containment of live virus, the relevant texts being the minimum standards adopted by the EUFMD Commission in 1993².

In EUFMD member countries that are not members of the EU, the bio-containment requirements should also meet or exceed the minimum standards for FMD laboratories adopted by EUFMD in 1993. These standards are in line (meeting or exceeding) with those of the relevant texts of the OIE (OIE, Manual of Standards for diagnostic tests and vaccines 2004; extracts s given in Appendix 1).

4. Collection and transportation of specimens for vesicular virus investigation to World Reference Laboratory for FMD

The World Reference Laboratory (WRL) for FMD undertakes the following main functions in diagnosis and control of vesicular diseases:

1. As a primary function undertakes the investigation of specimens from outbreaks of vesicular disease to establish whether FMD virus or one of the other vesicular viruses (swine vesicular disease virus, vesicular stomatitis virus) is involved. This diagnostic service for vesicular disease of animals is provided free to all member countries of the FAO, with results copied to FAO and OIE. Results of tests are reported rapidly, usually by Fax or E-mail. Reference virus strains and antisera to these strains can be purchased by other laboratories to enable them to undertake the identification of their own local isolates.

2. The accumulation of FMD virus isolates at the World Reference Laboratory provides a valuable library of different virus strains past and present, and allows for the rapid identification of suitable vaccine strains for the control of outbreaks. It also provides epidemiological data on the risk of spread of disease through regions and can warn those countries which vaccinate against FMD of the approach of emergent strains not adequately covered by their routine vaccination regime. It can also advise those countries that do not normally vaccinate of any approaching threat to their livestock.

3. The World Reference Laboratory can undertake the examination of sera for FMD, swine vesicular disease and vesicular stomatitis disease virus anti-bodies to determine freedom from infection, or otherwise, of animals involved in international trade movement. Probang (oesophageal-pharyngeal) samples and also semen samples can be tested for evidence of FMD virus. A charge is, however, made for the testing of sera, semen and probangs.

4.1. Instructions for collection and packaging of the specimens

Suitable samples for virus isolation and antigen detection are epithelium and vesicular fluid from unruptured and ruptured vesicles (a suggested minimum of 2 cm² of fresh tissue from recently affected

¹ Council Directive 2003/85/EC of 29 September 2003 on Community measures for the control of foot-and-mouth disease repealing Directive 85/511/EEC and Decisions 89/531/EEC and 91/665/EEC and amending Directive 92/46/EEC (Text with EEA relevance.)... (Eur-Lex - 32003L0085)

² Appendix 6 (ii), Report of the 30th Session of the European Commission for the Control of Foot-and-Mouth Disease, Rome, Italy, 27-30th April, 1993.

Online version: <http://www.fao.org/ag/againfo/commissions/docs/SecurityStandards.pdf>

areas), cardiac muscle, whole blood with anticoagulant, semen and probang samples. Sera only should be sent for estimation of vesicular virus antibody levels. Tissues such as muscle and lymph nodes from infected animals may contain virus but the amount of infectivity may be insufficient to isolate virus or identify antigen by in vitro procedures and, as negative results may be misleading, such specimens are not accepted for investigation.

EPITHELIUM/VESICULAR FLUID

Collection

The specimens should be suspended in a mixture of equal amounts of sterile glycerine and 0.04 M phosphate buffer pH 7.2.-7.6 (Appendix 2.1), preferably with added antibiotics (Appendix 2.2). There will be considerable loss of infectivity if samples are sent in buffer outside of this pH range.

Packaging (Fig. 1)



FIG.1
Items recommended for use in packaging epithelium, vesicular fluid, whole blood or serum

Starting with the innermost container, the recommended procedure for packing samples is as described below. Alternative methods of packing are acceptable if they provide a similar level of security against breakage and/ or leakage.

1. A strong glass container should be used with a metal screw cap fitted with a strong rubber washer or wad. The best container of this type is a 20 ml Universal bottle. Tape should be used around the cap in order to prevent leakage of fluid.
2. Sufficient information for the identification of the material should be written on a piece of waterproof adhesive tape attached to the bottle. The outside of the bottle should then be disinfected before proceeding further.
3. The bottle should be wrapped in absorbent cotton wool or lint or in corrugated paper, arranged to protect the ends as well as the sides of the bottle. The wrapping of the bottle in the various containers should be completed in clean surroundings.
4. The wrapped bottle should be inserted in a metal container in which it fits snugly. If collection takes place at a point which is some distance from the point of dispatch by air, the sample should be kept refrigerated. This container should also be labeled.

5. The metal container should be fluid-tight, preferably with a screw cap and a rubber washer. If such a container is not available, a tin with a tight-fitting lid which can be soldered on should be used.
6. The metal container should be placed in a solid outer covering to prevent distortion. A solid cardboard tube closed with a metal cap at each end is to be preferred but, as an alternative, a wooden box with a metal cap is equally satisfactory.
7. Sturdy wrapping paper secured by adhesive tape or string should be used and labels should be clear and comply with the International Transport Regulations.
8. Information that should be included on the label:

PATHOLOGICAL MATERIAL OF NO COMMERCIAL VALUE

World Reference Laboratory for Foot-and-Mouth Disease, Institute for Animal Disease Research, Pirbright Laboratory, Ash Road, Pirbright, Woking, Surrey GU24 0NF, UNITED KINGDOM.

PERISHABLE FRAGILE TO BE COLLECTED AT AIRPORT BY ADDRESSEE

KEEP AT 4° CENTIGRADE

WHOLE BLOOD

Collection

Blood samples should be collected under sterile conditions and mixed as soon as possible with the anticoagulant heparin (0.1-0.2 mg per ml of whole blood). Sequestrene EDTA can be used as an alternative anticoagulant (30 mg of Sequestrene EDTA in one ml of 0.7% aqueous solution sodium chloride per 20 ml of whole blood). The sample should be kept at 4°C until dispatched to the World Reference Laboratory.

Packaging

As described for specimens of epithelium/vesicular fluid.

PROBANG SAMPLES

Collection

Prior to sampling, a 2 ml amount of 0.08 M phosphate buffer^{3*} containing 0.01% bovine serum albumin, phenol red (0.002%) and antibiotics adjusted to pH 7.2 (Appendix 2.3) should be added to one bijou bottle or similar container for each of the animals to be sampled. Each bijou bottle should be identified with a waterproof label.

After collection, the sample should be poured from the "probang" cup into a wide-necked bottle such as a 20 ml Universal bottle and examined visually for quality. Two ml, which should contain some visible cellular material, should then be added to the previously prepared bijou bottle containing an equal volume of buffer and thoroughly mixed by gentle shaking. The final pH of a normal sample thus treated should be \pm pH 7.6.

Samples taken from some animals may be heavily contaminated with ruminal contents - these should be discarded and the animal's mouth should be flushed with water or physiological saline solution before repeat sampling.

Samples from sheep tend to be small, mucoid and difficult to detach from the probang. The easiest procedure is to insert the probang cup directly into a disposable Universal 20 ml bottle or similar container into which has been dispensed 3 ml of buffer solution. The probang is then shaken in the buffer solution to free the sample from the cup, and the sample together with the buffer should be poured into a previously labeled bijou bottle for transport.

³ Instead of phosphate buffer, tissue culture medium (e.g. Eagle's MEM or Earle's LYH) containing 0.04 M HEPES buffer has also been found satisfactory

Between collections from each animal, probangs should be disinfected in a bucket containing 4% Na₂CO₃ or 0.2% citric acid. After disinfection, the probang should be thoroughly rinsed in running tap water or at least three separate buckets of clean water placed in series.

Packaging

It is important to avoid thawing and refreezing at any stage, so packing should be done quickly and complete packages should be kept at -70°C or lower.

1. The outside of the bijou bottle containing the sample should be disinfected by immersion in 4% Na₂CO₃ or 0.2% citric acid, dried off, labeled and then frozen immediately in solid CO₂ within an insulated box or in a -70°C refrigerator.

2. For shipment the bijou bottles should be placed in a metal container within an insulated box of approximately 1 ft³ (0.3 m³), which is filled with dry ice. The container should be placed in the middle of the dry ice. The dry ice must be sufficient to last the journey to IAH. The insulated box should be wrapped and sealed and properly labeled. It should permit some escape of gas, otherwise there is a risk of explosion (see packaging of specimens of epithelium, paragraph 8.)

3. If insufficient dry ice has been used, and it has been exhausted by the time the samples are unpacked at IAH, further samples will be requested.

SEMEN SAMPLES

Collection and packaging

Semen should be collected under sterile conditions, frozen at -70°C or lower as soon as possible and packaged as described for probang samples.

SERUM SAMPLES

Collection

Serum, rather than whole blood, must be submitted for antibody testing. A minimum of 4 ml is essential. Should lesser amounts be submitted there is a risk that re-sampling may be required with subsequent delay.

It is essential that sterile containers be used. If sera have been collected under sterile conditions they can normally travel satisfactorily without refrigeration.

It is imperative that those submitting sera appreciate that a period of three weeks is required from the time sera are received at IAH before results will be available - this time period has been found necessary to allow for possible retesting or re-submission of sera. At least ten working days advance notice of submission of serum for testing is required.

Packaging

If sterile, samples can be submitted without refrigeration, but refrigeration or freezing is an added safeguard against spoilage. Samples of serum should be packaged as described for specimens of epithelium.

PROCEDURE FOR DISPATCH OF SPECIMENS TO THE WORLD REFERENCE LABORATORY

1. Dispatch from outside the United Kingdom should only be by air freight. The World Reference Laboratory makes arrangements to collect material from the London airports at Heathrow and Gatwick and has the necessary import permits to facilitate customs clearance.

Wherever possible, containers should be sent by British Airways so that airport clearance charges incurred by the World Reference Laboratory can be kept to a minimum.

2. The flight number, date and time of arrival and airway bill number should be sent by telex to the World Reference Laboratory in advance of arrival (Fax: +44 1483 232621, E-mail: chris.chisholm@bbsrc.ac.uk)

3. A letter should accompany the specimens giving as much history and epidemiological information as possible. In addition, forms similar to those shown in Appendix 2.4 and Appendix 2.5 should be sent with specimens for virus isolation or antibody assay, respectively.

4. Accompanying information must include the name, address and fax number, E-mail address of the sender and instructions for notification of results.

5. It is emphasized that the continued success of this service very much depends upon the active participation of all countries to ensure that the data bank of the Laboratory is up-to-date, thus making possible the provision of the most recent and relevant information on particular foot-and-mouth disease field situations.

5. IATA regulations ⁴concerning the Transport of FMDV by Air

The FMDV containing materials are defined Dangerous Goods which are capable of posing significant risk for animal health when transported by air and which are in the list of Dangerous Goods.

Dangerous Goods are classified into 9 Classes. Infectious substances are put into Class 6.2.

Definition of Infectious Substances:

Substances known to contain, or reasonably expected to contain pathogens. Infectious substances are subject to the Regulations only if they are capable of spreading disease when exposure to them occurs.

FMDV containing infectious substances were classified in Division 6.2 on the basis of their allocation to Risk Group 4 based on criteria developed by WHO.

Risk Group 4

For a pathogen that usually causes serious human or animal disease and can be readily transmitted from one individual to another directly and indirectly.

Diagnostic specimens must be assigned to UN 3373 unless the source animal has or may have a serious animal disease, which can be readily transmitted from one individual to another, directly or indirectly, in which case they must be assigned to UN 2900. Based on this criterion FMDV containing infectious substances and diagnostic specimens must be assigned identically to UN 2900.

Following entries can be found in the List of Dangerous Goods

UN	Proper Shipping Name	Class or Division	Passenger aircraft		CARGO Aircraft only	
			Packing Instr.	Max. Net Qty/ Package	Packing Instr.	Max. Net Qty/ Package
2900	Infectious substance affecting animals only (Liquid)	6.2	602	50 ml	602	4 L
2900	Infectious substance affecting animals only (Solid)	6.2	602	50 g	602	4 kg

5.1. Packaging for Infectious Substances

More detailed instructions for FMDV sampling can be found in section 4.1. above.

⁴ Dangerous Goods. Guidelines for the transport of infectious substances and diagnostic/clinical specimens by air. 44th edition of the IATA Dangerous Goods Regulation. EBSA Sixth Annual Conference, Lyon, France, 15-16 May 2003

Infectious substances must be packed in accordance with Packing Instructions 602.

Primary receptacle

A watertight receptacle containing the specimen

Secondary packaging

A second watertight leak – proof receptacle to enclose and protect the primary receptacle(s). Several wrapped primary receptacles may be placed in one secondary receptacle

Absorbent material

Absorbent material must be placed between the primary receptacle and secondary packaging. The absorbing material must be sufficient to absorb the entire content of all primary receptacles.

Outer packaging

The secondary packaging is placed in an outer packaging of sufficient strength, which protects it and its contents from external damage during the transport.

Special conditions apply if infectious substances are shipped refrigerated or frozen. The most frequently used refrigerants are Dry Ice and Liquid Nitrogen.

For Dry Ice the outer packaging must allow the release of carbon dioxide gas.

For Liquid Nitrogen, the shippers must use plastic primary receptacles, which can withstand very low temperatures.

The most frequently used refrigerant is the Dry Ice UN 1845. It must be placed outside the secondary packaging or alternatively in an overpack that meets the requirements of PI 904.

5.2. Marking and labeling the package

Packages containing infectious substances must be marked and labeled as follows:

Marking

- UN number and Proper Shipping Name(s) and Technical Name(s)
Example: UN 2900 Infectious substance affecting animals, Foot and Mouth Disease Virus, liquid
- Name and address of the shipper
- Name and address of the consignee
- Name and telephone number of a responsible person
- UN Specification markings

Labeling

- Primary hazard label
- Orientation labels on opposite sides on combination packages containing more than 50 ml of liquid in the package

If Dry Ice has been used, the package must also be marked with:

UN 1845,

Dry Ice or Carbon Dioxide solid

Net weight of Dry Ice

And must be labeled with the miscellaneous hazard label.

If the outer package is packed in an overpack, all the markings and labels must be repeated on the overpack, except that the “UN specification markings” will be replaced by the sentence:

Inner packages comply with prescribed specifications.

5.3. Documentation of the Transport

General principles

1. Two signed copies
2. Completed in English

For transport of FMDV containing materials the following documentations must be completed and enclosed:

1., Completion of the Shipper’s Declaration

Name and address of the shipper

Example:

Dr. X.Y. CVI, Budapest, Dept. of Virol. Tábornok utca 2., 1149 Budapest, Hungary

Tel: 00 36 1, Fax: 00 36 1, E-mail:

Name and address of the consignee

Example:

Dr. D. J. Paton, IAH ,Pirbright Laboratory, Ash Road , Pirbright, Woking, Surrey, GU 24 0NQ, UK

Tel: 00 44 1483 231012, Fax: 00 44 1483 232621, E-mail: david.paton@bbsrc.ac.uk

Name and Phone number of the responsible person: Dr. Z.A.,

00 44 1483...etc.

Air waybill number of the shipment:

Appropriate page number and total number of pages.

Page 1 of Page 1

Nature and Quantity of Goods

Proper Shipping Name: Infectious Substance affecting animals (FMDV)

Division Number: 6.2

UN number: 2900

Quantity and Type of packaging: 1 Fireboard box x 50 ml

Packing instructions number: 602

Alternatively:

Proper Shipping Name: Carbon dioxide, solid

Division number: 9

UN number: 1845

Quantity: 10

Packing instructions: 904

Handling information:

Statement: " Prior arrangements as required by the IATA Dangerous Goods Regulations 1.3.3.1. have been made

24 hr. Emergency Contact Tel. No: 00 36 1

Name/ Title of Signatory: Dr. X. Y. head of Dept.

Place and date:

Signature

2. Export / import license issued by the relevant veterinary authorities

Export licenses may be required under national regulations before diagnostic samples containing list A pathogens can be exported. For example, all shipments containing live virus to be exported from the UK must be accompanied by correctly completed Dangerous Goods Forms, an Individual Export Licence for that particular shipment, a copy of the receiving country's Import Permit and an Invoice. The package must also be marked with the name, address and telephone number of the recipient and the contact details of the sender (IAH). If the package contains dry ice it must be marked with a UN sticker. The sending officer should be in a position to rapidly obtain the necessary clearance/license.

Import licenses will be issued by the competent authorities in the receiving country. In the case of the WRL, Pirbright, an import license can be obtained very rapidly by contacting Chris Chisholm by telephone during normal working hours (Tel: 44 1483 231014) or else by Fax (No. 44 1483 232621) or E-mail (chris.chisholm@bbsrc.ac.uk).

3. Proforma invoice

- Name and address of consignee
- Name of the shipper
- Date
- Proforma Invoice
 1. Number of Pieces:
 2. Total Gross Weight:
 3. Total Net Weight:
 4. Carrier:
 5. Full description of contents :
 6. Value:
 7. Reason of export: For diagnostic purpose

Declaration:

I declare that the above information is true and correct to the best of my knowledge
Signature of the Shipper

5.4. Marking and labeling of the outer package

Package containing infectious FMDV must be marked and labeled as follows:

Infectious FMDV containing material must be packed in UN/UG/Class 6.2 package

Marking of the outer package:

From: (Shipper) Absolute identical with the Shipper's declaration

To: (Consignee) absolute identical with the Shipper's declaration

Infectious substance affecting animals (FMDV) , UN 2900

Name and telephone of responsible person:

Alternatively:

Dry Ice 10 kg, UN1845

Labeling of the outer package:

UN specific marking

Primary Hazard Label

Orientation label on combination packaging containing more than 50 ml of liquid in the package

Alternatively:

"Cargo Aircraft only "label if required

If Dry Ice has been used: " Miscellaneous hazard label".

If the outer pack age is packed in an overpack, all marking and labels must be repeated on the overpack, except that the UN specification marking will be replaced by the sentence:

"Inner packages comply with prescribed specifications "

Appendix 1. Overview of OIE standards for biocontainment of FMDV by diagnostic laboratories

According to OIE classification of animal pathogens (OIE, Manual of Standards of diagnostic tests and vaccines 2004, Appendix 1.1.5.1. D) FMDV has been classified as a Group 4 animal pathogen. FMDV is subject to official control and has a high risk of spread from the laboratory.

A laboratory should be allowed to possess and handle FMDV if it can satisfy the relevant authority that it can provide containment facilities appropriate to Containment Group 4.

The importation of FMDV pathological material or live virus should be permitted only under an import license issued by the relevant authority. The import license should contain conditions appropriate to the risk posed by FMDV. The import license for FMDV should only be granted to a laboratory that is licensed to handle the FMDV. The authority should first inspect the facilities to ensure that they are adequate and then issue a license specifying all relevant conditions. The authority should visit the laboratory periodically to ensure license conditions are being complied with.

Guidance on the requirements for containment level 4 laboratories are laid out in the OIE Terrestrial Animal Health Code (Chapter 1.4.6) and summarised in the following table.

REQUIREMENTS OF THE LABORATORY	Containment Group 4
A) Laboratory siting and structure	
1. Not next to known fire hazard	Yes
2. Workplace separated from other activities	Yes
3. Personnel access limited	Yes
4. Protected against entry/exit of rodents and insects	Yes
5. Liquid effluent must be sterilized	Yes and monitored
6. Isolated by airlock. Continuous internal airflow	Yes
7. Input and extract air to be filtered using HEPA or equivalent	Single for input, double for extract
8. Mechanical air supply system with fail-safe system	Yes
9. Laboratory sealable to permit fumigation	Yes
10. Incinerator for disposal of carcasses and waste	Yes on site
B) Laboratory facilities	
11. Class ½/3 exhaust protective cabinet available	Yes
12. Direct access to autoclave	Yes with double doors
13. Specified pathogens stored in laboratory	Yes
14. Double ended dunk tank required	Yes
15. Protective clothing not worn outside laboratory	Yes
16. Showering required before exiting laboratory	Yes
17. Safety Officer responsible for containment	Yes
18. Staff receive special training in the requirements needed	Yes
C) Laboratory discipline	
19. Warning notices for containment area	Yes
20. Laboratory must be lockable	Yes
21. Authorized entry of personnel	Yes
22. On entering all clothing removed and clean clothes put on	Yes
24. Individual must shower prior to transfer to clean side	Yes
25. All accidents reported	Yes

REQUIREMENTS OF THE LABORATORY	Containment Group 4
D) Handling of specimens	
26. Packaging requirements to be advised prior to submission	Yes
27. Incoming packages opened by trained staff	Yes
28. Movement of pathogens from an approved laboratory to another requires a license	Yes
29. Standard Operating Procedures covering all areas must be available	Yes

Appendix 2. Recommended materials, methods and template forms for submission of sample to the WRL, Pirbright

Appendix 2.1

COLLECTING MEDIUM FOR SPECIMENS OF EPITHELIUM

0.04 M phosphate buffer
 Add 3.05 gm Na₂HPO₄.2 H₂O
 0.39 gm KH₂PO₄
 to 500 ml sterile distilled water .
 Add 1 ml 1% phenol red.
 Add antibiotics -see Appendix 2.
 Adjust pH to 7.2-7.6 with HCl.

Appendix 2.2

ANTIBIOTICS

Reconstitution

Penicillin phial of 500,000 units add 2.5 ml sterile distilled water
 Mycostatin phial of 500,000 units add 10 ml sterile distilled water
 Neomycin phial of 500,000 units add 10 ml sterile distilled water
 Polymyxin phial of 350,000 units add 7 ml sterile distilled water

Probang and epithelium

To each 500 ml of 0.08 M or 0.04 M phosphate buffer add the following amounts of reconstituted antibiotics:

Penicillin 2.5 ml (final concentration 1000 units/ml)
 Mycostatin 1.0 ml (final concentration 100 units/ml)
 Neomycin 1.0 ml (final concentration 100 units/ml)
 Polymyxin 0.5 ml (final concentration 50 units/ml)

Appendix 2.3

COLLECTING MEDIUM FOR PROBANG SAMPLES

0.08 M phosphate buffer
 Add 6.11 gm Na₂HPO₄.2H₂O
 0.78 gm KH₂PO₄
 to 500 ml sterile distilled water .
 Add 1ml 1% phenol red
 Add antibiotics -see Appendix 2.
 Adjust pH to 7.2-7.4 with HCl.

Appendix 2.4
Specimen of Submission Form to accompany samples to the World Reference Laboratory for vesicular disease diagnosis

TO:
WORLD REFERENCE LABORATORY
 INSTITUTE FOR ANIMAL HEALTH
 PIRBRIGHT LABORATORY
 Ash Road, Pirbright,
 Surrey GU24 0NF
 ENGLAND
 Tel:01483 232441 Fax:01483 232621



FOOT-AND-MOUTH DISEASE
SAMPLE FOR TYPING FROM:-
 (Country of Origin)

For Laboratory Use Only

WRL REF No.

Batch No.

Received

DD/MM/YY

SENDER NAME: E-mail:	ADDRESS:	FAX:	TEL:	Are you a Gov't. Vet. Authority: Yes <input type="checkbox"/> No <input type="checkbox"/>
-------------------------	----------	------	------	---

Date Dispatched <u>DD/MM/YY</u>	Total Number of Samples	Year (s) of collection
---------------------------------	-------------------------	------------------------

OWNER	Local Farm or site Reference (Premises of Animals)	VILLAGE:	DISTRICT:	PROVINCE:	REGION:	GRID REF:	LAT/ LONG
-------	---	----------	-----------	-----------	---------	-----------	-----------

Sample Reference	Class of Animal	Age of Lesion	Material Sent	Date Collected <u>DD/MM/YY</u>	Time Collected HH : MM

Infection First Noticed <u>DD/MM/YY</u>	Date Reported <u>DD/MM/YY</u>	Outbreak Duration Days	Number of Herds Involved
---	-------------------------------	------------------------	--------------------------

Date of Previous Infection On farm <u>DD/MM/YY</u>	In Area <u>DD/MM/YY</u>	Serotype
--	-------------------------	----------

HUSBANDRY (please tick boxes)	Intensive <input type="checkbox"/>	Extensive <input type="checkbox"/>	Mixed <input type="checkbox"/>	Feedlot <input type="checkbox"/>	Nomadic <input type="checkbox"/>	Dairy <input type="checkbox"/>	Other <input type="checkbox"/>
COMMENTS:							

Possible Origin (please tick box)	Game Contact <input type="checkbox"/>	Adjacent Premises <input type="checkbox"/>	New Stock <input type="checkbox"/>	Other <input type="checkbox"/>
COMMENTS:				
(place/country/date)				
(method of spread)				
Control Measures (please tick box)	Slaughter Complete <input type="checkbox"/>	Partial <input type="checkbox"/>	Quarantine Measures <input type="checkbox"/>	Vaccination <input type="checkbox"/>
COMMENTS:				

Species	Breed	Sex	Number of Animals				Age of Affected Animals and Number				
			In Outbreak	Affected	Dead	Slaughtered	0-6m	6m-1y	1-2y	2-3y	>3y
Cattle											
Pigs											
Sheep											
Goats											
Other (please specify)											

Species	Vaccinated		Vaccination Regime				Vaccine Strains	Vaccine Producer	Batch Number
	Yes	No	Last Date	4m	6m	1y			

Appendix 2.5

Submission Form to accompany samples from animals for international trade purposes to World Reference Laboratory (Exportation of Animals) -

COUNTRY		DATE OF LETTER
		LABORATORY REF: SENDER'S REF: DATE RECEIVED: _____
	EXPORTATION OF ANIMALS	
OWNER OF ANIMALS ADDRESS:		
VETERINARIAN'S ADDRESS:		
TRADING COMPANY: ADDRESS:	TELEPHONE No. TELEX No. CABLE ADDRESS	
ANIMAL SPECIES _____ NUMBER COUNTRY OF ORIGIN DESTINATION	TYPE OF SAMPLES: _____	SERUM SEMEN PROBANG OTHER (SPECIFY)
DATE OF QUARANTINE EXPECTED DATE OF EXPORTATION		DATE OF SAMPLE COLLECTION
TEST REQUIRED: _____	SERUM ASSAY _____ FMD (SPECIFY TYPE) SVD VSV VIRUS ISOLATION: FMD _____ SVD _____ OTHERS (SPECIFY) _____	
TYPE OF TEST REQUIRED:	SERUM NEUTRALIZATION TEST ELISA	
NAME OF VETERINARY SURGEON SUBMITTING SAMPLES: _____		
ANIMAL No.	TUBE/SAMPLE No.	BREED SEX
AGE	LABORATORY No.	

Vaccine selection for the European banks

David Paton, WRL Pirbright

Issues

- Do we have adequate global information on outbreaks and their characteristics?
- Do we maximise information from available surveillance?
- Are the matching tests we do sufficient and reliable?
- How can we utilise data from other laboratories - collaboration versus competition?
- How do we prioritise risks from particular strains?
- How many doses of vaccines do we need?
- What useful collaboration can be undertaken between vaccine banks?
- Is there a prospect of more sharing of antigen reserves?
- Do we know enough about what vaccines are used in endemic countries and about their effectiveness?
- Tensions/conflicts between commercial vaccine producers and reference laboratories and vaccine banks.

Actions underway

- EU coordination action starting January 2005
 - Foster collaboration between reference labs
 - Establish systems for sharing information
 - Foster collaboration between vaccine bank managers
 - Develop model systems for evaluating risks of importing FMD
- EU Improcon and related research projects
 - Develop improved vaccine matching techniques
 - Measure cross-protection directly and evaluate correlation with in vitro matching techniques
- OIE ad hoc vaccine group
 - See below

The Mission statement for the OIE ad hoc vaccine group was adopted as:

"To facilitate information exchange and harmonisation of methods and standards to assist countries in the establishment of vaccine banks, with special emphasis on FMD; including the development of a network of banks for exchange of information on cross-protection of vaccine antigens and to resolve issues relating to potential supply of antigens and vaccines between banks and regions".

The following Terms of reference were agreed upon:

1. To develop guidelines for International Standards for vaccine banks for proposal as a Chapter of the *Terrestrial Manual*.
2. To develop guidelines for International standards, specific to FMD antigen and vaccine banks, to be proposed as an additional component within the FMD Chapter 2.1.1 in the *Terrestrial Manual*.
3. To develop guidelines on harmonisation of virus strain characterisation, to provide other information relating to cross-protection against infection with circulating FMD viruses and to assist in the identification and selection of antigens for inclusion in FMD vaccines and antigen banks.
4. To provide advice on future development and operation of a potential vaccine bank network.
5. To provide advice to the OIE on issues relating to networking of the OIE and FAO regional and international FMD Reference Laboratories.

Preliminary Report of a workshop on Comparative evaluation of FMD NSP antibody detection ELISAs

IZSLER, Brescia
3-15 May 2004

Purpose and limitations of the Workshop:

- Comparison of the currently available DIVA tests for antibodies against FMD non-structural proteins

Preliminary conclusions and recommendations: given at the end

Key to Coded database

A **B** **C** **D** **E** **F**
Bommeli **Cedi** **UBI** **IZS** **Panaftosa** **Svanova**

Test Methodologies

Company/Institute & NAME OF TEST	TEST DESCRIPTION
PANAFTOSA I-ELISA 3 ABC	a. Indirect ELISA
	b. Ag: Pre-coated with 3 ABC MS2, gel purified
	c. Conjugate: Anti-species IgG/HRPO
	d. Substrate: TMB
	e. Dilution of test serum: 1/20
	f. Interpretation: cut-off: 10 (PP); neg=<10; doubtful=>10 <15; pos=>20
	g. Control sera: C (-); C (+); C (++)
BRESCIA 3 ABC TRAPPING ELISA	a. Indirect-trapping ELISA
	b. Ag: 3 ABC MS2 trapped by a Mab
	c. Conjugate: Anti-ruminant IgG1 and IgG2 (Mab)/HRPO
	d. Substrate: OPD
	e. Final dilution of test serum: 1/100
	f. Interpretation: -cut-off: (PP); neg=<10; doubtful=>10/<15; pos=>15
	g. Control sera: C(-); C(+); C(++):
BOMMELI CHEKIT FMD-3 ABC ELISA	a. Indirect ELISA
	b. Ag: Pre-coated with 3 ABC GST immunopurified
	c. Conjugate: Anti-ruminant IgG1 (Mab)/HRPO
	d. Substrate: TMB
	e. Final dilution of test serum: 1/100

	f. Interpretation: -cut-off: 30 (PP) , pos.= >30 ; doubtful=>20/<30 ; neg=<20
	g. Control sera: C(+):less than2.0 OD; C(-):less than 0.5OD
CEDI DIAGNOSTIC CEDI TEST-FMDV-NS	a. Competitive ELISA
	b. Ag: 3 ABC (bocular) trapped by anti-3B Mab
	c. Conjugate: Anti-3B Mab/HRPO for competition
	d. Substrate: TMB
	e. Final dilution of test serum: 1/5
	f. Interpretation: -cut-off: 50% PI, >50 pos
	g. Control sera: C(-):<1.000 OD ;C(+):; >50% PI; C(++): >70%PI

SVANOVA SVANOVIR FMDV 3ABC-Ab ELISA	a. Indirect ELISA
	b. Ag: Pre-coated with 3 ABC purified
	c. Conjugate: Anti-bovine IgG1/HRPO
	d. Substrate: ABTS
	e. Final dilution of test serum: 1/40
	f. Interpretation: -cut-off: 48 (PP); neg=<48; pos=>48
	g. Control sera: C(+): ; C(-):
UBI FMD NSP ELISA	a. Indirect ELISA
	b. Ag: 3 B peptide, 50 aa of length
	c. Conjugate: recombinant protein A/G,/HRPO
	d. Substrate: TMB
	e. Final dilution of test serum: 1/21
	f. Interpretation: -cut-off: changed for each plate; neg=<1(T/C); pos=>1(T/C)
	g. Control sera: C(+): >0.7-<1.9OD; C(-): <0.150 OD

Table of values for threshold and interpretation of tests

		NEGATIVE	Inconclusive	POSITIVE
PANAFTOSA	PP	<10	≥10 & <20	≥20
BRESCIA	PP	<10	≥10 & <15	≥15
BOMMELI	PP	<20	≥20 & ≤30	>30
CEDI DIAGNOSTIC	P Inh.	<50	-	≥50
SVANOVA	PP	<48	-	≥48
UBI	T/C	<1	-	≥1

Information recorded in database of sera and codes used to input data

UN	COUNTRY	ANIMAL ID	EXP/FIELD	DUN/SEQ	SERUM ID	VAC STAT	Vac Type	INFECT	Route	Inf	Inf Virus	DPV	DPI	DPO
1	ITA		E	U		NV		I	IDL					
2	UK		F	S		V		NI	ID					
3	D					V2			IN					
4	BE					V4			CO					
5	DK					V8			AER					
6	NL					V16								
	ISR					V64								
	TUR													
	SA													

PROTECT	Clin Signs	AB	BOOSTER	VI	PCR	CARRIER	Age	Farm CLASSIF	Type	NoVac
P	S+	B		VI+	PCR+	C+	POS			
NP	S-	NB		VI-	PCR-	C-	NEG			
							INC			

CP %P	WB	CP PI	IZS	IZS PI	IZS comment	Bommeli	Bo Plate	Cedi	Ce PI	Svanova	Sv PI	UB I	Cut-off	T/C	UBI PI
PP			PP			PP		PI						T/C	

List of sera analysed and origin

CATTLE

	NEGATIVE	VACCINATED	INFECTED	VACC+INF	FIELD
ITALY	192	46	15	72	
BELGIUM	96	48			
GERMANY		191		78	
DENMARK	100				
UK	200	63	36	291	
NETHERLAND	91	77	2	140	
PANAFTOSA					32
TURKEY			13		161
ISRAEL					471

SHEEP

	NEGATIVE	VACCINATED	INFECTED	VACC+INF	FIELD
ITALY	96				
BELGIUM	96				
GERMANY					
DENMARK	96				
UK	100	6	9	6	100
NETHERLAND	19	6		16	
PANAFTOSA					
TURKEY					78
ISRAEL					65

PIGS

	NEGATIVE	VACCINATED	INFECTED	VACC+INF	FIELD
ITALY					
BELGIUM					
GERMANY					
DENMARK					
UK					
NETHERLAND					
PANAFTOSA					
TURKEY					
ISRAEL					

Comparison of Sensitivity values on sera derived from experimentally-challenged CATTLE

TABLE 1 Sensitivity for experimental sera from Non-vaccinated BUT Infected CATTLE. collected 11-72 days post-challenge (n =58)

	A	B	C	D	E	F
Tested	58	58	58	56	58	56
Positive	57	58	58	56	58	55
SE	98%	100%	100%	100%	100%	98%
95% CI						

TABLE 2 Sensitivity for experimental sera from Vaccinated and challenged CATTLE of unknown "Carrier" status. Sera were collected between 8 and 43 days post-challenge and multiple sera are included from some animals (n = 386)

	A	B	C	D	E	F
Tested	385	386	386	386	387	372
Positive	189	196	139	208	211	176
SE	49%	51%	36%	54%	55%	47%
95% CI	44-54%	46-56%	31-41%	49-59%	49-60%	42-53%

TABLE 3 Sensitivity for experimental sera from Vaccinated "Carrier" CATTLE. Sera were collected between 10 and 168 days post-challenge and multiple sera are included from some animals (n = 167)

	A	B	C	D	E	F
Tested	167	167	167	167	167	154
Positive	65	118	80	109	126	89
SE	39%	71%	48%	65%	75%	58%
95% CI	31-47%	63-77%	40-56%	58-72%	68-82%	50-66%

TABLE 4 Sensitivity for experimental sera from Vaccinated "Carrier" CATTLE. Sera were collected after 27 days post-challenge and multiple sera are included from some animals (n = 128)

	A	B	C	D	E	F
Tested	128	128	128	128	128	113
Positive	55	105	77	96	111	82
SE	43%	82%	60%	75%	87%	73%
95% CI	34-52%	74-88%	51-69%	67-82%	80-92%	63-81%

TABLE 5 Sensitivity for experimental sera from Vaccinated "Carrier" CATTLE single serum per animal, 28-100dpi (n = 49)

	A	B	C	D	E	F
Tested	49	49	49	49	49	46
Positive	34	43	40	43	45	39
SE	69%	88%	82%	88%	92%	85%
95% CI	55-82%	75-95%	68-91%	75-95%	80-98%	71-94%

Comparison of Specificity values on sera derived from non-infected CATTLE

TABLE 1 Specificity for field sera from Non-vaccinated, uninfected CATTLE (n = 610)

	A	B	C	D	E	F
Tested	610	609	608	610	609	585
Positive	1	12	2	17	15	9
Negative	609	597	606	593	594	576
SP	99.8%	98%	99.7%	97.2%	97.5%	98.5%
95% CI	99.1-100%	96.6-99%	98.8-100%	95.6-98.4%	96-98.6%	97.1-99.3%

TABLE 2 Specificity for experimental sera from vaccinated, uninfected CATTLE (n = 426)

	A	B	C	D	E	F
Tested	426	425	424	426	426	418
Positive	13	1	8	10	13	6
Negative	413	424	416	416	413	412
SP	96.9%	99.8%	98.1%	97.7%	96.9%	98.6%
95% CI	94.8-98.4%	98.7-100%	96.3-99.2%	95.7-98.9%	94.8-98.4%	96.9-99.5%

TABLE 3 Specificity for all sera from uninfected CATTLE (n = 1036) vaccinated [experimental sera] & unvaccinated [field sera]

	A	B	C	D	E	F
Tested	1036	1034	1032	1036	1035	1003
Positive	14	13	10	27	28	15
Negative	1022	1021	1022	1009	1007	988
SP	98.6%	98.7%	99.0%	97.4%	97.3%	98.5%
	A	B	C	D	E	F
TOTAL	471	471	471	471	471	462
Positive	98	104	73	119	120	97
%	21	22	16	25	25	21
95% CI	17-24	18-26	12-19	22-29	22-29	18-24
95% CI	97.7-99.3%	97.9-99.3%	98.2-99.5%	96.2-98.3%	96.1-98.2%	97.5-99.2%

Field Sera

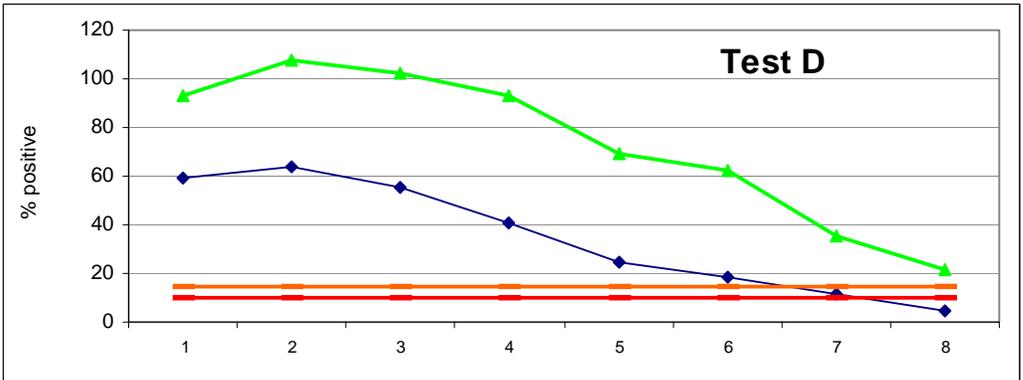
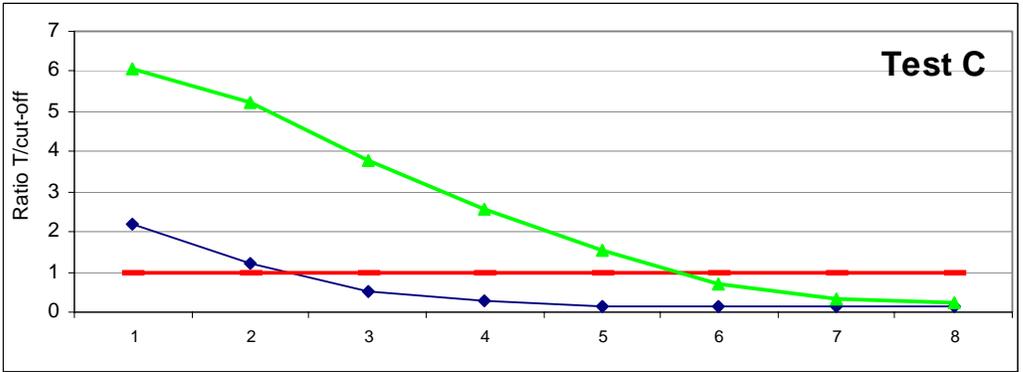
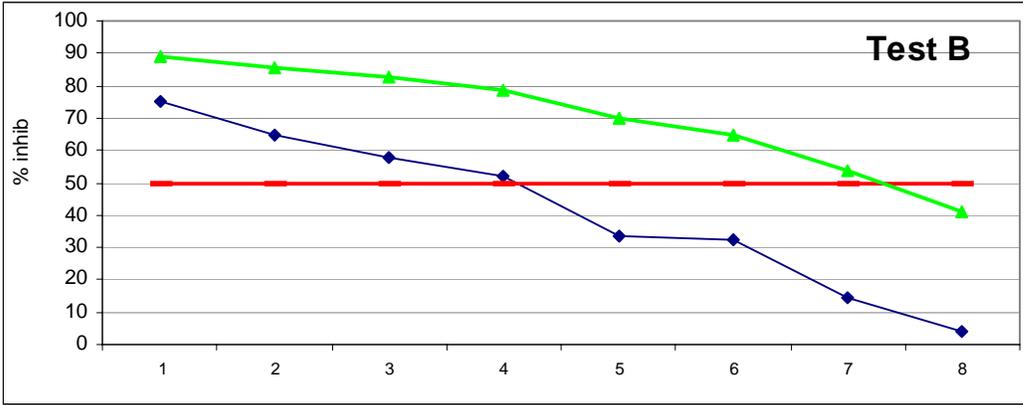
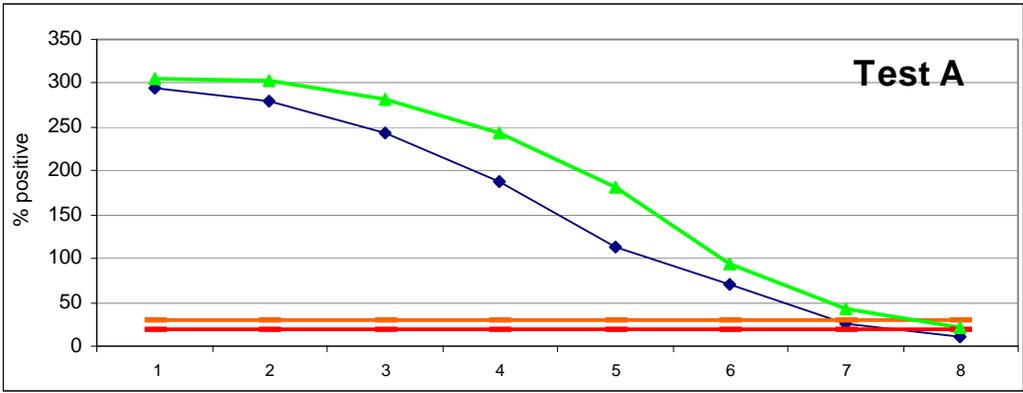
Table 1: Positive samples in a panel of 471 Israeli post-outbreak sera from vaccinated cattle.

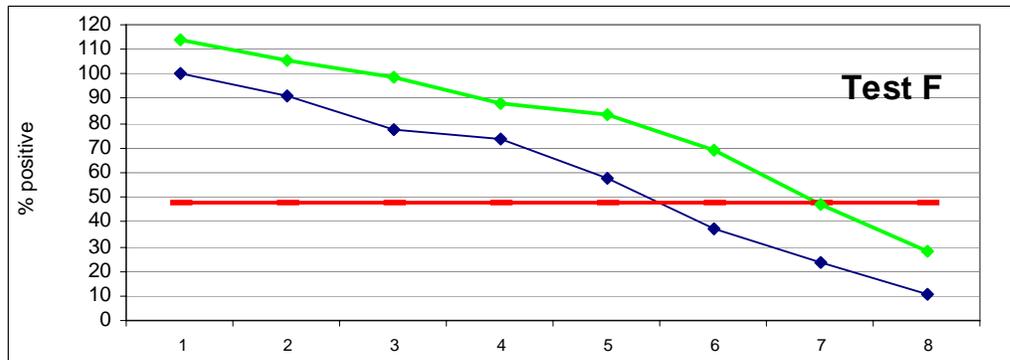
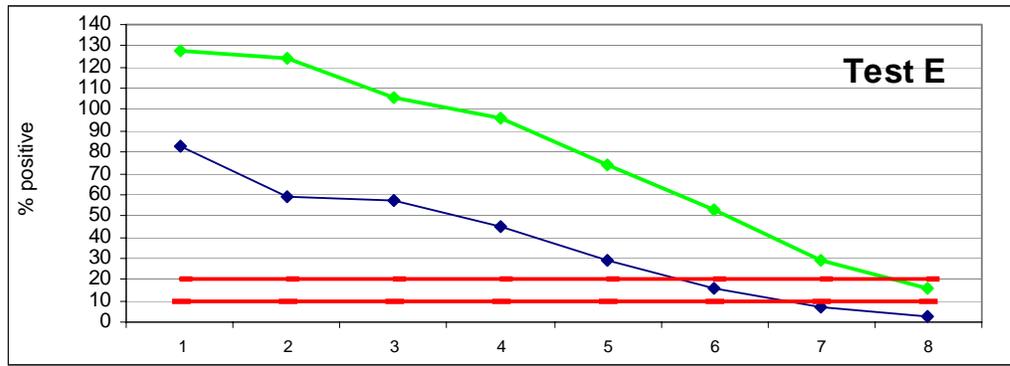
	A	B	C	D	E	F
TOTAL	174	174	174	173	174	173
Positive	122	113	63	123	118	110
%	70	65	36	71	68	64
95% CI	63-75	58-72	29-42	64-76	61-73	56-69

Table 2 A panel of selected NSP positive sera from vaccinated Turkish cattle (based on one of the tests included in the WS).

Discrepancy Analysis

Total	n	%						
Negative in all tests	1478	64,1						
Positive in all tests	344	14,9						
Discrepant in 1 or more tests	484	21						
	Total		A	B	C	D	E	F
	n		n	%	n	%	n	%
Neg. results for sera Pos in 4 tests (FN?)	88		42	47,7	15	17	56	63,6
Neg. results for sera Pos in 5 tests (FN?)	108		10	9,3	0	0	80	74,1
Neg. results for sera Pos in 4-5 tests (FN?)	196		52	26,5	15	7,7	136	69,4
					4	2	3	1,5
							47	>24





Quality Control Issues

There is an urgent requirement for procedures to ensure international acceptance of NSP test results. In some countries there are legal provisions for marketing authorization and batch release of diagnostic tests. In any case, accredited laboratories have to check the performance of tests and batches of kits they are using. Whereas such procedures for FMD NSP-test have to be implemented on the laboratory and national level, international harmonization of these procedures is crucial.

While the final objective is an agreement on the level of the OIE, an EU-wide agreement can probably be achieved much easier because of the common disease control strategy and the legal provisions in the Council Directive 2003/85 EC.

While the main concern of the trading partner's is sensitivity – necessitating international harmonization - specificity is mainly in the interest of the countries applying the test and different national panels to determine specificity are acceptable and may even be necessary because of different “background reactivities” and different control strategies. However, also specificity requirements may to some extent depend on the control strategies and the phase of eradication.

It is suggested to create a panel of internationally recognized reference sera to ensure a minimum sensitivity for the purpose of screening of areas. Since creating a definitive panel will take considerable time, it should be attempted to create a provisional panel for the needs of the EU using already existing sera.

Possibly a provisional panel of 10 – 30 sera, containing “early” and “late” post infection sera would be sufficient and the results could be analysed by a scoring system. A serum scored consistently positive in one test may be consistently negative in another test of equal diagnostic sensitivity.

National panels of negative sera, in which “problem sera” may be over represented, could be used to check specificity.

It is recommended, that laboratories should also run controls for repeatability until a batch of kits is accepted.

While producers should run their own tests and provide documents on the results, it is recommended that national labs perform batch testing before batches are used in disease control.

Purpose and limitations of the Workshop:

- Comparison of the currently available DIVA tests for antibodies against FMD non-structural proteins.
- The information on the status of the animals from which the sera used in the workshop, had been collected was not always complete or certain. In some cases more information may be available.

Preliminary conclusions and recommendations:

- Preliminary analysis of the cattle sera present at the workshop was discussed during the workshop. Further analysis of the results on the cattle sera is necessary, whilst sheep and pig data still have to be analysed.
- Discrepancy analysis showed a very good correlation between tests E and D and their results were more or less comparable results with those from test B. (The analysis could be repeated with a lower cut-off for test B). The sera showing discrepancy should be further analysed (e.g. source and results with tests against structural proteins, quantitative level of reactivity in DIVA tests).
- Similar specificity results were obtained with 610 field sera from non-vaccinated, non-infected cattle and with 426 experimental sera from vaccinated, non-infected cattle. In both cases the specificity of all tests was between 98 and 100% or 97 and 100% using lower cut-offs.
- Sensitivity results with experimental sera from vaccinated and challenged cattle known to be carriers ranged from approximately 70 to 90% with the more sensitive tests, depending on the time of serum collection after challenge and whether or not multiple sera were considered from the same animal. Much lower sensitivity values were obtained with some of the tests (30 to 60%).
- Lower sensitivity values (around 40 to 50%) were obtained with experimental sera from vaccinated and challenged cattle selected on the basis of challenge at 8 to 42 days before sampling and without regard to whether or not from known carriers. Low levels of virus replication and the short interval between challenge and sample collection probably contributed to the low sensitivity levels. The status of infection of some of the animals needs further analysis.
- Tests B and E were in general the most sensitive tests for the detection of antibodies in vaccinated and subsequently infected animals, followed closely by test D.
- A panel of approximately 60 experimental sera collected between 11 and 72 days post infection from non-vaccinated, experimentally infected cattle confirmed previous experience that the sensitivity of all tests was comparably high in the absence of prior vaccination.
- Using the lower test cut-offs, 15 to 25% of samples were positive for 471 field sera from vaccinated cattle in infected herds in Israel. Five of the 6 tests found 20 to 25% of the sera positive.
- More positive results (4 to 5 times more) in these Israeli sera were found in groups where clinical signs were observed. In the groups with clinical signs the time between last vaccination and infection was significantly longer than in the group without clinical signs.
- Preliminary results using ROC curves showed that the sera used during the workshop were a valuable addition to previously analysed sera. In the previous analysis sera of contact infected cattle were missing. More work will be done using this technique.
- An eight fold difference in analytical sensitivity was found between the test with the highest- and the one with the lowest analytical sensitivity, using two control sera from Panaftosa.
- However, the relative analytical sensitivity of the tests was not in accordance with the relative diagnostic sensitivity.
- It is recommended to develop a panel of sera for reference purposes, including samples collected from vaccinated infected cattle.
- Such an internationally recognized panel of reference sera is needed to ensure the required sensitivity of all batches of all the tests being used in the field. Since the creation of a definitive international panel will take considerable time, it should be attempted to create a provisional panel for use by national laboratories in the European community and by other parties willing to participate.
- Laboratories should check the sensitivity, but also specificity (on national panels of negative sera) and the reproducibility of their tests, even if they use commercial test kits.
- DIVA tests for FMD have a much higher sensitivity to detect a carrier in a single sample than probang tests currently in use.

Further points for discussion

Are the tests suitable for purpose?

- Screening after an outbreak using emergency vaccination
- Import-, export screening
- Regaining OIE status free of infection (with or without vaccination)

Further analysis of the data will include:

Establishment of likelihood ratios for CATTLE

ROC curves for use of the tests in CATTLE

Sensitivity and specificity values for sera from SHEEP

Sensitivity and specificity values for sera from PIGS

ADDENDUM

Please note that pages 56-65 (Appendix 6: *Preliminary Report of a workshop on Comparative evaluation of FMD NSP antibody detection ELISAs held at IZSLER, Brescia, 3-15 May 2004*) had been included in error in the Report, and have been removed from the final report after printing. At the Closed Session of the Research group (under item 3.3, pages 5-6 of this report) it had been concluded that *“Provisional figures for test performance will not be released until final quality checks have been completed”* and it was recommended that *“The working Group.....continue to finalise and publish the comparative analysis”*. The EUFMD Secretariat understands that scientific papers based on analysis of the data derived from the workshop and the final report of the workshop should be submitted for publication by 31 March 2005 and the latter should be completed by the end of May 2005.

Workshop on Validation of NSP-ELISAs: a comparison of 6 assays

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Introduction:

Validation of existing NSP tests is one of the tasks of the EU research project FMD_ImproCon (SSPE-CT-2003-503603). A workshop was organised in collaboration with the EC and the FAO EUFMD at the FMD laboratory of the IZSLER in Brescia (Italy) to compare the OIE NSP-ELISA index test with 5 other tests.

Materials and Methods:

The following tests were compared: PANAFTOSA I-ELISA 3 ABC, Brescia 3 ABC TRAPPING ELISA, Bommeli CHEKIT FMD-3 ABC ELISA, Cedi Diagnostics Ceditest FMDV-NS, Svanova SVANOVIR FMDV 3ABC-Ab ELISA, UBI FMD NSP ELISA. The 3829 sera tested came from Belgium, Denmark, Germany, Israel, Italy, South-America, the Netherlands, Turkey, UK. These sera came from naive, vaccinated, infected, vaccinated and infected, and field (post outbreak or post vaccination campaign) cattle, sheep or pigs. Analytical sensitivity of the 6 assays was tested on dilution series of two positive OIE candidate reference sera.

A databank with serum information was established. Diagnostic specificity was evaluated in naive and vaccinated populations. A preliminary analysis was made to compare the diagnostic sensitivity of the tests in several animal categories at different time points after vaccination and/or infection. A discrepancy analysis was performed on all results. Quality control of serials of kits by the users was discussed. Results were discussed with the test producers.

Discussion:

Comparison of test performance will be of interest to international organisations like EC, FAO and OIE to make serosurveillance guidelines and for decision makers to establish a scientific based sampling strategy. Further data analysis will include ROC and LHR analysis. More sera are needed from vaccinated and infected sheep and pigs.

Diagnostic Assays used for FMD surveillance: fitness for purpose revisited

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Application background

International context requires OIE regulation

- ▶ international trade, documenting disease freedom

Principles

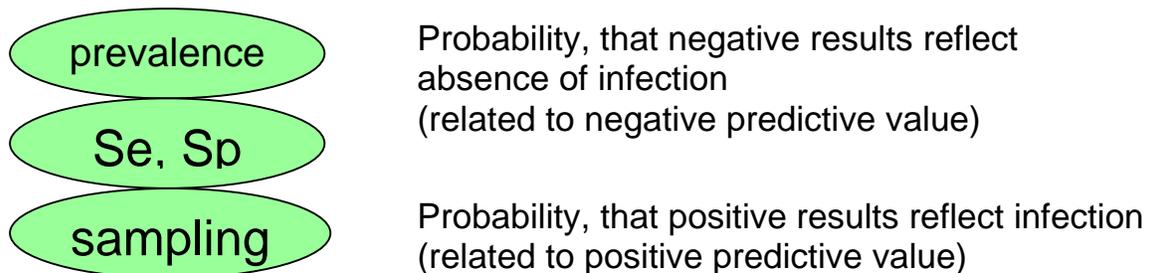
- ▶ science-based, all steps are documented

Epidemiological situations

- ▶ documenting disease freedom
- ▶ regaining free status without vaccination
- ▶ regaining free status after vaccination

Determinants of diagnostic performance

- ▶ animal-level → aggregate-level → country level
- ▶ performance of aggregate testing dependent on:



Fitness for purpose

Absolute performance criteria (Se, Sp)

- ▶ ... entails absolute upper limits of acceptable/feasible sample sizes (illustration)
- ▶ ... pre-supposes identical implementations of surveillance systems

Fitness criteria should be formulated in a more general way

- ▶ some ideas taken from the draft OIE template for assay validation

OIE validation template: level 1

Protocol development and feasibility

- ▶ analyte/reaction
- ▶ standardization/optimization
- ▶ analytical specificity*
- ▶ analytical sensitivity*
- ▶ accuracy and precision*

****performance parameters estimated***

- ▶ with scientifically valid method
- ▶ sufficient statistical precision, unbiased
- ▶ well-documented

OIE validation template: level 2

Diagnostic performance

- ▶ negative reference animals
- ▶ positive reference animals
- ▶ threshold determination
- ▶ diagnostic specificity/ sensitivity estimates (conventional estimates using a gold standard, a comparative test or no-gold standard methods)*

**performance parameters estimated as before*

OIE validation template: level 3

Technology transfer and reproducibility

- ▶ description of training requirements, standardization
- ▶ complete description of reproducibility study (serum panels, logistics, laboratory selection, analysis, performance requirements)

FMD specific performance criteria

Immunity

- ▶ differentiation between infection and vaccination
- ▶ detection of infection in vaccinated animals
- ▶ carrier status

Scope of validation

- ▶ cattle, swine, small ruminants, etc.
- ▶ virus-type
- ▶ geographical area

Epidemiology

- ▶ "absence of infection/disease" impossible to prove
- ▶ detectable levels of (design; threshold) prevalence; e.g. 0.2% among-herds; 5% within-herds ("virus circulation")
- ▶ established and circulating(?) infection at prevalence below design prevalence in vaccinated populations
 - how low? 5% in infected/vaccinated animals?
- ▶ Herd-level sensitivity (HSe) increases with prevalence

Illustration

Assume 80% sensitivity of NSP ELISA in carrier cattle and 99% specificity for vaccinated, non-infected cattle

For

- ▶ Herd size 1000; 5% within-herd prevalence; sample size 58

We obtain

- ▶ herd-level specificity (HSp) = 55.8%; HSe = 95%

But

- ▶ HSe = 66% and 47% for 1% and 0.1% prevalence, respectively

Discussion

We need reliable assay validation data (diagnostic Se and Sp)

- ▶ cattle, swine, small ruminants (and others)
- ▶ goals must be defined

***We need consensus about the levels of "design prevalence" for vaccinated populations
Fixed values for required sensitivity and specificity are not useful***

Post-vaccinal serosurveillance for FMD: a European perspective on progress and problems

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There has been much debate about the use of the so-called vaccinate-to-live policy for the control of FMD in Europe. According to this approach, spread of the FMD virus from future outbreaks could be controlled by a short period of "emergency" vaccination of surrounding herds, reducing the need for large-scale pre-emptive culling of at-risk animals. Since vaccinated ruminants may become subclinically and persistently infected with FMD virus following challenge exposure, it is necessary to either kill or slaughter under controlled conditions foreseen in the OIE Terrestrial Animal Health Code all vaccinates (vaccinate-to-kill) or to detect and kill or slaughter under controlled conditions all vaccinates that have become persistently infected (vaccinate-to-live), in order to rapidly regain the most favoured trading status of FMD-free without vaccination. The latter approach can be attempted by testing vaccinated animals for the presence of antibodies to certain non-structural proteins (NSP) of FMD virus, which are induced by FMD infection, but not by vaccination with purified vaccines. The numbers of herds and animals to be sampled and tested to be confident that infection has not been missed will depend upon the expected prevalence of subclinical infection amongst and within herds. This in turn will depend upon the manner in which infection is spread and on how vaccination is applied. The sensitivity of the tests used and the size of the herds will also influence the numbers of samples required to be collected and tested.

The new Council Directive 2003/85/EC on FMD takes account of these factors in its provisions for vaccination and for the use of post-vaccination serosurveillance to detect subclinical infection (Anon, 2003). According to the Directive, blood samples should be collected and tested from vaccinated animals and herds within a vaccination zone and from the unvaccinated offspring of vaccinated animals. Either all animals within vaccinated herds must be sampled and tested (Article 56, 3 (b)) or else sufficient numbers must be sampled and tested to enable a 5% prevalence of subclinical infection to be detected with 95% confidence (Annex III, point 2.2). Such sampling is not to take place until at least 30 days after the completion of emergency vaccination. EC legislation, taking into account that the necessary tests are regarded as herd tests and are not suited to verify the status of an individual animal, requires that herds within which at least one confirmed persistently infected animal has been detected must be slaughtered. However, difficulties in predicting the likely prevalence of post-vaccinal, subclinical infection and uncertainty over the performance of NSP tests has cast doubt over the suitability of these proposed sampling regimes.

There are now several commercially available NSP antibody ELISA tests. Under favourable sampling conditions, our estimates are that the sensitivity of these tests for detecting individual persistently infected vaccinated cattle can be as high as 90%, with a 99% specificity. This assumes that the vaccines used have been purified to remove traces of NSP and have been given in an emergency setting involving the application of a single vaccine dose. If all animals can be sampled, at least one animal should score positive to detect a herd. If a test with a 100% sensitivity is used in a herd of 100 animals a prevalence of 1% can be detected, but in a small herd of 10 animals 1 positive animal is equal to a prevalence of 10%. If the sensitivity is lower, e.g. 80%, the chance of missing positive animals is 0.2. To be sure we will detect a farm with 95% confidence the chance of missing animals (0.2^n) should be lower than 0.05. This means the number of positive animals should be at least $2^{(0.2 \log(0.05))} = \log(0.05)/\log(0.2) = 1.9$. In this case on a large farm (100 animals) a prevalence of 2% can be detected (95% confidence), but on a small farm (10 animals) only a prevalence of 20%.

This therefore sets a limit on the degree of certainty that can be achieved for detecting low levels of persistently infected animals. It has been difficult to obtain reliable information on carrier prevalence under field conditions following vaccine breakdowns and even where available may not be relevant to regimes of husbandry and vaccination intensity that would prevail under European conditions. Suttmoller and Gagero (1965) reported a 50% prevalence at four months after a vaccine trial breakdown in Brazil. In recent studies in which cattle that had been vaccinated 21 days earlier were exposed to five days of

direct contact with unvaccinated, infected cattle, nine out of 20 vaccinates became persistently infected with FMDV giving a prevalence of 45% (Cox et al., in press). On the one hand, this challenge was more even and severe than is likely under field conditions, but on the other hand field vaccination may be less reliable and in a field situation, challenge may occur before so much time has elapsed for the development of post-vaccination immunity. Therefore, scenarios can be envisaged in which the prevalence of carriers within a vaccinated population could be greater or smaller than 45%.

Measures can be taken to mitigate the risks from missing subclinical infections in vaccinated populations. If ring vaccination is used, then the animal population outside of the vaccination zone will have a higher susceptibility to FMD virus infection. Therefore, the EU Directive requires a buffer zone to be established around a vaccination zone and for animals to not be moved out of the vaccination zone until FMD-free status has been attained, which will be at least six months after the last outbreak or last vaccination, whichever is the longest. It would also be prudent to avoid the introduction of unvaccinated animals to vaccinated herds for the same time interval.

Another problem for post-vaccination serosurveillance is that testing large numbers of samples will lead to many false positive test results; on average at least one false positive result can be expected every time a herd of 100 or more animals is tested with a method that has a 99% specificity. No confirmatory tests have been introduced in European laboratories to verify whether or not such results are specific. However, recent findings suggest that some of the different 3ABC NSP ELISA tests do not score the same sera as false positive and therefore providing they are of sufficient sensitivity, they may be used to confirm each others results. Other solutions may be to analyse the test results on a herd-basis and to look for evidence of multiple seroconversions or sub-threshold increases in antibody levels that could be indicative of genuine infection. Another option is to retest all positive samples without changing the assay and then to resample and retest animals that continue to score positive, although this will marginally reduce the overall sensitivity of the assay. At the time of resampling reactors, additional blood collections should be made from neighbouring animals for delayed seroconversion that could have been missed at the first sampling. Herds testing negative at the second sampling would be considered uninfected, whilst herds showing an increased level of seroconversion would have to be culled. Individual reactors that remain seropositive would be culled. Based on the mathematics described above you need at least two positive animals to detect at least one with 95% confidence. If you cull only the one that reacted positive, you probably miss one carrier animal that was not NSP positive. The risk associated with carriers missed by NSP testing should therefore be quantified and compared to the risk related to the currently accepted non-vaccination policy in which clinically affected and at risk herds are destroyed along with those identified as infected by other means.

Evidently, the confidence with which available NSP tests can be expected to detect persistently infected vaccinated animals will be strongly linked to herd size. Since it is hard to predict the likely prevalence of infection in vaccinated herds, an approach for large herds could be to begin by sampling sufficient animals to detect a low prevalence of infection with 95% confidence and then to relax the stringency of sampling if warranted by the initial results. Small herds are more difficult to deal with. One could (1) accept the risk associated with a potentially inadequate level of sampling, (2) use only a vaccination-to-kill policy in small herds, (3) have additional biosecurity restrictions on small herds after an outbreak is over or (4) avoid vaccinating such herds in the first place. This last solution is attractive on a number of grounds including the fact that small herds generally pose a relatively low risk to neighbours and are not therefore a high priority for vaccination. Secondly, it is extremely important to implement emergency vaccination as quickly as possible in order to reach a critical proportion of vaccinates in the susceptible population, whereas vaccinating small herds is slow on a per capita basis. However, it can be anticipated that such a policy would be unacceptable to the owners of small herds and therefore politically difficult to implement.

Vaccinate-to-live in pigs, potentially an attractive option following the adoption of the proposed amendments to the OIE Terrestrial Animal Health Code, may be less troublesome for NSP-based exit testing. Firstly carriers do not occur. Secondly, large herd sizes mean that low level virus circulation can be detected with confidence by NSP serosurveillance, even if test sensitivity is low. Specificity however becomes a greater problem, but since pigs are kept in pens, one could discriminate between false positives and true reactors by whether or not test positive results are spatially clustered.

Taking account of technical progress and recognising the difficulty of proving the absence of a low level of persistently infected animals within a vaccinated population, the OIE, at its General Session in May 2004, has altered the requirements for countries using long-term vaccination programmes for FMD control. In

this situation, vaccination takes place in the entire country or geographic zone and is applied continuously. Instead of being required to prove absence of persistently infected animals, the veterinary authorities in such countries will be required to show that virus is no longer circulating and will then gain the trading status of FMD-free with vaccination. Countries that are FMD-free with vaccination cannot easily export live ruminants due to the risk of some being persistently infected. So far the EU requires that meat derived from such animals must be de-boned, matured and pH-controlled before being exported, however the OIE recently modified its rules to allow such meat without restrictions..

To prove absence of virus circulation by serosurveillance is much easier than to prove absence of infection since no definite decision needs to be taken as to whether NSP reactors are genuine or not; it will suffice for herds with NSP seroreactors to be resampled and retested to show absence of seroconversion. The OIE is still finalising its FMD surveillance guidelines and an Epidemiology subgroup was formed to discuss this issue in June 2004. More detailed guidance has been provided on the follow-up procedures necessary when NSP reactors are discovered in vaccinated animals, but this guidance does not apply to the situation envisaged in Europe – i.e. emergency vaccination and then rapid recovery of the status of FMD-free without vaccination.

In conclusion, problems associated with the imperfect specificity of NSP tests may be overcome by a resampling and retesting programme, but the requirement to use serosurveillance to prove complete absence of any persistently infected cattle in a vaccinated population cannot be met. However, the risk associated with a low level of undetected carriers will be low. This risk needs to be better quantified and compared to the risks of other control policies, including the previously accepted one of non-vaccination. The revision of the OIE guidelines for serosurveillance requirements in countries wishing to attain the status of FMD-free with vaccination and the transposition of the new EC Directive on FMD with its effects also on imports might conceivably lead European countries to include in their contingency planning an approach to disease control in which emergency vaccination could be followed by serosurveillance to detect virus circulation rather than infection (including detection of all carriers).

However, there is currently no provision in the OIE code for a country that was previously FMD-free without vaccination to use emergency vaccination to help control an outbreak and then move rapidly to the status of FMD-free with vaccination; only countries that were FMD-free with vaccination in the first place can regain this status within 6 or 18 months, depending on the scenario.

Therefore, following an outbreak of FMD in a previously free-without vaccination country or zone there is the imperative need to regain free status followed by a decision whether this status will in future be maintained with or without regular prophylactic vaccination. While this situation may hypothetically become relevant also for certain peripheral parts of the Community where the FMD situation in a neighbour country deteriorates and intervention by the Community on the territory of that country would not be admitted, the disadvantages including difficulties in maintaining an adequate separation of livestock and their products within the two zones and possible trade disincentives for the zone which was free without vaccination (including the rest of the European Community) still make this a questionable option. Furthermore, such an approach is not in line with EU legislation.

References

Anon (2003). Council Directive 2003/85/EC on Community measures for the control of foot-and-mouth disease repealing Directive 85/511/EEC and Decisions 89/531/EEC and 96/665/EEC and amending Directive 92/46/EEC. Official Journal of the European Union L306, Volume 46, 22 November 2003.

Cox, SJ, Voyce, C, Parida, S, Reid, SM, Hamblin, PA, Paton, DJ and Barnett, PV (In press) Protection against direct contact challenge following emergency FMD vaccination of cattle and the effect on virus excretion from the oropharynx. Vaccine.

Sutmoller P and Gaggero A (1965) Foot and mouth disease virus carriers. Veterinary Record 77, 968-969.

Authors Conclusions

- Problems associated with the imperfect specificity of NSP tests may be overcome by a resampling and retesting programme.

- It will not be possible to prove complete absence of infection (including detection of every carrier) by serosurveillance and this problem will be greatest in small herds.

Authors Recommendations

- More detailed guidelines are required for use of NSP serosurveillance in support of emergency vaccination-to-live policies in countries currently FMD-free without vaccination.
- Consideration could be given to use of an approach to disease control in which emergency vaccination in previously FMD-free countries could be followed by serosurveillance to detect virus circulation rather than infection (including detection of all carriers).

On the issue of documenting small herds as free from disease^{1,2}

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Summary

Small herds may present a problem in animal disease surveillance because typical levels of a within-herd design prevalence are not applicable. Small herds may be defined on the basis of having not more than one animal expected to be diseased. Consequently, the probability of detecting such herds cannot be improved by choosing a larger sample size. However, establishing evidence for freedom from disease for the subpopulation of small herds is possible and the level of statistical confidence for this assignment exceeds the probability of correctly classifying the individual infected herd. This paper also shows that the animal-level sensitivity of the diagnostic procedure is a limiting factor for the confidence that can be reached. Methods for establishing the necessary number of small tests to be sampled are shown.

Introduction

International trade regulations require that countries document the absence of transboundary animal diseases in the susceptible population(s) at a specified level of statistical certainty. This can be achieved by sampling primary epidemiological units such as herds and subsequent testing of individual animals from the herd. If all animals test negative, the herd will be classified as negative. The country (or zone) can be classified as disease free at the level of confidence (C) if all tested herds are classified as negative and if the following provisions hold. Firstly, valid estimates must be available for the probability of correctly classifying a herd as positive, given that the herd is affected at a specified level of within-herd prevalence ("design" prevalence). This probability is referred to as herd-level sensitivity and is a function of the animal-level sensitivity and specificity of the test and of the within-herd design prevalence. A typical value for the within-herd design prevalence used for such calculations is 5%. Secondly, the sampling of herds and animals from herds must be unbiased and representative. The underlying statistical principles are well documented. Obviously, the minimum, non-zero prevalence for small herds can be greater than the within-herd design prevalence. Consider for example that the smallest non-zero prevalence in a herd of size 5 is 20%. We are interested to explore the first provision with regard to specific issues presented by small herds. Specifically, we derive from first principles the maximum attainable sensitivity for the detection of small infected herds, the definition of a threshold for "small herds", the confidence of freedom in small herds and the required sample sizes for documenting disease freedom in a subpopulation of small herds.

The calculations will make use of the herd size (N), herd sample size (n), number of truly diseased animals in the herd (m), number of test-positive animals in the sample (k), animal-level diagnostic sensitivity (Se), herd-level diagnostic sensitivity (SeH), within-herd design prevalence (pa), among-herd design prevalence (ph), total number of small herds in the population (H), number of small herds in the sample (h), number of infected small herds in the population (J) and number of infected small herds in the sample (j). Unless stated otherwise, we assume that the diagnostic procedure has perfect diagnostic specificity. The confidence C is defined as the probability that the surveillance system detects at least one diseased animal (i.e. identifies a country or zone as affected) given that the disease is prevailing at the specified level of design prevalence (within and among herds).

¹ Paper prepared to support discussion in the EU FMD Research Group, 11-16 October 2004, Chania, Greece.

² As this text is a generic formulation of the problem, the term "disease" can be replaced with "FMDV infection" without loss of applicability.

Results

Result 1: We consider the herd-level sensitivity under assumption of perfect sensitivity, $Se = 1$, and diagnostic testing of the whole herd, $n = N$. The minimum, non-zero prevalence is $1/N$, i.e. $m = 1$. Thus we have that

$$\begin{aligned} SeH &= \Pr(k > 0 \mid m=1; N) \\ &= 1 - \Pr(k=0 \mid m=1; N) \\ &= 1, \end{aligned}$$

ie, the probability of not missing a single affected animal is 100% if a perfect test is used and all animals in the herd are tested. Clearly, this does not present an issue in relation to the small-herd-problem, although $1/N > pa$. Result 1 holds regardless of the herd size N .

Result 2: In extension of result 1 we assume $Se < 1$ and again $n = N$. Now

$$\begin{aligned} SeH &= 1 - \Pr(k=0 \mid m=1; N) \\ &= 1 - (1-Se) = Se, \end{aligned}$$

which shows that the maximum attainable confidence SeH is constrained by the sensitivity of the diagnostic test. Again, this result holds for all herd sizes N .

Result 3: The only way to improve SeH for small herds is to improve the diagnostic procedure. One possibility for this is to use a parallel testing scheme involving two independent diagnostic tests with a sensitivity of the combined testing, given by $Se = 1 - (1-Se_1)(1-Se_2)$. This approach is complicated by the fact that the occurrence of false negative results may be correlated. If this dependence is expressed in terms of the sensitivity co-variance γ_{Se} , we have $Se = 1 - (1-Se_1)(1-Se_2) - \gamma_{Se}$ as shown by Gardner et al. (Gardner et al. 107-22). The challenge is to select two tests with Se_1, Se_2, γ_{Se} such that Se reaches an acceptable value.

Result 4: It is of interest to investigate minimum values for Se that would comply with values for SeH fixed at some desired level, given that there are at least two truly infected animals in the herd, $m = 2$. We solve

$$\begin{aligned} SeH &= \Pr(k > 0 \mid m=2; N) \\ &= 1 - \Pr(k=0 \mid m=2; N) \\ &= 1 - (1-Se)^2, \end{aligned}$$

for Se and obtain

$$Se = 1 - (1-SeH)^{1/2},$$

which can be used for any specified values for SeH . For example, we obtain the set of minimum values for Se of (0.68, 0.78, 0.90) for values of SeH of (0.9, 0.95, 0.99). This illustrates that a level of 95% SeH can be reached with diagnostic procedures that have moderate Se of 0.78 given there are two or more infected animals in the herd.

Result 5: We are interested in the minimum herd size compliant with result 4, i.e. the presence of $m = 2$ truly diseased animals in the herd. This can be derived from setting the expected number of diseased animals in the herd $E(m)$ equal to 2 and solving for N .

$$\begin{aligned} E(m) &= N pa \\ N &= 2 / pa. \end{aligned}$$

The minimum herd size for $p_a = 0.05$ for which result 4 holds is $N = 40$. Thus, herds with a size smaller than $2 / p_a$ may be referred to as "small herds" for the purpose of animal disease surveillance using the expected number of infected animals of less than two as criterion.

Result 6: We explore, how a constrained SeH would compromise the confidence C for correct classification of small herds. Note that the SeH for the small herd is just Se. Let us first consider that all small herds are tested, i.e. $h = H$.

$$\begin{aligned} C &= \Pr(\text{at least one small infected herd test positive} \mid p_h, h) \\ &= 1 - \Pr(\text{all small infected herds test negative} \mid p_h, h) \\ &= 1 - (1 - \text{SeH})^J \end{aligned}$$

where J is the greatest integer smaller than or equal to $H p_h$. Note that J is not stochastic. For example consider a SeH of 80% and that all $H = 155$ small herds were tested in the surveillance. The among-herd design prevalence p_h is fixed at 2%. For this scenario and conditional on $h = H$, the confidence C for freedom from disease in the subpopulation of small herds is 99.2%.

Result 7: If only a sample of $h < H$ herds is tested, the number of positive small herds in the sample of h herds is a random variable, denoted with j. An appropriate model for j is the Poisson distribution with the rate parameter $r = h p_h$. In this case, the confidence can be given as

$$\begin{aligned} C &= \sum_{j=0}^{\infty} C(j) \Pr(j \mid r) \\ &= \sum_{j=0}^{\infty} \frac{\exp(-r) r^j (1 - \alpha^j)}{j!} \end{aligned}$$

i.e. as sum of the confidence given discrete values for $j = 0, 1, \dots$ with $\alpha = 1 - \text{SeH}$, weighted with the probability of observing j, derived from the Poisson distribution. Note that C is a function of the sample size h through the rate parameter r. Lets use the example above but now assume that we have only a sample of $h = 155$ herds out of the population of H herds. The expected number of infected herds in the sample is $r = 3.1$. Evaluating C for $j = 0, 1, \dots, 999$ yields a confidence estimate of 91.6%, which is considerably lower than the value obtained in result 6.

Result 8: Finally, the necessary number of small herds to be sampled in order to reach a specified confidence is considered. This is only a useful consideration if $h < H$. Thus, the first part of result 6 can be applied only if the number of infected herds in the sample $j = J$ is known. Then the required sample size h can be given as the smallest integer greater than or equal to

$$\frac{\log(1 - C)}{\log(1 - \text{Se}_H) p_h}$$

For example, h should be at least 93 to achieve $C = 0.95$ when assuming 2% among-herd prevalence in the sample and 80% herd-level sensitivity. This means, the population was over-sampled when 155 small herds were chosen, given the number of truly infected herds in the sample was known. Note that C was greater than 95% for $H = h = 155$ herds.

Result 9: Unfortunately, there is no closed form solution available analogue to result 8 but accounting for the number of infected herds in the sample being unknown. However, a numerical procedure can be implemented in spreadsheet programs that have a numerical solver function (available from the author). The procedure is to find h such that C, evaluated as shown in result 7 equals 0.95 (or the desired confidence). For the example scenario described in result 7 we obtain $C = 0.9506$ with the corresponding sample size $h = 188$.

Discussion and conclusions

"Small herds" in the context of animal disease surveillance could be defined as those herds in which the expected number of infected animals is less than 2 based on the within-herd design prevalence (ph), i.e. herds with the size smaller than $2/ph$. For those herds we have that the probability of correct classification as infected cannot be greater than the animal-level sensitivity of the diagnostic procedure used, given that the number of infected herds in the sample is known (or fixed by the among-herds design prevalence). In this situation a problem arises if the sensitivity of the diagnostic procedure is less than the desired level of the statistical power (typically 95%). If only a sample of all small herds is tested, the number of infected herds in the sample is a random variable. This introduces additional variability and compromises the level of confidence that can be reached with a given diagnostic process.

This paper demonstrates that the absence of disease in small herds can be documented unambiguously (with a herd-level sensitivity of 100%) if the diagnostic procedure has perfect sensitivity and if all animals are tested (result 1). Values of sensitivity less than 1 are the upper limit of the herd-level sensitivity that can be reached in small herds if all animals are tested (result 2). Parallel application of at least two diagnostic tests can be a remedy to achieve a sufficient level of SeH (result 3). It should be noted that the inclusion of a second test in the diagnostic procedure may not always be a realistic option in a given application. If this option is chosen, estimates of the dependence between the tests must be available to avoid overestimation of the overall sensitivity. Another option to improve the sensitivity may be multiplexing, i.e. pooling of diagnostic reagents for simultaneous detection of a range of analytes. Multiplex tests, even when composed using well-characterised diagnostic reagents should be regarded as new tests and be subjected to recommended assay validation procedures.

If the herd size is large enough to have two or more animals expected to be diseased, a high level of SeH (for example 0.95) can be reached with non-perfect diagnostic sensitivity (for example 0.78) (result 4). "Small herds" can be defined based on the within-herd design prevalence p_a (threshold is <40 animals for $p_a = 0.05$) with the rationale of having a minimum expected number of two diseased animals in the herd (result 5).

Since the classification of disease-freedom is to be made for the population rather than for the individual herd, the confidence will depend on the among-herd design prevalence for small herds. The calculation of the confidence can be made on the basis that all small herds are tested or that the number of infected herds in the sample is known otherwise (result 6) or for the general situation that the actual number of infected herds in the sample is unknown (result 7). The latter approach is more realistic but leads to a loss of power (i.e. smaller values for the confidence).

The number of small herds to be tested for a given among-herd design prevalence can be calculated readily under the assumption that the number of infected herds in the sample is fixed by the design prevalence (result 8). No sample size formulas exist for the situation that the number of infected herds in the sample is treated as random variable. However, the required sample size can be found by numerical methods (result 9).

Other approaches for dealing with the small-herd-problem include (a) to consider small herds as diseased or (b) to consider the questionable herds along with other (small) neighbouring herds as one epidemiological unit, for which the small-herd-problem does not apply. Option (a) seems unacceptable because in consequence small herds cannot be allowed to exist in free countries or regions unless the provisions for compartmentalisation are fulfilled. Option (b) may be justified in situations, where small herds are individually registered but are in fact no epidemiological entities.

It should also be noted that the assumption of a perfect specificity is made because it is assumed that any positive results is confirmed by follow-up epidemiological and/or laboratory investigation. If the assumption of resolving all positive test results doesn't hold, the level of SeH will increase, all other factors being held constant. This will compromise the specificity of the herd classification, which is beyond the scope of this paper.

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References

- Cannon, R. M. 2001. "Demonstrating disease freedom -- combining confidence levels." *Preventive Veterinary Medicine* 52.3-4: 227-49.
- Gardner, I. A., Stryhn, H., Lind, P. & Collins, M.T. 2000. "Conditional dependence between tests affects the diagnosis and surveillance of animal diseases." *Preventive Veterinary Medicine* 45: 107-22.

FAO Phase XVIII FMD serological standardisation; progress and future prospects

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Abstract

The aims of Phase XVIII are 1) The introduction of the solid phase competition ELISA (SPCE); 2) The preparation of secondary standards by national laboratories (based on the reference sera derived from Phase XVII); 3) The use of calibrated tests to examine local negative serum panels and proficiency panels; 4) Standardisation of quality control procedures. To this end, steps have been taken to prepare and validate the SPCE for detection of serotypes A and Asia 1 (See Anderson et al., 2003; Paiba et al., 2004). Secondly, large batches of the new reference sera selected in Phase XVII have been prepared for distribution to a wider range of testing laboratories, along with a proficiency test panel. Test sera and SPCE reagents were distributed to 22 laboratories and their results collated and compared. Prospects for future studies are discussed.

Introduction

FAO has supported the FMD WRL in conducting a series of standardisation exercises for FMD serology known as the "Phase" studies. Currently, Phase XVIII is approaching completion. These studies have attempted to improve the reliability and consistency of serological testing for FMD carried out by EUFMD member states. Recent Phase exercises have combined the establishment of reference and proficiency sera both of which have been distributed to participating laboratories. The intention has been that the reference sera (RS) would be used to calibrate the various tests in use by member countries and then the proficiency sera would be tested to assess how effectively the tests performed.

At the end of Phase XVI, a set of RS were established and OIE adopted them as official standards. These RS were derived from cattle that had been experimentally infected with FMD virus of serotypes O (Manisa), A (A22 Iraq) or C (Noville). As well as a single negative RS, a strong positive, weak positive and cut-off serum was designated for each serotype. During Phase XVII new RS were prepared to cover Asia 1 virus and additional strains of serotype O (specific for the important PanAsia strain) and A. This was done by vaccinating or infecting cattle with FMDV O SKR, A Iran 96 and Asia 1 Shamir. The conclusion of a preliminary round of evaluation by different laboratories (Paton et al., 2002) was that the weak and cut-off RS were too weak, and it was proposed to strengthen, redistribute and retest them; this was reported at the Gerzensee meeting of the EUFMD Research Group in September 2003 (Paton et al., 2003). Data sheets for the new RS were sent to the OIE Standards Commission in June 2004. Two weak positives were designated (weak 1 and weak 2) according to the purpose of testing; for herd-based serosurveillance or for certification of individual animals.

Phase XVIII runs from January 2003 to December 2004 with objectives that were agreed at the EUFMD Research Group meetings in Izmir, 2002 and Gerzensee, 2003:

- Introduction of the solid phase competition ELISA (SPCE).
- Preparation of secondary standards based on reference sera to O, A and Asia 1, including an analysis of what is done in this respect by participating laboratories.
- Use of calibrated tests to examine local negative serum populations.
- Use of calibrated tests to examine a proficiency panel.
- Standardisation of internal quality control procedures.
- Evaluation of non-structural protein (NSP) ELISAs (added in Gerzensee).

Materials and Methods

Twenty-two laboratories agreed to participate in Phase XVIII (Table 1). During July 2004, each participant was supplied with the ten RS prepared in Phase XVII (Table 2), a proficiency panel of 12 sera of undisclosed characteristics (Table 3), a set of reagents and instructions for SPCE for serotypes O, A and Asia 1, a request to test 500 naïve field sera

by SPCE and a template for recording results. Participants were also requested to supply protocols for the tests used in their laboratories. The proficiency panel was established using sera from cattle that were either FMDV naïve or had been vaccinated and/or infected with serotypes O, A or Asia 1. They were selected/prepared to have a low/moderate reactivity in homologous virus neutralisation test (VNT), some being diluted in negative serum to achieve this.

The tests used by the participating laboratories were the VNT, liquid phase blocking ELISA (LPBE) and SPCE as described in the OIE Manual (OIE, 2004); three commercially available NSP ELISAs (Bruderer et al., 2004; Chung et al., 2002; Shen et al., 1999) and a number of in-house assays for detecting antibodies to FMDV NSP (Bergmann et al., 2000; De Diego et al., 1997) or structural protein (SP) antibodies (Brocchi et al., this proceedings; unpublished methods).

Results and Discussion

Results of tests carried out on the RS and proficiency panel are summarised in Figs 1-4 and 5-8 respectively; in-house assay results being shown at the bottom of each figure. Testing of negative serum populations by SPCE is summarised in Figs 9-10. All of the laboratories except one supplied results within the required timeframe of approximately three months after materials had been despatched to them.

Overall, a high level of consistency was observed in the results obtained by laboratories for both reference sera and proficiency panel sera using both commercial and other tests. However, antigenic variability of type A strains reduced the sensitivity of “structural protein” tests that use “heterologous” virus/antigens. For example, use of A22 Iraq (and some other) test virus or antigen lowered the sensitivity for detection of the Type A RS derived from animals vaccinated with A Iran 96, with VNT, LPBE and SPCE (Figs 1-3). A similar effect was observed with proficiency serum #4, derived from an animal infected with A24, when tested against a heterologous strain of type A – see for example Fig 7. At the same time, some degree of cross-serotype reactivity was also observed with all of the structural protein tests (Figs 5-7). Most participating laboratories tested samples in duplicate for NSP serology although this is not recommended by the companies that supply the kits. NSP test results were not affected by antigenic diversity and this means that for serotypes with a high antigenic diversity, e.g serotype A, the sensitivity of the method might be as good or superior than SP tests for import certification, where it may not be possible to select a “homologous” test virus or antigen. Compare, for example, the NSP (Fig 8) and SP (Figs 5-7) results for detecting FMDV A24 antibodies in proficiency sera 4, 7 and 10 from unvaccinated but infected animals. More analysis of this type is needed for sera from animals vaccinated and infected with serotype A viruses. Fig 4 shows that only the type O RS are applicable to NS testing, the other two series (A and Asia 1) having been derived from vaccinated, but not infected animals. The type O RS would be useful as NSP RS, since the strong and Weak 1 positive sera were consistently detected, whereas the Weak 2 serum was more borderline. Some of the proficiency panel sera from vaccinated and subsequently challenged cattle were too weak to be suitable for NSP evaluation, having been collected too soon after infection (#1, #5) or from an animal in which there was insufficient virus replication to stimulate a NSP response (# 3).

Useful specificity data was generated for SPCE tests, using panels of locally prepared naïve field sera; mostly from cattle, but with small numbers of pig and sheep sera in some laboratories (Figs 9-10). Test specificity inconsistencies reported by different laboratories might have resulted from either difference in laboratory technique or in the sera, but some variability in SPCE results for naïve sera does appear to be due to operator differences. Serotype A and Asia 1 tests behaved similarly to the previously validated serotype O test (Paiba et al., 2004) and for cattle, specificity at 50% and 60% cut-off points were $\geq 99\%$ and $\geq 99.9\%$ respectively. Different cut-offs will be applicable for different test purposes and the single 60% cut-off recommended in the OIE Manual (OIE, 2004) is appropriate for herd-based serosurveillance, but not for individual certification, where a level of around 40% is likely to be more appropriate. For quantitative studies of antibody levels, for example to evaluate post-vaccinal immunity, it will be necessary to titrate sera before testing in the SPCE and this approach should be studied in the future.

Other information disclosed by participating laboratories revealed that a wide range of different tests were in use but that some laboratories tested few sera in the previous year. Not all laboratories could handle live virus and do VNT, whilst the range of virus serotypes available for VN testing was also variable. Most participating laboratories aspire to the ISO 17025 standard for quality accreditation, but few have achieved this for all of their testing. Preparation and use of secondary RS and use of trend analysis to monitor test performance was rather patchy.

Future direction of serological standardisation work

Successful aspects of the Phase studies have been:

- A regular testing exercise and proficiency monitoring is very valuable
- Reference sera have been successfully established for SP tests for serotypes O, A and Asia 1
- The SPCE trial has introduced this method to a wider circle of laboratories
- The testing of locally obtained naïve sera as part of an interlaboratory comparative exercise has been very successful and could be used in the future

However, there have also been problems:

- It has proved difficult to keep to timetables for carrying out this work. Contributory factors have included the limited resource committed to this project, the time taken to obtain permits to send materials and the complex nature of the procedure for selection and processing of the sera.
- The process of deriving the RS is complex and perhaps overly consensual. The main requirement for an OIE reference serum is quite simple in principle, being to produce a set of uniform test calibrators, i.e. reference standards that can be used to monitor the performance of a test over time and give comparability between laboratories. The most difficult issue is the decision on the threshold of positivity for weak positive sera and this issue is made much more complicated if the standard has to be used for different tests in which different cut-off points are applied depending on the purpose of testing. Generation of cut-off sera requires even greater precision and is inherently more difficult.
- Preparation of the RS is much more difficult and time consuming than preparation of the proficiency panel, but actually there is little evidence that the RS are being used for their intended purpose, rather than as a de facto proficiency panel. How many laboratories are using the RS for routine test calibration via production of secondary RS? What use is actually being made of the RS?
- Countries may have difficulty to prepare secondary standards because of a lack of suitable bulk sera from animals of a known, suitable infection history.
- Secondary standards may be used as or in addition to internal test control sera and some clarification of options is required. Some of the primary RS appear to be not ideally suited for use as internal control standards in all tests. For example, the weak 1 RS for Asia 1 Shamir (Table 2) appears too strong for use as a weak positive control in the SPCE.
- Procedures for inactivation of sera have not been closely defined. OIE recommends gamma irradiation, but this is not very practical and potentially harmful to the quality of the RS. The WRL FMD prefers inocuity testing followed by BEI inactivation and if necessary further inocuity testing.
- There is now uncertainty over the priority for introduction of the SPCE. On the one hand there is a shift towards the use of NSPE to detect infection and on the other hand, there is a lack of insight into interpretation of SPCE titres for post-vaccinal surveillance, creating a continued demand for LPBE test kits.

Solutions proposed and future objectives:

It would be beneficial to follow-up all Phase exercises with a working group meeting to discuss the results in detail and agree on future studies.

The purposes and use of RS should be clarified. The development of cut-off sera should be a second priority after weak positive sera have been established for all of the serotypes. The process of creating RS should be simplified by establishing only strong positive and negative sera and deriving dose response curves for different tests by testing serial dilutions of the strong positive serum in the negative serum. For each test and test

purpose one or more given dilution(s) could be identified for use as weak positive serum/sera. This would hugely simplify the preparation, distribution, evaluation and re-evaluation of RS. It would give flexibility to readily derive further weak positive sera for new tests and test purposes.

The development and use of secondary standards should be clarified and it would be useful if a position paper could be prepared on this subject. Provision should be made for supply of alternative hyperimmune sera for creation of secondary standards to laboratories that cannot infect animals themselves. This could be arranged by bilateral agreement with laboratories that regularly perform animal experiments.

The issues of establishing RS and proficiency testing should be separated and an annual proficiency testing exercise should be introduced. This could be the main focus of future Phase studies. There should also be more emphasis on quality control issues, such as trend analysis. Growing awareness by laboratories of the need to participate in externally organised quality assessment exercises will lead to more wanting to participate in exercises of this kind and consideration needs to be given to criteria for participation and cost recovery.

With respect to NSP testing, OIE has set up an ad hoc group to look at progress with validation of NSP serological tests and this group has met three times, most recently in August 2004. The group recommended the adoption of the PANAFTOSA 3ABC ELISA as the index method. Data was supplied by PANAFTOSA on the performance of the test with sera from persistently infected cattle and this has been augmented by testing carried out at the Brescia workshop of the EU Improcon project in May 2004. This workshop also showed that some other tests were comparable to the index method in terms of their sensitivity and specificity. PANAFTOSA have supplied serial dilutions of two positive bovine sera as putative NSP RS. One of these serum samples is derived from a field case in an area where vaccination was practised and the other is from an experimentally infected animal. During the Brescia workshop the dilution series was tested with all of the available NSP ELISAs and dose response curves plotted. There were considerable differences in the shapes of the curves for some of the tests and there was no clear relationship between the diagnostic sensitivity of tests and their ability to detect higher dilutions of the RS. A specific dilution of one of the sera was chosen as the strong positive RS and a specific dilution of the other serum as the weak positive RS. It was agreed that the next priority was to establish NSP test RS from pigs and sheep since some of the NSP tests are species-specific. An improved proficiency panel is also required for NSP test evaluation and monitoring.

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References

Anderson, J., Corteyn, M., Gibson, D., Hamblin, P. & Paton, D. 2003. Further validation of the solid-phase competitive ELISA for FMDV types A, C & Asia 1. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Gerzensee, Switzerland, 16-19 September 2003. Rome: FAO, Appendix 24: 157-165.

Brocchi, E., Grazioli, S., Yadin, H. & De Simone, F. 2004. Validation of a Solid Phase Competitive ELISA based on the use of one neutralising monoclonal antibody for the measurement of antibodies to FMDV type Asia 1. This proceedings; Appendix 45.

Bergmann, I.E., Malirat, V., Neitzert, E., Beck, E., Panizzutti, N., Sanchez, C., Falczuk, A. 2000. Improvement of a serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. Arch Virol.145: 473-489.

Bruderer, U., Swam, H., Haas, B., Visser, N., Brocchi, E., Grazioli, S., Esterhuysen, J.J., Vosloo, W., Forsyth, M., Aggarwal, N., Cox, S., Armstrong, R. & Anderson, J. 2004. Differentiating infection from vaccination in foot-and-mouth-disease: evaluation of an ELISA based on recombinant 3ABC. *Vet Microbiol.* 101: 187-197.

Chung, W.B., Sorensen, K.J., Liao, P.C., Yang, P.C. & Jong, M.H. 2002. Differentiation of foot-and-mouth disease virus-infected from vaccinated pigs by enzyme-linked immunosorbent assay using nonstructural protein 3AB as the antigen and application to an eradication program. *J Clin Microbiol.* 40: 2843-2848.

De Diego, M., Brocchi, E., Mackay, D., De Simone, F. 1997. The non-structural polyprotein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. *Arch Virol.* 142: 2021-33.

Office International des Epizooties, World Organisation for Animal Health. 2004. Foot and mouth disease, in: OIE Standards Commission (Ed.), Manual of standards for diagnostic tests and vaccines, 5th ed., Office International des Epizooties, Paris, France, Chapter 2.1.1. www.oie.int

Paiba, G. A., Anderson, J., Paton, D. J., Soldan, A. W., Alexandersen, S., Corteyn, M., Wilsden, G., Hamblin, P., Mackay, D. K. J. & Donaldson, A. I. 2004. Validation of a Foot-and-mouth disease antibody screening Solid-phase competition ELISA (SPCE). *J. Virol. Methods* 115: 145-158.

Paton, D.J., Armstrong, R.M., Turner, L.S., Hamblin, P.A., Corteyn, M. & Anderson, J. 2002. FAO Collaborative Study Phase XVII: Standardisation of FMD Antibody Detection. European Commission for the Control of foot-and Mouth Disease, Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease. Cesme, Izmir, Turkey, Sept 2002, pp226-234.

Paton, D., Armstrong, R. & Anderson, J. 2003. An update on progress with the FAO Collaborative Studies for FMD Serology Standardisation, Phases XVII and XVIII. European Commission for the Control of foot-and Mouth Disease, Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease. Gerzensee, Switzerland, Sept 2003, pp102-115.

Shen, F., Chen, P.D., Walfield, A.M., Ye, J., House, J., Brown, F. & Wang, C.Y. 1999. Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA. *Vaccine.* 17 (23-24): 3039-49.

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Table 2. Description of Reference Sera

Serum	Source	Dilution	VNT	LPBE	SPCE
Negative	Naïve adult bovine	n/a	neg	neg	neg
O SKR 2000	Infected adult bovine				
<i>strong pos</i>	34 dpc	2:3	1/237	1/708	92 PI
<i>weak 1</i>	34 dpc	1:3.5	1/119	1/596	85 PI
<i>weak 2</i>	34 dpc	1:12	1/44	1/178	58 PI
A Iran 96	Twice vaccinated adult bovine				
<i>strong pos</i>	39 dpv1, 13 dpv2	1:3	1/399	1/11,585	89 PI*
<i>weak 1</i>	39 dpv1, 13 dpv2	1:40	1/127	1/724	39 PI*
<i>weak 2</i>	39 dpv1, 13 dpv2	1:76	1/78	1/181	46 PI*
Asia 1 Shamir	Twice vaccinated adult bovine				
<i>strong pos</i>	47 dpv1, 21 dpv2	1:2	1/270	1/2048	98 PI
<i>weak 1</i>	47 dpv1, 21 dpv2	1:6	1/119	1/794	93 PI
<i>weak 2</i>	47 dpv1, 21 dpv2	1:16	1/50	1/316	73 PI

* heterologous antigen used

Table 3. Proficiency Panel Sera

ID	FMD STRAIN	Status¹	EXP DETAILS	Animal(s)²	Clinical signs³	Virus Recovery⁴	VNT⁵	LPBE	SPCE	CEDI	UBI	BOM
1	Asia 1 Shamir	V, I	21 dpv + 12 dpc	US 64 50% naïve 50%	None	nt	1/112	1/562	87%	neg	neg	POS
2	Adult Bovine	N	naïve	ex NZ	n/a	n/a	neg	neg	neg	neg	neg	neg
3	O UKG 34/01	V, I	21 dpv O Man "+" 37 dpc	UV 3 Neat	None	PCR +ve at 7dpc	1/32	1/457	96%	neg	neg	neg
4	A24 Cruzeiro	I	28 dpc	UV 79 25% naïve 75%	F&M	nt	1/60	1/447 x O	43%	POS	POS	POS
5	Asia 1 Shamir	V, I	21 dpv + 12 dpc	US 64 30% naïve 70%	None	nt	1/98	1/316	81%	neg	neg	INC
6	Asia 1 Shamir	V, I	21 dpv + 12 dpc	US 60 Neat	None	nt	1/360 x A	>1/2896 x O,A	96% >50% x O,A	POS	neg	POS
7	A24 Cruzeiro	I	28 dpc 14 dpc	UV 79 82.5% UV 80 17.5%	F&M F&M	nt	1/320 x As1	1/1778 x O, As1	62%	POS	neg	POS
8	Adult Bovine	N	naïve	ex NZ	n/a	n/a	neg	neg	neg	neg	neg	neg
9	O UKG 34/01	I	37 dpc only	UV 25 Neat	F&M	2, 4, 7 & 14dpc	1/126	1/1412	88%	POS	POS	POS
10	A24 Cruzeiro	I	28 dpc 14 dpc	UV 79 25% UV 80 75%	F&M F&M	nt	1/407 x As1	>1/2896 x O, As1	70% >50% x O, As1	POS	POS	POS
11	Adult Bovine	N	naïve	ex NZ	n/a	n/a	neg	neg	neg	neg	neg	neg
12	O UKG 34/01	I	28 dpc	UV 59 Neat	F&M	8 & 9 dpc	1/151	1/1698 x A	100%	POS	POS	POS

¹ V=vaccinated, I=infected, N=Naïve; ² Animal ID and proportions of sera utilised; ³ F&M=signs of FMD; ⁴ nt=not tested, dpc=days post challenge; ⁵ x A=cross-serotype reaction

Fig 1 REFERENCE SERA VIRUS NEUTRALISATION TEST (VNT) HOMOLOGOUS RESULTS

IAH-P	Type O VNT				Type A VNT				Type Asia 1 VNT			
	"=POS"		"=INCONC"		"=POS"		"=INCONC"		"=POS"		"=INCONC"	
	"=>1/45"	"1/16-1/32"	"=>1/45"	"1/16-1/32"	"=>1/45"	"1/16-1/32"	"=>1/45"	"1/16-1/32"	"=>1/45"	"1/16-1/32"	"=>1/45"	"1/16-1/32"
	or as locally applied			or as locally applied			or as locally applied			or as locally applied		
	O SK S+	O SK W1	O SK W2	Virus	A IR S+	A IR W1	A IR W2	Virus	A1 Sh S+	A1 Sh W1	A1 Sh W2	Virus
	1/237	1/119	1/44	O SKR	1/399	1/127	1/78	A IRAN 96	1/270	1/119	1/50	AS1 SHAMIR
2	1/32	1/16		O MANISA	1/28			A22 IRAQ	1/216	1/76	1/32	AS1 SHAMIR
3	1/512	1/128	1/64	O SKR	1/724	1/128	1/91	A IRAN	1/362	1/128	1/64	ASIA 1
6	1/1024	1/512	1/64	UKG 31/01	1/90	1/16		A22 IRAQ	1/512	1/256	1/45	NEP 29/97
6					1/128	1/64	1/22	A IRAN 96				
9	1/1024	1/362	1/128	O MANISA	1/6816	1/852	1/362	A IRAN 97	1/192	1/128	1/45	AS1 SHAMIR
12	1/264	1/168	1/84	O MANISA	1/264	1/36		A22 IRAQ	1/1056	1/384	1/96	AS1 SHAMIR
12					1/4608	1/576	1/384	A IRAN 96				
014	1/256	1/45	1/32	O MANISA	1/1000	1/256	1/96	A IRAN 96	1/1000	1/256	1/64	CAM '80
16												
17	1/128	1/80	1/24	O MANISA	>1/1280	1/224	1/56	A IRAN 96	1/1024	1/128	1/64	AS1 SHAMIR
19					1/1280	1/80	1/40	A IRAN 96				

014 uncertain local diagnosis

FIG 2 REFERENCE SERA LIQUID-PHASE BLOCKING ELISA (LPBE) HOMOLOGOUS RESULTS

	Type O LPBE			Type A LPBE				Type Asia 1 LPBE			"INCONC" ">1/40- <1/90"	
	"=POS"	"=POS"	"=POS"	"INCONC"	"=POS"	"=POS"	"INCONC"	"=POS"	"=POS"			
	"=>1/90"	"=>1/90"	"=>1/90"	">1/40-<1/90"	"=>1/90"	"=>1/90"	">1/40-<1/90"	"=>1/90"	"=>1/90"			
	O SK S+	O SK W1	O SK W2	Virus	A IR S+	A IR W1	A IR W2	Virus	A1 Sh S+	A1 Sh W1	A1 Sh W2	Virus
IAH- P	1/708	1/596	1/178	O SKR	1/11585	1/724	1/181	A IRAN 96	1/2049	1/794	1/316	AS1 SHAMIR
3	1/726	1/303	1/96	O MANISA	1/235			A22 IRAQ	1/328	1/103		SHAMIR
4	1/650	1/420	1/95	O MANISA	1/480	1/45		A22 IRAQ	1/2250	1/1180	1/420	AS1 SHAMIR
5	1/1024	1/64	1/256	O	1/128	1/45	1/64	A	1/2048	1/512	1/64	ASIA 1 AS1
9	1/708	1/588	1/178	O MANISA	1/7943	1/575	1/295	A IRAN 97	1/2042	1/794	1/316	SHAMIR
14	1/320	1/160		O MANISA	1/160			A22	>1/320	1/160		ASIA 1
15	1/712	1/712	1/128	O MANISA	1/8192	1/192	1/96	A96	1/3096	1/512	1/128	SHAMIR
16	1/483	1/231	1/96	O MANISA O	1/674	1/67	1/55	A22 MAHMATLI	1/1838	1/614	1/241	AS1 SHAMIR
18	1/479	1/116	1/32	O CAMPOS	1/155			A24 CRUZ				NR
\$2	80	73	57	O MANISA	91	75	63	A22 IRAQ	93	93	92	AS1 SHAMIR
†6	1/205	1/80	1/26	O MANISA	1/61			A5 PARMA				NR
#7	93	89	62	O BFS	92			A5 WEST				NR
‡13	69	55	35	O				NR				NR
19	0.1	0.17	0.46	O MANISA	0.21			A22 IRAQ				

NR=Not reported
 \$SPBE PI
 #% Blocking at 1/32
 ‡13 CEDI O Blocking
 †6=Mab-based LPBE; final diln giving 50% blocking

FIG 3 REFERENCE SERA SOLID-PHASE COMPETITION ELISA (SPCE) HOMOLOGOUS RESULTS
 "O" SPCE "=>60PI" "A22" SPCE "=>60PI" "Asia 1" SPCE "=>60PI"
 local doubtful local doubtful

IAH-P	O SK			Virus	A IR			Virus	A1 Sh			Virus
	S+	W1	W2		S+	W1	W2		S+	W1	W2	
2	92	85	58	O MANISA	89	39	46	A22 IRAQ	98	93	73	AS1 SHAMIR
3	91	80	51	O MANISA	81	17	14	A22 IRAQ	96	88	72	AS1 SHAMIR
4	97	91	72	O MANISA	83	30	18	A22 IRAQ	96	89	73	AS1 SHAMIR
5	96	86	64	O MANISA	90	45	34	A22 IRAQ	98	93	79	AS1 SHAMIR
6	86	75	67	O MANISA	84	84	61	A22 IRAQ	95	91	78	AS1 SHAMIR
7	98	94	74	O MANISA	97	70	52	A22 IRAQ	99	94	86	AS1 SHAMIR
8	94	88	67	O MANISA	87	41	19	A22 IRAQ	99	96	89	AS1 SHAMIR
9	98	92	67	O MANISA	85	28	21	A22 IRAQ	98	93	75	AS1 SHAMIR
11	93	84	59	O MANISA	86	28	16	A22 IRAQ	98	93	79	AS1 SHAMIR
12	90	78	65	O MANISA	89	43	31	A22 IRAQ	93	92	83	AS1 SHAMIR
13	79	65	43	O MANISA	81	31	28	A22 IRAQ	94	87	70	AS1 SHAMIR
14	84	72	35	O MANISA	67	-36	-18	A22 IRAQ	104	98	63	AS1 SHAMIR
15	87	67	40	O MANISA	91	43	40	A22 IRAQ	93	88	71	AS1 SHAMIR
16	97	82	62	O MANISA					105	92	77	AS1 SHAMIR
18	91	84	62	O MANISA	89	59	45	A22 IRAQ	95	91	79	AS1 SHAMIR
20	83	67	39	O MANISA	78	21	14	A22 IRAQ	98	89	70	AS1 SHAMIR
21	74	64	40	O MANISA	74	22	21	A22 IRAQ	95	87	72	AS1 SHAMIR
21	89	78	48	O MANISA	88	41	29	A22 IRAQ	98	95	80	AS1 SHAMIR
†6					1/1892	1/115	1/58	A IRAN 96	1/500	1/180	1/60	NEP 29/97
†6					1/190	1/10		A22 IRAQ				
Ω9	88	81	61	O MANISA	95	87	81	A IRAN 97	94	91	85	AS1 SHAMIR
12					93	77	64	A IRAN 96				
Φ21	0.32	0.98	0.70	O1	0.04	1.76	1.98	A				

Ω9=In house SPCE
 †6=in house Mab-based SPBE; final diln giving 50% blocking

locally applied diagnosis
 Φ21=liquidPCE

FIG 4	REFERENCE SERA			NON-STRUCTURAL PROTEIN ASSAYS							TEST
	O SK S+	O SK W1	O SK W2	A IR S+	A IR W1	A IR W2	A1 Sh S+	A1 Sh W1	A1 Sh W2		
IAH-P	127	78	18							Bommeli PP	
3	145	80	33							Bommeli PP	
4	90	38	14							Bommeli PP	
5	141	63	13							Bommeli PP	
14	81	48	11							Bommeli PP	
16	154	65	15							Bommeli PP	
20	107	55	7							Bommeli PP	
IAH-P	57	51	38							CEDI PI	
2	93	87	75							CEDI PI	
4	94	87	78							CEDI PI	
7	94	89	76							CEDI PI	
8	89	86	78							CEDI PI	
9	90	83	70							CEDI PI	
12	94	90	82							CEDI PI	
13	91	89	75							CEDI PI	
14	91	85	68							CEDI PI	
16	91	84	70							CEDI PI	
9	92	86	72							new CEDI PI	
4	0.82	0.35	0.1							De Diego	
18	R	R	R							EITB	
18	82	55	31							I-ELISA	
6	120	87	47							Trapping	
IAH-P	1.17	0.6	0.2							UBI OD	
Ж16	0.58	0.22								UBI OD	
20	1.16	0.56								UBI OD	
19	1.35	0.82	0.3							"3ABC ELISA"	

locally applied diagnosis

ЖInconsistent with <0.232 scored as negative

R=Reactive

FIG 5 Proficiency Panel VNT

"=POS" "=INCONC"
"=>1/45" "1/16-1/32" or as locally applied

PANEL	1	2	3	4	5	6	7	8	9	10	11	12	Virus used
History	V+I	NEG	V+I	I	V+I	V+I	I	NEG	I	I	NEG	I	
Type O VNT													
IAH-P			1/32						1/126			1/151	UKG 10/01
2			1/28						1/44			1/76	MANISA S
3			1/91				1/32		1/512	1/32		1/1024	KOREA UKG
6			1/512				1/11		1/1024	1/16		1/3072	31/01
9			1/512						1/362	1/32		1/1024	MANISA
10			1/128				1/11		1/128	1/16		1/724	MANISA
12			1/96				1/72		1/432	1/168		1/1152	MANISA
014			1/360				1/45	1/16	1/180	1/45		1/180	MANISA
17			1/256						1/224	1/24		1/768	MANISA
Type A VNT													
IAH-P				1/60		1/17	1/320			1/407			A24 CRUZ
2				1/12		1/216	1/40			1/20			A IRAQ
3				1/45		1/45	1/91			1/256			A IRAN
6				1/22		1/90	1/177			1/44			A IRAQ
6						1/11	1/22			1/60			A IRAN
9						1/19	1/78			1/128			A IRAN 97
10				1/28		1/1024	1/91			1/181			A22
12				1/48		1/768	1/144			1/216			A22 IRAQ
12				1/72		1/144	1/288			1/576			A IRAN 96
014				1/22		1/45	1/180			1/360			A IRAN 96
17				1/24		1/28	1/160			1/256			A IRAN 96
19				1/20		1/80	1/160			1/160			A IRAN 96
Type Asia 1 VNT													
IAH-P	1/112				1/98	1/360				1/16			AS1 SHAM
2	1/64				1/64	1/1024							AS1 SHAM
3	1/128				1/64	1/512						1/45	ASIA 1 NEP
6	1/450				1/64	1/1024	1/45					1/90	29/97
9	1/362				1/128	1/3920							AS1 SHAM
12	1/216				1/240	1/1056						1/66	AS1 SHAM
014	1/180				1/400		1/64		1/45	1/64			CAM '80
17	1/160				1/112	1/1280							AS1 SHAM

014Diagnosis inconsistent with local criteria

"V+I=Vaccinated then infected"

"I=Infected only"

FIG 6 Proficiency Panel LPBE

"=POS"
"=>1/90"

"INCONC"
">1/40-<1/90"

PANEL	1	2	3	4	5	6	7	8	9	10	11	12	Virus used
History	V+I	NEG	V+I	I	V+I	V+I	I	NEG	I	I	NEG	I	
Type O													
IAH-P			1/457	1/178		1/630	1/128		1/1412			1/698	UKG 10/01
1													O MANISA
3			1/421				1/49		1/951	1/110		1/1593	O MANISA
4			1/820				1/64		1/850	1/180		1/2800	O MANISA
5			1/256			1/64	1/128		1/192	1/128		1/1024	Unknown
9	1/52		1/933	1/108	1/43	1/426	1/295		1/2691	1/467		1/708	O MANISA
14			1/320						>1/320	1/80		>1/320	O MANISA
15			1/512						1/362	1/45		1/2048	O MANISA
16			1/415	1/112		1/811	1/404		1/1018	1/1462		1/3070	O MANISA
18	1/29		1/97	1/56	1/22	1/889	1/439		1/597	1/879		1/550	O CAMPOS
\$2			85			60	59		72	60		87	O MANISA
Σ6			1/70			1/130	1/40		1/180	1/70		1/260	O MANISA
Σ6			1/151	1/10		1/652	1/470		1/260	1/658		1/679	O SWITZ
#7			93				60		92	86		95	O BFS 1860
‡13			88			80	65		84	71		94	O CAMPOS
19			0.29						0.1	0.28		0.08	O MANISA
Type A													
IAH-P				1/447		1/79	1/1778			1/1024		1/71	A24 CRUZ
1													A22 IRAQ
3				1/77		1/1144	1/243			1/546			A22 IRAQ
4				1/90		1/2650	1/620			1/650		1/50	A22 IRAQ
5				1/45		1/512	1/256			1/256			Unknown
9			1/43	1/174		1/1122	1/832		1/191	1/1412		1/166	A Iran '97
14						>1/320	1/160			1/320			A22
15				1/64		1/128	1/512			1/3096			A96
16				1/332		1/7435	1/1382		1/156	1/7368		1/312	A22 M'matli
18	1/29		1/71	1/153	1/23	1/726	1/1145		1/254	1/2259		1/338	A24 CRUZ
\$2				76		93	88			87			A22 IRAQ
Σ6				1/110		1/20	1/750			1/1500			A5 PARMA
#7				90		72	92			94			A5 WEST
19				0.47		0.09	0.14			0.12			A22 IRAQ
Type Asia 1													
IAH-P	1/562				1/316	1/2048	1/56			1/158			AS1 SHAM
1													AS1 SHAM
3	1/62				1/43	1/562	1/43		1/51	1/59			AS1 SHAM
4	1/750				1/450	1/4680	1/85		1/64*	1/480		1/55	AS1 SHAM
5	1/256				1/213	1/2024	1/45			1/128			UNKNOWN
9	1/1737				1/1288	1/1660	1/162		1/118	1/588			AS1 SHAM
14	1/160				1/80	>1/320				1/80			ASIA 1
15	1/192			1/96	1/362	1/4096	1/128			1/256			SHAMIR
16	1/443				1/256	1/4096	1/115		1/57	1/354			AS1 SHAM
\$2	87				88	93				76			AS1 SHAM

"V+I=Vaccinated then infected"

"I=Infected only"

\$SPBE PI

#% Blocking at 1/32

Σ6= Mab-based LPBE; final diln giving 50% blocking

‡13 CEDI O Blocking

FIG 7 Proficiency Panel SPCE

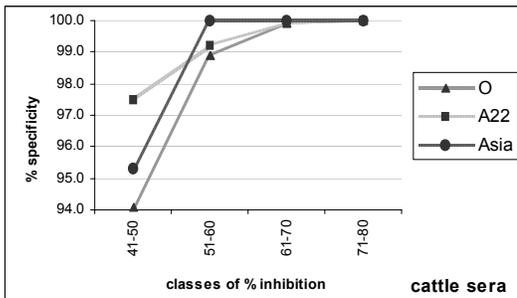
PANEL	Pos "=>60PI" Results "=>30PI"												Virus used
	1	2	3	4	5	6	7	8	9	10	11	12	
History	V+I	NEG	V+I	I	V+I	V+I	I	NEG	I	I	NEG	I	
O Manisa SPCE													
IAH-P			96			54	40		88	62		100	
1													
2			81						75			93	
3			97			67	56	30	92	57		98	
4			95	34		58	52		94	68		98	
5			86			78	55		83	63		87	
6		34	98	47		75	71	40	97	84		101	
7		32	94	43	36	68	65		89	72	34	98	
8			98			46	40		96	44		100	
9			93			44	41		90	44		98	
Ω9			77			57	49		89	41		93	
10													
11	31		90	47		71	65		90	69		99	
12			58			26	29		69	27		81	
14			87			40	43		84	38		93	
15		42	110	48		68	72		102	65		102	
16			92	33		44	44		87	51		96	
18			88	35		75	60		88	64		96	
20			82	33		67	66		78	72		91	
21			86			36	31		84	40		93	
Φ21			0.04						0.11	0.46		0.04	O1
A22 Iraq SPCE													
IAH-P				43		87	62			70		46	
1													
2				34		89	64			64		33	
3				57		96	82			81		37	
4				58		96	76			83		46	
5				59		87	70			73		50	
6	43	31	53	79	41	100	94		54	97	32	69	
7			44	55		92	75		36	84		65	
8				37		92	72			75		43	
9				49		95	75		33	80		52	
Ω9				50		78	80			84		32	
10													
11			41	63		95	87		54	91		61	
12			33	42		90	70		33	83		41	
14			31	40	40	96	73			76		48	
16	33		30	66		92	85		45	85		56	
18				35		92	64			69		31	
19				31		67	38			52		30	
20				44		90	66		30	72		39	
21				49		95	80		33	73		48	
†6				1/33		1/600	1/185			1/270			A22 IRAQ
†6				1/24		1/50	1/130			1/243			A Iran '96
12				31		52	56			69			A Iran '96
Φ21				0.09		0.59	0.01			0.02			A
Asia 1 Sham SPCE													
IAH-P	87				81	96	31			60			
1													
2	83				79	96	46			61			
3	94				94	99	72			55			
4	96				91	98	54			68			
5	87		35	40	83	97	66		50	78		48	
6	92			48	88	100	75		46	89		35	
†6	1/120			1/10	1/90	1/800	1/100		1/28	1/220			
7	92		33	52	92	100	67		43	79	30	48	
8	89				79	98	43		41	48			
9	93				90	100	56		40	71			
Ω9	92				90	96	49		33	69			
11	89		49	54	87	95	73		72	74		64	
12	88				84	95	50			71			
14	85				65	95	38			38			
15	102	32	31		98	94	78		45	80			
16	90			35	87	95	56		41	68			
18	88			37	81	98	50		34	61		32	
20	79				73	95	49		38	63		45	
21	93	31	40	37	92	99	59	39	56	79		35	

Fig 10. Field sera: SPCE specificity data



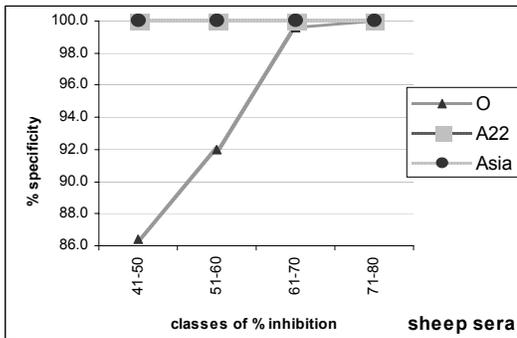
frequency distribution - NAIVE total sera

classes % inhib	O	A22	Asia	
41-50	50	95.2	97.6	96.1
51-60	60	98.7	99.3	99.2
61-70	70	99.8	99.9	100.0
71-80	80	100.0	100.0	100.0



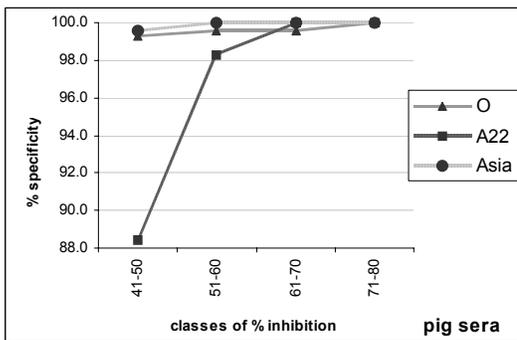
frequency distribution - NAIVE cattle sera (~5,000)

classes % inhib	O	A22	Asia	
41-50	50	94.1	97.5	95.3
51-60	60	98.9	99.2	100.0
61-70	70	99.9	99.9	100.0
71-80	80	100.0	100.0	100.0



frequency distribution - NAIVE sheep sera (~250)

classes % inhib	O	A22	Asia	
41-50	50	86.4	100.0	100.0
51-60	60	92.0	100.0	100.0
61-70	70	99.6	100.0	100.0
71-80	80	100.0	100.0	100.0



frequency distribution - NAIVE pig sera (~250)

classes % inhib	O	A22	Asia	
41-50	50	99.3	88.4	99.6
51-60	60	99.6	98.3	100.0
61-70	70	99.6	100.0	100.0
71-80	80	100.0	100.0	100.0

Proposals for Phase XIX

The main aim of this should be the organisation of two rounds of annual proficiency testing, one in 2005 and the other in 2006.

A strong positive sheep and a strong positive pig serum will be prepared from infected but unvaccinated animals along with a negative serum from each species. For each species, a titration series will be prepared and tested by different NSP methods. If acceptable dose response curves are obtained, aliquots of the strong and weak positive sera will be sent to laboratories, for use as NSP standards.

Efforts will be made to obtain bulk strong positive sera for the SAT serotypes. If necessary, these could be prepared at the WRLFMD, provided additional funding was made available. These strong positive sera could be distributed in the second year of the study for local titration and use as in-test control sera.

In addition, a position paper should be drafted on the purpose and use of secondary standards and in-test control sera.

Proposal for 2005:

1. Concentrate on proficiency serum panel with serotypes O, A and Asia 1.
2. Aim to provide sheep and pig NSP strong positive sera.
3. Include more sera from vaccinated and infected animals, especially of serotype A.
4. Do not specify particular tests to be used, but rather invite labs to test sera with the methods that they would normally use.
5. Request that interpretation of results be given as if these samples were from animals being examined prior to import (i.e. when serotype/strain of infecting virus would be unknown).
6. Ask EUFMD Secretariat to identify participants and draw up agreement on terms of participation.
7. Make sera available to other participants on request, subject to payment for sera and shipping.
8. Send out serum panel in April 2005.
9. Prepare analysis of results in time for next closed meeting in September 2005.

Proposal for 2006:

1. To be finalised at closed meeting in September 2005.
2. Aim to include SAT serotype proficiency sera.
3. Send out serum panel in April 2006.
4. Prepare analysis of results in time for next open meeting in October 2006.

Progress and future prospects for standardisation of FMD tests

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Progress with serological standardisation

FAO has supported the FMD WRL in conducting a series of standardisation exercises for FMD serology known as the "Phase" studies. Currently, Phase XVIII is approaching completion. These studies have attempted to improve the reliability and consistency of serological testing for FMD carried out by EUFMD member states. Recent Phase exercises have combined the establishment of reference and proficiency sera both of which have been distributed to participating laboratories. The intention has been that the reference sera (RS) would be used to calibrate the various tests in use by member countries and then the proficiency sera would be tested to assess how effectively the tests performed.

At the end of Phase XVI, a set of RS were established and OIE adopted them as official standards. These RS were derived from cattle that had been experimentally infected with FMD virus of serotypes O (Manisa), A (A22 Iraq) or C (Noville). As well as a single negative RS, a strong positive, weak positive and cut-off serum was designated for each serotype. During Phase XVII additional RS were prepared by vaccinating or infecting cattle with FMDV O SKR, A Iran 96 and Asia 1 Shamir. This work was interrupted by the FMD outbreak in UK in 2001, but preliminary results were presented at the EUFMD Research Group meeting in Izmir in 2002. It was considered that the weak and cut-off RS were too weak, and it was proposed to strengthen them. A workplan was agreed for completion of Phase XVII, incorporating a six months extension to the project, until the end of June 2003.

Phase XVII had been largely completed by the time of the Gerzensee meeting of the EUFMD Research Group in September 2003, by which time a new set of candidate RS for strains O SKR, A Iran 96 and Asia 1 Shamir had been prepared, distributed and tested by 9 testing laboratories. The distribution included a new range of RS dilutions and two weak positive sera were selected for each serotype. Data sheets for the new RS were sent to the OIE Standards Commission in June 2004.

The aims of Phase XVIII were agreed at the EUFMD Research Group meetings in Izmir, 2002 and Gerzensee, 2003:

- Introduction of the solid phase competition ELISA (SPCE).
- Preparation of secondary standards based on reference sera to O, A and Asia 1, including an analysis of what is done in this respect by participating laboratories.
- Use of calibrated tests to examine local negative serum populations.
- Use of calibrated tests to examine a proficiency panel.
- Standardisation of internal quality control procedures.
- Evaluation of NSP ELISAs (added in Gerzensee).

The workplan was to run from Jan 2003 to December 2004.

There are 24 participating laboratories in Phase XVIII. During July 2004, each participant has been supplied with the ten RS prepared in Phase XVII, a proficiency panel of 12 sera of undisclosed characteristics, a set of reagents and instructions for SPCE for serotypes O, A and Asia 1, a request to test 500 naive field sera by SPCE and a template for recording results. Participants were also requested to supply protocols for the tests used in their laboratories.

Results have so far been received from 13 countries (30th Sept). A summary of the results available to date will be sent by email to participants who have submitted results on 4th October and will be presented at the EUFMD Research Group Meeting along with provisional conclusions and recommendations from the study.

Future direction of serological standardisation work

Successful aspects of the Phase studies:

1. Proficiency aspect has been of great benefit ensuring regular assessment of serological competence for many laboratories.

2. RS have been established for serotypes O, A, C and Asia 1 and large stocks of these sera are available on request.

Problems with the Phase exercises:

1. It has proved difficult to keep to timetables for carrying out this work. Contributory factors have included the limited resource committed to this project, the time taken to obtain permits to send materials and the complex nature of the procedure for selection and processing of the sera.
2. The process of deriving the RS is complex and perhaps overly consensual. The main requirement for an OIE reference serum is quite simple in principle, being to produce a set of uniform test calibrators, i.e. reference standards that can be used to monitor the performance of a test over time and give comparability between laboratories. The most difficult issue is the decision on the threshold of positivity for weak positive sera and this issue is made much more complicated if the standard has to be used for different tests in which different cut-off points are applied depending on the purpose of testing. Generation of cut-off sera requires even greater precision and is inherently more difficult.
3. Preparation of the RS is enormously more difficult and time consuming than preparation of the proficiency panel, but actually there is little evidence that the RS are being used for their intended purpose, rather than as a de facto proficiency panel. How many laboratories are using the RS for routine test calibration via production of secondary RS? What use is actually being made of the RS?
4. Countries may have difficulty to prepare secondary standards.
5. Procedures for inactivation of sera have not been closely defined. OIE recommends gamma irradiation, but this is not very practical and potentially harmful to the quality of the RS. The WRL FMD prefers inocuity testing followed by BEI inactivation and if necessary further inocuity testing.
6. There is now uncertainty over the priority for introduction of the SPCE. On the one hand there is a shift towards the use of NSPE to detect infection and on the other hand, there is a lack of insight into interpretation of SPCE titres for post-vaccinal surveillance, creating a continued demand for LPBE test kits.

Solutions proposed and future objectives:

1. Simplify the process of creating RS. Establish strong positive and negative sera only and derive dose response curves for different tests by testing serial dilutions of the strong positive serum in the negative serum. For each test and test purpose a given dilution could be identified for use as a weak positive serum. This would hugely simplify the preparation, distribution, evaluation and re-evaluation of RS. It would give flexibility to readily derive further weak positive sera for new tests and test purposes.
2. Make provision for supply of alternative hyperimmune sera for creation of secondary standards to laboratories that cannot infect animals themselves.
3. Abandon the concept of cut-off sera for the time being until weak positive sera are established for all of the serotypes.
4. Completely separate the issue of establishing RS from proficiency testing.
5. Develop projects for further validation of SPCE

Progress with NSP serology standardisation

OIE set up an ad hoc group to look at progress with validation of NSP serological tests and this group has met three times, most recently in August 2004. The group recommended the adoption of the PANAFTOSA 3ABC ELISA as the index method. Data was supplied by PANAFTOSA on the performance of the test with sera from persistently infected cattle and this has been augmented by testing carried out at the Brescia workshop of the EU Improcon project in May 2004. This workshop also showed that some other tests were comparable to the index method in terms of their sensitivity and specificity. PANAFTOSA have supplied serial dilutions of two positive bovine sera as putative NSP RS. One of these serum samples is derived from a field case in an area where vaccination was practised and the other is from an experimentally infected animal. During the Brescia workshop the dilution series was tested with all of the available NSP ELISAs and dose response curves plotted. There were considerable differences in the shapes of the curves for some of the tests and there was no clear relationship between the diagnostic sensitivity of tests and their ability to detect higher dilutions of the RS. A specific dilution of one of the sera was chosen as the strong positive RS and a specific dilution of the other serum as the weak positive RS. It was agreed that the next priority was to establish NSP test RS from pigs and sheep since some of the NSP tests are species-specific. Some large stocks of sera are available at the WRL from unvaccinated, experimentally infected sheep and pigs and these will be examined for suitability. The WRL also agreed to establish a NSP proficiency panel with a limited availability for evaluation of new tests and batch control of existing tests.

Progress with FMD Virus detection proficiency panel

At the closed meeting of the EUFMD Research Group in Gerzensee, it was agreed that the WRL would prepare a proficiency panel for virus detection tests. This panel would be distributed to a small number of laboratories as a trial and if satisfactory would then be issued more widely. The panel, comprising 20 epithelial samples has been assembled and tested blind at the WRL with good results. It is being distributed to laboratories in Brescia, Lelystad, Riems and Brussels. Letters of undertaking have been requested from the participating laboratories to enable the infectious virus materials to be exported. Some samples have now been sent out, whilst some letters of undertaking are still awaited.

**Field Evaluation of Rapid “penside” Tests for FMDV antigen and FMDV-NSP antibody
FAO-EUFMD pilot study in Erzurum, Turkey; 12-25 September 2004**

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Introduction

At a training workshop held in Athens in December 2003 under an FAO technical co-operation project (TCP/RER/2903), participants from Turkey and other countries discussed the steps that need to be taken when investigating FMD outbreaks and the information that needs to be captured for tracing dangerous contacts. A set of revised guidelines and forms for use during outbreak investigation was eventually drafted. It was decided at the 70th Session of the EUFMD Executive Committee (Dublin, June 2004) that the Secretariat proceed with the first phase of a proposed pilot study to “field-test” the revised guidelines and forms in Eastern Anatolia. This was also seen by the Secretariat as an opportunity to conduct a field evaluation of rapid “penside” diagnostic tests (for FMD viral antigen and NSP antibody) during an outbreak investigation, thereby following up one of the recommendations of the 2003 Closed Session of the EUFMD Research Group (Gerzensee, September 2003).

Materials and methods

Implementation of revised guidelines - field investigation of an FMD outbreak

An investigative team (comprising local veterinary officers and veterinary expertise from Ankara and from overseas) was assembled in Erzurum and a local disease control centre (LDCC) was established at the Veterinary Control and Research Institute (VCRI). When a suitable FMD outbreak was identified, a field investigation was conducted at the outbreak location following the steps laid down in the revised guidelines mentioned above; clinical and epidemiological information was recorded on redesigned forms. Follow-up investigations in neighbouring villages and at more remote locations were then prioritised based on a logical assessment of the risk associated with proximity to the index village and different types and timing of contacts. In each village the same procedures were followed: epidemiological information was gathered by interviewing the Muthar (Head of the village) and livestock owners, a sample of animals were clinically examined and specimens were collected for diagnostic testing.

Clinical specimens:

A total of 144 sera were collected for testing by rapid test. These were collected from 81 cattle, which were either clinically-affected or in close contact with affected animals; 63 of these cattle were resampled 5-7 days later for retesting. Oesophago-pharyngeal (OP) fluid or “probang” specimens were collected from 38 cattle, all of which were either clinically-affected or in close contact with affected animals. Vesicular Fluid was obtained from two clinically-affected cattle with early lesions. Such intact vesicles were infrequently observed even in recently-infected groups of animals and when seen were very easily ruptured on handling (Figure 1).

Diagnostic test kits

The rapid test devices for both viral antigen and NSP antibody were supplied by Princeton Biomedical Corporation. These were both immunochromatographic strip test devices, one of which was designed to test either whole blood or serum for FMDV-NSP antibody and the other to test either OP fluid or vesicular fluid for FMDV antigen.

Sera were tested for antibodies to FMD virus non-structural proteins (NSP) using an ELISA test-kit, *Ceditest FMD-NS* (CEDI Diagnostics BV, Netherlands). A liquid phase blocking ELISA (LPBE) was also used to test sera for antibodies to the structural protein antigens of FMD virus; this was performed for each of three serotypes (O, A and Asia 1) with reagents supplied by the Institute of Animal Health (IAH), Pirbright, UK.

Vesicular fluids were tested by an Indirect Sandwich ELISA which was performed with reagents supplied by IAH as described by Ferris and Dawson (1988).

Rapid testing for both antigen and antibody was conducted at VCRI, Erzurum. The same specimens were subsequently transported to the Şap Institute, Ankara and retested using laboratory-based ELISA test-kits. Each of the tests was performed according to the instructions provided by either the manufacturer (Rapid tests and Cedi-diagnostics test) or the supplier of reagents (LPBE and ACE).



Figure 1: an intact vesicle (arrow in A) is ruptured during handling (B)

Results

Field investigation of an FMD outbreak

A suspected FMD outbreak in the village of Ozbek (which is located just over 20 km from the city of Erzurum) was reported on the day after the investigative team arrived in Erzurum province. A preliminary investigation confirmed the clinical suspicion of FMD. However a number of visits had to be conducted (over a four day period) to obtain the information required on each of the different grazing groups of animals in Ozbek. It was not until then that the neighbouring villages were investigated (the results of which are not presented in this paper).

The owner of a group of 40 calves which had recently been purchased, reported a suspicion of FMD. On clinical inspection of these calves, many were severely lame and some were recumbent and reluctant to rise. In addition to intra-oral lesions consistent with FMD, they had extensive ulceration of the interdental space and underrunning of the bulbs of the heel in one or more feet which was complicated by secondary bacterial infection and in some cases blowfly myiasis. Thirty-one of the calves in this "index" group (Group 1) were examined in detail; 25 having FMD lesions estimated as between 4 and 10 days-old. Serum and OP fluid were collected to test for antibody (Table 1) and FMDV antigen (Table 2), respectively. Seven days later when most of the intra-oral lesions had healed; serum was again collected from this group to test for antibody.

Four other distinct epidemiological groups of cattle were identified in Ozbek:

Group 2 consisted of 17 "yearlings", 16 of which had FMD lesions ranging from 3 to 7 days-old. Serum was collected to test for NSP antibody by rapid test on that occasion and again six days later.

Group 3 was composed of more than 300 cattle in a single grazing group of which 60 were randomly-selected for examination. 22 of the 60 had oral lesions but only in three animals were these considered to be specific for FMD (in those animals the lesion age varied from 1 to 7 days). In the remaining 19 animals there were abrasions and scarring on the dental pad and gums suggestive of traumatic injury. Although serum was collected from all 60 animals, only in the case of the 22 animals with oral lesions was serum tested for NSP antibody by rapid test.

Group 4 also consisted of more than 300 cattle which were accompanied by 30 buffalo whilst at pasture. 42 cattle were sampled from this group, five of which had FMD lesions ranging from 1 to 7 days-old. Sera were collected from all 42 animals but only 11 were tested by rapid test; five days later nine of the 11 animals were rebled to be tested by rapid test for NSP antibody. In addition, OP fluid, collected from 10 of the 11 animals on the second sampling day, was tested for FMDV antigen by rapid test. Some of the animals in this group were reported by their owner to have shown clinical signs of FMD in May 2004. A separate subgroup of animals belonging to one owner were also examined; these cattle had been grazing with Group 4 until housed approximately one week before they were examined (at the time that the owner first noticed one of his cattle to be ill); when first examined, 18 of 31 calves had early FMD lesions (0-3 days-old) and vesicular fluid was obtained from one of the calves; five days later yearling cattle were also severely-affected with swollen lips/muzzle, profuse salivation and 3-5 day-old intra-oral lesions.

Group 5 consisted of 38 housed, fattening bulls which when examined showed no clinical signs of FMD; these animals had been kept indoors for more than one month and had no contact in that time with any of the other cattle kept in the village; no specimens were collected from this group of cattle.

Evaluation of a rapid test for detection of antibody to FMDV-NSP

When first sampled, five of the 81 cattle tested by rapid test were strongly NSP seropositive whilst another 20 cattle were weakly seropositive (6-35 percent seropositive depending on whether or not weak positives are included). When sera from the same animals was tested by a laboratory-based ELISA for NSP antibody, 86% were seropositive. Repeat sera collected 4-7 days later from 63 of these animals were also tested by both methods. Nine of the resampled cattle were strongly NSP seropositive and 19 were weakly seropositive by rapid test (11-48% seropositive) whilst 98% were NSP-seropositive by laboratory-based ELISA. A breakdown of the serological results obtained from the different epidemiological groups of animals identified during the field investigation is provided in table 1. The test was relatively easy to perform and the result was easily read by the naked eye (Figure 2)

Table 1 % NSP seropositive in each group comparing rapid test and laboratory-based ELISA

<i>GROUP</i>	<i>n</i>	<i>% with FMD lesions</i>	<i>Estimated age of lesions</i>	<i>RAPID TEST (day 0)</i>	<i>Lab ELISA (day 0)</i>	<i>resampling RAPID TEST (retest)</i>	<i>Lab ELISA (retest)</i>
1	31	81%	4-10 days	10-35%	100%	+7 days (n = 37) 14-48%	97%
2	17	94%	3-7 days	0-18%	94%	+6 days 19-44%	100%
3	22	12%	1-7 days	6-27%	73%	ND -	-
4	11	0%	-	-	74%	+5 days (n = 9) 11-44%	100%



Figure 2: a weak positive test result (T) and two strong positive test results for NSP-antibody

Evaluation of a rapid test for detection of FMDV Antigen

Both vesicular fluid samples were positive for both SP and NSP antigens (Figure 3). Both were also positive when tested by laboratory-based antigen capture ELISA, one specimen for serotype O virus and the other specimen for serotype A virus.

Thirty-eight OP fluid specimens were tested using rapid test devices. Only three specimens gave any indication of positivity but this was a very weak, barely-visible or "trace" reaction on the membrane and in each case the control line did not develop properly on the test device such that the result had to be considered inconclusive. A similar problem was encountered with many of the OP fluid specimens that were tested; control lines did not develop presumably because the viscosity of OP fluid (even when diluted 50:50 with PBS) prevented capillary action or "wicking" through the membrane.

Table 2 Results of testing for viral antigen by rapid test and by laboratory-based ACE

Group	OPF or VF ¹	Rapid test for FMDV-Ag				ACE +ve
		Strong +	Weak +	Inconclusive (no control)	Negative	
Group 1	OPF (n=28)	0	0	13	15	ND
Group 3	VF (n=1)	1	0	0	0	1/1
Group 4	OPF (n=10)	0	0	0	10	ND
	VF (n=1)	1	0	0	0	1/1

¹OPF = oesophago-pharyngeal fluid, VF = vesicular fluid; ²the % positive values for the rapid test method ranges from a lower value where only the strong positives are considered "positive" to a higher value where weak/trace positives are also considered "positive"; ND = not done.

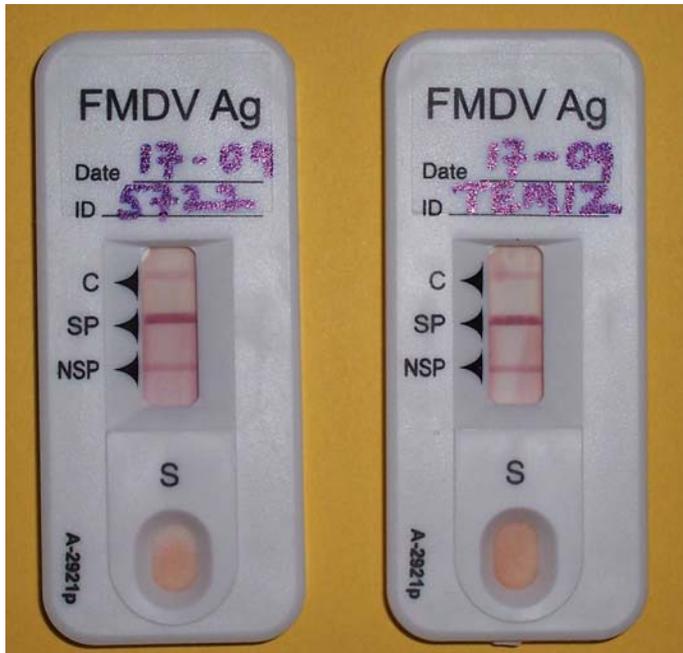


Figure 3: Vesicular fluid specimens gave a strong positive test result for both structural protein (SP) and non-structural protein (NSP) FMDV antigens.

Discussion

FMDV infection and clinical disease were widespread in the index village and all age groups were affected although the most severe disease, including lameness and the presence of interdigital lesions, was only evident in juvenile cattle. The onset of clinical FMD in Ozbek in September 2004 would appear to be associated with the recent purchase of young cattle from Erzurum market, most probably because of introduction of virus with these animals. However, there was also both epidemiological and serological evidence that an FMD outbreak had occurred in the village in the recent past, approximately 5 months before the present outbreak. Therefore it is also possible that the bought-in calves which were the most severely-affected group in the present outbreak may have developed disease because of being exposed to virus which was already present in the village in persistently-infected or subclinically-infected animals.

For practical reasons neither of the rapid tests evaluated in this pilot study were actually applied "penside" but both were immediately used on return of the investigative team to the LDCC. Both tests were relatively easy to perform although a steady surface and pipetting were required.

The test for detection of FMD viral antigen worked very well when it was used to test vesicular fluid and both specimens were strongly positive for both SP and NSP antigens. However, intact vesicles from which vesicular fluid could be obtained are infrequently observed in field cases of FMD. Furthermore, when "classical" vesicles are recognisably present, the clinical diagnosis is relatively certain and there may be little reason to perform a rapid test. The test performed poorly with OP fluid specimens in that many of the test-strips did not register a positive control line even where the specimen was diluted to reduce its viscosity. Although three specimens gave a very weak positive reaction ("trace" positives), these results had to be considered inconclusive given the absence of a control line on the testing device in each case. In addition it should be remembered that probang-sampling is unlikely to be performed during an outbreak investigation and requires the ready-availability of probang devices and considerable experience on the part of the sampler.

The usefulness of a rapid test for FMD antigen, which is only designed to test vesicular fluids and OP fluids and which in fact is only effective in testing the former, must be questioned. To be of use in field investigation it should be possible with a rapid testing method to use epithelial fragments from the edge of lesions as the clinical specimen under test; this would require that a suspension could be prepared from such specimens under field conditions that would be capable of diffusing through the membrane of an immunochromatographic test device. In addition the test devices would have to be sufficiently sensitive to detect the much smaller concentrations of FMD viral antigen that might be expected in such test materials derived from FMDV-infected animals.

Compared with results obtained when the same sera were retested using a laboratory-based ELISA test-kit the rapid test devices for detection of NSP-antibody did not detect very many seropositive cattle. The rapid test was therefore much less sensitive for detection of NSP antibody than the laboratory-based ELISA. However these devices may still be of some use during initial epidemiological investigations at an infected premises or village. In such a situation it is important to estimate the time elapsed since introduction of infection for the purposes of tracing the most likely source of the infection and also to determine the risk of spread associated with different contacts. Establishing which was the first group of animals in a herd or village to become infected can be attempted by estimating the age of lesions in different epidemiological groups within the herd/village and thus identifying the oldest lesion present. This option is no longer available if lesions have healed. However, as a stronger serological response might be expected from animals with healed lesions (due to earlier exposure to the virus) a random-sample could be selected from each group of animals and their serum tested for the presence of NSP antibody. In addition, healing intra-oral lesions which are observed during an outbreak investigation may be caused by trauma or something else other than FMDV infection, as suspected in one of the groups examined during the present study. If the healed lesions were caused by FMD virus some indication of a serological response would be expected whilst no such response would be expected if the injury arose otherwise. Sera from such suspect animals could be collected and tested "penside" for this purpose.

Conclusions

- Rapid tests may be a useful tool during FMD outbreak investigation but they are NOT a substitute for careful clinical and epidemiological investigation
- Available tests need further field evaluation and their use "penside" should be attempted.

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EUFMD/EC Workshop on Contingency Planning for Foot-and-Mouth Disease Laboratory Diagnostic Activities

Universidad de Córdoba, 28-30 April 2004

Output 1: General conclusions and recommendations

Recognising that:

1. Even a single confirmed outbreak of FMD in a single European country will create a high and urgent demand for diagnostic tests to be performed in the country concerned and in other European countries, and may require hundreds of thousands of serological tests to be performed.
2. The scaling up of diagnostic activities following a confirmed outbreak will be constrained by factors including the limited size of available space in the high security containment laboratories, of biological resources required for the tests, of available competent technical staff, financial resources and other factors.
3. Other options need to be reviewed to reduce the cost of maintaining a high standing capacity for performance of FMDV diagnostic tests within days of first requirement.

The workshop reaches the following general conclusions and recommendations:

1. Each European country should before 2005 develop, evaluate, and update on a yearly basis a contingency plan, to elaborate the strategy, organisation and resources required to be maintained during non-outbreak periods and each phase of an FMD emergency in Europe.
2. The model laboratory contingency plan should be further developed by mid-June 2004, following the review of the WG5, and thereafter circulated and made available on-line to assist the NLs in developing their own plans.
3. The LCP should address the issue of rapid scaling up of virus diagnostic capacity. The report (of working group 1) provides a guide for major elements to be considered and addressed.
4. Countries should urgently put into place arrangements for transport of specimens for FMD diagnosis, and sera, to reference laboratories in the European region.
5. The guidelines being developed by the EUFMD working group on transport of specimens for FMD diagnosis should be developed further by October 2004 and updated on a yearly basis by the EUFMD research group.
6. The LCP should address the issue of rapid scaling up of serological diagnostic capacity. The report (of working group 3) provides a guide for major elements to be considered and addressed.
7. The guidelines developed by the EUFMD working group on biosecurity requirements for sero-diagnostic laboratories, as modified by the working group during the workshop, were accepted in principle. The group is encouraged to complete the review by the EUFMD research group by October 2004 with a view to early acceptance at the European level.
8. Portable diagnostic devices have a potential to increase the speed of detection and confirmation of FMD virus infection, particularly in countries without national reference laboratories, and to reduce the scale of diagnostic activity required of reference laboratories. Guidelines are required to address issues relating to test performance, authorisation of personnel, indications for use, and on the subsequent submission to and use of reference laboratories. A WG should be established.
9. The creation of a European FMD diagnostic reagent bank is urgently required. The guidelines on the development of the bank should cover the arrangements for drawing rights, the necessity of laboratory contingency plans which address the provision of the resources required which are not provided by the bank. The technical specification of kits in the bank should be reviewed at least on an annual basis.
10. The subject of evaluation of LCPs should address at the next meeting of the EUFMD RG.

Virus Inactivation Kinetics

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At the session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease in Gerzensee, Berne, Switzerland in September 2003 a review of methods for describing the effect of temperature and time upon virus survival in products was presented (Have, 2003). The Research Group recommended that "specific studies on heat inactivation should be designed to support further risk assessments for those identified as "high" risk commodities. These studies should make use of existing experimental data on D and Z-values or involve further experiments to fill any gaps" and that "the available data on inactivation of FMDV in milk and milk products should be reviewed in the light of current international trade standards. If necessary, additional studies on inactivation by heat treatment or lowering pH should be carried out". It was decided to develop study plans for assessing D-values and Z-values for heat treatment of milk and pork from FMD-infected animals and to consult the industry in regard to the planning and funding of such studies.

As mentioned by Have (2003) the scientific literature regarding inactivation kinetics of FMDV from relevant sources and under relevant conditions is fragmented and sporadic and the difficulties of making quantitative assessments from various data sources on virus inactivation e.g. in the environment has been reviewed by Bartley et al (2002). As explained (Have, 2003); the effect of heating is determined by a combination of time and temperature and the inactivation kinetics are often assumed to be first-order although this is not always the case. The decimal reduction time D_T is the time needed to reduce the viable population by 90% at the temperature T. Semi-log plots of D-values against temperature often yield linear relationships, from which z-values can be calculated as the number of degrees temperature required to change D by one log unit. The z-value is often considered to be constant for a given strain of microorganism in a given product. Heat treatment includes a heating phase and a cooling phase and to account for the combined effect during heating and cooling, the temperature/time relationship data can be used to calculate lethal rates over the entire process and integrating into a cumulated lethal effect, expressed relative to a standard treatment at a chosen reference temperature and taking advantage of knowing the value of z (Peleg, 2003).

In regard to FMDV, clearly also other conditions, apart from the temperature, such as product type, pH, ionic strength and a number of other factors are also of importance for the kinetics of inactivation.

Conclusions

- Inactivation studies of FMDV need to be done in a systematic way employing kinetic data under relevant conditions.

Recommendations

- The recommendations of the 2003 meeting need to be taken immediately forward. It is suggested to make a small working group with the responsibility of urgently drawing up a study plan and to take this plan to the industry for consultation in regard to the practical planning and funding of such studies.

References

- Bartley, L. M., Donnelly, C.A. & Anderson, R.M. 2002. Review of foot-and-mouth disease virus survival in animal excretions and on fomites. *Vet. Rec.* 151:667-669.
- Have, P. An assessment of guidelines for treatment of meat from a FMD vaccination zone. Session of the Research Group of the Standing Technical Committee, European Commission for the Control of Foot-and-Mouth Disease 2003: 149-152.
- Peleg, M. 2003. Calculation of the non-isothermal inactivation patterns of microbes having sigmoidal isothermal semi-logarithmic survival curves. *Crit Rev. Food Sci. Nutr.* 43:645-658

Screening for FMD virus in vaccinated herds affected by field infection

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Introduction

Israel is located in the Middle East in an FMD endemic area and since August 1999 had been free of FMD outbreaks. In January 2004 the country faced numerous FMD outbreaks, eight farms were affected; two dairy and fattening herds, four calf feedlots, one sheep flock and one farm keeping game animals. Although morbidity approached 30% in the feedlots, there were no clinically affected animals in the dairy operations. The objective of this study was to estimate the prevalence of subclinical infection in vaccinated herds by identifying NSP seropositive animals.

Material and methods:

Five FMD-affected farms were blood sampled between 30 and 80 days after the outbreak had occurred on each premises; two of these were dairy farms with associated feedlots, two were stand-alone feedlot operations and one was a sheep-rearing enterprise (Table 1). As controls, animals were also blood-sampled in three dairy farms, one feedlot and one sheep farm, all of which were unaffected. Commercial ELISA kits were used for evaluation of the presence of NSP antibodies; a direct ELISA for Bovine sera (Svanova) and an indirect ELISA for both ovine and bovine sera (Cedi-diagnostics).

Table 1 Sampled animals in feedlots, dairy farms and sheep farms

Date of outbreak	FARM	Enterprise Type	Morbidity	Last FMD Vacc ⁿ
19/01/04	2. Givati I	Feedlot	65/360	May 2003
		Dairy (n = 331)		
23/01/04	3. Givati II	Feedlot	37/365	May 2003
26/01/04	5. Ein Hashofet	Feedlot	80/650	April 2003
		Dairy (n = 550)		
11/02/04	7. Beit Zarzir	Sheep farm	5/80	No vaccination
18/03/04	8. Arab Zbeidat	Feedlot	4/80	June 2003

Controls:

1 x Feedlot, n = 110 calves (7 groups on 3 farms)

3 x Dairy farms, n = 398 cows (Elifaz, n=319; K. Warburg I, n=43; K. Warburg II, n=36)

1 x sheep farm, n = 131 dairy sheep (Vulcany center)

Summarised interpretation of the presence of NSP Antibody in serum

NSP + Disease - virus multiplication - (\pm vaccination).

NSP + Subclinical Disease - virus multiplication (\pm vaccination)

NSP + No disease - No virus multiplication - multiple vaccination with unpurified vaccine.

NSP - No disease - No virus multiplication - efficient vaccination.

Results

Although the two dairy farms were free of clinical disease, calves in the adjacent feedlots developed typical signs of FMD. On those dairy farms, 27% of the animals were sampled (239 of 881 animals; 132 of 456 cows and 107 of 425 calves and heifers) and eight of the sampled animals were NSP positive; seven were cows aged more than three years which had been vaccinated between five and eight times and one was a heifer which had received three vaccinations. At the four feedlots, 194 calves were sampled (from a total population of 1400) and 137 of these were NSP seropositive. Ninety-two of the seropositive calves were between six and ten months of age and were between four and seven months after receiving a single vaccination. In the unvaccinated flock of 80 sheep, four clinical cases were seen and 60 of the 69 sheep that were sampled were NSP seropositive. From multiply-vaccinated but clinically-unaffected dairy herds, only one of the 398 cows that were tested was NSP seropositive whilst only a single seropositive sheep was detected in a control flock of 131 dairy sheep.

Table 2: Screening for NSP antibody in dairy herds

(i) FMD vaccinated dairy herds after outbreak

Farm No. 2

Age	Morbidity	Days post- outbreak	NSP Ab- Svanova	NSP Ab- Ceditest
7m-5y	0/331	80	2/119	2/119
<6m	0/135	70	1/58	
>6m	0/196	31	1/61	

Farm No. 5

Age	Morbidity	Days post- outbreak	NSP Ab- Svanova	NSP Ab- Ceditest
2m-5y	0/550	80	6/120	6/120
<6m	0/290	70	0/49	
>6m	0/260	31	6/71	

(ii) FMD-vaccinated control dairy herds (No outbreak)

Farm No.		Age	NSP Ab- Svanova
I	43	2y-5y	0/43
II	36	2y-5y	0/36
III	319	2y-5y	0/319

(iii) FMD vaccinated feedlots after outbreak

Farm No. 2

Age	Morbidity	Days post- outbreak	NSP Ab- Svanova
2m-10m	65/360	31,56	38/128
<6m	1/120	56	4/61
>6m	64/240	31	34/67

Farm No. 5

Age	Morbidity	Days post- outbreak	NSP Ab- Svanova
< 7m	4/33	56	9/29
> 8m	37/41	56	32/37

Farm	FMD	Enterprise	Age	Morbidity	Days post- outbreak	NSP Ab- Svanova	NSP Ab- Ceditest
3	YES	Feedlot	6 – 7m	35/365	30	10/44	9/44
8	YES	Feedlot	7 - 8m	4/80	28	32/37	
1	NO	Feedlot (Control)	4.5m	0		0/110	

(iv) Screening in sheep flocks after outbreak (FMD vaccination status known or not?) vaccinated

Farm	FMD	Enterprise	Age	Morbidity	post-outbreak	NSP Ab- Ceditest
5	YES	sheep	6 – 7 m	5/80	31 days	61/64
IV	NO	sheep (control)	2 – 85 m	0/131	56 days	1/131

Discussion

Dairy farms were well protected by vaccination against clinical as well as subclinical infection and very few animals were NSP seropositive. It seems that even the young calves before first vaccination are well protected by colostral antibody. However, feedlots with imported calves from FMD free-countries were susceptible to infection as early as four month after a single dose of vaccine had been administered. Unvaccinated lambs may be considered as potential vectors of FMD virus as they may spread the infection without showing clinical signs of disease.

Conclusions

- Sheep are a high risk factor for disease spread as are feedlots which have practical problems with implementation of vaccination.
- Revaccination of calves in feedlots (every four months) is essential if the policy of prevention by vaccination is to be effective.
- Multiply-vaccinated cows may be falsely NSP seropositive, masking truly infected NSP reactors.

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Serological responses in relation to vaccination and infection in Zimbabwe cattle following outbreaks of FMD

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Abstract

Objectives: The aim of this study was to evaluate the performance of serological tests in the detection of SAT-type FMDV infection in cattle, particularly ELISAs for detection of antibodies to non-structural proteins of FMDV (NSPE) and solid phase competition ELISAs (SPCE). Secondary aims were: to compare virus detection rates by virus isolation and RT-PCR tests on both oesophago-pharyngeal (OP) fluids and nasopharyngeal (NP) brush swabbings; to evaluate salivary IgA responses and to examine NSP seroconversion rates in vaccinated cattle that had been exposed to infection.

Materials and Methods: Epidemiological information and clinical specimens were collected from six cattle herds in two different regions of Zimbabwe (404 cattle). One herd was thought not to have been infected, whilst the other five had had outbreaks of disease 1-5 months previously. A trivalent SAT 1, 2 and 3 vaccine had been used in some of these herds at various times either before and/or after the recent outbreaks of FMD. Sampled animals were clinically inspected at the time of sampling for the presence/absence of hoof lesions as clinical evidence of FMD convalescence. All specimens were collected over a ten day period and shipped to the Pirbright Laboratory to be tested for: (1) FMDV-specific serum antibody (by NSPEs, SPCEs and virus neutralisation); (2) the presence of virus and viral RNA in OP fluids and NP swabs and (3) salivary FMDV-specific IgA.

Results: SAT 2 viruses were isolated in cell culture from OP fluids collected at two outbreak locations in Mashonaland provinces (Northern Zimbabwe) whereas SAT 1 viruses were isolated from three FMD-affected herds in Masvingo province (Southern Zimbabwe). Combining the results for OP fluids of virus isolation and RT-PCR, the prevalence of persistently infected animals ranged from 14-38%. In contrast, NP swabs yielded only two virus positive samples. The overall seroprevalence varied with different NSPEs from 48% to 67% whilst 70% and 82% seropositivity was apparent by homologous SPCE and VNT respectively. Approximately 75% to 90% of carriers scored seropositive in different NSPEs. Salivary IgA results are awaited.

Discussion: Two different serotypes were involved in FMD outbreaks in Zimbabwe in 2003/2004. Infection with either type could be readily detected by available tests for virus or antibody, but NP swabs were not a very sensitive method for virus detection.

Introduction:

Recent outbreaks of SAT-type FMD in Zimbabwe provided an opportunity to carry out a field study, collecting clinical specimens to evaluate different laboratory tests which had not so far been fully assessed with such serotypes. Tests for antibodies to non-structural proteins of FMDV (NSPE) have been developed to detect antibodies induced by infection, but not vaccination. They target antibodies to antigens that are highly conserved amongst FMDV serotypes, but the SAT viruses are the most distantly related to other FMDV serotypes and therefore the sensitivity of NSPE for detecting antibodies induced by SAT types of FMDV should be evaluated. Since it has been shown that the sensitivity of NSPE for detecting infected animals may be lower in previously vaccinated cattle, herds with a history of vaccination followed by infection were specifically sought for this study. A particular interest was to investigate the sensitivity of NSPE for the detection of FMDV carriers amongst vaccinated and subsequently infected cattle. Therefore, full virological work-up was essential on all of the sampled cattle.

The solid phase competition ELISA (SPCE) is being introduced as a replacement for the liquid phase blocking ELISA (LPBE) due to the higher specificity of the SPCE (Paiba et al., 2004). SPCE is serotype-

specific, so it has been necessary to develop SAT-specific tests, but there has been a shortage of sera containing SAT-induced antibodies with which to evaluate the performance of these assays.

Other aims of the study were: (1) To compare the sensitivities of RT-PCR methods and virus isolation in detection of SAT viruses in oesophago-pharyngeal (OP or "probang") samples. (2) To investigate if long nasopharyngeal brush swabs could provide suitable samples for detection of FMDV carriers. (3) To determine the prevalence of FMDV carriers in vaccinated and subsequently infected herds. (4) To evaluate an assay for detection of salivary IgA as a means of identifying FMDV carriers.

Materials and Methods:

Sample collection and shipment

All necessary sample buffers, sampling tubes and probang rods were shipped to Zimbabwe from the WRL FMD, along with packaging to enable the materials to be returned safely and in compliance with IATA regulations.

Herd Selection

The criteria for herd selection were: (i) that clinical FMD had occurred between 1 and 6 months previously and (ii) that convalescent or in-contact animals could be identified for sampling.

Sampling protocol

The following protocol was applied at each sampling location:

- (i) relevant epidemiological information was gathered
- (ii) the identity of each sampled animal was recorded; dentition was examined to estimate age and the hooves were examined for evidence of "linear breaks".
- (iii) specimens were collected in the following order: 50 ml of blood was taken from the jugular vein; two different types of swab were used to collect saliva; a guarded brush swab was passed via the nares to collect nasopharyngeal mucus/cells and fluid from the oropharynx and oesophagus was collected with a probang cup.
- (iv) a field "laboratory" was established at each sampling location to process specimens on-site; saliva was expressed from swabs; specimens collected from the pharynx (OP fluid and nasopharyngeal swabbings) were divided into an aliquot for virus isolation and one to which a Lysis/Binding Buffer (Roche) was added for RT-PCR
- (v) all specimens were labeled and with the exception of blood, which was allowed to clot at ambient temperature, were immediately stored on ice.

Further processing of specimens prior to shipment

On return from the field, specimens were further processed at the Central Veterinary Laboratory, Harare. Serum (20-25 ml) was harvested from each clotted blood specimen, heat-treated for 30 minutes at 56 °C and then stored at -80 °C. All of the other specimens (in 2ml cryotubes) were sealed with parafilm and stored at -80 °C.

For air shipment of specimens to WRL the following steps had to be taken:

- (i) arrangements were made with an airline accepting "infectious substances" (categorised by IATA as "dangerous goods") as specimens for delivery to WRL must be sent by air-freight direct to London Heathrow and **not** by courier.
- (ii) packaging was sufficient to allow the inclusion of 50kg of dry ice as a refrigerant and sufficiently well-insulated to keep specimens frozen for at least 48 hours.
- (iii) a shipper's declaration and other documentation (including labels) was completed in accordance with IATA shipping regulations and the instructions provided by WRL (to ensure UK customs clearance).
- (iv) a contact person at the WRL was notified of the flight details and the airway bill number for the shipment.

Virus detection tests

Probang fluids and nasopharyngeal swab eluates were inoculated onto primary bovine thyroid cell cultures for virus isolation. Supernatant fluids from cultures showing cytopathic effects were tested for the presence of FMDV by a serotyping ELISA (Ferris and Dawson, 1988). A part of the 5'UTR and the

complete VPI gene were sequenced for a selection of the isolated viruses using RT-PCR amplification and cycle sequencing. The derived 5'UTR sequences were used to modify the forward primer employed in the diagnostic RT-PCR used at the WRL, so as to achieve perfect complementarity with the viruses that had been detected by virus isolation. The VP1 sequences were used to compare the detected viruses to previously analysed FMDVs of equivalent serotype. Probang fluids and nasopharyngeal swab eluates that had been collected in Lysis/Binding Buffer were tested by real-time RT-PCR using the procedure of Reid et al. (2003) with both the original and the newly modified diagnostic primers and TaqMan probe. RT-PCR CT values of ≤ 45 are considered positive, values of >45 to <50 are considered inconclusive and ≥ 50 are considered negative. However, for data analysis in this study, inconclusive results have been treated as negative.

Serology

Sera were tested for neutralising antibodies by virus neutralisation test (VNT) and for antibodies reactive in solid phase competition and liquid phase blocking ELISAs (SPCE and LPBE) as well as for non-structural protein (NSP) antibodies by ELISA (NSPE).

The VNT, SPCE and LPBE were performed using SAT 1 and SAT 2 viruses and the procedure described in the OIE Manual. For the VNT and LPBE, the FMDV isolates used had been obtained from the present study. The SPCE employed antigens prepared from viruses isolated in the present study (SAT 1) or previously (SAT 2 Cameroon) and was also carried out using antigens and polyclonal antibody reagents prepared from serotypes O, A, Asia 1 and C (Paiba et al., 2004; Anderson et al., 2003). The specificity of the SAT 1 and SAT 2 SPCE was assessed by testing approximately 100 UK sera from each of cattle, sheep and pigs. Specificity was 100% at the 60% inhibition cut-off and approximately 99% at the 50% cut-off.

The NSPEs used were the Ceditest FMDV-NS (Cedi-Diagnostics), the FMDV NSP ELISA (UBI) and the CHEKIT-FMD-3ABC (Bommeli). They were carried out according to the manufacturer's instructions.

Results:

Summary - collection of specimens from FMD-convalescent cattle in Zimbabwe

Clinical specimens (serum, saliva, nasopharyngeal brush swabbings and oesophago-pharyngeal fluid from probang-sampling) were collected between 27 April and 7 May 2004, from 344 cattle at five outbreak locations in two different regions of Zimbabwe. Virus isolation and typing had not been attempted on specimens collected from clinically-affected cattle during the outbreaks at these locations. Therefore, at the time of sampling, it was not known which virus strain or serotype was responsible for each outbreak. At some of these outbreak locations, cattle had been vaccinated with a trivalent vaccine (comprising SAT-1, SAT-2 and SAT-3 components) before and/or during the outbreak. As repeated vaccination might induce either serum antibody to NSPs of FMD virus and/or FMD-specific IgA in saliva, this would be a complicating factor in the interpretation of test results. Therefore serum and saliva were collected from a further 60 cattle in a herd in which there had been no evidence of clinical FMD but in which all cattle had been vaccinated on four separate occasions. All specimens were labeled, catalogued and stored as they were collected. When sampling was completed at all locations a single shipment was sent to the WRL.

Information on "sampled" herds and animals

The location of the sampled herds is shown in Figure 1 whilst summary information on sampled animals at each location, including age, breed, vaccination status and estimated time since infection, is given in Table 1.

Herd A

Location: Harare South district, Mashonaland East; S 17° 59.590'; E 030° 53.174'

Enterprise type: intensive dairy farm

Census: 1300 cattle; 550 milking cows plus calves, followers and replacement heifers

Breeds: mostly Red Danish breed

Vaccination status: as this farm was within 10km of a previous FMD outbreak in July 2003 all cattle had been vaccinated twice in July/August 2003 with trivalent vaccine (SAT1, SAT2 and SAT3). In addition, in an attempt to control FMD, the entire herd (including animals that were clinically-affected with FMD) was vaccinated twice with the same vaccine in February/March 2004.

History of most recent FMD outbreak: FMD was reported on 23 February 2004; 30 animals in a group of replacement heifers were very severely lame. The following day the disease was noticed in milking cows; 20 were clinically affected on that occasion and a further 20 cows were affected the following day. The daily milk yield of the herd (460 lactating cows) was more than 9000 litres just before the outbreak and decreased within days to less than 3000 litres. Forty calves, all of which were less than one week of age, died within the first two days after the outbreak was reported and approximately 100 lactating cows died or were culled due to severe secondary mastitis. The most likely source of infection was a kudu which had entered the paddock in which the first clinically-affected group of cattle (replacement heifers) was grazing; this occurred about 2-3 weeks before clinical FMD was observed in cattle and the kudu is thought to have been present in the paddock with the cattle for about one week. However it was also reported that a cow from a nearby premises had escaped from a truck near the entrance to this premises and had contact with replacement heifers in a paddock close to the entrance before being recaptured.

Sampling and examination of animals: A total of 130 cattle were sampled, all of which had been clinically-affected during the outbreak; the sampled animals included 20 cows (each of which, as a sequel to clinical FMD, had lost a quarter due to severe mastitis) and two groups of replacement heifers (10-18 months-old). Linear breaks in the continuity of the horn forming the wall of the hoof were observed in one or more feet of 11/20 cows and 16/30 heifers. The distance of these lesions from the coronary band was estimated as approximately 20 mm in most cases.

Herd B

Location: Zvimba district, Mashonaland West; S 17.42050 E 30.38789

Enterprise type: communal cattle-rearing on a resettled land holding

Census: 75 cattle

Breeds: mostly indigenous breed (Mashona) with some European and Brahman crossbreeds

Vaccination status: uncertain; it was not clear as to whether or not animals on this premises were vaccinated at any time, either before, during and/or after the outbreak.

History of most recent FMD outbreak: 11 cattle were reported to have clinical FMD on 31 March 2004. There was no indication as to the likely source of infection but uncontrolled movement of livestock from other communal farming areas was most probably a factor.

Sampling and examination of animals: A total of 42 cattle were sampled; most had full permanent dentition (but the "sample" also included two juveniles with no permanent teeth and nine adult cattle with 2-6 permanent incisors). Indistinct linear breaks were observed in one or more hooves in at least 10 cattle but handling facilities did not favour photography or measurement of these lesions.

Herd C

Location: Chiredzi district, Masvingo; S 21^o 07.512'; E 031^o 28.401'

Enterprise type: commercial beef fattening

Census: 307 cattle

Breeds: mostly Beefmaster with some Mashona crossbreeds

Vaccination status: this herd was last vaccinated on 26 October 2001. Vaccination was **not** used during or after the outbreak in this herd.

History of most recent FMD outbreak: outbreak reported on 7th April 2004; 112 clinical cases of FMD were observed; most of the affected animals had healing lesions at that time. The most likely source of infection was a feedlot owned by the same private company, from which cattle were moved to this premises (on 25 February) as FMD had been reported in the feedlot on 9 January 2004.

Sampling and examination of animals: A total of 65 cattle were sampled; all had exhibited clinical signs at the time of the outbreak. All except two of these cattle had 4-6 permanent incisors, the other two animals having full permanent dentition. Linear breaks were observed in one or more hooves in 43/65 cattle (Figure 2). The distance measured between these breaks and the coronary band varied between 11 and 18 mm.

Herd D

Location: Mwenezi district, Masvingo; S 21^o 14.617'; E 030^o 44.129'

Enterprise type: communal cattle-rearing

Census: 85 cattle

Breeds: mostly Brahman crossbreeds

Vaccination status: there are no records of this herd ever having been vaccinated for FMD (before, during or after the most recent outbreak).

History of most recent FMD outbreak: 40 animals were observed with clinical FMD on 8 January 2003. The most likely source of infection was a nearby ranch in which an outbreak had occurred; animals from both premises shared common grazing because of disruption of fences between them.

Sampling and examination of animals: A total of 42 animals were sampled; most of which had full permanent dentition (but the "sample" also included six juveniles with no permanent teeth and ten adult cattle with 2-6 permanent incisors). Linear breaks were observed in one or more hooves in 22/42 cattle. The distance between the coronary band and these breaks varied from 20-35 mm.

Herd E

Location: Mwenezi district, Masvingo; S 21° 26.442'; E 030° 51.637'

Enterprise type: commercial cattle-rearing (a Government-owned farm)

Census: 767 cattle

Breeds: mostly Brahman crossbreeds

Vaccination status: Before the outbreak, this herd had last been vaccinated in 1994. However, all cattle were vaccinated twice after the outbreak (January/February 2004).

History of most recent FMD outbreak: FMD was reported on 8th December 2003; 37 clinical cases were observed when the disease was first reported but 90-95% morbidity was evident within a further 24 hours. The most likely source of infection was cattle which had been moved from a different section of the same ranch (and in which FMD had previously been reported).

Sampling and examination of animals: A total of 42 cattle were examined; all were adult animals with full permanent dentition. Distinct hoof lesions were not apparent in any of these animals at the time of sampling (but the underfoot conditions in the handling facilities may have obscured some lesions).

Herd F

Location: Harare South district, Mashonaland East; S 18° 03.054'; E 030° 52.452'

Enterprise type: commercial beef fattening at grass; same ownership as Herd A

Breeds: mostly European crossbred

Vaccination status: as for Herd A; both farms were within 10km of a previous FMD outbreak and were therefore vaccinated twice (July/August 2003). In addition this herd was re-vaccinated at the time of the outbreak in Herd A (twice; February/March 2004).

There has been **no** clinical evidence of FMD in this herd. In addition to re-vaccination at the time of the outbreak in Herd A, strict biosecurity measures were applied to prevent transmission of infection which included a complete ban on movement of personnel and vehicles between the two premises.

Sampling and examination of animals: 60 heifers, all of which were 14-16 months-old, were sampled; only serum and saliva were collected from these animals.

Virus detection tests

SAT 2 virus was recovered from herds A and B in northern Zimbabwe, whilst SAT 1 virus was recovered from herds C, D and E in southern Zimbabwe. VP1 gene sequences were obtained from isolates SAT2/ZIM/P1/2004[A2761], SAT2/ZIM/P1/2004[B7], SAT1/ZIM/P1/2004[C10], SAT1/ZIM/P1/2004[D19], SAT1/ZIM/P1/2004[E45] from herds A, B, C, D, E, respectively and their relationship to other SAT 1 and 2 viruses from southern Africa are described by Valarcher et al., elsewhere in this proceedings. Sequencing in the 5'UTR revealed 2 mismatches with the forward primer used in the WRL RT-PCR test. Therefore, a new primer was designed which gave 100% complementarity. The details of virus detection are shown in Table 2. Virus isolation combined with antigen detection ELISA yielded only one positive result from 330 nasopharyngeal swab samples and only one positive result was obtained by RT-PCR out of 140 nasopharyngeal swab samples that had been collected in Lysis/Binding Buffer (Roche). The two positive samples came from different animals within herd D. A total of 314 probang samples were examined by virus isolation and 300 that had been collected in Lysis/Binding Buffer were examined by RT-PCR. Thirty-seven samples were virus isolation and antigen detection ELISA positive (12%) and the proportion of positive animals ranged from 6-26% in the different herds. Using our original diagnostic RT-PCR reagents, thirty-two samples (11%) were positive, whereas with the improved primer, this figure rose to 67 samples (22%), with a range of 8-31% between herds. Only about half of individual samples positive by virus isolation were also positive by RT-PCR, whilst around a third that were positive by the more sensitive of the RT-PCR methods were also positive by virus isolation.

Serology

VNT results for each of the six herds are summarised in Fig 3, which shows frequency distributions for SAT 1 and 2 VNT titres by herd. Cattle in herd F had apparently been vaccinated four times during the

previous nine months using a trivalent SAT vaccine but had apparently not been infected. However, only 9 of 60 cattle had SAT 1 neutralising antibody titres of greater than or equal to 1 in 45 (8 being at 1 in 45 or 1 in 64), which suggests a rather low level of vaccine induced immunity to the challenge strain from nearby herd A. The SAT 2 profile was different and suggested that some of the animals had been more effectively vaccinated, vaccinated with a strain more closely matching that used in the VNT or else infected with a SAT 2 virus. NSP testing demonstrated that between 2 and 20% of cattle were seropositive, depending on the test used, and it is therefore likely that some cattle in this herd had been previously infected with a SAT 2 virus. This would be consistent with the geographical location of the farm. In herds A, B, D and E there was a clear difference in the neutralising antibody profiles to SAT 1 and 2 viruses; in each case the highest level of antibodies being found when tested against the virus serotype isolated in the probang samples. Herd D, which had not been vaccinated, but had been infected with SAT 1, was largely seronegative to SAT 2. Samples from herd C neutralised both serotypes to a similar extent.

SPCE reactivity profiles were similar to those of the VNTs with either a SAT 1 or SAT 2 response predominating except in herd C. A summary of the reactivity of the sera in all of the different serological tests is given in Table 3. VNT with the homologous virus detected the most seropositive animals (82%), followed by SPCE (70%), Cedi (67%), UBI (53%) and Bommeli (48%). The frequency distribution curves for the Cedi and the homologous SPCE were rather similar in all herds (data not shown). In herds D and E, sera from a significant proportion of animals had percentage inhibitions in the 50-60% range which were scored negative in SPCE, but positive in Cedi, giving the latter a higher sensitivity for these sample sets (Table 3). Table 4 shows the sensitivity of the various serological tests to detect presumed carrier animals identified by viral recovery from probang samples collected 1-5 months after outbreaks of disease and tested by virus isolation or RT-PCR. Results were similar, regardless of which method was used for virus detection. The VNT using homologous virus, detected 100% of carriers, although in some cases, the titres of antibody were as low as 1 in 45 (Fig 4). SPCE and Cedi tests detected approximately 90% of carriers, whereas for the Bommeli and UBI tests, the figure was around 75%.

Discussion:

The main aim of this study was to evaluate the sensitivity of various laboratory methods to detect infection of cattle with SAT serotypes. Efforts were made to obtain the fullest possible history of the herds and cattle investigated and a wide range of samples were collected so that both the virological and serological status of the animals could be comprehensively assessed. In order to be as sure as possible that samples came from infected animals, herds A-E were selected on the basis that they had all experienced outbreaks of clinical FMD in the preceding 1-5 months and sampling was targeted towards animals that had either been clinically affected or had been in close contact with affected animals. Examination of feet for hoof growth arrest lines was used to help target sampling to clinically affected groups. Information was not available on the potency of the vaccines that had been used in some herds, or on the relationship between the FMD virus vaccine strains and the virus isolates recovered from the herds. Although herds A and E had a history of prior vaccination, this had occurred more than 3 months before the outbreaks and was unable to prevent clinical disease. Herds B, C and D had no history of recent vaccination, whilst Farm F, in which clinical disease was not observed, had been vaccinated at the same time as farm A, i.e. in mid 2003 and early 2004.

Hoof lesions like those observed in the present study have previously been described in cattle in Southern Africa which have recovered from clinical FMD (Thomson, 1994). Although not specific for FMD, the presence of "linear breaks" in one or more hooves of cattle at outbreak locations in this study was taken as an indicator of convalescence. It was presumed that acute infection with FMD virus at the time of the outbreak and viral replication in the horn-producing cells of the coronary band caused cessation of growth in the hoof wall such that when horn growth resumed after recovery from disease, a break appeared in the continuity of the hoof wall. If the rate of growth of the hoof wall is known, the distance between such lesions and the coronary band can be used to roughly estimate the time which has elapsed since acute infection occurred (Dekker et al., 2004). However when using this method it is important to realise that hoof horn growth rates vary considerably in cattle of different ages and even between the fore feet and hind feet of the same animal (Prentice, 1973). In the present field study, the distance from the coronary band of "linear breaks" in hooves of cattle at different sampling locations did correlate with the clinical history of when FMD outbreaks occurred at those locations.

Two different serotypes of FMDV were isolated from the clinically affected herds; SAT 2 from the two northern herds and SAT 1 from the three southern herds. These viruses were genetically very similar to other SAT viruses obtained from Zimbabwe in 2003 (Valarcher et al., these proceedings). The infectivity status of individual cattle was assessed by probang sampling and testing at a single time point and although this was successful in identifying many carrier animals, it is likely to have underestimated the prevalence of such animals, since it has been estimated that without multiple sampling, up to 50% of carriers can be missed. Virus was isolated from 6-22% of probang samples (12% overall). A wide range of prevalence of carriers has been described in the literature at different times after outbreaks occurred (Salt, 2004). The prevalence of carrier cattle detected by VI and RT-PCR combined in herds A-E was between 14 and 38%; the lowest prevalence being found in herds D and E (17% and 14%) where the longest interval had elapsed between infection and sampling. The relative sensitivity of virus isolation and RT-PCR were compared and the results show that the conventional RT-PCR method in use at WRL approaches the sensitivity of virus isolation using highly sensitive primary bovine thyroid cell cultures (and in fact matches it, if inconclusive positive samples are considered as positive). Furthermore, using a RT-PCR that had been optimized for detection of SAT 1 and 2 viruses, a significantly greater proportion of cattle was scored as carriers than by virus isolation. The relatively poor concordance between virus isolation and RT-PCR may reflect the fact that the levels of virus present in probang samples are close to the lower limit of detection for both tests.

The probang sampling cup collects mucus and cells from the back of the pharynx and the upper oesophagus where FMDV is known to persist. Because the collection method requires the use of a specialist sampling rod, considerable expertise and a means of cleaning the rod between animals, it was considered worthwhile to investigate an alternative sampling method.

Guarded nasopharyngeal brush swabs have been used in cattle for many years as a means of detecting respiratory viruses. Provided that the swab is sufficiently long, it can be passed to the back of the nasopharynx and used to collect mucus and cells from the bovine tonsillar region (Nuttall et al., 1980). However, this study did not find the method very successful for detecting FMDV carriers and the nasopharyngeal sampling procedure was also less well tolerated by the cattle than was insertion of the probang sampling cup.

A representative of each FMDV SAT serotype was grown in cell culture and used for virus neutralization tests and to prepare antigens for SPCE. However, the SAT 2 virus selected did not generate a suitable antigen for use in SPCE and therefore was substituted with another antigen prepared from SAT 2 Cameroon 2000. The prevalence of seroreactors varied substantially between tests with the highest level being detected by homologous VNT used at a cut-off of 1 in 45 (Table 3). At this cut-off, the specificity of the VNT probably approaches 100% (Paiba et al., 2004). The test does not distinguish antibodies due to infection from those attributable to vaccination. However, the level of vaccine-induced neutralizing antibodies appeared to be low; for example there was little difference between the low levels of SAT 2 neutralising antibody found in herds D and E (Fig 2), although animals in the former herd had never been vaccinated, whilst those in herd E had been vaccinated twice approximately three months prior to the recent outbreak of FMD. The occurrence of SAT 1 neutralising antibody seroreactors in herds A, B and F, which had higher titres of SAT 2 antibodies may be attributed to either vaccination, undisclosed SAT 1 infection or cross-reactivity of SAT 2 antibodies. In herd C, where there had been no recent vaccination, the presence of high levels of SAT 1 and SAT 2 neutralising antibodies is suggestive of previous infection by SAT 2 as well as with the SAT 1 viruses that were recovered from probang samples. The finding of significant numbers of SAT 2 neutralising antibody seroreactors in herd F, is suggestive of previous SAT 2 infection rather than vaccination and this could be explained by the occurrence of subclinical infection around the time that SAT 2 was infecting neighbouring herds A and B. It would therefore be useful to examine sera from herd F for IgM antibodies to see if there was evidence that infection was recent or not.

The SAT 1 and SAT 2 SPCEs showed similar specificity profiles when tested with naïve sera from the UK, and these were also similar to the profiles seen with SPCEs for other serotypes (Paiba et al., 2004; Anderson et al., 2003; Paton et al., this proceedings). The use of a 60% cut-off is likely to result in a test with specificity close to 100% and therefore, a lower cut-off of around 50% could probably have been used to boost sensitivity. Even at the 60% cut-off, the test was quite sensitive, scoring 70% of sera as positive, compared to 82% for VNT.

All of the NSPE tests evaluated were able to detect SAT 1 and 2 antibodies. However, there were considerable differences in the seroprevalence recorded with each test; overall, the Cedi test scored 67% of samples as positive, compared to 48% and 53% with the NSPE from Bommeli and UBI. Similarly, more carriers were scored positive by the Cedi test (89-91%) compared to the Bommeli (74-76%) and UBI (70-75%) tests (Table 4). Figures of 92% and 47% were reported for detection of carriers (mixture of serotypes SAT 2 and A) in unvaccinated cattle from the Cameroon using the precursor to the Cedi test and the Bommeli test respectively (Bronsvort et al., 2004). There is considerable interest in estimating the prevalence of FMDV carrier animals that can be identified by NSPE tests in vaccinated herds that have been infected with FMDV. This investigation should complement studies with similar aims that have been carried out using experimentally vaccinated and/or infected animals, since the experimental models yield fewer samples and are unlikely to fully mimic natural transmission conditions. However, not all of the herds had been vaccinated and even in those that had, their levels of protective immunity as assessed by neutralizing antibody titre and protection against disease were low.

Authors conclusions:

1. "Linear breaks" in the hooves of cattle at known outbreak locations may be used as both an indicator of convalescence and to roughly estimate the time that has elapsed since infection occurred.

2. This study has provided data on the prevalence of SAT 1 and 2 virus carriers in cattle herds 1-5 months after FMDV infection and on their ease of detection by different virological and serological methods.
3. Virological tests on nasopharyngeal brush swabs scored very few cattle as infected compared to the conventional approach of testing samples obtained with a probang sampling cup.
4. Optimised RT-PCR was more sensitive than virus isolation for the detection of SAT 1 and SAT 2 FMDV in probang cup samples.
5. SPCE and NSPE tests readily detected antibodies to SAT 1 and SAT 2 FMD viruses.
6. Sensitivity estimates of NSPE for detection of FMDV carriers (75-90%) were similar to those obtained from a study of unvaccinated Cameroon cattle and accord with analyses of experimentally derived sera from a recent NSPE workshop (DeSimone et al., this proceedings).
7. However, none of the herds from which virological data were available had been optimally vaccinated and the study therefore provides limited insight into the prevalence of carriers likely following subclinical infection in such herds.

Authors recommendations:

1. Some outstanding work remains to be finished, concerning antibody detection tests on saliva samples, use of RT-PCR internal standards and completion of data analysis.
2. It would be useful to conduct similar exercises involving herds with a more certain vaccination status and preferably following use of emergency vaccination in a previously disease-free region; also in areas where disease has occurred in vaccinated pigs and sheep.

References

Anderson, J., Corteyn, M., Gibson, D., Hamblin, P. & Paton, D. 2003. Further validation of the solid-phase competitive ELISA for FMDV types A, C & Asia 1. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Gerzensee, Switzerland, 16-19 September 2003. Rome: FAO, Appendix 24: 157-165.

Bronsvort, B. M. D., Sorensen, K. J., Anderson, J., Corteyn, A., Tanya, V. N., Kitching, R. P. & Morgan, K. L. 2004. Comparison of two 3ABC enzyme-linked immunosorbent assays for diagnosis of multiple-serotype foot-and-mouth disease in a cattle population in an area of endemicity. *J. Clin. Microbiol.* 42: 2108-2114.

Dekker, A., Moonen, A. & Pol, J.M.A. Linear hoof defects discovered in foot-and-mouth disease infected sheep; a case report. *Veterinary Record.* in press.

DeSimone, F., DeClercq, K., Brocchi, E., Grazioli, S., Paton, D., Dekker, A., Haas, B., Yadin, H., Bulut, N., Tjornehoj, K. and Sammin, D. *Preliminary report of a workshop for comparative evaluation of NSP antibody ELISAs, IZSLER, Brescia, 4-15 May 2004.* Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Chania, Crete, Greece, 12-15th October 2004. Rome: FAO, Appendix 6 (*This proceedings*).

Reid, S. M., Grierson, S. S., Ferris, N. P., Hutchings, G. H. & Alexandersen, S. 2003. Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J. Virol. Methods* 107: 129-139.

Ferris, N. P. & Dawson, M. 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet. Microbiol.* 16: 201-209.

Nuttall, P. A., Stott, E. J., & Thomas, L. H. 1980. Experimental infection of calves with two strains of bovine virus diarrhoea virus: virus recovery and clinical reactions. *Res. Vet. Sci.* 28: 91-95.

Office International des Epizooties, World Organisation for Animal Health. 2004. Foot and mouth disease, in: OIE Standards Commission (Ed.), Manual of standards for diagnostic tests and vaccines, 5th ed., Office International des Epizooties, Paris, France, Chapter 2.1.1.

Paiba, G. A., Anderson, J., Paton, D. J., Soldan, A. W., Alexandersen, S., Corteyn, M., Wilsden, G., Hamblin, P., Mackay, D. K. J. & Donaldson, A. I. 2004. Validation of a Foot-and-mouth disease antibody screening Solid-phase competition ELISA (SPCE). *J. Virol. Methods* 115: 145-158.

Paton, D.J., Armstrong, R.M., Fernandez, R., Hamblin, P.A., Turner, L., Corteyn, M, Gibson, D, Parida, S., Wright, C. & Anderson, J. FAO Phase XVIII FMD serological standardisation; progress and future prospects. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Chania, Crete, Greece, 12-15th October 2004. Rome: FAO, Appendix 10 (*This proceedings*).

Prentice, D. E. 1973. Growth and wear rates of hoof horn in Ayrshire cattle. *Res. Vet. Sci.* 14: 285-290.

Salt, J. 2004. Foot and Mouth Disease, Current Perspectives, edited by Francisco Sobrino and Estaban Domingo, Horizon Bioscience, Wymondham, England. Chapter 6, Persistence of Foot-and-Mouth disease. Chapter 6, pp 103-143.

Thomson, G.R. 1994. Foot and mouth disease. In: *Infectious diseases of livestock with special reference to Southern Africa*. eds. J. A. W. Coetzer, G. R. Thomson & R. C. Tustin. Cape Town, Oxford University Press. pp 825-852

Valarcher J. F., Knowles, N. J., Fernandez, R., Davies, P. R., Midgley, R. J. Statham, B., Hutchings, G., Newman B. J., Ferris N. P. & Paton D. J. Global foot-and-mouth disease situation 2003-2004. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Chania, Crete, Greece, 12-15th October 2004. Rome: FAO, Appendix 21 (*This proceedings*).

Zhang, Z. & Alexandersen, S. 2003. Detection of carrier cattle and sheep persistently infected with foot-and-mouth disease virus by a rapid real-time RT-PCR assay. *J. Virol. Methods* 111: 95-100.

Table 1. Summary of the “sampled” animals in each herd

Herds	Province	No. of cattle sampled and tested	Breed	Age	Vaccination status	Time elapsed since outbreak	Hoof Lesions*	Serotype of virus detected in probang samples
A	Mashonaland East	130	Red Danish	110 < 18 m. 20 adults	x2 pre-outbreak x2 post-outbreak	>2 months	27/50; approx. 20mm	SAT 2
B	Mashonaland West	42	Mashona	Adult	unknown	≥1 month	10/42; not measured	SAT 2
C	Masvingo	65	Beef-master	2.5-3.5 years	Not since 2001	~1 month	43/65; 11-18 mm	SAT 1
D	Masvingo	42	Brahmin X	Adult	Never	~4 months	22/42; 20-35 mm	SAT 1
E	Masvingo	65	Brahmin X	Adult	x2 post-outbreak	~5 months	NOT seen	SAT 1
F	Mashonaland East	60	European X	14-16 m.	x2 Jul/Aug 03 x2 Feb/Mar 04	No history of infection	NOT seen	No probang samples collected

* the number of animals in which “linear breaks” were apparent in one or more hooves/the number of animals whose feet were examined; distance of “linear breaks” in the hoof-wall from the coronary band

Table 2. Virus detection results

Herds [§]	Serotype of virus detected in probang samples	Number of nasopharyngeal swab samples tested by virus isolation/RT-PCR*	Number of probang samples tested by virus isolation/RT-PCR	Number and percentage of probang samples found positive by virus isolation	Number and percentage of probang samples found positive by RT-PCR ¹	Number and percentage of probang samples found positive by RT-PCR ²	Number of probang samples found positive by virus isolation and RT-PCR / positive by any method	Prevalence of probang sample positive animals detected by one or more detection methods
A	SAT 2	129 / 51	127 / 117	8 / 6%	19 / 16%	36 / 31%	4 / 40	34%
B	SAT 2	36 / 17	36 / 37	4 / 11%	2 / 5%	5 / 14%	1 / 8	22%
C	SAT 1	62 / 23	61 / 51	16 / 26%	8 / 16%	16 / 31%	9 / 23	38%
D	SAT 1	40 / 15	35 / 36	4 / 11%	1 / <1%	3 / 8%	1 / 6	17%
E	SAT 1	63 / 34	55 / 59	5 / 9%	2 / 3%	7 / 12%	4 / 8	14%
Overall		330 / 140	314 / 300	37 / 12%	32 / 11%	67 / 22%	19 / 85	28%

[§] no probang samples collected from herd F; * only one nasopharyngeal swab sample (from herd D) was found positive by virus isolation and one (other) sample (from herd D) was found positive by RT-PCR; RT-PCR¹ = RT-PCR using diagnostic primers of Reid et al. (2003); RT-PCR² = using modified forward primer with enhanced match to isolated SAT viruses

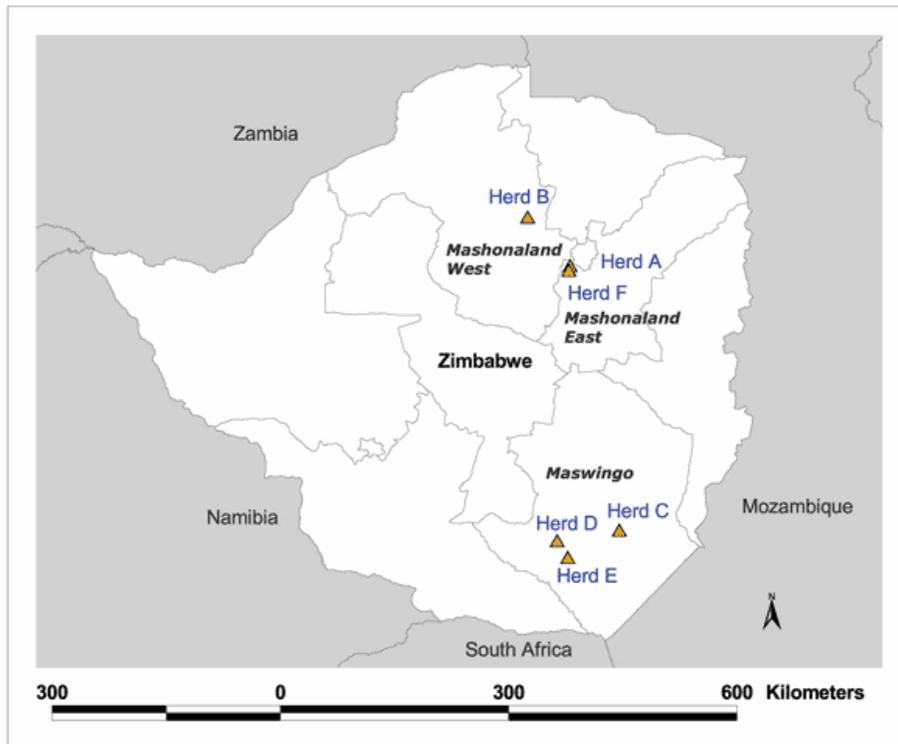
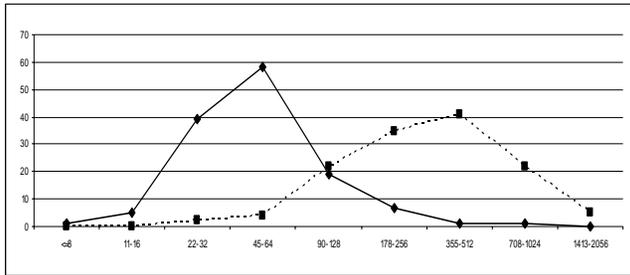


Figure 1. Map of Zimbabwe showing herd locations

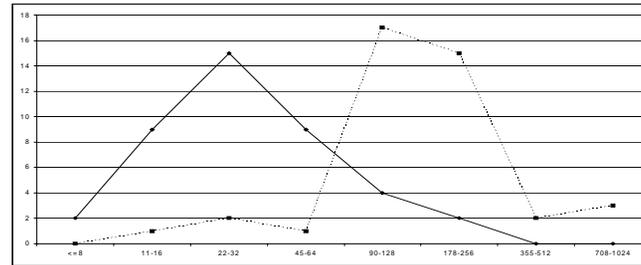


Figure 2. Linear breaks 11 mm from the coronary band in the hoof of a convalescent animal

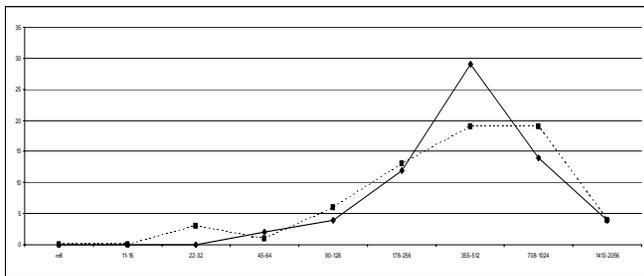
Herd A



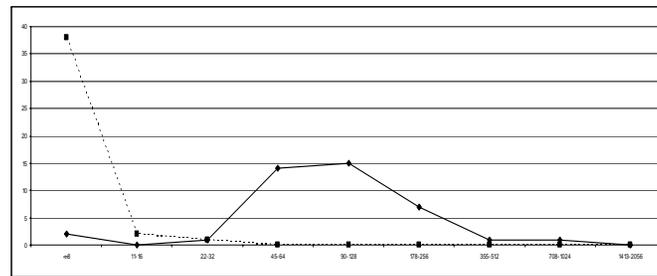
Herd B



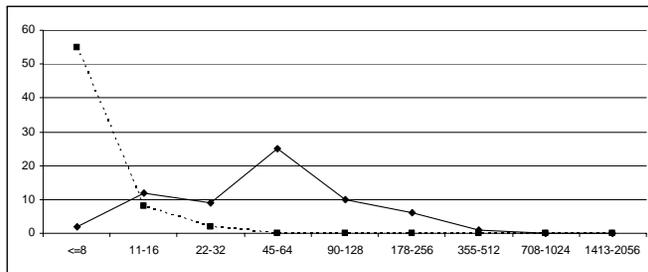
Herd C



Herd D



Herd E



Herd F

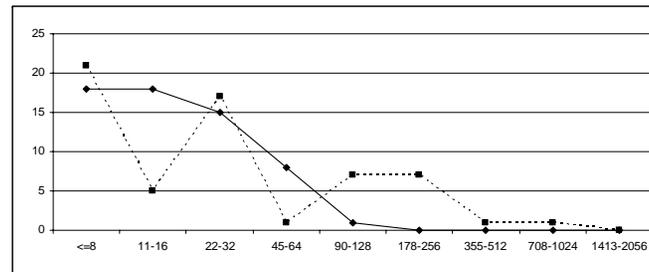


Fig 3. VNT results presented as frequency (y axis) versus titre expressed as reciprocal serum dilution (x axis) plots. SAT 1 VNT = solid line; SAT 2 VNT = dotted line

Table 3. Comparative numbers of seroreactors and seroprevalence within herds

Herd	VNT*	SPCE*	Cedi	Bommeli	UBI
A	128 (98%)	129 (98%)	120 (91%)	86 (66%)	101 (77%)
B	39 (95%)	39 (95%)	30 (74%)	20 (49%)	25 (61%)
C	65 (100%)	65 (100%)	63 (97%)	65 (100%)	65 (100%)
D	38 (93%)	9 (22%)	16 (39%)	10 (24%)	7 (17%)
E	42 (65%)	21 (32%)	30 (46%)	10 (15%)	10 (15%)
F	17 (28%)	20 (33%)	12 (20%)	1 (2%)	7 (12%)
Combined	329 (82%)	283 (70%)	271 (67%)	192 (48%)	215 (53%)

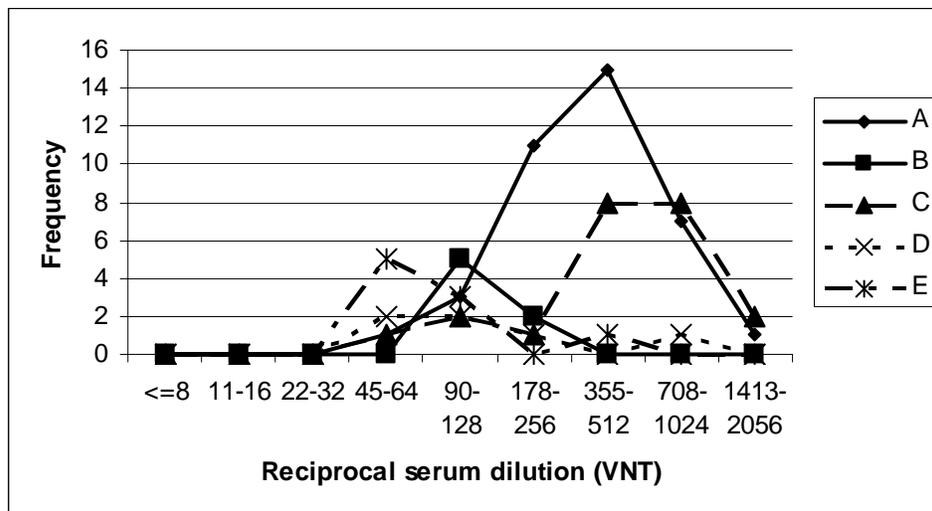
* Using virus or antigen of the same serotype as that isolated from probang samples
 Sera were scored positive at the following cut-offs: VNT, ≥ 1 in 45; SPCE, $>60\%$; Cedi, $\geq 50\%$; Bommeli, ≥ 20 percent positivity; UBI, OD ≥ 0.23 x positive control

Table 4. Comparative serological detection of virus carriers

	VNT*	SPCE*	Cedi	Bomme li	UBI
% of 37 VI carriers detected	100	86	89	76	70
% of 65 RT-PCR carriers detected**	100	94	91	74	75

*Using virus or antigen of the same serotype as that isolated from probang samples
 ** No matching blood sample found for two cattle with RT-PCR positive probangs
 Sera were scored positive using the same criteria as in Table 3.

Fig 4. Homologous VNT results by herd for carrier animals detected by virus isolation or RT-PCR, presented as frequency (y axis) versus titre (x axis) plots.



**Performance of the new oil adjuvanted vaccine and conventional vaccines produced by the
SAP Institute in 2004**

Nilay Ünal, Şap Institute, Ankara, Turkey

Vaccine production method

- Virus production
- Cell cultures
- Virus cultures
- Clarification of vaccine virus
- Filtration of vaccine virus
- Virus inactivation
- Concentration and purification
- Preparation of the vaccine (formulation)

Controls applied within the Şap Institute:

A. Pre-production controls

- Raw material controls
 - all chemicals controlled for cell growth properties
 - disposable material controlled for cell toxicity
- Sera controls
- Water controls
- Cell controls
 - contamination controls(bacteria,mycoplasma and virus)
 - cell growth
 - cell characteristics
 - virus sensitivity

B. In-process controls

- Sterility controls (media, cells and virus)
- Antigenity (CFT and ELISA)
- Infectivity (plaque test)
- 146s content (ultracentrifugation)
- Inactivation

C. Final product controls

- Sterility controls
 - FTM
 - TSB
- Safety controls
 - inactivation kinetics for each batch of virus
 - elution test in cell culture

Cattle protection and challenge test in 3 cattle for each monovalent vaccine batch.

Herd immunity test: at least 20 cattle negative for FMD antibodies are vaccinated and then the level of antibody is assayed by LPB-ELISA, between 21 and 28 days post-vaccination.

A pilot study to assess the performance of an oil-adjuvanted FMD vaccine

This was a double oil emulsion (water/oil/water) vaccine which contained Montanide ISO 206 (Seppic) as adjuvant and incorporated FMD viral strains: O₁ Manisa, A₉₈ Aydin (homologous to A₉₆ Iran) and Asia-1. The antigenic component was controlled by ELISA measurement, by determination of the infective titre and by assessing the 146s content. After inactivation, inactivation kinetics, sterility, safety in tissue culture and 146s content were measured and after concentration, sterility, 146s content and safety in tissue culture were again assessed.

Potency tests:

This formulation was then applied to animals in the field (both cattle and small ruminants) in Denizli, Ankara and Kastamonu. The results obtained for cattle are graphically represented in figures 1 and 2 whilst those obtained in sheep are illustrated in figure 3. In sheep, 3 different doses of vaccine (0.5, 1 and 1.5 ml) were used. There were 30 animals (10 per group) which received vaccine and 10 control animals. The serological results by both ELISA and SNT suggested a 70-100 % protection

level regardless of the dose of vaccine. However, a dose of 1ml per sheep was recommended as this gave more than 85% protection.

Vaccination programme 2004

Spring (March/April)

- Thrace and Marmara region; vaccination of all ruminants with the trivalent vaccine.
- In other regions of Turkey; vaccination of all large ruminants with the trivalent vaccine

Autumn (September/October) :

- Thrace and Marmara region; vaccination of all ruminants with the bivalent vaccine
- In eastern and southeastern border areas; vaccination of large ruminants with trivalent vaccine
- In other regions of Turkey; vaccination of all large ruminants with the bivalent vaccine

Vaccine production in 2004

The Şap institute planned to produce oil-adjuvanted vaccine for the vaccination campaign in 2004 but there were delays in obtaining a virus purification and concentration system, which has been expected since the end of the 2002. Installation of this system is being supported by an EU project. It is now hoped that the system will be in place before 2005 such that concentrated antigens can be stored, thus overcoming the problems of storing formulated vaccine. This will also enable production of oil-adjuvanted vaccine to prolong the duration of immunity in vaccinated animals.

In 2004, a total of 11.8 million monovalent vaccine doses of O Manisa 69; A Aydin 98 (homologous to A Iran 96) and Asia 1 were incorporated into aluminium hydroxide-adjuvanted vaccine. In addition the Şap Institute produced 3.1 million trivalent doses and 2.1 million bivalent doses of oil-adjuvanted vaccine (equivalent to a total of 13.5 million monovalent doses of the antigens in question)

The Şap Institute also exported formulated vaccine to Jordan (1.1 million monovalent doses and 12,000 trivalent doses) and Georgia (575,000 bivalent doses and 500,000 trivalent doses cattle of oil adjuvanted vaccine plus a donation of 120,000 trivalent doses of oil adjuvanted vaccine)

Herd immunity test in the field

Sera were taken from cattle in the state farm after the vaccination and tested by LPB-ELISA.

Sero-survey in 2004

Following the spring vaccination campaign, a sero survey was carried out in the Thrace region to determine the immunity level of animals vaccinated with trivalent vaccine and to monitor for antibodies against non structural proteins (NSP) of FMD virus. Another sero survey was carried out in order to monitor antibodies to non structural proteins (NSP) of FMD virus in small ruminants in eastern and south eastern border provinces.

Control of FMD vaccines in Turkey

Studies to control the quality of veterinary vaccines, both those produced in Turkey and imported vaccines, are conducted at the Bornova Veterinary Control and Research Institute (BVCRI); these studies are still in progress for FMD vaccines. The Şap (FMD) Institute continues to control the sterility, safety and potency of vaccines as usual. BVCRI inspected the safety and potency tests conducted at the Şap Institute. In addition, the Şap Institute sends a dossier to BVCRI detailing the results of QA tests for each batch of vaccine produced. Quality control checks are also conducted on randomly selected batches of vaccine in the presence of BVCRI staff.

Figure 1 Denizli (Cattle), protection %

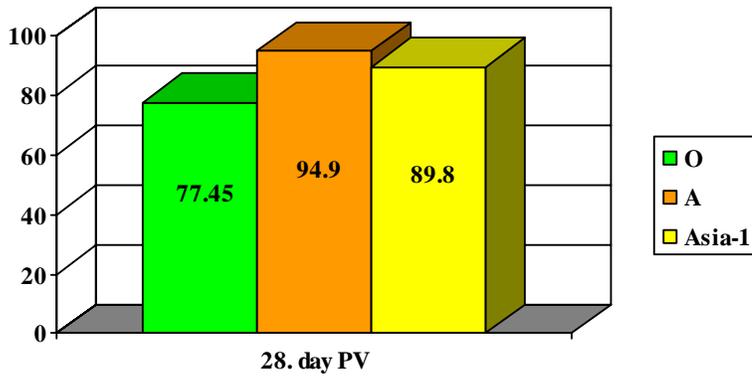


Figure 2 Denizli

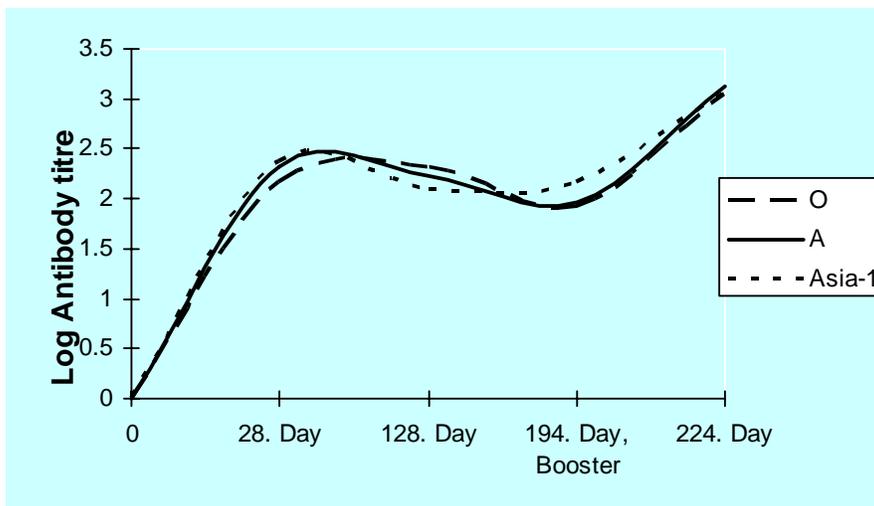


Figure 3 Ankara-Fevziye Köyü (sheep) protection %

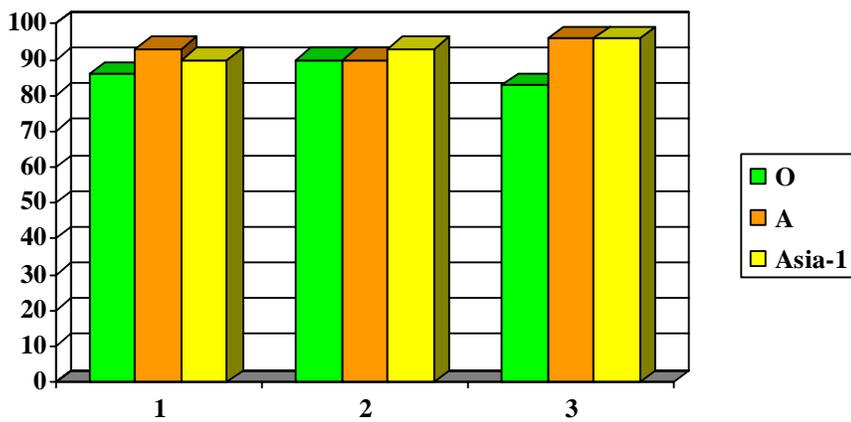
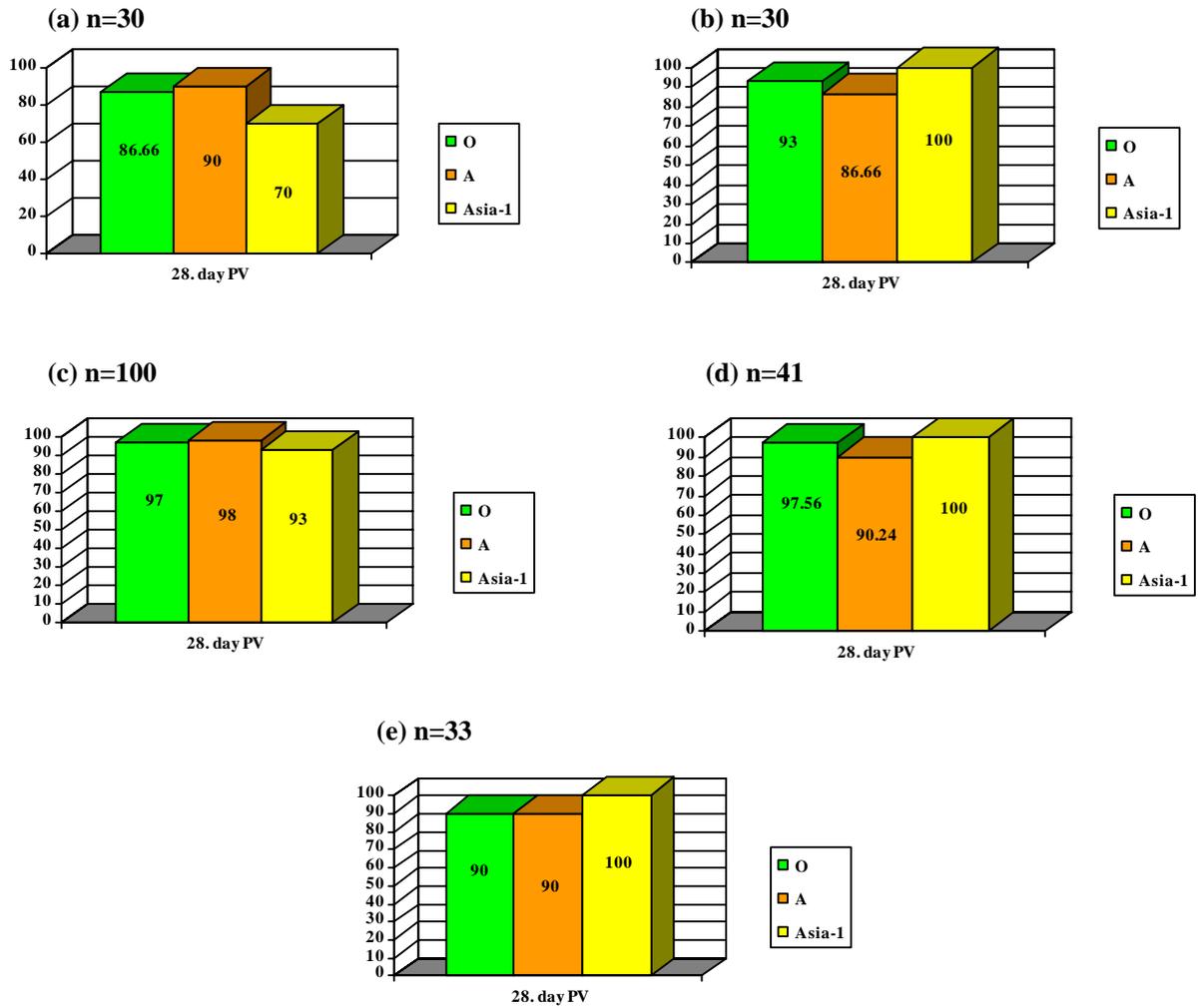


Figure 4 Protection rates (%) for cattle in (a) Sivas-Ulas; (b) Ankara-Bala; (c) Urfa-Ceylanpinar; (d) Aksaray and (e) Samsun-Gelemen.



Discussion paper on guidelines for control of Foot-and-Mouth Disease (FMD) vaccine quality and performance in the field

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Abstract

In many parts of the world with endemic FMD or intrusions from neighboring countries, control of the disease is relying on vaccination of cattle and other susceptible species. In principle, vaccines should be produced and should perform according the directions of the European Pharmacopoeia (E.P.) with a guarantee of safety (correct inactivation plots) and sufficient potency. Also, ideally, vaccines should be purified and not induce antibodies against non-structural proteins. The last will enable the verification of freedom of FMD in vaccinated herds. In particular if the numbers of doses that are needed are difficult to predict, or if purified vaccine is required we recommend purchasing concentrated antigen. The formulation and bottling can be carried out locally. If the purchased antigen is stored frozen at ultra-low temperature (vaccine bank principle), the quantities needed can be produced at any time. Also, it will reduce the price of the vaccine (low transport costs), and guarantee a shelf life of at least 5 years. The veterinary authorities of the receiving country must, independently, control the vaccine efficacy. Therefore, it is important that veterinary services have clear guidelines on how to control and verify the quality of the vaccines, their performance in the field, and vaccine coverage. Israel has a long standing experience in this respect. Tests must be simple and relatively easy to perform and the use of live virus in the test should be avoided (no VNT!). The LPB-ELISA (with inactivated antigen) offers the internationally accepted test to verify these conditions. The World Reference Laboratory and the National Control Laboratories in South America consider a LPB titer of 1:100 (2 logs) as protective and – with some variation between countries - in South America 12 out of 16 cattle must reach that level shortly (6 weeks) after vaccination. Such titers are considered to be protective against (the heavy) challenge under laboratory conditions. The Russian FMD Institute FGI-ARRIAH considers 1:40 (1.6 logs) as sufficient protective. Cattle with these levels are probably protected under field conditions. This paper discusses the various aspects of FMD vaccine control and performance in the field with emphasis on vaccine control in Israel.

Introduction

In many countries there is a continuous threat from neighboring countries or from foci within the country where vaccination is not well possible (e.g. wild-life reservoirs). In particular for areas with a high cattle density FMD will spread rapidly. Even though mortality can be high in young animals, in adult cattle it usually is not more than 5%. However, recovered cattle may suffer serious sequels, such as unthrift ness, mastitis, abortion, infertility, and chronic heat intolerance syndrome (HIS, also known as “panthers”). Also, in areas where oxen are used to plough and harvest of crops FMD outbreaks can have serious socio-economic consequences because recovered oxen often loose their ability for hard labor. Dependent on the local husbandry systems FMD can –like in South America and in Europe in the past – be controlled by the vaccination of cattle only. However, in other areas like the Middle East the roaming herds of sheep and goats and the intensive marketing system of these animals enables FMD virus to remain circulating in those herds. Under such circumstances vaccination of sheep and goats is necessary as well. When pig herds are endangered, the boars and sows could be vaccinated (with oil emulsion vaccines), however, around outbreaks all pigs must be vaccinated. Because oil emulsion vaccines induce, in general, a longer lasting immunity, and can be used for cattle, sheep, and pigs, this type of vaccine is recommended. For successful control it is essential that the vaccine is of good quality and contains the correct vaccine strains that cover the local field strains. Logistics of transport (cold chain) must guarantee that the quality of the vaccine is maintained until used in the field. To reach sufficient herd protection and to stop spreading of disease, it is essential that more then 80% of the animals be vaccinated. Because producers, whether private or governmental, want to market their products, independent control of the quality of the vaccines is required. Also, duration of immunity and herd coverage are important elements to make sure that FMD cannot spread and is effectively controlled. Here we will give guidelines to perform all steps in a proper way.

Guidelines for control of FMD by vaccination

1. General, zonal, or incidental vaccination

1.1. Situations where general vaccination is recommended:

- Frequent occurrence of outbreaks all over the country
- Continuous and frequent threat from foci at the borders or within the country (e.g. wild-life reservoirs);
- Sufficient financial means to enable general vaccination programs;
- Low incidence but large (valuable) cattle population;
- Good infrastructure, with consistent FMD surveillance ;
- In the context of a regional eradication program e.g. like in South America.

1.2. Situations where incidental or zonal vaccination is recommended:

- Limited financial means;
- Incidental threat from outside, buffer zone vaccination at the borders to prevent outbreaks inside the country;
- Consistent surveillance systems all over the country, a pen-side test may contribute in rapid diagnosis (Kitching, 2004);
- Good contingency plans (including vaccine bank) for ring-vaccination around outbreaks available.

2. Species that must be vaccinated

2.1. All susceptible species:

- In the face of an outbreak (s);
- When small ruminants are regularly brought to markets or when they are not pastured at fixed places (like in the Middle East) and when pigs are kept in large holdings. In the latter case breeding stock (boars and sows) must be vaccinated; However, in the face of outbreaks vaccination of all pigs is required. Depending on the type of oil adjuvant, local reactions may occur and cause problems up to one month after vaccination. Suckling piglets can be vaccinated with a (double) oil emulsion vaccine intra-peritoneally to avoid local lesions at time of slaughter (Augé de Melho et al. 1987 and 1988). Double emulsions, in general, cause little local reaction.

2.2. Cattle only if:

- There is a small population of small ruminants;
- Small ruminants housed or pastured (mainly) at fixed places. E.g. in Uruguay only cattle are vaccinated. They graze in open pastures mixed with sheep and sometimes pigs. In general they live there until slaughter.

3. Vaccine strains needed

Effective vaccination campaigns can be carried out with vaccines containing standard vaccine strains. Only occasionally has the incorporation of an emerging field strain been necessary. In general, such emerging strains will only be identified if other reasons for vaccination failure can be excluded. Breakthroughs in vaccinated livestock can often be controlled by re-vaccination with a potent vaccine containing standard antigens. The difficulties of adapting field isolates to the conditions of vaccine production and all the required testing must not be underestimated e.g. with regard to industrial growth of the virus in cell systems, proper inactivation and purification, antigen stability and shelf life of vaccines.

Vaccine strains incorporated in the vaccine can be selected on the basis of:

- Advice of local laboratory (if available and capable);
- Advice of WRL;
- Advice of (potential) producers;

- Strains selected in the context of regional co-operation (favored situation).

4. Purchasing vaccine or concentrated antigen?

Conditions and arguments for buying concentrated antigen:

- Ultra-low temperature (ULT) storage facilities (+ back-up power unit)
- Facilities for formulation and bottling available (e.g. at local laboratory or pharmaceutical plant)
- Flexibility, counts in particular when vaccines are incidentally required;
- Possibility to increase the payload of the vaccines for rapid control of outbreaks;
- Economical.

Note: Purchasing of commercial completely formulated vaccine has the advantage that the producer is responsible for failures. If vaccine is formulated from concentrated antigen that is bought elsewhere, the local laboratory that carries out the formulation and bottling of the vaccine becomes responsible for eventual failures or other problems.

5. Type of adjuvant:

- Aqueous (Al(OH)₃ – saponin) for ruminants only (not for pigs);
- Oil emulsion for all species.

6. Tender:

- No tender when there is a fixed relationship with a producer (e.g. a national laboratory);
- Tender for commercial producers (at least 3), preferably working according to (internationally approved and certified) Good Manufacturing Practice (GMP). If a company or laboratory claims to work according to a Quality-based Operational System (Q-BOS) – not all requirements for GMP (e.g. for equipment) fulfilled, however, fixed procedures and controls and quality of product guaranteed (Barteling, 2004) – this must be verified by an audit (e.g. once every 3 year) if possible by an independent inspector/specialist in the field of (FMD) vaccine production;
- Judgment of obtained data;
- Selection of producer (price per dose).

7. Testing / verifying the quality of vaccine batches to be purchased:

“You get what you inspect not what you expect” is a slogan that certainly holds true in the field of veterinary vaccines. Also, it is essential to make sure that the vaccine can do the job. If this testing is not incorporated one is always behind the facts. This testing is necessary for successful control of FMD as the history from South America (and other places) has learnt us. If the vaccine is of insufficient quality, there might be incidental cases of FMD in the vaccinated population and the farmers will lose their believing in vaccination and will become less cooperative.

If sufficient quality of the vaccine is verified only by monitoring the campaign it may become a frustrating business in case of poor quality vaccine. One can only hope for the best and that next time the producer will provide better quality, however, one has no guarantee. Also, negative results in the survey may be explained by the producer by failures in the campaign or cold-chain and breakthroughs by strain differences.

- Checking safety test results of the antigen batches that are incorporated in the vaccine batch (es), as presented by the producer: correct inactivation plots, and *in vitro* (cells) safety tests.
- Testing of overall protein concentration of the vaccine or of the concentrated antigen (must be in accordance with producers purity estimations/guarantee). This can be carried out by using a Lowry test, or a commercial test kit (e.g. from Pierce).
- Testing for absence of non-structural proteins (NSP) by ELISA (if required). In principle, the α -NSP ELISA can be used in a direct mode for testing concentrated (purified) antigen for the presence of traces of NSP. It is possible that this test can also be applied for testing vaccines directly.

- Anyhow animals that have repeatedly (up to 5 times) been vaccinated with vaccine from the same producer must remain negative (if absence of NSP is claimed by the producer).
- Testing of potency: Vaccinate 16 "clean" calves (no significant maternal antibody levels). In 3 (aqueous) or 6 weeks (oil) time 13 out of 16 must reach antibody levels (LPB-ELISA, Hamblin et al., 1987) of 2.0 logs (to guarantee 1.6 later on after vaccination).

8. Monitoring quality upon arrival: see 7

9. Storage and cold-chain facilities

Upon arrival the vaccine should be stored in a very reliable cool house with technical backup. Also, there must be cold-chain provisions for transportation of the vaccine to the local veterinary posts and for the distribution of the vaccine in the field.

10. Vaccination campaigns

Experience from the Middle East and from Europe in the past learned that FMD outbreaks occur (or start) in the winter time February/March. Therefore, it is recommended that the annual vaccination campaign (s) should be carried out at least 3 month in advance which means October / November and vaccination of new born young calves (3 month old) in January. By this schedule one may face the FMD period with completely vaccinated livestock.

11. Monitoring vaccination campaign(s)

Monitoring of vaccination campaigns is very dependent on local facilities: presence of sufficiently large farms (with at least 20 clean calves in one age group) located in different area's of the country (to make it unlikely that these farms become all involved in outbreaks) and laboratories (+staff) and logistics and other infrastructural requirements to do all the tests. The same counts for the sheep and/or goats.

In general, it will be difficult to find such farms. In fact on such farms veterinary services / laboratory must take over the responsibility of all FMD vaccination on the farm. Farmers also want guarantees in case anything happens.

The sera of the animals from the test can also be used to monitor protection against outbreak strains.

For an overall picture one can take at random samples at slaughterhouses that are representative for the different districts. If monthly a total of 1.000 (?) sera of slaughtered animals are tested one may get a fair impression of overall herd immunity throughout the year. After all, every animal will end up in the slaughterhouse.

Vaccination schedule: The first 3 vaccinations (of calves) every 6 months, thereafter once a year.

Verifying endurance of immunity: Not less than 3 groups of 20 calves not previously vaccinated, on farms located in different areas of the country, will be followed during 6 months after vaccination. After that they will be revaccinated and followed for another 6 months. Serum samples will be taken every month.

Evaluation of herd immunity (can be carried out on the same farms as mentioned under 2):

- Method: Testing of serum samples by LPB-ELISA. Titer of >1.6 (1:40) can be considered protective against a first take.
- When? Testing should preferably be carried out shortly before and 3 – 6 weeks after vaccination campaigns.
- What?
 - Testing of serum samples from slaughterhouses;
 - Testing of serum samples from "controlled farms (see 2) and, if possible, randomly selected (representative) dairy, beef, and sheep (if applicable) farms;
 - Districts represented in the testing;
- Requirement: at least 80% of the animals within herds must be protected throughout the year.

12. Surveillance for sub-clinical infections in vaccinated herds by a-NSP antibodies

- Routinely, e.g. on slaughterhouse sera;
- Around outbreaks.

13. Annual evaluation and reporting

- Evaluation of monitoring and surveillance systems;
- Evaluation of the results of vaccination campaigns;
- Reporting to:
 - The ministry;
 - The farmers organization (s);
 - The press;
 - The regional FMD organization (if applicable);
 - The FAO / OIE and/or sponsoring organizations like the EU

Evaluation and annual planning

On the basis of the results of the past year and the current situation (outbreaks in surrounding countries (new field strains etc.) the FMD situation must be evaluated as well as current monitoring and surveillance systems. If necessary these systems must be improved. In accordance, the annual vaccination campaigns must be planned.

Discussion

The guidelines above are a step by step approach of what is – in our views – required in order to control FMD by vaccination in an endemic area. Israel is a good example of how a consistent vaccination policy can control FMD in an environment of continuous external disease pressure and their procedures stood model for the above guidelines.

Situations in many countries with a continuous threat of FMD may differ, however, with those in Israel:

- Not all countries have highly valuable livestock like in Israel, with an highly organized veterinary infrastructure.
- In many countries farmers cannot afford to pay the world market prizes for FMD vaccine.
- For above or other reasons veterinary services may be bound to the use of vaccines that are locally produced often by a national laboratory of the country itself or of a neighboring country.
- Vaccine producers in developing countries often cannot afford or don't have access to the high standards of equipment or of building facilities that are internationally required for GMP accreditation. However, their procedures might be according to GMP with fixed production steps in a closed system described in standard operating procedures and adequate controls of each production step (e.g. sterility). Such a Quality-based operational system carried out by well-trained operators can guarantee the production of quality vaccines as well. If, the production unit is large enough the vaccine can probably be produced for competitive prizes.
- An alternative can be to buy concentrated (purified) antigen from the big international producers against competitive prizes. The formulation and bottling being carried out locally. Tenders can be made per virus strain. E.g. O1 Campos, a vaccine strain with a proven broad protection (e.g. against O1 Taiwan) can be bought from one of the South American producers working under a GMP regime while other strains are bought elsewhere.

Whatever the situation, history has learned that, in general, the quality of the vaccines must be tested by a laboratory or institution that is independent from the producer (or formulator) of the vaccine. In addition, reporting of results of tests (like in South America) and evaluation of the performance in the field (+ reporting) are other elements that will stimulate producers to make the quality of the vaccines to the highest standards.

References

Augé de Mello, P., Gomes, I., Alonso Fernandez, A. & Mascarenhas, J.C. 1978. Foot-and-mouth disease oil-adjuvanted vaccine for pigs. Intraperitoneal vaccination of young pigs with double emulsion vaccine. Bol. Centro Panamericano fiebre Aftosa 31-32: 21-27.

Augé de Mello, P. 1979. Reflections on the prevention of foot-and-mouth disease in swine. Bol. Centro Panamericano fiebre Aftosa 39-36: 59-61.

Barteling, S.J. 2004. Modern inactivated Foot-and-Mouth Disease (FMD) vaccines: Historical background and key elements in production and use. In: "Foot-and –Mouth Disease. Current perspectives", Sobrino, F. and Domingo, E. editors, Horizon Bioscience, Wymondham, Norfolk, U.K. p.305-334

Hamblin, C., Kitching, R.P., Donaldson, A.I., Growther J.R. & Barnett, I.T. 1987. Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. III. Evaluation of antibodies after infection and vaccination. Epidemiol. Infect. 99, 733-744

Kitching, R. P. 2004. Diagnosis and control of foot-and-mouth Disease In: : "Foot-and – Mouth Disease. Current perspectives", Sobrino, F. and Domingo, E. editors, Horizon Bioscience, Wymondham, Norfolk, U.K. p.411-424.

Shimshoni, A. 2002. Foot-and-mouth disease: The Israeli approach.
http://www.israel-embassy.org.uk/web/pages/fmd_isr.htm

ADDENDUM

A model of technical specifications for FMD vaccines for a tender (as applied in Israel)

A. Requirements for Trivalent FMD vaccine for cattle

1. Antigen composition: trivalent vaccine should be composed of the following serotypes and isolates:
 - 1.1 O₁ Manisa, + O₁ Isr. 2/85 (Geshur), + O₁ 3039.
 - 1.2 A₂₂ Iran 87, +A turkey1/98 or equivalent.
 - 1.3 Asia₁ Isr. 3/89 (Shamir).
2. Antigen purification. It is required that the antigen will be purified for 90% and higher of (non-FMD) proteins. The purification process should use PEG or ultra-filtration (or both).
3. Adjuvant: the adjuvant should be double oil emulsion (w/o/w) Isa 206 or equivalent.
4. Safety.
 - 4.1. The producer /Supplier should present the inactivation curves of The antigens presented in the vaccine.
 - 4.2. The vaccine should be accompanied with results of safety control In cell culture and cattle.
 - 4.3. The producer /Supplier should present evidence that the vaccine does not cause any allergic reactions in cattle and pregnant cows, even after repeated vaccinations.
5. Efficacy.
 - 5.1. The vaccine documents should contain the results of efficacy test in cattle. The vaccine should contain not less than 6 PD₅₀ per type per dose.
 - 5.2. The antigen load expressed as 146S per type per dose should be mentioned in the vaccine documents.
 - 5.3 The vaccine documents should contain the results of virus load before inactivation expressed as TCID₅₀ per 1 ml.
6. Expiry: expiry date should be 18 month started at the last day of production.
7. Doses for cattle: 2 ml for trivalent cattle vaccine
8. Packaging: 100 doses per bottle.
9. Supply date will be fixed.
10. The label on each bottle should contain: producer name, place of production, batch number, quantity of dose, volume vaccine in ml per bottle, production date and expiry date.

11. Responsibility: the producer/supplier of the vaccine will be responsible for any harm caused by neglecting the indicated requirements.

B. Requirements for Monovalent O1 FMD vaccine for Sheep and goats.

1. Antigen composition.
The vaccine (against sero-type O) should be composed of the following vaccine strains:
O₁ Manisa, + O₁ Isr. 2/85 (Geshur), + O₁ 3039.
2. Antigen purification.
It is required that the antigen will be purified for 90% and higher of non-FMD proteins. The purification should be carried out by PEG-precipitation and/or by ultra-filtration methods.
3. Adjuvant.
The adjuvant should be double oil emulsion (w/o/w) Isa 206 or equivalent.
4. Safety.
 - 4.1. The producer /Supplier should present the inactivation curves of The antigens presented in the vaccine.
 - 4.2. The vaccine should be accompanied with results of safety control In cell culture and sheep.
 - 4.3. The producer /Supplier should present evidence that the vaccine does not cause any allergic reactions in sheep and pregnant sheep, even after repeated vaccinations.
5. Efficacy.
 - 5.1. The vaccine documents should contain the results of efficacy test in sheep, it should contain not less than 6 PD₅₀ for sheep and goat per dose.
 - 5.2. The results of antigen load expressed as 146S per dose should be added to the vaccine documents.
 - 5.4 The vaccine documents should contain the results of virus load before inactivation expressed as TCID₅₀ per 1 ml.
6. Expiry
Expiry date should be 18 month started at the last day of production.
7. Dose
Doses, for sheep/goat: 1ml for monovalent sheep/goat vaccine.
12. Packaging: 100 doses per bottle (100ml).
13. Supplying date will be fixed.
14. The label on each bottle should contain: producer name, place of production, quantity of dose, volume of vaccine in ml per bottle, batch number, production date and expiry date.
15. Responsibility;
The producer/supplier of the vaccine will be responsible for any harm caused by neglecting the indicated requirements.
The producer/supplier of the vaccine will be responsible for any harm caused by neglecting the indicated requirements.

Recommendations for tests for induction of antibodies to NSP antigens by FMD vaccines

Extract from source: EMEA/CVMP/775/02-FINAL. Committee for Medicinal Products for Veterinary Use (CVMP) Position Paper on Requirements for Vaccines against Foot-And-Mouth Disease

Position Paper adopted by CVMP on 16 June 2004, with date of coming into effect of 16 December 2004. <http://www.emea.eu.int>

Quality requirements in support of information related to non-structural proteins

Manufacturers should supply data to substantiate information provided on the Summary of Product Characteristics (SPC) that a vaccine does not induce antibody to one or more NS proteins. To support such information:

(i) The manufacturing process should include one or more steps to purify the virus from cellular or other contaminants, including NS proteins that are produced during virus growth in cell culture. As part of the authorisation dossier, the manufacturer should present data from immunochemical tests such as SDS-PAGE, immunoblotting, or some form of competitive based assay to demonstrate that the purification process reduces the level of NS proteins in the final purified, concentrated antigen. However, it is unlikely that any purification process can completely remove NS proteins and there is currently no method of predicting the immunogenicity of any proteins remaining. Therefore demonstrating that the vaccine does not induce significant levels of NS antibody in immunised animals currently represents the only means of supporting information on the SPC relating to NS antibody.

(ii) The manufacturer should conduct a test to demonstrate that repeated immunisation of one or more of the indicated species with vaccines formulated to contain the maximum permitted amount and number of antigens does not result in seroconversion to NS proteins. Such a test should be required to be conducted at least once for each FMD vaccine (as defined above) for which information is provided (i.e the information relates to the formulation of both adjuvants and antigens together and not to the antigens alone). In order to reduce the use of animals, it may be possible to conduct this test in the animals used for demonstrating the safety of the administration of a single dose, an overdose and a repeat dose in Part III of the application dossier. A recommended immunisation and testing program is as follows:

Prior to Day 0: Collect a sample of blood from a minimum of 10 animals to verify freedom from antibody to FMD virus structural and non-structural proteins. The animals should have no history of exposure to FMD, should not have been previously vaccinated and should be free of non-specific antibody to FMD virus NS antibodies.

Day 0: Administer to a single site a minimum of two doses of a vaccine containing the maximum permitted amount of antigen of each of the maximum permitted number of antigens (i.e. if the authorisation permits up to 1 5ug of up to four different antigens then the vaccine should contain at least 1 5ug of each of at least four different antigens)

Day 14-28: Administer a second, identical injection after the interval recommended in the basic vaccination schedule, usually two to four weeks

Day 42 onwards: Administer a third, identical injection between a minimum of one and a maximum of six months after the second injection.

Day 56 onwards: Collect serum samples between two and four weeks after the last vaccination and test for antibody to NS proteins. The antibody levels against specified NS proteins should be lower than those considered as positive in a validated test.

(iii) Manufacturers may use alternative immunisations and testing schedules provided that they can justify that the schedule used is the most likely to induce an antibody response to NS proteins.

(iv) The manufacturer should use a test that has been adequately validated and, where possible, one that has been recognised by an international organisation such as Office International des Epizooties (OIE). In the absence of internationally recognised standards for NS antibody serology, the manufacturer should justify the validation performed to the satisfaction of the competent authority by reference to published data, by independent validation of the test by an internationally recognised FMD reference laboratory and/or by conducting suitable 'in-house' validation studies.

Where possible, the data from experimental animals should be supported by field data derived under actual conditions of use. Data available to date indicate that some animals respond particularly well to some NS proteins but not to others and information on the prevalence of such animals can only be derived from field data. As stated under General Comments, this type of field data is considered as supportive rather than obligatory as it is likely to be generated under relatively uncontrolled conditions.

Care needs to be taken to ensure that the product information provided by the manufacturer is fully supported by the data presented. The test(s) used to detect antibody to NS proteins should always be indicated on the SPC together with the conditions under which lack of immunogenicity was proven (e.g. number of vaccinations, potency and valency of the vaccine, intervaccination interval). This will enable Competent Authorities to make an informed choice of the NS antibody test to be used as part of a surveillance program when NS antibody testing is to be included in a control strategy that involves emergency vaccination. As Competent Authorities for marketing authorisation of vaccines, have no competence in the EU with regard to diagnostic tests, it is inappropriate for manufacturers to propose claims related to 'marker' vaccines or 'differentiation of infection from vaccination' in Section 5 of the SPC. Freedom with respect to NS proteins is a quality issue and therefore the information provided should be restricted to defining precisely the nature of the immune response induced under Section 4 of the SPC 'Immunological Properties'.

Global Foot-and-Mouth Disease Situation 2003-2004

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Abstract:

The FAO World Reference Laboratory for Foot-and-Mouth Disease (WRL/FMD) is involved in FMD surveillance by characterising FMD isolates from around the world and by collecting epidemiological information. This paper describes the global FMD situation over the last 21 months.

WRL/FMD collects FMD epidemiological information through Promed, OIE website and by direct contacts with countries and references laboratories. Between January 2003 and September 2004, 870 clinical samples or isolates collected in 31 countries were submitted to WRL/FMD. From clinical samples, FMDVs were detected by cell culture, by ELISA and by RT-PCR. Serotypes were defined and a selection of isolates were further characterised both antigenically and genetically.

From the beginning of 2003 and up to now (30/09/04), FMD outbreaks were reported by 62 countries (49 countries in 2003 and 43 same or different countries in the first 9 months of 2004). No FMD outbreak was reported in FMD-free countries that did not practice vaccination. However, outbreaks occurred in FMD-free zones which did or did not practice vaccination. All other reported outbreaks occurred in countries in which FMD is endemic. Over a period of 21 months, serotypes O, A, C, Asia1, SAT 1, SAT 2 and SAT 3 were reported, at least once, by 44, 17, 3, 4, 11, 16 and 5 countries respectively; whereas FMDV involved in outbreaks in eight countries were never characterised. There were also unconfirmed reports that serotype C FMDV had been detected in Pakistan and Ethiopia. Among clinical samples received at WRL and subjected to phylogenetic analysis, the serotype O/PanAsia strain was the most prevalent strain detected and serotype A FMDVs maintained a very high degree of divergence. Serological matching tests suggested that classical vaccine strains will provide a good coverage against serotype O field isolates, whereas for serotype A more variable matching results were obtained. Selection of vaccine strains against FMDV serotype A appears to be not as straightforward as against serotype O.

Ongoing efforts are being made by WRL to coordinate and improve the global surveillance for FMD.

Introduction:

The FAO World Reference Laboratory for Foot-and-Mouth disease (WRL/FMD) is involved in FMD surveillance by receiving clinical samples or isolates from around the world and by compiling epidemiological information. Even if not all countries report their FMD outbreaks, a trend of the global FMD situation can be estimated. The characterisation of FMDVs that are sent to WRL/FMD helps national laboratories to confirm their diagnoses and allows us to further characterise these FMD isolates by phylogenetic studies and by evaluating their antigenic relationships with vaccine strains. The outcomes of these studies are helpful in vaccine strain selection. This paper describes the global FMD situation over the last 21 months as well as the characteristics of FMDV strains involved in these outbreaks.

Materials and Methods:

The WRL/FMD not only compiles epidemiological information accompanying submitted clinical samples, but also gathers information from Promed (<http://www.promedmail.org>), the OIE website (www.oie.int), the coordination project against FMD in South East Asia website (<http://www.seafmd-rcu.oie.int>) or by direct contact with countries that report FMD outbreaks. Outbreaks, origins of clinical samples and epidemiological information were mapped by using Arcview software. Maps presented during these meeting will be available on http://www.iah.bbsrc.ac.uk/primary_index/current_research/virus/Picornaviridae/Aphthovirus/index.html. FMDV was detected in clinical samples received at WRL/FMD by cell culture isolation, by antigen ELISA and/or by RT-PCR, as described by Ferris and Dawson (1988) and Reid *et al.* (2003). The antigenic relationships ('r' value) between field isolates and vaccine strains were evaluated by ELISA and/or virus neutralisation test (VNT) (Kitching *et al.*, 1988; 1989; Samuel *et al.*, 1990). Sequencing and phylogenetic analysis were performed as previously reviewed by Knowles and Samuel (2003).

Results and discussion:

During 2003 and until September 2004, FMD remained largely confined to traditionally infected areas. No outbreaks were officially reported in FMD-free countries that did not practice vaccination. However, outbreaks occurred in FMD-free zones which did not practice vaccination or in surveillance zones around the FMD-Free zones (Botswana, South Africa), in zones that were free with vaccination (Argentina) and in regions where FMD has not recently been shown to circulate (Libya, Russia, Mongolia, Peru, Brazil and Colombia). Many of these outbreaks have been brought under control. All other reported outbreaks have involved countries in the Middle East, Asia, Africa and South America, in areas where FMD was already endemic. In total, FMD outbreaks occurred (OIE website, or clinical samples were sent to WRL/FMD) in 50 and 44 countries during 2003 and 2004 (to 30/09/2004), respectively (Table 1). Whereas FMDV serotypes were never characterized in eight of these countries during this period; serotypes O, A, C, Asia1, SAT 1, SAT 2 and SAT 3 were reported by 35, 15, 1, 3, 10, 13 and 3 countries in 2003, and by 23, 9, 2, 2, 5, 7 and 2 in 2004. In addition, serotype C may have been detected in Pakistan and Ethiopia. The presence of two or more different serotypes was observed in 23 countries. Serotypes SAT 1, SAT 2 and SAT 3 remained localised in Africa and serotype Asia 1 in Asia. However, the presence of serotype C in South America, Africa and in Asia as well as the occurrence of serotype SAT 3 in West, Central and East African countries needs to be confirmed by a FMD reference laboratory and /or their origins must be determined.

Eight hundred and seventy six clinical samples or FMD isolates collected between 2000 and 2004 in 32 countries, located in Africa, Europe, Middle-East and Asia, were submitted to the WRL/FMD for virological examination during these last 21 months (Tables 2 and 3). No samples or isolates were received from South America, or former Soviet Union countries. By isolation in cell culture and/ or antigen ELISA, FMDV serotypes O, A, Asia 1, SAT 1 and 2 were detected in 219, 53, 10, 15 and 43 samples. One hundred and seventy six samples are still under process or characterisation (Sudan, Vietnam and Yemen, Zambia). Some improvements have been made in the diagnostic service offered by the WRL/FMD. An RT-PCR for detection of FMDV in real time, based on the conserved IRES region, has been developed (Reid *et al.*, 2003). The assay has been evaluated on samples received at WRL/FMD and has been compared to virus isolation and FMDV detection by ELISA. Compared to these two conventional techniques, the RT-PCR appeared to be highly sensitive and specific and allowed detection of more positive samples (Nigel *et al.* 2004; Shaw *et al.*, in press).

Some of the FMDV isolates were further characterised by phylogenetic analysis or by studying their relationships with vaccine strains.

African FMD isolates, received at WRL/FMD during 2003 and 2004, were collected in eleven countries (Botswana, Burundi, Libya, Malawi, Mozambique, Namibia, Rwanda, Sudan, Uganda, Zambia and Zimbabwe) between 2000 and 2004, and were characterised by phylogenetic analysis of the VP1 gene. These isolates belonged to serotypes O, SAT1 and SAT2. FMDV serotype O collected in Burundi, Uganda, Rwanda and Zambia belonged to a recently characterised toptotype, EA-2 (Knowles *et al.*, 2004). Isolates collected in Sudan are intermediate between WA and EA-1 toptotypes (Knowles *et al.*, 2004). SAT 1 FMDVs, collected in Malawi, Namibia, Zambia, and Zimbabwe, showed a high degree of divergence. However, all SAT 1 isolates from Zimbabwe, except one, were closely related. One SAT1 isolate collected in Zambia was closely related to viruses collected in Malawi and a second Zambian isolate was closely related to a virus collected in Namibia, in line with their geographical origins within different areas of Zambia (Fig. 1). SAT2 FMDVs were submitted from Botswana, Libya, Malawi, Mozambique, Rwanda and Zimbabwe. A high degree of genetic diversity was found (Fig. 2). Libya submitted FMD viruses that belonged to the SAT 2 serotype, which has not been detected in North Africa since 1950. A close genetic relationship with viruses collected in Cameroon in 2000, Saudi Arabia in 2000 and Eritrea in 1998 was demonstrated, but these viruses were distinct from those analysed from Southern Africa (Fig. 2) Four distinct lineages were found in Zimbabwe; one included viruses from Botswana, and another included an isolate from Mozambique, while the other two appeared to be unique to Zimbabwe. Two isolates from Malawi in 2003 were distinct from all the other viruses examined (Fig. 2). By virus neutralisation it appeared that serotype O field isolates collected in Burundi were matching well to O₁ Manisa (n=3). The evaluation of relationships between FMDV SAT 1 and 2 with vaccine strains are still under investigation. However, according to 'r' values obtained by ELISA, SAT 2 isolates from Libya were matching well to SAT 2 Sau 2000 (n=2) and moderately to Zim 7/83 (n=1) and Zim 11/89 (n=1).

FMD isolates obtained from the **Europe** and **Middle East** were collected in one and six countries (Turkey; Iran, Israel, Lebanon, Palestinian Autonomous Territories, Saudi Arabia and the United Arabia Emirates) between 2002 and 2004, respectively. Phylogenetic analyses showed that serotypes O and A from different lineages and sub-lineages were co-circulating (Figs. 3 and 4). For Serotype O, the PanAsia strain was detected in Turkey, Iran, Israel, Lebanon and Saudi Arabia. Isolates belonging to the Irn2001 and Ind2001 strain were also detected, the former in Iran and United Arabia Emirates

(data not shown) and the latter in Palestinian Autonomous Territories (2002). Isolates Serotype A was only detected in Iran and in Turkey; but in each of these countries, several sub-lineages were present. In Turkey, FMD isolates of Irn96 or Irn99 strains were present and in Iran, some isolates belonged to the Irn96 strain and others to different sub-lineages that have not yet been named. Considering antigenic relationships between field isolates and vaccine strains, FMDVs from serotype O were matching well to O Manisa by VNT (n=8), and also by ELISA with O Manisa (n=3), 3039 (n=3), Geshur Isr 2/85 (n=3) and Dalton Isr 2/88 (n=3). For serotype A according to r values obtained by ELISA, a poor matching with A₂₂ Irq 24/64 (n=4) was obtained that was confirmed by VNT (n=3); a moderate matching with A Sau 95 (n=4); a good to moderate matching with A Irn 96 (n=4), A Sau 23/86 (n=4), A Irn 87(n=4) and a good matching with A Irn 99 (n=4) and A May 97 (n=4).

FMDVs from **Southern Asia** were collected in Afghanistan, Bhutan, Nepal and Pakistan between 2002 and 2004. Serotypes O, A and Asia 1 and sublineages were detected and with particular frequency in Pakistan (Figs. 3 to 5). For serotype O, the PanAsia strain (Fig.3) was detected in Afghanistan, Bhutan, Nepal and Pakistan (not shown). Isolates that belonged to the strains Ind2001, Irn2001 and Pak98 were also found in Pakistan (Fig. 3). Serotype A isolates were detected in Pakistan and Bhutan but in distantly related sub-lineages (Fig. 4). The Asia 1 isolates obtained from Pakistan during 2003 were closely related to, but distinct from, Pakistani isolates obtained in 2002 (Fig. 5). Both these groups belonged to the same genetic lineage as viruses responsible for the incursion of Asia 1 into Greece in 2000, which can be traced back to the Indian subcontinent and the Middle East as far back as 1994 (data not shown). Considering 'r' values obtained by VNT (n=10) a good matching was obtained with O Manisa and by ELISA for different sublineages of serotype O (n=12) a good matching was obtained with O Manisa, 3039, Phi 95, Tai 189/87 and O TNN 24/84, whereas a poor to moderate matching was obtained with 4174 or BFS. For isolates belonging to serotype A (n=7) a good matching was obtained with A Irn 99, 4164, A May 97, moderate with A Sau 95, A Sau 23/86 and no or poor with 5925, A Irn 96, A 22 Irq 24/64 (which was confirmed by VNT), A Irn 87 and Ind 17/82. For serotype Asia 1 (n=3), a good matching 'r' value was obtained with Asia1 Shamir 3/89 and WBN 117/85.

Strains collected in **South East Asia** between 2002 and 2004 were obtained from Hong Kong, Lao PDR, Malaysia, Philippines, Thailand and Vietnam. Considering serotype O, ME-SA toptotype/ PanAsia strain was again the most prevalent virus (except in Hong Kong and Philippines where only the pig adapted Cathay toptotype strains were detected) (Fig. 3). However, in Lao PDR and in Malaysia, a few isolates belonging to SEA toptotype were detected (Mya98 and Cam94 strains, respectively). It is noteworthy that some isolates related to the PanAsia strain obtained from Lao PDR, collected in the Bokeo region, showed a certain degree of genetic divergence (Fig. 3). It remains unexplained if these isolates are the result of a particular evolution of the PanAsia strain or if their origin can be traced to different countries, like India or China. Isolates of serotype A, detected in Malaysia, were clustered in the toptotype Asia/ sublineage SEA and were closely related to isolates from Thailand collected in 2003 (Fig. 4). According to 'r' values obtained by ELISA and/or virus neutralisation, field strains isolated in southeast Asia from serotype O (n=10) were matching well with most of the vaccine isolates (O Manisa, Phi 95, TNN 24/84, Tai 189/87, 3039 and 4174). Isolates of serotype A (n=7) showed a good matching with May 97, moderate to good with Sau 23/86, Irn 87, Tai 118/87 and Tai ASK 99 and no or poor with A₂₂ Irq24/64 and A5925.

In the near future, the global surveillance performed by WRL/FMD will be improved through efforts performed in molecular epidemiology and also through collaborations and a coordination action that is to be funded by the European Community, hopefully from January 2005.

Conclusions:

- FMDV is still active in many parts of the world; however, only some countries report their FMD outbreaks or try to characterise FMDV isolates.
- The PanAsia strain of serotype O remains the most prevalent FMDV strain in the Middle East, Southeast Asia and the Far East, but not in Africa. FMDV strains of serotype A that are present in Middle East and southern Asia have a very high degree of sequence variation.
- Serological matching tests suggest that available vaccine reserves for serotype O are still appropriate, but as in previous years, serotype A viruses from the Middle East and Asia exhibit considerable antigenic variation. The strains that are circulating in Iran and Pakistan are poorly matched by traditional vaccine strains such as A₂₂ Iraq, but some newly evaluated vaccine strains (such as A May 97 or A Irn 99) show promise. Vaccine recommendations for antigen banks, reviewed at the Research Group Meeting in Gerzensee, September 2003, seem to remain relevant.

- To improve global surveillance performed by WRL FMD, efforts continue to solicit sample submissions, however the cost and difficulties of sending infectious goods by air remains a considerable constraint. An increased effort is also made in WRL/FMD to sequence the complete VP1 gene of all received FMDV isolates and all capsid genes for selected isolates. A web based system and a new database is under development for processing, analysing and storing sequence data, to make them available more efficiently to the scientific community. This will also help in the tracing of outbreaks in endemic countries and in prediction of antigenic changes.

Recommendations:

- Efforts must be made to make information available on the antigenic and genetic characteristics of isolates collected in South America, China and former Soviet Union countries.
- Considering the information on the occurrence of FMD that is reported on the OIE website (Handistatus II), it would be useful to have an indication of which laboratory has confirmed the clinical diagnosis and the techniques that have been used for this purpose. The presence of serotype C and the occurrence of serotype SAT3 in some West and East African countries must be confirmed by a reference laboratory and their origin determined.
- A better coordination between reference laboratories will improve the global surveillance of FMD. Antigenic and genetic data could be shared between reference laboratories on a website.
- A more comprehensive system of vaccine matching is required for global surveillance.

Acknowledgements

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References:

- Ferris, N.P. & Dawson, M.** 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet. Microbiol.*, 16: 201-209.
- Ferris, N.P., Reid, S.M., King, D.P., Hutchings, G.H. & Shaw, A.E.** 2004. Prospects for improved laboratory diagnosis of FMD using real-time RT-PCR. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Chania, Crete, Greece, 12-15th October 2004. Rome: FAO, Appendix 42 (*This proceedings*).
- Kitching, R.P., Rendle, R. & Ferris, N. P.** 1988. Rapid correlation between field isolates and vaccine strains of foot-and-mouth disease virus. *Vaccine*, 6: 403-408.
- Kitching, R.P., Knowles, N.J., Samuel, A.R. & Donaldson, A.I.** 1989. Development of foot-and-mouth disease virus strain characterisation - a review. *Trop. Anim. Hlth Prod.*, 21: 153-166.
- Knowles, N.J. & Samuel, A.R.** 2003. Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.*, 91: 65-80.
- Knowles, N.J., Davies, P.R., Midgley, R.J. & Valarcher, J.-F.** 2004. *Identification of a ninth foot-and-mouth disease virus type O toptotype and evidence for a recombination event in its evolution.* Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Chania, Crete, Greece, 12-15th October 2004. Rome: FAO, Appendix 24 (*This proceedings*).
- Reid, S.M., Grierson, S.S., Ferris, N.P., Hutchings, G.H. & Alexandersen, S.** 2003. Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J. Virol. Methods*, 107: 129-39.
- Samuel, A.R., Ouldrige, E.J., Arrowsmith, A.E.M., Kitching, R.P & Knowles, N.J.** 1990. Antigenic analysis of serotype O foot-and-mouth disease virus isolates from the Middle East, 1981 to 1988. *Vaccine*, 8: 390-396.
- Shaw, A.E., Reid, S.M. King, D.P., Hutchings G.H. & Ferris. N.P.** In Press. Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chain reaction. *OIE Scientific and Technical Review*.

Table 1. Countries that have reported FMD outbreaks in 2003 and in 2004 (up to 30/09/04) and FMD serotypes related to those outbreaks (Sources: OIE website or results determined at WRL/FMD).

Country	FMDV serotypes	
	2003	2004
Afghanistan	O	
Argentina	O*	
Barhain	O*	
Bangladesh	(?)	
Benin	O*, SAT 1*, 2*, 3*	(?)
Bhutan	O, A	O
Bolivia	O*	
Botswana	SAT 1*	
Brazil		O*, C*
Burkina Faso	(?)	(?)
Burundi	O	
Cambodia	(?)	(?)
Cameroon	O*, A*, SAT 1*, 2*, 3*	
Chad	O*, A*, C*, SAT 1*, 2*, 3*	(?)
Colombia		A*
Ecuador	O*	O*
Eritrea	(?)	(?)
Ethiopia	(?)	(?)
Georgia		(?)
Ghana	O*	(?)
Hong Kong (China)	O	O
India	O*, A*, Asia 1*	O*, A*, Asia 1*
Iran	O, A, Asia 1	O, A
Israel		O
Kenya	O*, A*, SAT 1*, 2*	O*, A*, C* SAT 1*, 2*
Kuwait	O*	(?)
Kyrgyzstan		(?)
Lao PDR	O	O*, A*
Lebanon	O	
Libya	SAT 2	
Malaysia	O, A	O, A*
Malawi	SAT 2	SAT 2*
Mali	(?)	(?)
Mongolia		O*
Mozambique	SAT 1*	
Myanmar	O*	O*
Nepal	O, A	O*, A*, Asia 1*
Niger	O*, SAT 1*, 2*	SAT 1*
Nigeria	O*, A*, SAT 1*, 2*	
Oman	O*	
Pakistan	O, A, Asia 1	
Paraguay	O*, A*	
Peru		O*
Philippines	O	O
Russia		O*
Rwanda		O, SAT 2
Saudi Arabia		O
South Africa	SAT 2*	SAT 2*
Sri Lanka	O*	(?)
Sudan		<i>Clinical samples in process (O)</i>
Tajikistan	A*	
Tanzania	O*, SAT 1*, 2*	O*, SAT 1*, 2*, 3*
Thailand	O, A	O*, A*
Togo	SAT 2*	
Turkey	O, A	
Uganda	O*, SAT 2*	O
United Arab Emirates	O	
Venezuela	O*, A*	A*
Vietnam	O	(?)
Yemen	<i>Clinical samples in process</i>	<i>Clinical samples in process</i>
Zambia		O*, SAT 1*, 2*, 3*
Zimbabwe	SAT 1, 2	SAT 1, 2

*, not confirmed at WRL

(?), FMD outbreak(s) reported but FMDV not characterised

Table 2. Clinical samples received for FMD diagnosis received at the WRL/FMD* in 2003

Country	No. of samples	ELISA/Virus isolation in cell culture							RT-PCR for FMDV				
		FMDV serotypes							SVDV (a)	NVD (b)	Positive	Negative	Not tested
		O	A	C	SAT 1	SAT 2	SAT 3	Asia 1					
AFGHANISTAN	57	8	-	-	-	-	-	-	-	49	16	41	-
BHUTAN	46	7	7	-	-	-	-	-	-	32	35	11	-
BOTSWANA	20	-	-	-	-	-	-	-	-	20	-	18	2
BURUNDI	7	5	-	-	-	-	-	-	-	2	5	2	-
CHINA (HONG KONG)	7	3	-	-	-	-	-	-	-	4	7	-	-
IRAN	41	21	9	-	-	-	-	-	-	11	31	10	-
ITALY	10	-	-	-	-	-	-	-	10	-	10**	-	-
LAOS PDR	35	33	-	-	-	-	-	-	-	2	35	-	-
LEBANON	2	2	-	-	-	-	-	-	-	-	2	-	-
LIBYA	10	-	-	-	-	2	-	-	-	8	1	9	-
NEPAL	6	5	-	-	-	-	-	-	-	1	5	1	-
PAKISTAN	81 ^(c)	29	8	-	-	-	-	4	-	42	56	25	-
PHILIPPINES	23	9	-	-	-	-	-	-	-	14	23	-	-
PORTUGAL	2	-	-	-	-	-	-	-	2	-	2 ^(d)	-	-
THAILAND	13	3	10	-	-	-	-	-	-	-	13	-	-
TURKEY	10	4	3	-	-	-	-	-	-	3	9	1	-
UNITED ARAB EMIRATES	3	3	-	-	-	-	-	-	-	-	3	-	-
VIETNAM	1	1	-	-	-	-	-	-	-	-	1	-	-
TOTAL	374	133	37	0	0	2	0	4	12	188	254	118	2

The following samples were additionally received by the WRL/FMD in 2003:

Country	Sample year	No. of samples	ELISA/Virus isolation in cell culture							RT-PCR for FMDV				
			FMDV serotypes							SVDV (a)	NVD (b)	Positive	Negative	Not tested
			O	A	C	SAT 1	SAT 2	SAT 3	Asia 1					
BHUTAN	2002	16	2	1	-	-	-	-	-	-	13	7	9	-
IRAN	2002	4	-	2	-	-	-	-	-	-	2	2	1	1
PALASTINAN AUTONOMOUS TERRITORIES	2002	1	1	-	-	-	-	-	-	-	-	1	-	-
ITALY	2001	1	-	-	-	-	-	-	-	1	-	-	-	1
	2002	37	-	-	-	-	-	-	-	37	-	-	-	37
LEBANON	2002	2	2	-	-	-	-	-	-	-	-	2	-	-
PAKISTAN	2002	23	5	5	-	-	-	-	6	-	7	16	4	3
TURKEY	2002	10	4	3	-	-	-	-	-	-	3	8	2	-
VIETNAM	2002	7	7	-	-	-	-	-	-	-	-	7	-	-
TOTAL		101	21	11	0	0	0	0	6	38	25	43	16	42

* Institute for Animal Health, Pirbright Laboratory, Woking, Surrey, GU24 0NF, UK.

FMDV foot-and-mouth disease virus

(a) swine vesicular disease virus

(b) no foot-and-mouth disease, swine vesicular disease or vesicular stomatitis virus detected

(c) two samples from Pakistan contained a mixture of foot-and-mouth disease virus types O and A

(d) positive by RT-PCR for SVDV but not FMDV genome

Table 3. Clinical Samples or isolates received at the WRL/FMD, Pirbright* between January 2003 and 30th of September 2004.

Country	Year of sample collection	No. of samples	Virus isolation in cell culture/ELISA								RT-PCR for FMD				
			FMD virus serotypes								SVD virus	NVD	Positive	Negative	Not tested
			O	A	C	SAT 1	SAT 2	SAT 3	Asia 1						
BHUTAN	2003-2004	52	18	-	-	-	-	-	-	-	34	31	21	-	
BOTSWANA	2002	2	-	-	-	-	2	-	-	-	-	-	-	2	
HONG KONG	2004	12	9	-	-	-	-	-	-	-	3	11	1	-	
IRAN	2004	9	2	1	-	-	-	-	-	-	6	-	-	-	
ISRAEL	2004	6	3	-	-	-	-	-	-	-	3	6	-	-	
ITALY	2004	18	-	-	-	-	-	-	-	18	-	-	-	18	
MALAYSIA	2002-2004	13 ^(a)	10	4	-	-	-	-	-	-	-	13	-	-	
MALAWI	2000-2001 2003-2004	7	-	-	-	5	2	-	-	-	-	-	-	7	
MOZAMBIQUE	2002	1	-	-	-	-	1	-	-	-	-	-	-	1	
NAMIBIA	2000	1	-	-	-	1	-	-	-	-	-	-	-	1	
PORTUGAL	2004	2	-	-	-	-	-	-	-	2	-	-	-	2	
RWANDA	2004	3	2	-	-	-	1	-	-	-	-	-	-	3	
SAUDI ARABIA	2004	1	-	-	-	-	-	-	-	-	1	1	-	-	
SUDAN	2004	37 ^(b)	+	-	-	-	-	-	-	-	-	-	-	-	
UGANDA	2004	60	8	-	-	-	-	-	-	-	52	10	50	-	
YEMEN	2003-2004	111 ^(b)	-	-	-	-	-	-	-	-	-	-	-	-	
ZAMBIA	2000-2004	20 ^(b)	2	-	-	2	-	-	-	-	-	-	-	4	
ZIMBABWE	2001-2004	40 ^(c)	-	-	-	7	35	-	-	-	1	-	-	40	
TOTAL		395	54	5	0	15	41	0	0	20	100	72	72	78	

* Institute for Animal Health, Pirbright Laboratory, Woking, Surrey, GU24 0NF, UK.

FMD foot-and-mouth disease

SVD swine vesicular disease

NVD no FMD, SVD or vesicular stomatitis virus detected

RT-PCR reverse transcription polymerase chain reaction for FMD (or SVD) viral genome

(a) one sample from Malaysia contained a mixture of FMD virus types O and A

(b) testing in progress

(c) three samples from Zimbabwe contained a mixture of FMD virus types SAT 1 and SAT 2

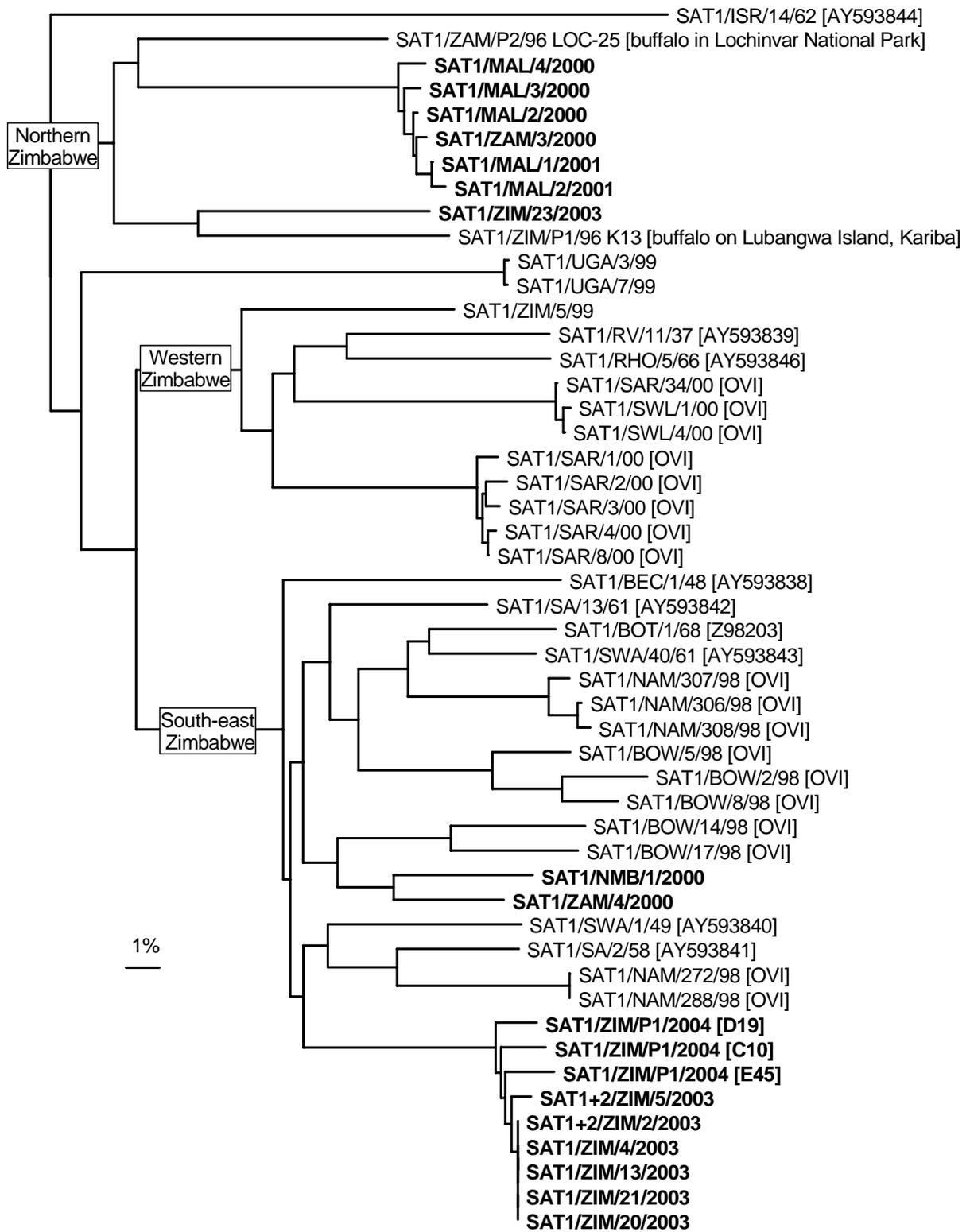


Fig.1. Neighbor-joining tree comparing the complete VP1-coding sequences of type SAT 1 FMDVs.

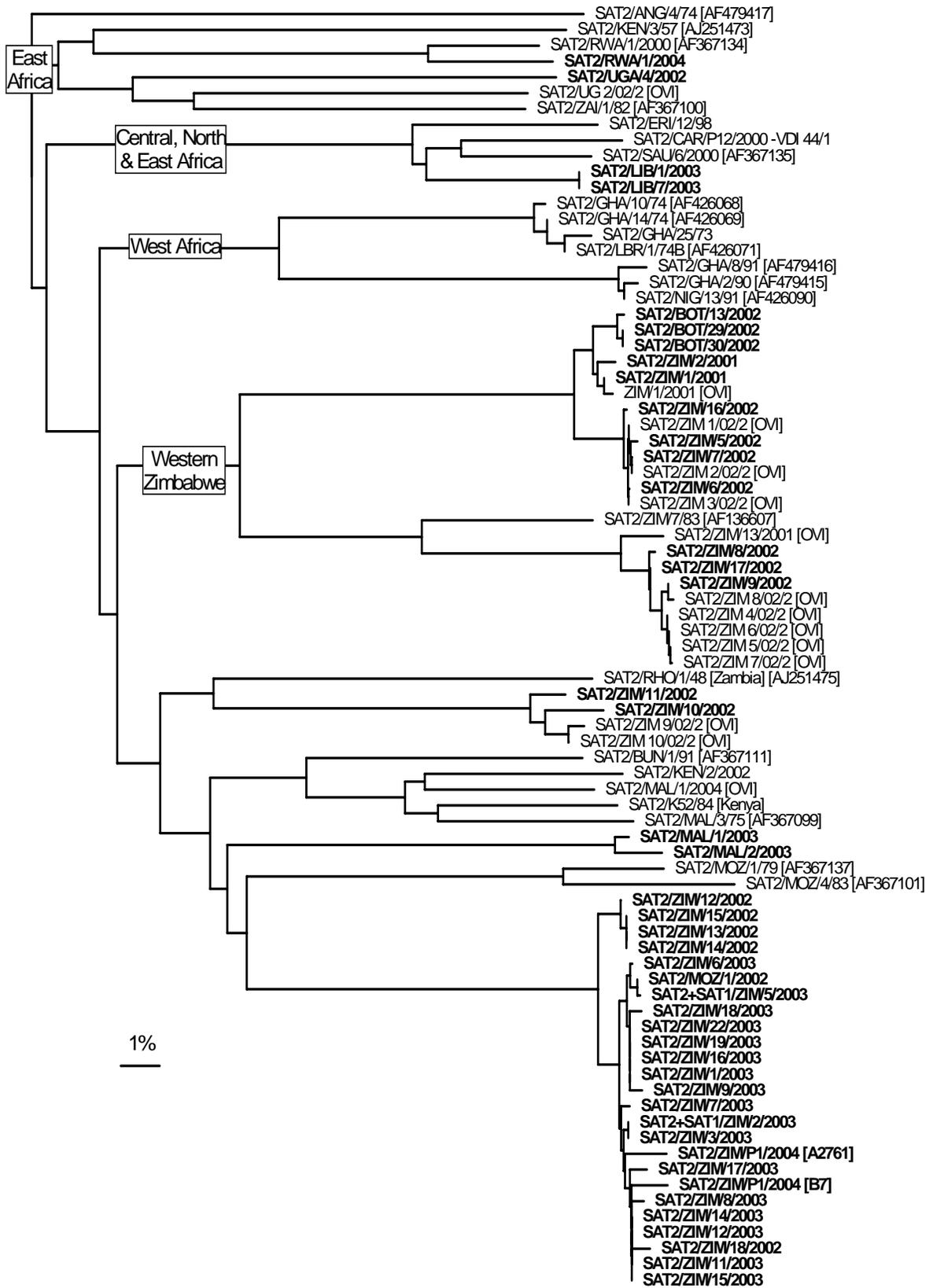


Fig.2. Neighbor-joining tree comparing the complete VP1-coding sequences of type SAT 2 FMDVs.

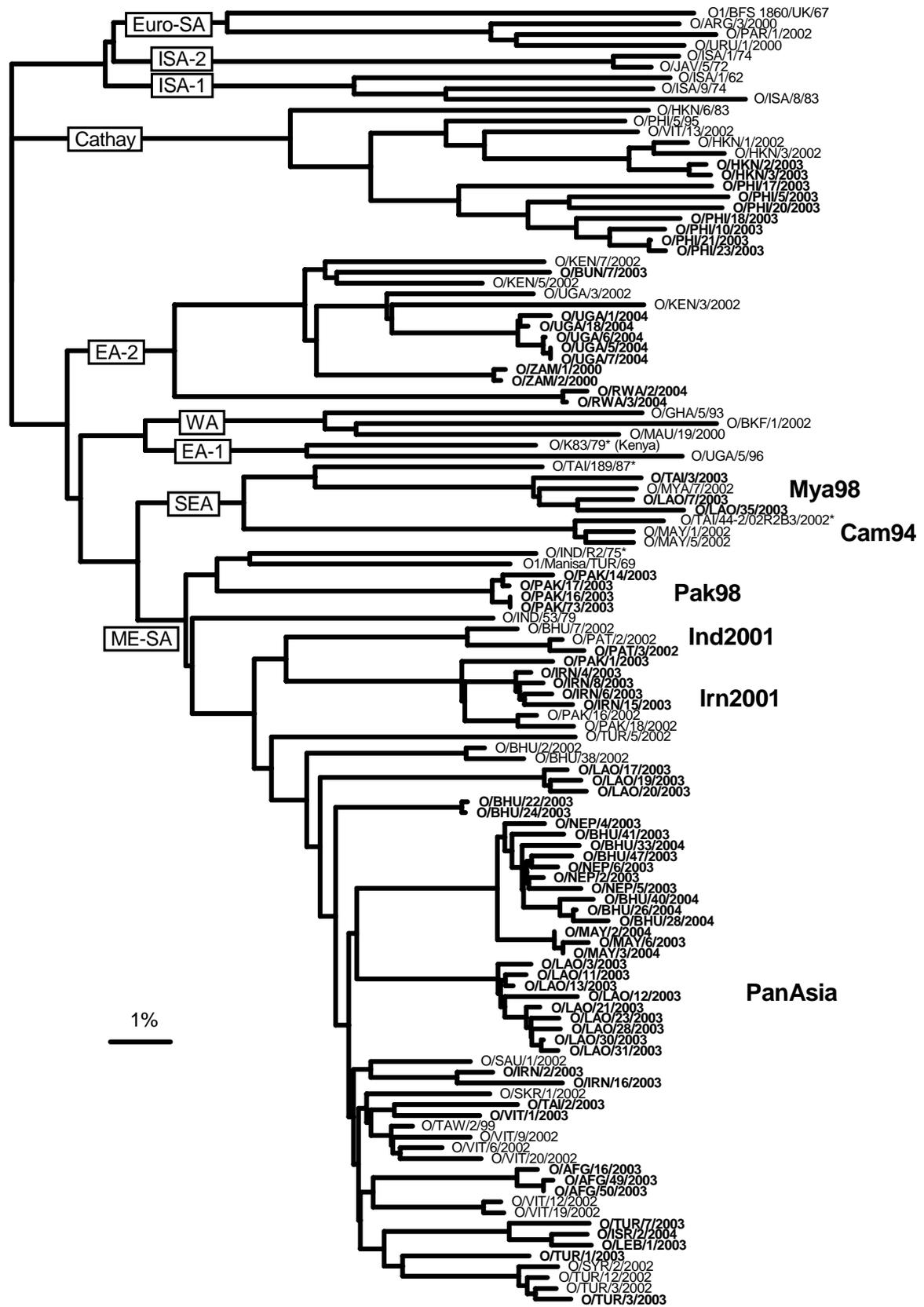


Fig. 3. Neighbor-joining tree comparing the complete VP1-coding sequences of type O FMDVs.

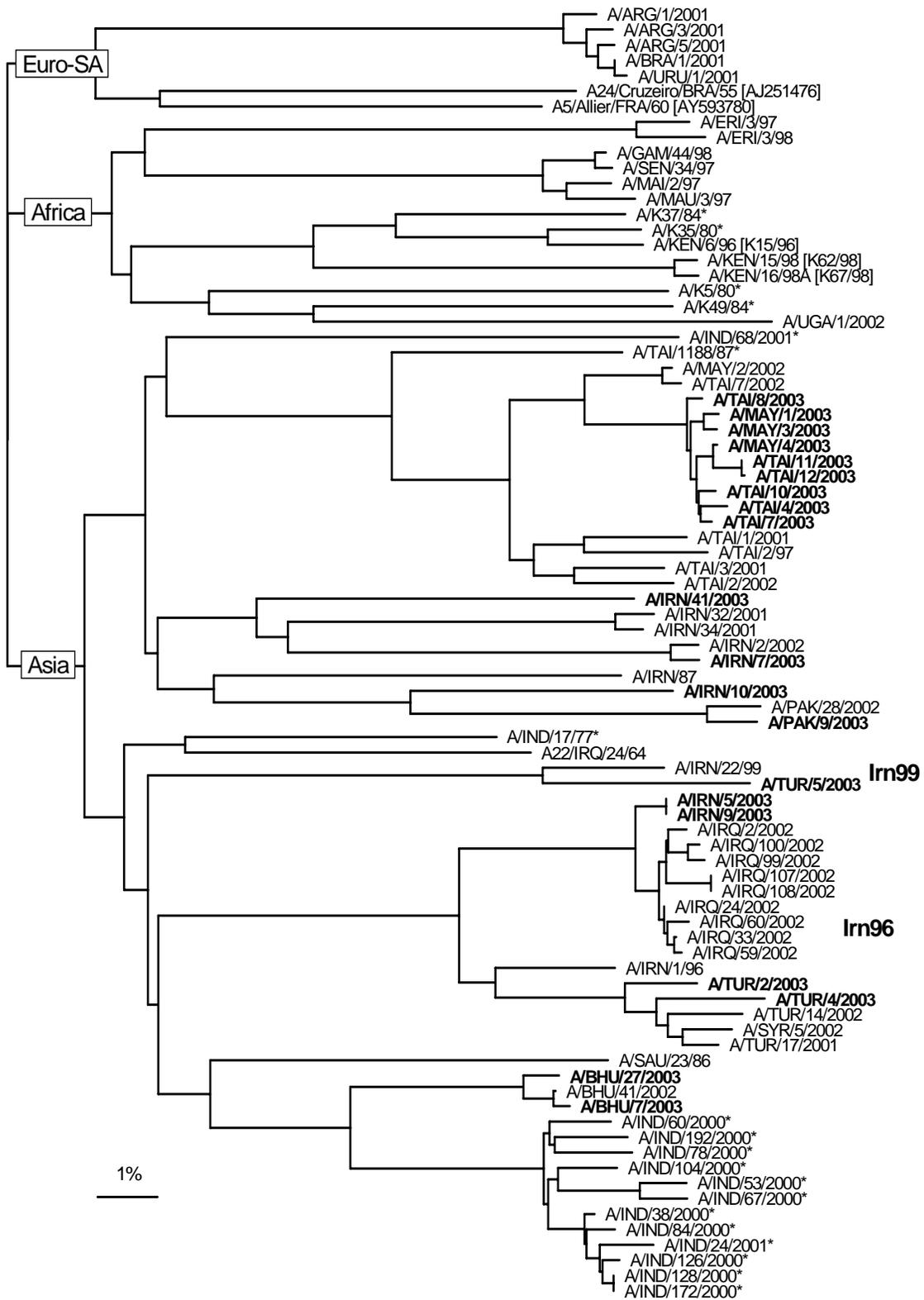


Fig. 4. Neighbor-joining tree comparing the complete VP1-coding sequences of type A FMDVs.

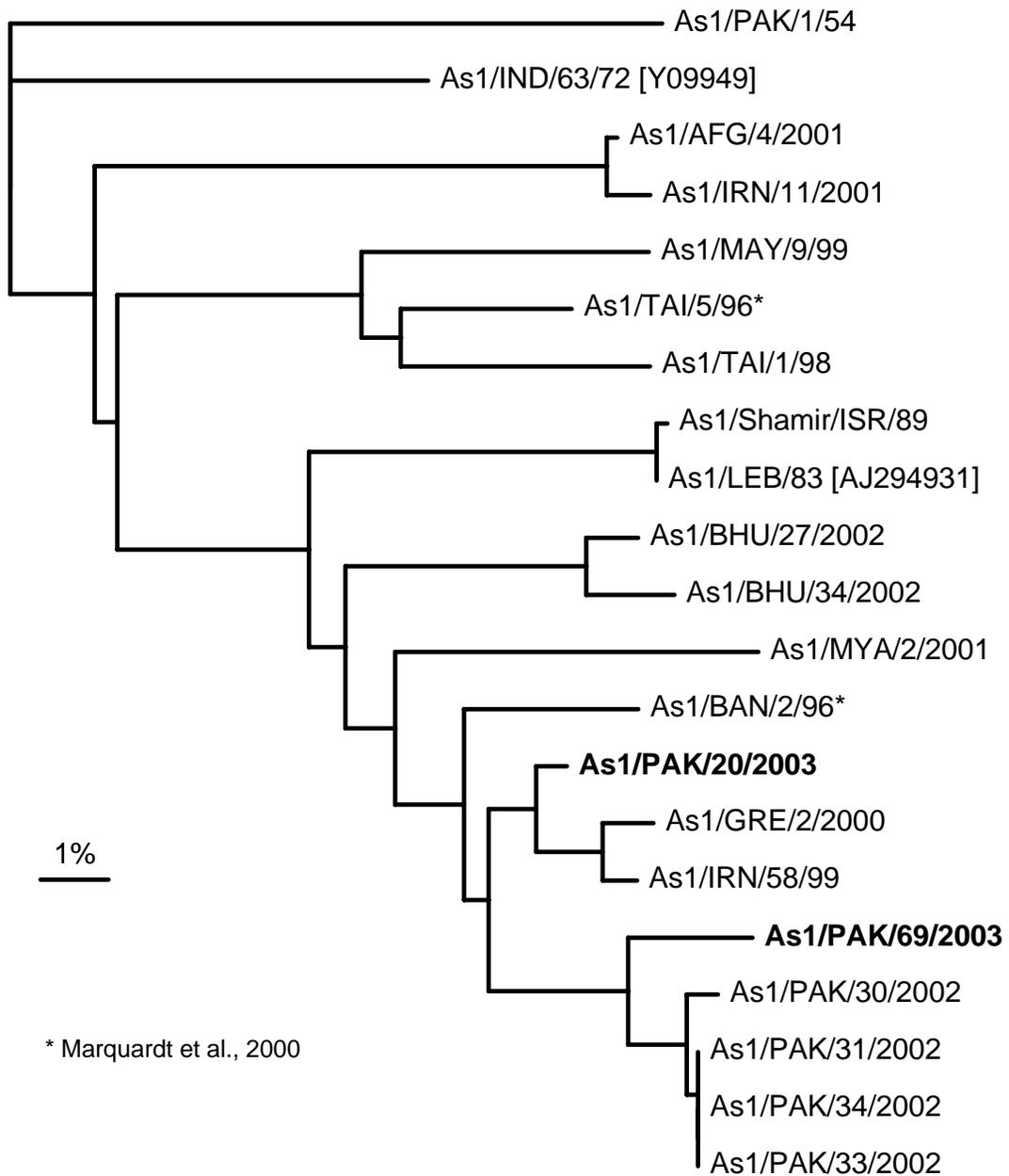


Fig. 5. Neighbor-joining tree comparing the complete VP1-coding sequences of type Asia 1 FMDVs.

Molecular epidemiological studies of Foot-and-Mouth disease virus in sub-Saharan Africa indicate the presence of large numbers of topotypes: implications for local and international control

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Abstract:

Six of the seven serotypes of foot and mouth disease (FMD) virus occur on the African continent and numerous topotypes occur for each serotype. Due to underreporting of FMD, the current strains circulating throughout sub-Saharan Africa are in most cases not known. For both SAT-1 and SAT-2 the genetic diversity is reflected in antigenic variation and indications are that vaccine strains may be needed for each topotype. This has serious implications for control using vaccines and for choice of strains to include in international vaccine banks. The epidemiology of FMD is further complicated by the presence of large numbers of persistently infected African buffalo (*Syncerus caffer*) and other wildlife species which together with largely uncontrolled movement of domestic animals may spread the disease over vast distances. This dearth of knowledge on FMD in Africa poses a serious threat to regions free of FMD in the face of increased international travel and the possible smuggling of illegal bushmeat and other livestock products.

Introduction:

Foot and mouth disease (FMD) virus probably originated from Africa since greater genetic variation occurs in the SAT types (Vosloo *et al.*, 1995; Bastos *et al.*, 2000; Bastos, 2001; Bastos *et al.*, 2001; Bastos *et al.*, 2003a and b) and a sub-clinical cycle occurs in African buffalo (*Syncerus caffer*) where the virus can persist in a single animal for up to five years (Condy *et al.*, 1985). This is the only species for which long-term maintenance of FMD virus has been described (Hedger, 1972; Hedger *et al.*, 1972; Hedger, 1976; Condy *et al.*, 1985; Thomson, 1994; Thomson *et al.*, 2001; Thomson *et al.*, 2003). During persistent infection in buffalo, the SAT type viruses undergo high rates of mutation, giving rise to genetic and antigenic variants (Vosloo *et al.*, 1996).

The disease is endemic to most countries in sub-Saharan Africa (Vosloo *et al.*, 2002) and will not be eradicated from southern and East Africa while infected buffalo are present. Disease-free areas are recognised mainly in southern Africa, where a number of countries have been able to control FMD by separating infected buffalo from livestock and by limited use of vaccination (Brückner *et al.*, 2002; Thomson *et al.*, 2003). Lack of movement control within countries and across international borders for both wildlife and domestic animals aggravates the problem, and gives credence to the fact that FMD will remain a problem on the sub-continent for the foreseeable future. With the increase in international travel, the threat from illegally smuggled bushmeat and other livestock products cannot be ignored, and it is imperative to understand the current epidemiology of FMD to predict what strains are currently most likely to pose a threat to disease-free regions.

Six of the seven serotypes of FMD virus occur on the African continent (Vosloo *et al.*, 2002), with the exception of Asia-1, which complicates control of the disease by vaccination. In sub-Saharan Africa, two cycles of FMD occur, one where virus circulates between wildlife hosts and domestic animals and the other where the virus spreads among domestic animals, without the involvement of wildlife (Vosloo and Thomson, 2004). In southern Africa and to a large extent, eastern Africa, the cycle between wildlife and domestic animals occurs, while in West Africa, due to the low numbers of wildlife, the disease is maintained predominately in domestic animals. However, once disease crosses from wildlife into domestic animals, a domestic cycle could be maintained without the involvement of wildlife. As it is costly to sample wildlife, very little is known about the FMD virus populations circulating in these animals and most information outside southern Africa is based on isolates obtained from domestic animals.

Molecular epidemiological studies have contributed in planning control strategies by elucidating historical and current disease transmission patterns within and between countries. Furthermore, it is

important to have data on the viral topotypes in both wildlife and domestic animals, information that should be heeded when planning FMD vaccination strategies (Vosloo *et al.*, 1992; Vosloo *et al.*, 1995; Bastos, 1998; Bastos *et al.*, 2001; Bastos *et al.*, 2003a and b; Sangare *et al.*, 2003; Sangare *et al.*, 2004). High levels of genetic diversity will most likely be reflected in antigenic differences and it has been shown that for vaccination to be effective, the viruses incorporated into vaccines need to be antigenically related to viruses circulating in the field (Hunter *et al.*, 1996; Hunter, 1998).

This paper summarises the current knowledge of genetic variation of FMD virus in sub-Saharan Africa and discusses the antigenic relationships of SAT-1 and SAT-2 isolates from various topotypes measured against vaccine strains obtained in southern Africa.

Materials and Methods:

Phylogenetic analysis:

Most viruses included in this study were propagated on IB-RS-2 cells before RNA extraction, cDNA synthesis and amplification of the 1D gene using primers and methodology as described in the references presented in Table 1. The 1D gene was partially sequenced and phylogenetic analysis performed as described in the various references. The summary of topotypes in Table 1 was performed by relating similar strains in various papers and not by comparative analysis of sequencing data and may be different when actual data are utilised.

Determination of r-values:

The antigenic relationships were determined using cross-neutralisation assays in IB-RS-2 cells and microtitre plates against cattle sera prepared by two consecutive vaccinations (vaccinated at day 0, boosted at day 28 and bled at day 38) with alhydrogel containing vaccines with either one strain per serotype or a combination of both vaccine strains per serotype (SAT-1: SAR9/81 and KNP196/91/1; SAT-2: ZIM7/83 and KNP19/89/2) as described by Rweyemamu *et al.* (1978). The r-values were calculated as:

$$r = \text{serum titre against heterologous virus} / \text{serum titre against homologous virus}$$

Interpretation of r-values:

r-values were interpreted as proposed by Samuel *et al.* (1990). Briefly, values between 0 – 0.19 indicated highly significant antigenic variation from the vaccine strains and another vaccine strain should be chosen, values of 0.20 - 0.39 showed a significant difference, but a potent vaccine may provide protection, while r-values of 0.40 – 1.0 demonstrated that the vaccine and field strains are similar and the vaccine would provide good protection.

Results:

Topotype diversity in sub-Saharan Africa:

Within each serotype viruses grouped together in distinct clusters which were supported by high bootstrap values and these clades corresponded to geographically defined regions in agreement with the FMD topotype concept as described for the European and SAT serotypes (Bastos *et al.*, 2001; Samuel and Knowles, 2001). The number of topotypes identified in sub-Saharan Africa is summarised in Table 1. For SAT-1 eight topotypes were identified throughout the region of which most had localised geographic distribution. However, isolates from Zambia, Malawi, Tanzania, Kenya and northern Zimbabwe clustered in topotype III, demonstrating that related viruses were found in southern and East Africa and describing a link between the two regions. Three of the topotypes may be extinct (see below) since no recent viruses belonging to these were isolated. However, FMD is endemic to most of these regions and samples are submitted infrequently for further investigation, suggesting that these topotypes may still be present. Further research on this aspect is urgently needed. Topotype VI consisted of isolates obtained during 1974 only from Uganda, while topotype VII had isolates from Nigeria and Sudan between 1974-1981 and finally topotype VIII consisted of viruses from Nigeria and Niger isolated between 1975-1976. Uganda had three topotypes exclusive to that country (IV, V and VI) similar to Zimbabwe, but the latter shares topotypes with neighbouring countries (I [South Africa, Mozambique and southern Zimbabwe], II [Botswana, Namibia, Zambia and western Zimbabwe] and III [Zambia, Malawi, Tanzania, Kenya and northern Zimbabwe]; Table 1).

SAT-2 demonstrated the most genetic diversity with a total of 14 topotypes with five of these possibly extinct (Table 1). Topotypes VI (Gambia and Senegal 1979-1983), XI (Angola 1974), XII (Uganda 1975-1976), XIII (Sudan 1977) and XIV (Ethiopia 1991) did not consist of any recent isolates. Two

topotypes occurred in West Africa (IV [Burundi, Malawi, Kenya, Tanzania and Ethiopia] and V [Nigeria, Senegal, Liberia, Ghana, Mali and Cote d'Ivoire]), with topotype IV including countries from East and southern Africa. Four were found in southern Africa (I [South Africa, Mozambique and southern Zimbabwe], II [Namibia, Botswana, northern and western Zimbabwe], III [Botswana, Zambia and Zimbabwe] and XI [Angola]; Table 1). All the other topotypes were found in East Africa, with a single isolate from the Democratic Republic of Congo grouping with a virus from Uganda (topotype X; Table 1). Most of the topotypes in East Africa consisted of single isolates from one country (VII Eritrea; VIII Rwanda; IX Kenya, Uganda; XII Uganda; XIII Sudan; XIV Ethiopia) and could have wider geographic distribution if more isolates were available from these regions. Zimbabwe shared three different topotypes with neighbouring countries (I, II and III), Botswana shared two (II and III), while three topotypes were found in Uganda (IX, X, and XII), two consisting solely of isolates from this country (Table 1).

Although SAT-3 has the most restricted distribution and is the type least frequently recovered from African buffalo, 6 topotypes were found with 25 genotypes of which four occurred in southern Africa and two were unique to East Africa (Bastos *et al.*, 2003a). The latter two (V and VI) consisted of one virus each obtained from a single game park in Uganda isolated 37 years apart (Bastos *et al.*, 2003a). Zimbabwe had the most diversity with three topotypes (I [South Africa, southern Zimbabwe], II [Namibia, Botswana and western Zimbabwe] and III [Malawi and northern Zimbabwe]; Table 1). The topotype distribution of SAT-3 demonstrated similarities with SAT-1 and SAT-2 distribution and as more information becomes available, better correlation will become evident. The SAT-3 topotype IV consisted of isolates obtained solely from Zambia since the genetic diversity is of such an extent that they were assigned to a separate topotype, while with both SAT-1 and SAT-2, the Zambian viruses grouped with neighbouring countries (Table 1).

The number of topotypes and the geographical distribution was concluded by extracting information from various publications (Knowles and Samuel, 2003; Sahle, 2003; Samuel and Knowles, 2001; Sangare *et al.*, 2001) and the sequencing data needs to be evaluated to ensure the conclusions are correct. However, in the absence of these analyses, it was found that eight topotypes for serotype O exist in Africa of which a number could be ascribed to exotic introductions from elsewhere in the world. During 2000 South Africa experienced an outbreak caused by the Pan-Asian serotype O virus (topotype VII; Table 1) which was also closely related to the strain causing the later outbreak in the United Kingdom (Sangare *et al.*, 2001; Knowles and Samuel, 2003). Similarly, the outbreak that occurred in Angola during 1974-1975 (topotype VIII; Table 1) grouped with a virus isolate in Venezuela during 1951 (Sangare *et al.*, 2001). A number of the isolates from East Africa grouped with North Africa in topotype I (Ethiopia, Eritrea, Kenya, Somalia, Sudan, Tunisia, Egypt, Algeria, Morocco, Libya and Tanzania) while viruses from West Africa grouped together in a single topotype similarly with north African countries (topotype II [Algeria, Cote d'Ivoire, Guinea, Morocco, Niger, Ghana, Burkina Faso, Tunisia and Sudan]). All other topotypes consisted of viruses from East African countries (III [Uganda, Kenya, Sudan], IV [Uganda], V [Uganda], VI [Tanzania, Uganda]). Uganda demonstrated the most diversity with four topotypes, while Sudan formed the link between East and West Africa in topotype II (Table 1).

Sudan also acted as link between East and West Africa when investigating serotype A distribution by grouping in the same topotype which consisted of all the West African viruses investigated so far (topotype I [Mauritania, Mali, Cote d'Ivoire, Ghana, Niger, Nigeria, Cameroon, Chad, Senegal, Gambia, Sudan] Table 1; Knowles *et al.*, 1998). Topotype II consisted of isolates from southern Africa (Angola and Malawi) and North Africa (Algeria, Morocco, Libya and Tunisia) but it is not clear whether there is a link between these outbreaks and should be investigated further. Type A is not endemic to southern Africa and outbreaks have only been documented in Namibia, Malawi and Zambia of which a number were most probably introductions from outside the continent (Knowles *et al.*, 1998). The largest number of topotypes were identified in East Africa (III [Tanzania, Burundi, Malawi]; IV [Kenya, Somalia, Ethiopia]; V [Sudan, Eritrea]; VI [Uganda, Kenya, Ethiopia]).

Serotype C isolates from Africa grouped into three distinct topotypes (I [Kenya]; II [Ethiopia, Kenya] and III [Angola]; Table 1), but it is possible that this serotype is extinct and recent outbreaks are vaccine related.

Antigenic relationships between FMD virus from various topotypes:

Vaccine strains from southern Africa (SAT-1 and SAT-2) were antigenically compared to viruses from various topotypes in sub-Saharan Africa to determine whether these vaccines would be applicable to other regions of the sub-continent. The two SAT-1 viruses (SAR9/81 and KNP196/91/1) both belong to topotype I (Table 1) and demonstrated r-values generally higher than 0.4 when compared with other isolates belonging to the same topotype (Figure 1). However, differences were observed between the two vaccine strains when compared to isolates such as KNP41/95/1 where antiserum to

SAR9/81 had an r-value of 0.24 while the KNP196/91/1 antiserum had a value of 0.57. Antiserum from animals vaccinated with a combination of the two vaccine strains had higher values on most comparisons (Figure 1). The r-values were markedly lower when viruses from other topotypes were investigated and were generally below 0.2. The two isolates from topotype II (BOT14/98/1 and NAM307/98/1) both had r-values of approximately 0.1, demonstrating highly significant antigenic differences between the vaccine strains and field isolates. Similarly low values were observed with one of the topotype III viruses, ZIM14/90/1, but the SAR9/81 antiserum had better, albeit low r-values than the antiserum to KNP196/91/1 when compared to other topotype III viruses (ZIM25/90/1, ZIM6/94/1, ZAM2/93/1, ZAM18/96/1 and TAN1/99/1; Fig. 1). An exception was ZIM 6/94/1, a buffalo isolate from Hwange National Park in the west of Zimbabwe where values of 0.35 (against SAR9/81 antiserum), 0.36 (KNP196/91/1) and 0.48 (combination of the two vaccine strains) were observed. The isolate from Uganda (UGA1/97/1 topotype V) demonstrated values of 0.23 (SAR9/81) and 0.16 (KNP196/91/1), while one from Nigeria (NIG5/81/1 topotype VI) had lower values (0.05 against SAR9/81 antiserum and 0.07 against KNP196/91/1) indicating that a high potency vaccine may still protect animals in the field in Uganda, but not in Nigeria.

The two SAT-2 vaccine strains belong to different topotypes, KNP19/89/2 to topotype I and ZIM7/83 to II (Table 1). The antigenic variation for SAT-2 was more pronounced with r-values below 0.4 even when vaccine and field strains from the same topotype were compared (Figure 2). As with the SAT-1 data, the antiserum prepared with both vaccine strains presented higher r-values, but antiserum prepared against ZIM7/83 alone reacted better against all other viruses compared to antiserum prepared against KNP19/89/2 alone (Figure 2).

Discussion:

Due to the endemic nature of the disease, outbreaks are often not reported in sub-Saharan Africa and samples not submitted for further study. Therefore the results do not represent the current situation and some of the topotypes may be extinct, but this can only be confirmed with improved sampling strategies. The results indicate that the genetic variation is significant with each of the six serotypes displaying numerous topotypes. Consequently, vaccination strategies should not only consider the fact that numerous serotypes could be occurring simultaneously, but that the genetic variation within serotypes can have serious implications on the choice of strain to include in antigen banks and vaccines. It also demonstrates that frequent surveillance investigating circulating strains is necessary.

Although the topotypes demonstrated discrete geographic distribution, overlaps occurred with several countries having more than one topotype circulating within their borders. Uncontrolled movement within countries and across borders could lead to greater dissemination of different topotypes, further complicating control by vaccination. Using molecular data it was shown that Zimbabwe suffered four different outbreaks of SAT-2 since 2001 that belonged to the three topotypes occurring in that country (Records of the Exotic Diseases Division, Onderstepoort Veterinary Institute) and vaccines containing strains from various topotypes were used to ensure sufficient protection (S. Hargreaves, personal communication). However, the use of different vaccines in various districts confounded logistics significantly. It has also been shown that sequence data of FMD viruses can be used under certain conditions to trace the origin of animals (Vosloo *et al.*, 2001), but not when movement is unrestrained therefore affecting the possible tracing of outbreaks negatively and confounding control strategies.

The topotype distribution also indicated that movement between southern and East Africa as well as between East and West Africa has occurred in the past. Since wildlife does not occur in large numbers between East and West Africa, dissemination of disease most likely occurred through uncontrolled movement of livestock. Large numbers of African buffalo and other antelope move between the northern borders of southern and East Africa, and the most likely explanation would be due to persistently infected buffalo or other infected species moving between the two regions. However, movement of livestock cannot be ruled out completely.

Uganda demonstrated the most diverse genetic range of viruses with three SAT-1, three SAT-2, two SAT-3, four O and one A topotypes with several of these occurring in Uganda only. The reason for this diversity is not clear and it could be that a more intensive study of the region will find similar results in neighbouring countries. Currently there is a dearth of knowledge in countries with large cattle populations such as Sudan, Somalia and further afield in Nigeria and Chad and it is possible that the topotypes found in Uganda is a reflection of what is circulating in other countries or alternatively that many more topotypes are present of which there is no information. It is therefore imperative that the situation in sub-Saharan Africa be studied more intensively with emphasis on countries in the Horn of Africa and North Africa, the regions posing the biggest threat to southern Europe and the Middle East.

It is generally accepted that r-values higher than 0.4 demonstrate a good antigenic relationship and vaccine strains would protect against those field isolates (Samuel *et al.*, 1990). The antigenic variation

within SAT-1 was less marked than that of the SAT-2 viruses. The SAT-1 toptype I vaccine strains demonstrated good antigenic relationships with viruses from the same toptype, but significantly poorer against viruses from other topotypes. The SAT-2 vaccine strains had poor antigenic relationships with most other isolates, even within the same toptype. It was shown during persistent infection in buffalo that a plaque-purified SAT-2 virus demonstrated higher rates of change over time than a SAT-1 isolate and the genetic changes in SAT-2 were reflected in greater antigenic changes than for SAT-1 (Vosloo *et al.*, 1996). With both SAT-1 and SAT-2 one strain reacted consistently better with different viruses, albeit not at a high enough value to guarantee protection. This could indicate that certain strains may have better cross-reactivity across topotypes and these should be investigated or developed to include into vaccines. A combination of more than one strain per serotype presented better r-values and the possibility of combinations of strains should be further investigated. The amount of antigenic variation has serious implications on the choice of vaccine strain, as it seems that each toptype may need to have its own vaccine strain. However, more data is needed where viruses from different topotypes are compared before reaching a final conclusion.

The topotypes that have occurred in the Horn of Africa and North Africa up to 1994 – 2003 are summarised in Table 2. Uganda is included in this list as it may represent disease occurrence in neighbouring countries in the Horn of Africa. For SAT-1 three topotypes have occurred in Tanzania and Kenya (III) and 2 in Uganda (IV and V) up to 1999. No antigenic profile has been performed for toptype IV viruses by the authors of this paper and data is limited for the other topotypes present in these regions. More focus should be placed on these topotypes by countries at risk from introduction from this area. Similarly, 5 topotypes of SAT-2 may threaten disease safety (Table 2) and limited antigenic data is available for only 3 (Fig. 2). The distribution of SAT-3 is more limited and only one toptype has been found in Uganda during 1997 from buffalo (Bastos *et al.*, 2003a, Table 2). Serotype O is currently the most prevalent in the risk areas and 5 topotypes may be present. Serotype A demonstrates the most genetic and antigenic variation of the classical European serotypes and four topotypes may still be circulating in the North, West and East African regions (Table 2). Antigenic data and cross protection studies of the strains included in the antigen banks need to be performed to ensure that they will confer protection to the various topotypes present in the field.

Most outbreaks in southern Africa can be attributed to SAT-2, followed by SAT-1 and then SAT-3 (Thomson, 1994). This information is not available for the rest of the sub-continent, but SAT-2 has recently occurred in Cameroon, a country where this serotype has not occurred before (Bronsvort *et al.*, 2004). SAT-2 has also caused outbreaks in the Middle East in 1990 and 2000, while incursions of SAT-1 has occurred during 1961-65 and 1970 (Ferris and Donaldson, 1992; Bastos *et al.*, 2003b). Currently there is widespread occurrence of serotype O in East Africa, with reports from Kenya, Uganda, Rwanda and southern Sudan (records of the Exotic Diseases Division, Onderstepoort Veterinary Institute, D. Paton and G.R. Thomson, personal communication). However, despite the genetic variation observed for serotype O viruses world-wide, the antigenic variation is not extensive and the current vaccine strains can protect against most outbreaks (Knowles and Samuel, 2003). Therefore, it seems probable that SAT-2 may pose a greater threat to the rest of the world than other serotypes in terms of ability to cause outbreaks and together with the antigenic data presented here, more attention should be given to this serotype to determine what strains should be included in international antigen banks to protect countries currently free from disease.

The endemic nature of FMD on the African continent and the low priority for controlling a disease that does not cause high rates of mortality is a serious threat to regions free of FMD.

References:

- Bastos, A.D., Anderson, E.C., Bengis, R.G., Keet, D.F., Winterbach, H.K. and Thomson, G.R. 2003a. Molecular epidemiology of SAT3-type foot-and-mouth disease. *Virus Genes*. 27: 283-90.
- Bastos, A.D.S., Boshoff, C.I., Keet, D.F., Bengis, R.G. and Thomson, G.R. 2000. Natural transmission of foot-and-mouth disease virus between African buffalo (*Syncerus caffer*) and impala (*Aepyceros melampus*) in the Kruger National Park, South Africa. *Epid. Infect.* 124: 591-598.
- Bastos, A.D.S. 1998. Detection and characterisation of foot-and-mouth disease virus in sub-Saharan Africa. *Onderstepoort J. Vet. Res.* 65: 37-47.
- Bastos, A.D.S. 2001. Molecular epidemiology and diagnosis of SAT-type foot-and-mouth disease in southern Africa. PhD thesis. University of Pretoria, pp 1-148.
- Bastos, A.D.S., Haydon, D.T., Forsberg, R., Knowles, N.J., Anderson, E.C., Bengis, R.G., Nel, L.H. and Thomson, G.R. 2001. Genetic heterogeneity of SAT-1 type foot-and-mouth disease viruses in southern Africa. *Arch. Virol.* 146: 1537-1551.

- Bastos, A.D.S., Haydon, D.T., Sangare, O., Boshoff, C.I., Edrich, J.L. and Thomson, G.R. 2003b. The implications of viral diversity within the SAT-2 serotype for control of foot-and-mouth disease in sub-Saharan Africa. *J. Gen. Virol.* 84: 1595-1606.
- Bronsvort, B.M., Radford, A.D., Tanya, V.N., Nfon, C., Kitching, R.P. and Morgan, K.L. 2004. Molecular epidemiology of foot-and-mouth disease viruses in the Adamawa province of Cameroon. *J. Clin. Microbiol.* 42: 2186-2196.
- Brückner, G.K., Vosloo, W., Du Plessis, B.J.A., Kloock, P.E.L.G., Connaway, L., Ekron, M.D., Weaver D.B., Dickason, C.J., Schreuder F.J., Marais, T. and Mogajane, M.E. 2002. Foot and mouth disease: the experience in South Africa. *Rev. Sci. Tech. OIE.* 21: 751-764.
- Condy, J.B., Hedger, R.S., Hamblin, C. and Barnett, I.T.R. 1985. The duration of the foot-and-mouth disease carrier state in African buffalo (i) in the individual animal and (ii) in a free-living herd. *Comp. Immunol. Microbiol. Infect. Dis.* 8: 259-265.
- Ferris, N.P. and Donaldson, A.I. 1992. The World Reference Laboratory for Foot and Mouth Disease: a review of thirty-three years of activity (1958-1991). *Rev. Sci. Tech.* 11: 657-684.
- Hedger, R.S. 1972. Foot-and-mouth disease and the African buffalo (*Syncerus caffer*). *J. Comp. Path.* 82: 19-28.
- Hedger, R.S. 1976. *Foot-and-mouth disease in wildlife with particular reference to the African buffalo (Syncerus caffer)*. In: Wildlife Diseases. L.A. Page, ed. Pp. 235-244. New York, Plenum Publishing.
- Hedger, R.S., Condy, J.B. and Golding, S.M. 1972. Infection of some species of African wildlife with foot-and-mouth disease virus. *J. Comp. Pathol.* 82: 455-546.
- Hunter, P. 1998. Vaccination as a means of control of foot-and-mouth disease in sub-Saharan Africa. *Vaccine.* 16: 261-264.
- Hunter, P., Bastos, A.D.S., Esterhuysen, J.J. and van Vuuren, C. de W.J. 1996. Appropriate foot-and-mouth disease vaccines for southern Africa. All Africa Conference on Animal Agriculture, Pretoria, South Africa. 2.2.7: 1-4.
- Knowles, N.J. and Samuel, A.R. 2003. Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.* 91: 65-80.
- Knowles, N.J., Ansell, D.M. and Samuel, A.R. 1998. Molecular comparison of recent foot-and-mouth disease type A viruses from West Africa with historical and reference virus strains. *Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of foot-and-mouth disease, Pirbright, UK, 14-18 September, 1998.*
- Reid, S.M., Ferris, N.P., Hutchings, G.H., De Clercq, K., Newman, B.J., Knowles, N.J. and Samuel, A.R. 2001. Diagnosis of foot-and-mouth disease by RT-PCR: use of phylogenetic data to evaluate primers for the typing of viral RNA in clinical samples. *Arch Virol.* 146: 2421-2434.
- Rweyemamu, M.M., Booth, J.C., Head, M. and Pay, T.W.F. 1978. Microneutralization tests for serological typing and subtyping of Foot-and-Mouth Disease virus strains. *Journal of Hygiene, Cambridge.* 81, 107-123.
- Sahle, M. 2003. An epidemiological study of the genetic variants of foot and mouth diseases viruses in East Africa. PhD thesis. University of Pretoria.
- Samuel, A.R. and Knowles, N.J. 2001. Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *J. Gen. Virol.* 82: 609-621.
- Samuel, A.R., Ouldrige, E.J., Arrowsmith, A.E.M, Kitching, R.O. and Knowles, N.J. 1990. Antigenic analysis of serotype O foot-and-mouth disease virus isolates from the Middle East, 1981-1988. *Vaccine,* 8: 390-196.
- Sangare, O. 2002. Molecular epidemiology of foot-and-mouth disease virus in West Africa. Pp.1-114. PhD thesis. University of Pretoria.
- Sangare, O., Bastos A.D.S., Venter, E.H. and Vosloo, W. 2003. Retrospective genetic analysis of SAT-1 type foot-and-mouth disease outbreaks in West Africa (1975-1981). *Vet. Microbiology.* 93: 279-289.
- Sangare, O., Bastos A.D.S., Venter, E.H. and Vosloo, W. 2004. A first molecular epidemiological study of SAT-2 type foot-and-mouth disease viruses in West Africa. *Epidemiology and Infection,* 132: 525-32.
- Sangare, O., Bastos, A.D., Marquardt, O., Venter, E.H., Vosloo, W. and Thomson, G.R., 2001. Molecular epidemiology of serotype O foot-and-mouth disease virus with emphasis on West and South Africa. *Virus Genes.* 22: 343-350.
- Thomson, G.R. 1994. *Foot-and-mouth disease*, In: Infectious Diseases of livestock with special reference to southern Africa. J.A.W. Coetzer, G.R. Thomson and R.C. Tustin, eds. Pp. 825-952. Cape Town, London, New York: Oxford University Press.

- Thomson, G.R., Bengis, R.G. and Brown, C.C. 2001. *Picornaviruses*. In: Infectious diseases of wild mammals, 3rd Edn. E.S. Williams and I.K. Barker, eds. Pp. 119-130. Iowa University Press, Ames.
- Thomson, G.R., Vosloo, W. and Bastos, A.D.S. 2003. Foot and mouth disease in wildlife. *Virus Res.* 91: 145-61.
- Vosloo, W. and Thomson, G.R. 2004. *Natural Habitats in which Foot-and-Mouth Disease Viruses are Maintained*. In: Foot-and-mouth disease. Domingo, E. and Sobrino, F. eds. Horizon Scientific Press, pp. 383-410.
- Vosloo, W., Bastos A.D.S., Michel, A. and Thomson, G.R. 2001. Tracing movement of African buffalo in southern Africa. *Rev. Sci. Tech. OIE.* 20: 630-639.
- Vosloo, W., Bastos, A.D., Kirkbride, E., Esterhuysen, J.J., Janse van Rensburg, D., Bengis, R.G., Keet, D.F. and Thomson, G.R. 1996. Persistent infection of African buffalo (*Syncerus caffer*) with SAT-type foot-and-mouth disease viruses: rate of fixation of mutations, antigenic change and interspecies transmission. *J. Gen. Virol.* 77: 1457-1467.
- Vosloo, W., Bastos, A.D.S., Sangare, O., Hargreaves, S.K. and Thomson, G.R. 2002. Review of the status and control of foot and mouth disease in sub-Saharan Africa. *Rev. Sci. Tech. OIE.* 21: 437-449.
- Vosloo, W., Kirkbride, E., Bengis, R.G., Keet, D.F. and Thomson, G.R. 1995. Genome variation in the SAT types of foot-and-mouth disease viruses prevalent in buffalo (*Syncerus caffer*) in the Kruger National Park and other regions of southern Africa, 1986-1993. *Epidem. Infect.* 114: 203-218.
- Vosloo, W., Knowles, N.J. and Thomson, G.R. 1992. Genetic relationships between southern African SAT-2 isolates of foot-and-mouth disease virus. *Epidem. Infect.* 109: 547-558.

TABLE 1 Summary of the toptype distribution of FMD serotypes O, A, C and SAT types 1-3 in Africa

Serotype	Topotype	Representative Country / Countries	References
SAT-1	I	South Africa, southern Zimbabwe, Mozambique	Vosloo <i>et al.</i> 1995
	II	Botswana, Namibia, Zambia, western Zimbabwe	
	III	Zambia, Malawi, Tanzania, Kenya, northern Zimbabwe	Bastos <i>et al.</i> 2001
	IV	Uganda	Reid <i>et al.</i> 2001
	V	Uganda	
	VI	Uganda	Sahle 2003
	VII	Nigeria, Sudan	
	VIII	Nigeria, Niger	Sangare <i>et al.</i> 2003
SAT-2	I	South Africa, Mozambique, southern Zimbabwe	Bastos <i>et al.</i> 2003b
	II	Namibia, Botswana, northern & western Zimbabwe	
	III	Botswana, Zambia, Zimbabwe	
	IV	Burundi, Malawi, Kenya, Tanzania, Ethiopia	Vosloo <i>et al.</i> 1995
	V	Nigeria, Senegal, Liberia, Ghana, Mali, Cote d'Ivoire	
	VI	Gambia, Senegal	Sangare 2002
	VII	Eritrea	
	VIII	Rwanda	Sahle 2003
	IX	Kenya, Uganda	
	X	Democratic Republic of the Congo, Uganda	Sangare <i>et al.</i> 2004
	XI	Angola	
	XII	Uganda	
	XIII	Sudan	
	XIV	Ethiopia	
SAT-3	I	South Africa, southern Zimbabwe	Vosloo <i>et al.</i> 1995
	II	Namibia, Botswana, western Zimbabwe	
	III	Malawi and northern Zimbabwe	Bastos <i>et al.</i> 2003a
	IV	Zambia	
	V	Uganda	Reid <i>et al.</i> 2001
	VI	Uganda	
O	I	Ethiopia, Eritrea, Kenya, Somalia, Sudan, Tunisia, Egypt, Algeria, Morocco, Libya, Tanzania	Samuel and Knowles 2001
	II	Algeria, Côte d'Ivoire, Guinea, Morocco, Niger, Ghana, Burkina Faso, Tunisia, Sudan	
	III	Uganda, Kenya, Sudan	Sangare 2002
	IV	Uganda	
	V	Uganda	Sahle 2003
	VI	Tanzania, Uganda	
	VII	South Africa	Sangare <i>et al.</i> 2001
	VIII	Angola	
A	I	Mauritania, Mali, Côte d'Ivoire, Ghana, Niger, Nigeria, Cameroon, Chad, Senegal, Gambia, Sudan	Knowles and Samuel 2003
	II	Angola, Algeria, Morocco, Libya, Tunisia, Malawi	
	III	Tanzania, Burundi, Malawi	Knowles <i>et al.</i> 1998
	IV	Kenya, Somalia, Ethiopia	
	V	Sudan, Eritrea	
	VI	Uganda, Kenya, Ethiopia	
C	I	Kenya	Reid <i>et al.</i> 2001
	II	Ethiopia, Kenya	
	III	Angola	Knowles and Samuel 2003

TABLE 2 Topotypes occurring recently in the Horn of Africa and West Africa that may be a threat to the Mediterranean and Middle East

Serotype	Topotype	Year of isolation	Representative Country / Countries
SAT-1	III	1977-1999	Zambia, Malawi, Tanzania, Kenya, northern Zimbabwe
	IV	1999	Uganda
	V	1997	Uganda
SAT-2	IV	1975-1999	Burundi, Malawi, Kenya, Tanzania, Ethiopia
	VII	1998-2000	Eritrea, Saudi Arabia
	VIII	2000	Rwanda
	IX	1957-1998	Kenya, Uganda
	X	1982-1998	Democratic Republic of the Congo, Uganda
SAT-3	VI	1997	Uganda
O	I	1977-2001	Ethiopia, Eritrea, Kenya, Somalia, Sudan, Tunisia, Egypt, Algeria, Morocco, Libya, Tanzania
	II	1976-1999	Algeria, Côte d'Ivoire, Guinea, Morocco, Niger, Ghana, Burkina Faso, Tunisia, Sudan
	III	1974-1996	Uganda, Kenya, Sudan
	IV	1998-2003	Uganda
	VI	1980-2003	Tanzania, Uganda
	A	I	1973-1998
IV		1979-1996	Kenya, Somalia, Ethiopia
V		1977-1998	Sudan, Eritrea
VI		1975-1994	Uganda, Kenya, Ethiopia
C	II	1971-1996	Ethiopia, Kenya

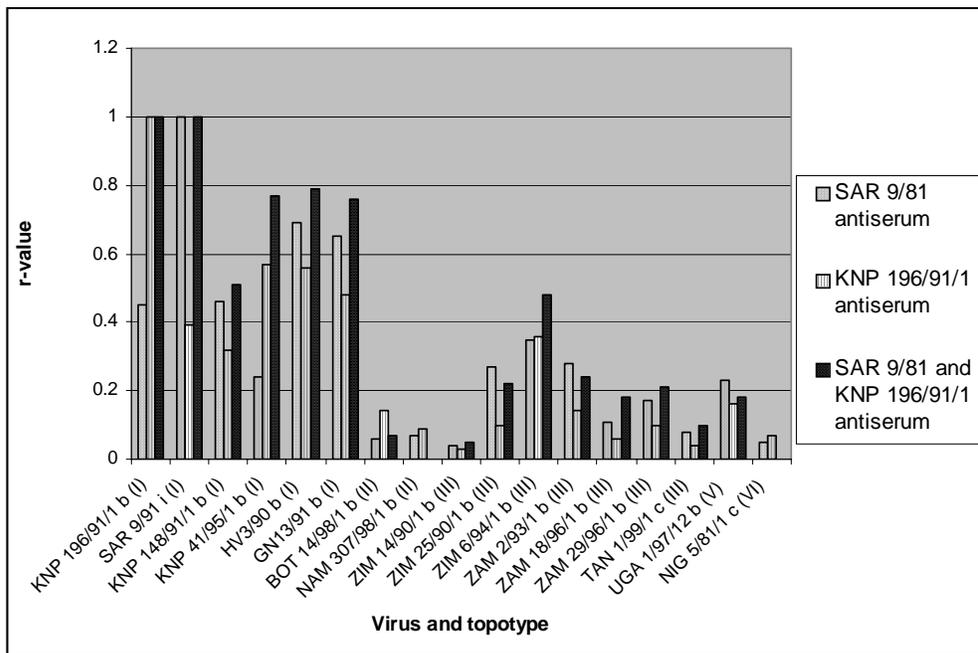


Figure 1: : Chart to indicate the r-values of various SAT-1 FMD virus isolates from different toptypes in sub-Saharan Africa compared to antiserum prepared to the vaccine strains SAR9/81 and KNP196/91/1 (both toptype I). The toptype of each virus is indicated in brackets after the virus name. b indicates an isolate obtained from buffalo, i from impala and c from cattle. KNP and SAR indicate isolates made in the Kruger National Park (South Africa), GN Ghonerazhou National Park, HV Hippo Valley (both Zimbabwe), BOT Botswana, NAM Namibia, ZIM Zimbabwe, ZAM Zambia, TAN Tanzania, UGA Uganda and NIG Nigeria.

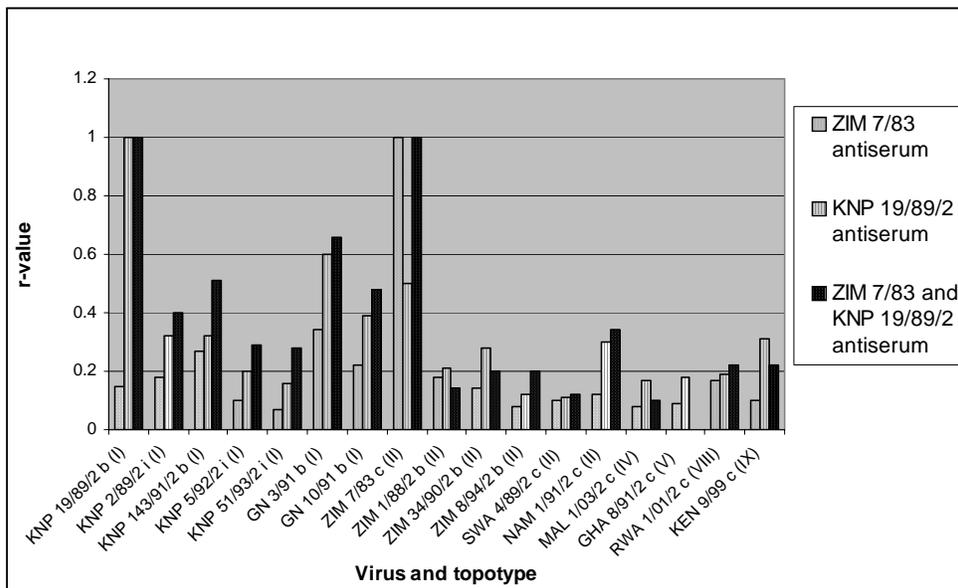


Figure 2: Chart to indicate the r-values of various SAT-2 FMD virus isolates from different toptypes compared to antiserum prepared to the vaccine strains KNP19/89/2 (topotype I) and ZIM7/83 (topotype II). The toptype of each virus is indicated in brackets after the virus name. b indicates an isolate obtained from buffalo, i from impala and c from cattle. KNP indicates isolates made in the Kruger National Park (South Africa), GN Ghonerazhou National Park (Zimbabwe), ZIM Zimbabwe, SWA and NAM Namibia, MAL Malawi, GHA Ghana, RWA Rwanda and KEN Kenya.

Characterisation of a new type O lineage of FMDV from Uganda with atypical clinical manifestations in domestic cattle

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Abstract

During 2000-2003 a disease appeared in domesticated cloven-hoofed animals in Uganda characterised by photophobia, lethargia, abnormal growth of hair, and reduced or arrested lactation. The syndrome became known among farmers and veterinarians as "Otolimo" for "seeking the shade" and it was concluded to be a chronic sequelae of FMDV infection. Isolates from 2002 outbreaks of FMD in Uganda were characterised by full-length sequencing, discriminating antigen ELISA and cross neutralisation tests, which revealed that the isolates were type O. The branch lengths of dendrograms between the Uganda strains and other type O strains were of the same order of magnitude as the ones between the other type O lineages. The appearance of a new type O lineage in domestic cattle in Uganda raises the question if type O was always present on the African continent or if this lineage was reintroduced – and if reintroduced – from where. The continuous identification of new lineages of FMDV also addresses the question if our knowledge of the global diversity of FMDV is still so limited – or if new lineages emerge by distinct jumps from pre-existing ones.

Introduction

From 2000 to 2003 Uganda has reported between 28 and 38 annual outbreaks of Foot and mouth disease, which is an increase from the 1-15 annual outbreaks reported during 1996-1999. The 2000-2002 outbreaks included 1200-3100 annual cases, while 27000 cases were reported in 2003 and about 18000 in the first half of 2004. The outbreaks have been caused by strains of serotypes A, O, SAT1, SAT2 and SAT3.

In collaboration with the Ministry of Agriculture, Animal Industry and Fisheries in Uganda and FAO, we have had the opportunity to study a number of bovine probang samples collected in 2002 in Kumi district in Uganda. In this paper we present the preliminary results of the characterisation of a new type O lineage.

Materials and Methods

Clinical signs

The clinical description is a summary of local records.

Samples

Twelve probang samples (EL1 through EL12) were collected from bovines during an outbreak in Kumi district, Uganda, in 2002. Virus was isolated on primary porcine kidney monolayer cell culture (PK cells), and six isolates were subjected to discriminating antigen ELISA according to Have, Lei and Scherning-Thiesen (1984).

Production of guinea pig sera

Guinea pigs were injected two times intradermally in the footpad of one hind limb with one of two Ugandan isolates (EL7 and EL11). The injections were separated by nine weeks. The inocula consisted of a 1:5 dilution of second passages of the isolates in PK cells, which contained $10^{4.9}$ TCID₅₀/ml (EL7) and $10^{4.7}$ TCID₅₀/ml (EL11). Serum samples were obtained after 4 weeks, and tested for antibodies towards FMDV strains O-Manissa, A-Iraque and themselves. Furthermore, the EL7/O-Manissa titre ratio was determined for five guinea pig anti-EL7 or anti-EL11 sera, a pool of these and a guinea pig anti-O-Manissa serum in three separate tests.

Cross-neutralisation test

A cross-neutralisation study including all seven FMDV serotypes and the Ugandan isolates was performed. Virus included O-Manissa, A-Iraque, C-Noville, Asia1/Shamir, SAT1/Bot1/68, SAT2/Zim5/81, SAT3/Zim4/81 and EL7. The sera included were guinea pig sera raised towards the viruses and a pool of five positive guinea pig anti-EL7 or anti-EL11 sera. Quadruplicates of sera were two-fold serial diluted seven times starting from 1:4 or 1:10 in 96-well culture plates (Nunc). Each

well was added 10^2 TCID₅₀ of the FMDV strain in question, and the plates were left for 1 hour at 37°C. The wells were then added 50 µl of a suspension of 400000 PK cells/ml, and left for 72 hours at 37°C. Each test included a ten-fold dilution of the FMDV strain in question. Finally, the plates were read for CPE, and 50%-end point titres calculated according to Reed and Muench (1938).

Characterisation of isolates by full-length PCR

RNA was purified from cell culture supernatants and sequenced using a pan-O serotype FMDV 24-hour full-length sequencing method to be published in details elsewhere. According to this method, the entire genome is amplified by PCR using sets of overlapping primer pairs located in conserved domains. Sequences are edited by reading in both directions of the PCR products using the PCR primers and, when required, an internal pair of primers. In >80% of the genome including the entire VP1 coding region, overlapping PCR products or overlapping sequencing reactions from internal primers result in an additional 2-fold sequencing to be included as raw data in the sequence editing process. Multiple alignments and phylograms of the sequences generated during the present study and sequences retrieved from the Genbank sequence database were done with ClustalX (EMBL, Heidelberg, Germany, May 1994) (Thompson et al., 1997) using the default parameters and 1000 bootstrap replications. The dendrograms were visualized with TREEVIEW (Page, 1996), version 25.

Results

At the beginning of the outbreak, the clinical signs were typical for of FMD: salivation, high temperatures, formation of vesicles in the mouth, nares, muzzle, feet, teats and udder. In most cases, pigs and small ruminants were not or only slightly affected, while the African buffalo showed clinical signs. After about two weeks, the animals recovered from the lesions, however, secondary infections were common during the rainy season. After recovery, some of the animals showed the following clinical signs: Lethargia, stiry hair, long hairs – especially on the back and head, abortions, reduced or arrested lactation, and as the most significant finding, these animals were photophobic and would only graze early in the morning and late at night, seeking the shade during the day. This syndrome became known among farmers and veterinarians as “Otolimo” for “seeking the shade”. Seven of the twelve probang samples were found positive for FMDV in cell culture, and was shown to belong to serotype O by discriminating antigen ELISA.

Seven of the ten injected guinea pigs developed neutralising antibodies towards the injected isolates, five of them with titres above 32 (2^5). The response towards the homologous Ugandan isolate equalled the response towards other Ugandan isolates, hence, there was no serological difference between the isolates.

The cross neutralisation study confirmed that the Ugandan isolates belonged to serotype O.

The geometric mean EL7/O-Manissa titre ratios were 1.5-3.4 for the five guinea pig anti-EL7 and the pool of these, while the mean was 0.4 for guinea pig anti-O-Manissa, indicating that the Ugandan isolates differed serologically from O-Manissa.

The serotype was confirmed by alignments of full-length (Fig. 1) as well as VP1 (not shown) sequences. The branch lengths between the Uganda strain and other type O strains were of the same order of magnitude as the one between type O1 and PanAsia both for full-length sequences and for VP1 sequences.

Discussion

The Ugandan isolates were characterised as serotype O, but were as different from strain O1 as the PanAsia strain. The clinical syndrome “Otolimo” was identified as photophobia but could be related to heat-intolerance (HI) syndrome, which has been described as a late sequelae of FMDV infection (S. Alexandersen, personal communication, 2004, referring to old studies e.g. mentioned in Scott, Cottral and Gailiunas, 1965). The association between HI and FMD has recently been confirmed in cattle belonging to pastoralists and agropastoralists in Tanzania (Catley et al., 2004), who described HI as a chronic disease sign of FMD. However, “Otolimo” was not recognized as a clinical entity in Uganda until recently, and its appearance may therefore signal an altered epidemiological FMD situation in Uganda with increasing severity of the disease giving rise to more secondary infections and long-term sequelae.

The appearance of a new type O lineage in domestic cattle in Uganda raises the question if type O was always present on the African continent or it was reintroduced – and if reintroduced – from where. The continuous identification of new lineages of FMDV also addresses the question if our

knowledge of the global diversity of FMDV is still so limited or if new lineages emerge by distinct jumps from pre-existing ones.

Authors conclusions

- The 2002 FMDV outbreak in Kumi District of Uganda was caused by a virus belonging to serotype O.
- The strain represents a new lineage of type O.

Authors recommendations

- From the perspective of the serious nature of the PanAsia pandemic it is recommended to further study the type O strains developing in Africa.

References

Catley, A., Chibunda, R.T., Ranga, E., Makungu, S., Magayane, F.T., Magoma, G., Madege, M.J., & Vosloo, W. (2004) Participatory diagnosis of a heat-intolerance syndrome in cattle in Tanzania and association with foot-and-mouth disease. *Prev. Vet. Med.*, 65: 17-30.

Have, P., Lei, J.C. & Scherning-Thiesen, K. (1984) An enzyme linked immunosorbent assay (ELISA) for the primary diagnosis of foot-and-mouth disease. Characterization and comparison with complement fixation. *Acta Vet. Scand.*, 25: 280-296.

Page, R.D. Tree view: an application to display phylogenetic trees on personal computers. *Comp Appl Biosci* 1996; 12: 357-8

Reed, L.J. & Muench, H. (1938) A simple method of estimating fifty per cent endpoints. *Am. J. Hyg.*, 27 (3): 493-497.

Scott, F.W., Cottral, G.E. & Gailiunas, P. (1965) Presence of foot-and-mouth disease virus in the pituitary and central nervous system of experimentally infected cattle. *Proc. Annu. Meet. U. S. Livestock Sanit. Assos.*, 69: 87-93).

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.J. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res* 1997; 24: 4875-82.

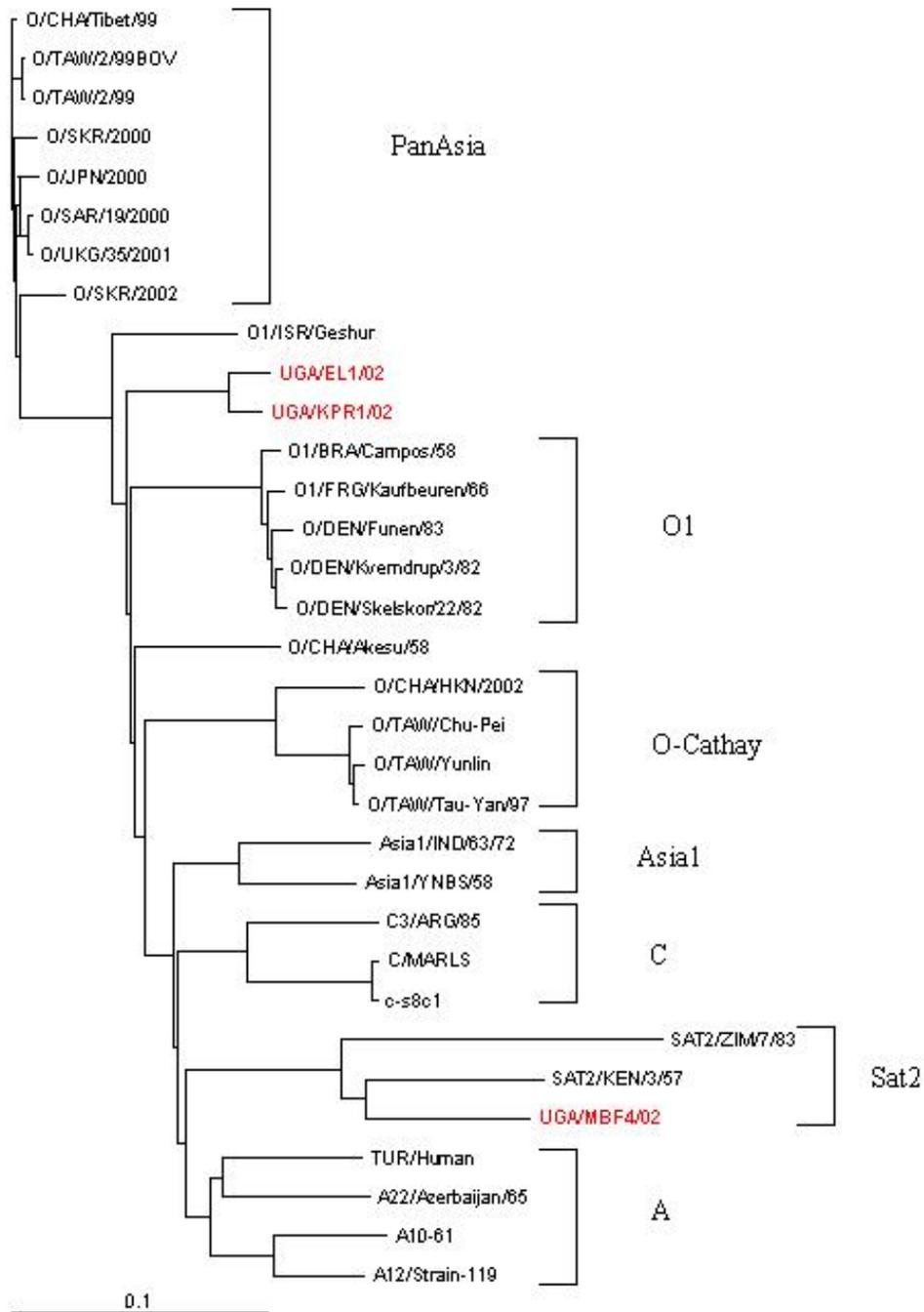


Fig 1: Alignment of full-length sequences of FMDV isolates from Uganda, shown in red, with representatives of full-length sequences available at GenBank and representatives of full-length sequences generated at our laboratory (unpublished data).

Identification of a ninth Foot-and-Mouth disease virus type O topotype and evidence for a recombination event in its evolution

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Abstract:

Introduction: Eight geographically distinct genotypes (topotypes) were previously identified for foot-and-mouth disease virus (FMDV) type O using partial VP1 sequences. These findings were confirmed using a smaller dataset of complete VP1 sequences. During routine molecular epidemiological surveillance we have identified a ninth topotype present in East Africa. **Materials and Methods:** Complete VP1 or P1 sequences were determined following RT-PCR amplification of vRNA and phylogenetic trees constructed using the Neighbor-joining algorithm. Scanning analysis was performed using SimPlot. **Results:** The complete VP1 sequences for 30 FMDV-O isolates from East Africa were determined and compared to representatives of the existing eight topotypes. These comparisons demonstrated the presence of a ninth FMDV-O topotype in Tanzania (1996, 1998), Malawi (1998), Kenya (2002), Uganda (2002, 2004), Burundi (2003) and Rwanda (2004). Further comparisons of the complete P1 capsid-coding region of two representatives of the new topotype, named East Africa 2 (EA-2), with representatives of each of the other eight topotypes confirmed these relationships. Scanning analysis of the P1 region showed that all the topotypes were distinct from each, apart from EA-2 and ME-SA which were more closely related at the 3' end of VP1. **Discussion:** The presence of a previously unrecognised FMDV-O topotype in East Africa had been missed during the original work on topotype classification since, when the partial 3' end sequences of these viruses was compared to other type O viruses, they appeared to be part of the ME-SA topotype. We conclude that at some time in its history a member of the EA-2 topotype underwent genetic recombination with a ME-SA virus with a cross-over point somewhere near the end of the VP1 gene.

Introduction:

Phylogenetic analysis using partial or complete nucleotide sequence data of the VP1 gene is now an internationally accepted method of studying the epidemiology of foot-and-mouth disease (OIE, 2004). Foot-and-mouth disease type O viruses have previously been divided into eight topotypes (geographically distinct genotypes) (Samuel and Knowles, 2001). In general, nucleotide differences between members of each FMDV O topotype exceeded 15%. The topotypes were named by the geographic region in which they occurred: viz. Europe-South America (Euro-SA), Cathay, Middle East-South Asia (ME-SA), southeast Asia (SEA), East Africa (EA), West Africa (WA), Indonesia-1 (ISA-1) and Indonesia-2 (ISA-2).

There have been very few studies on the European FMDV serotypes in Africa (Knowles *et al.*, 1998; Samuel *et al.*, 1999; Samuel and Knowles, 2001; Sangare *et al.*, 2001; Sahle *et al.*, 2004; Bronsvort *et al.*, 2004). However, these studies have shown that there are distinct genetic lineages of both FMDV-O and FMDV-A circulating in East and West Africa and that viruses occurring in North Africa may be introduced from a variety of sources (Europe, South America, Middle East and West Africa). Outbreaks of FMD types O and A in southern Africa are very infrequent and are usually introduced from outside the continent (Knowles *et al.*, 1998; Knowles *et al.*, 2001; Sangare *et al.*, 2001).

In this study we have examined 40 FMDV type O isolates from Africa and classified them by phylogenetic comparison with previously characterized viruses. Since submission of the title and abstract of this paper, nucleotide sequence data published by Sahle *et al.* (2004) has been released and some of our conclusions have therefore been modified.

Materials and Methods:

Viruses

All the virus isolates were obtained from the FAO World Reference Laboratory for Foot-and-Mouth Disease (WRLFMD) strain collection either as 10% epithelial suspensions or as cell culture passaged material (Table 1).

RNA extraction

RNA was extracted directly from these samples using RNeasy spin-columns (Qiagen) as per the manufacturer's instructions. A total volume of 0.46 µl was extracted and resuspended in 50 µl nuclease-free water.

RT-PCR

One-step reverse transcription and PCR was performed as previously described by Knowles and Samuel (1995) using Ready-to-Go One-step RT-PCR beads (Amersham Pharmacia Biotech) and 5 µl of RNA on a PTC-100™ thermal cycler (MJ Research). The thermal profile for all the RT-PCR's was 42 °C for 30 min, 94 °C for 5 min, 35 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1.5 min and finally a single cycle of 72 °C for 5 min. These were then held at 4 °C until processed or stored at -20 °C. The RT-PCR amplicons were analyzed by agar gel electrophoresis and the amount of DNA estimated by comparison with standard markers of a known concentration (GeneRuler 100 bp DNA Ladder Plus, ready-to-use; MBI Fermentas). The amplicon sizes for the primer pairs O-1C244F/NK61, O-1C272F/NK61 and O-1C283F/NK61 (Table 2) were 1162 to 1165, 1132 to 1135 and 1121 to 1124 bp, respectively.

Sequencing

Following treatment with ExoSAP-IT (Exonuclease I and Shrimp Alkaline Phosphatase; USB Corporation) the amplicons were cycle-sequenced with the primers NK72, O-1C499F and O-1C583F (Table 2) using a DTCS Quick Start kit (Beckman Coulter) and run on a CEQ8000™ automated sequencer (Beckman Coulter).

Phylogenetic analysis

Sequences were compiled and edited using BioEdit v5.0.9 (Hall, 1999) and the EpiSeq 2.0 package (N.J. Knowles, unpublished data). Nucleotide distance matrices were generated and used to build Neighbor-joining trees (Saitou and Nei, 1987) using the program Clustal X (Thompson *et al.*, 1997). The trees were drawn using TreeView 1.6.6 (Page, 1996). Confidence levels on the tree branches were assessed by bootstrap analysis (Felsenstein, 1985) as implemented in Clustal X.

Scanning analysis

Aligned sequences in the form of a FASTA format file were analyzed using SimPlot v3.2 (Lole *et al.*, 1999). The nucleotide sequence of O/UGA/3/2002 (EA-2) was compared to the consensus sequences of representatives of each toptype, as follows: Euro-SA (O1/Kaufbeuren/FRG/66, O/Brescia/ITL/47 and O3/VEN/51), Cathay (O/HKN/14/82, O/HKN/6/83 and O/PHI/5/95), ME-SA (O/A/CHA/58, O/IND/53/79, O/SAR/1/2000, O/Manisa/TUR/69), SEA (O/CAM/2/98, O/TAI/189/87, O/TAI/4/99), EA-1 (O/K83/79, O/UGA/5/96), EA-2 (O/BUN/7/2003, O/UGA/3/72, O/RWA/2/2004), WA (O/ALG/2/99 and O/BKF/1/2002), ISA-1 (O/ISA/1/62, O/ISA/8/83) and ISA-2 (O/ISA/1/74 and O/JAV/5/72). A slightly different three-letter code (see the legend in Fig. 4) was used to designate the toptypes for purposes of grouping within the program. The Kimura 2-parameter distance model was used with a window value of 160 and a step of 20.

Results:

All the RNAs extracted in this study were amplified by one or more of the three primer sets used (Table 2). In general the O-1C272F/NK61 primer set was used initially and the others tried if this set failed. The complete VP1 sequences for 40 FMDV-O isolates from Africa were determined and compared to representatives of the existing eight toptypes. Comparison of the complete VP1 sequences was in general agreement with the previous toptype classification (Samuel and Knowles, 2001), except for the presence of a novel lineage present in East Africa, from Uganda and Kenya in the north to Malawi and Zambia in the south (Fig. 1). We have named this new toptype East Africa 2 (EA-2) and renamed the original East Africa toptype as East Africa 1 (EA-1). Additionally, some virus isolates that had been included in the EA-1 toptype based on partial VP1 data (O/ETH/8/95 and O/ERI/1/96) and some newly sequenced viruses (four from Sudan in 2004) fell on a branch slightly more closely related to the WA toptype (Fig. 1) than to the EA-1 viruses. However, bootstrap confidence levels were very low on some of these branches (e.g. 186 and 230 out of 1000 pseudo-replicates) (Fig. 1). Another virus, O/UGA/3/72, was difficult to classify, but may be an early example of the EA-2 toptype (Fig. 1).

On the recent release of nucleotide sequence data generated by Sahle et al. (2004) we have been able to construct an additional tree based on nts 145-642 of VP1 (Fig. 2). On this tree branches leading to the EA-1, EA-2 and the Ethiopia/Eritrea/Sudan group were all well supported by bootstrapping (988, 832 and 829, respectively). In this tree the latter group was most closely related to the EA-1 topotype, however, we tentatively suggest that these viruses may form a new topotype occupying a different geographical area (Ethiopia, Eritrea and Sudan) distinct from EA-1 which is found in Uganda and Kenya. Further sequencing of viruses from these geographic regions may clarify the situation.

To examine the reason why these new lineages had been missed in earlier studies, the sequences used to construct the tree presented in Fig. 1 were truncated to 165 nt at the 3' end of VP1 (nt 478 to 642) and re-analyzed. This tree revealed that some viruses had shifted position (Fig. 3). Essentially, members of the ME-SA, SEA, WA, EA-1, EA-2 and candidate EA-3 topotypes became jumbled. The bootstrap confidence values on most of these branches were very low indicating a poor reliability of the tree.

A sub-set of these sequences were compared to O/UGA/3/2002 using SimPlot (Lole *et al.*, 1999). This program uses a window which is moved across the sequence in specified steps calculating at each point the relationship between the test sequence and other chosen virus sequences (or a consensus of a number of virus sequences). The exact sequences used are listed in materials and methods. The resultant graph (Fig. 4) clearly shows a closer relationship between O/UGA/3/2002 and the ME-SA at the 3' end of VP1.

Discussion:

The sequence comparisons presented demonstrate the existence of a ninth, and possibly a tenth, FMDV-O topotype. One of these new lineages, which we have named East Africa 2 (EA-2) was found in Tanzania (1996, 1998), Malawi (1998), Zambia (2000), Kenya (2002), Uganda (2002, 2004), Burundi (2003) and Rwanda (2004) and possibly earlier in Uganda (1972) while the second, East Africa 3 (EA-3), was found in Ethiopia, Eritrea and Sudan. More extensive studies are required to determine the distribution of the various FMDV O lineages present in Africa, both historically and in recent years. The historical occurrence of FMDV O topotypes in Africa is shown in Fig. 5.

The presence of a previously unrecognised FMDV-O topotype in East Africa had been missed during the original work on topotype classification since, when the partial 3' end sequences of these viruses was compared to other type O viruses, they appeared to be part of the ME-SA topotype. Based on the scanning analysis, we conclude that at some time in its history a member of the EA-2 topotype may have undergone genetic recombination with a ME-SA virus with a cross-over point somewhere near the end of the VP1 gene. Alternatively, it may be that all the African lineages evolved after the introduction of Asian FMD viruses into Africa and more mutations have subsequently accumulated in certain parts of the capsid-coding region while other parts may have remained more conserved. Further characterization is required to clarify the situation with the candidate EA-3 viruses.

Conclusions:

- Two previously unrecognised genetic lineages of FMDV O were identified in East Africa, each having a distinct geographic distribution.
- Recombination near the 3' end of VP1 may have played a role in the evolution of the EA-2 topotype.

Recommendations:

- Future phylogenetic analyses for molecular epidemiological purposes should be based on complete VP1 sequences.
- More extensive molecular epidemiological studies of the European FMDV serotypes in Africa should be undertaken (this is in progress in our laboratory for serotypes O and A).
- More extensive genome analyses need to be performed to assess the role of recombination in the natural evolution of FMD viruses.

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References:

- Bronsvoort, B.M.D., Radford, A.D., Tanya, V.N., Nfon, C., Kitching, R.P. & Morgan, K.L.** 2004. Molecular epidemiology of foot-and-mouth disease viruses in the Adamawa province of Cameroon. *J. Clin. Microbiol.* 42: 2186-2196.
- Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 39: 783-791.
- Forss, S., Strebel, K., Beck, E. & Schaller, H.** 1984. Nucleotide sequence and genome organization of foot-and-mouth disease virus. *Nucl. Acids Res.* 12: 6587-6601.
- Hall, T.A.** 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucl. Acids. Symp. Ser.* 41: 95-98.
- Knowles, N.J. & Samuel, A.R.** 1995. *Polymerase chain reaction amplification and cycle sequencing of the 1D (VP1) gene of foot and mouth disease viruses.* Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease held jointly with the FMD Sub group of the Scientific Veterinary Committee of the Commission of the European Community, Mödling, Vienna, Austria, 19-22 September, 1994. Rome: FAO. Appendix 8: 45-53.
- Knowles, N.J., Ansell, D.M. & Samuel, A.R.** 1998. *Molecular comparison of recent foot-and-mouth disease type A viruses from West Africa with historical and reference virus strains.* Report of the session of the Research Group of the European Commission for the Control of Foot-and-Mouth Disease, Aldershot, United Kingdom, 14-18 September 1998. Rome: FAO, Appendix 4: 41-48.
- Knowles, N.J., Samuel, A.R., Davies, P.R., Kitching, R.P. & Donaldson, A.I.** 2001. Outbreak of foot and mouth disease virus serotype O in the UK caused by a pandemic strain. *Vet. Rec.* 148: 258-259.
- Knowles, N.J. & Samuel, A.R.** 2003. Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.* 91: 65-80.
- Lole, K.S., Bollinger, R.C., Paranjape, R.S., Gadkari, D., Kulkarni, S.S., Novak, N.G., Ingersoll, R., Sheppard, H.W. & Ray, S.C.** 1999. Full length human immunodeficiency virus type 1 genomes from subtype C infected seroconverters in India, with evidence of intersubtype recombination. *J. Virol.* 73: 152-160.
- OIE.** 2004. *Foot and mouth disease* (Chapter 2.1.1). Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 5th Edition. 1178 pp. (also available at www.oie.int).
- Page, R.D.M.** 1996. TREEVIEW: An application to display phylogenetic trees on personal computers. *Computer Applic. Biosci.* 12: 357-358.
- Samuel, A.R. & Knowles, N.J.** 2001. Foot-and-mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *J. Gen. Virol.* 82: 609-621.
- Samuel, A.R., Knowles, N.J. & Mackay, D.K.J.** 1999. Genetic analysis of type O viruses responsible for epidemics of foot-and-mouth disease in North Africa. *Epidemiol. Infect.* 122: 529-538.
- Saitou, N. & Nei, M.** 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Mol. Biol. Evol.* 4: 406-425.
- Sahle, M., Venter, E.H., Dwarka, R.M. & Vosloo, W.** 2004. Molecular epidemiology of serotype O foot-and-mouth disease virus isolated from cattle in Ethiopia between 1979-2001. *Onderstepoort J. Vet. Res.* 71: 129-138.
- Sangare, O., Bastos, A.D.S., Marquardt, O., Venter, E.H., Vosloo, W. & Thomson, G.R.** 2001. Molecular epidemiology of serotype O foot and mouth disease virus with emphasis on West and South Africa. *Virus Genes* 22: 345-351.
- Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G.** 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* 25: 4876-4882.

Table 1. The origin of FMD O viruses used in this study

WRLFMD Ref. No.	Geographical origin	Date of collection	Species	Topotype	Accession no.	Reference
O/ALG/2/99	Bir touta, Blida, Algeria	02/1999	Bovine	WA	-	This work
O/BKF/1/2002	Traore Siaka, Kologo (Kokoro), Du Kene Dougou, Burkina Faso	2002	Bovine	WA	-	This work
O/BUN/3/2003	Nshinyabigoye Ic, Buganda, Cibitoke, Uganda	28/08/2003	Bovine	EA-2	-	This work
O/BUN/6/2003	Ndikumagenge, Bujumbura, Mairie, Uganda	08/09/2003	Bovine	EA-2	-	This work
O/BUN/7/2003	Ndikumagenge, Bujumbura, Mairie, Uganda	08/09/2003	Bovine	EA-2	-	This work
O/CAM/2/98	Kâmpông Spey, Cambodia	05/01/1998	Bovine	SEA	AJ294909	Knowles et al., 2001
O/CAR/6/89	Adamaoua, Cameroon	04/1989	Bovine	WA	-	This work
O/CAR/16/2000	Vina, Ngaoundere, Cameroon	20/09/2000	Porcine	WA	-	This work
O/CAR/17/2000	Vina, Ngaoundere, Cameroon	20/09/2000	Porcine	WA	-	This work
O/A/CHA/58	Akesu, XinJiang, P.R. China	1958	Bovine	ME-SA	AJ131469	Q. Zhao et al., unpublished ^a
O/CIV/8/99	Côte d'Ivoire	1999	Bovine	WA	AJ303485	Samuel and Knowles, 2001
O/ERI/1/96	Asmara, Eritrea	19/01/1996	Bovine	EA-3?	-	This work
O/ETH/8/94	Highland area of eastern Ethiopia	02/02/1994	Bovine	EA-3?	-	This work
O ₁ /Kaufbeuren/ FRG/66	Kaufbeuren, Federal.R.epublic of Germany	1966	Bovine	Euro-SA	X00871	Forss et al., 1984
O/GHA/5/93	Kintampo, Ghana	06/01/1993	Bovine	WA	AJ303488	Samuel & Knowles, 2001
O/HKN/6/83	Pokfulam, Hong Kong Island	18/12/1982	Bovine	Cathay	AJ294919	Knowles et al., 2001
O/HKN/14/82	Hei Ling Chau Island, N.T., Kowloon, Hong Kong	25/02/1982	Porcine	Cathay	AJ294917	Knowles et al., 2001
O/IND/R2/75*	Tamil Nadu, India	1975	NK	ME-SA	AF204276	Hemadri et al., unpub. ^b
O/IND/53/79	Tamil Nadu, India	1977	Bovine	ME-SA	AF292107	Hemadri et al., unpub. ^c
O/IRQ/30/2000	Iraq	09/04/2000	Bovine	ME-SA	AJ303499	Samuel & Knowles, 2001
O/ISA/1/62	Bali, Indonesia	07/1962	Bovine	ISA-1	AJ303500	Samuel & Knowles, 2001
O/ISA/1/74	Bali Quarantine Station, Indonesia	1974	Bovine	ISA-2	AJ303501	Samuel & Knowles, 2001
O/ISA/9/74	Bali, Indonesia	30/10/74	Bovine	ISA-1	AJ303502	Samuel & Knowles, 2001
O/ISA/8/83	East Java, Indonesia	1983	Bovine	ISA-1	AJ303503	Samuel & Knowles, 2001
O/JAV/5/72	Java, Indonesia	1972	NK	ISA-2	AJ303509	Samuel & Knowles, 2001
O ₂ /Brescia/ITL/47	Brescia, Italy	1947	NK	Euro-SA	M55287	Krebs et al., 1991a
O ₉ /KEN/102/60	Nanyuki, Kenya	1960	NK	EA-1	-	This work
O/K83/79*	Mweiga, Nyeri Dist., Central Province, Kenya	1979	Bovine	EA-1	AJ303511	Samuel & Knowles, 2001
O/KEN/2/95	Homa Bay, Kenya	1995	NK	EA-1	AJ303514	Samuel & Knowles, 2001
O/KEN/3/2002	Mundulusia location, Busia, Kenya	29/07/2002	Bovine	EA-2	-	This work
O/KEN/5/2002	Kiratina Location, Nakuru, Kenya	09/10/2002	Bovine	EA-2	-	This work
O/KEN/7/2002	Rumuruti, Kenya	04/11/2002	Bovine	EA-2	-	This work
O/MAL/1/98	Mwongulukulu dip tank, Karonga, Northern Province, Malawi	06/11/1998	NK	EA-2	-	This work
O/MAL/2/98	Mwongulukulu dip tank, Karonga, Northern Province, Malawi	06/11/1998	NK	EA-2	-	This work
O/MAU/19/2000	Nieleba, Guidimakha, Mauritania	10/2000	NK	WA	-	This work
O/NGR/11/2001	Tchimoumouna, Niger	19/09/2001	Bovine	WA	-	This work
O/PHI/5/95	Bauang, La Union, Philippines	09/02/1995	Porcine	Cathay	-	This work
O/PHI/7/96	Mahabang Parang, Angono, Philippines	1996	Porcine	Cathay	AJ294926	Knowles et al., 2001
O/RWA/2/2004	Rwanda	2004	NK	EA-2	-	This work
O/RWA/3/2004	Rwanda	2004	NK	EA-2	-	This work
O/SAR/1/2000	KwaZulu-Natal Province, South Africa	2000	Bovine	ME-SA	-	This work
O/SUD/16/2004	Turalei, Turalei, Twic, Sudan	04/09/2004	Bovine	EA-3?	-	This work
O/SUD/25/2004	Pindit, Luanyaker, Sudan	07/09/2004	Bovine	EA-3?	-	This work
O/SUD/26/2004	Pindit, Luanyaker, Sudan	07/09/2004	Bovine	EA-3?	-	This work
O/SUD/30/2004	Pindit, Luanyaker, Sudan	07/09/2004	Bovine	EA-3?	-	This work
O/TAI/189/87*	Udonthani, NE region, Thailand	1987	Bovine	SEA	-	This work
O/TAI/4/99	Mae Hong Son, Thailand	01/03/1999	Bovine	SEA	AJ303536	Samuel & Knowles, 2001
O/TAN/3/96	Maili Moja, Kibaha, Tanzania	25/07/1996	Bovine	EA-2	-	This work
O/TAN/7/98	Kyela, Mbeya, Tanzania	22/10/1998	Bovine	ME-SA	AJ296320	Samuel & Knowles, 2001
O/TAW/81/97	I-lan, Taiwan Province of China	17/04/1997	Porcine	Cathay	AJ296321	Samuel & Knowles, 2001
O/TAW/2/99	Kinmen Island Prefecture, Taiwan Province of China	1999	Bovine	ME-SA	AJ539137	Mason et al., 2003
O ₁ /Manisa/TUR/69	Manisa, Turkey	01/04/1969	Bovine	ME-SA	AJ251477	Aktas and Samuel, 2000
O/UGA/3/72	Uganda	1972	NK	EA-2?	-	This work
O/UGA/5/96	Mbarara, Uganda	17/01/1996	Bovine	EA-1	AJ296327	Samuel & Knowles, 2001

O/UGA/3/2002	Nakasongola, Uganda	2002	NK	EA-2	-	This work
O/UGA/5/2002	Uganda	2002	NK	EA-2	-	This work
O/UGA/6/2002	Nakasongola, Uganda	2002	NK	EA-2	-	This work
O/UGA/1/2004	Wakiso, Uganda	20/01/2004	Bovine	EA-2	-	This work
O/UGA/3/2004	Wakiso, Uganda	20/01/2004	Bovine	EA-2	-	This work
O/UGA/4/2004	Mukono, Uganda	04/02/2004	Bovine	EA-2	-	This work
O/UGA/5/2004	Nakasongola, Uganda	12/02/2004	Bovine	EA-2	-	This work
O/UGA/6/2004	Nakasongola, Uganda	12/02/2004	Bovine	EA-2	-	This work
O/UGA/7/2004	Nakasongola, Uganda	12/02/2004	Bovine	EA-2	-	This work
O/UGA/8/2004	Nakasongola, Uganda	12/02/2004	Bovine	EA-2	-	This work
O/UGA/18/2004	Kampala, Uganda	23/03/2004	Bovine	EA-2	-	This work
O/UKG/12/2001	Essex, United Kingdom	19/02/2001	Porcine	ME-SA	-	This work
O ₃ /VEN/51	Venezuela	1951	NK	Euro-SA	AJ004645	Leister et al., 1993
O/VIT/7/97	Har, Yrongpa, Gialai, Vietnam	31/08/1998	Bovine	SEA	AJ296328	Samuel & Knowles, 2001
O/ZAM/1/2000	Mpulungu, Zambia	25/02/2000	Bovine	EA-2	-	This work
O/ZAM/2/2000	Mpulungu, Zambia	25/02/2000	Bovine	EA-2	-	This work

a) Zhao Q., Chang H., Liu Z., Liu W. and Xie Q. Nucleotide sequence of VP1 encoding regions of foot-and-mouth disease virus type O strains isolated from pig. Unpublished.

b) Hemadri, D., Venkataramanan, R., Sanyal, A., Tosh, C., Manju, G. and Gurumurthy, C.B. Nucleotide sequence of structural gene coding region of FMDV serotype O (R2/75). Unpublished.

c) Hemadri, D., Venkataramanan, R., Gurumurthy, C., Sanyal, A. and Tosh, C. Comparison of the structural gene coding region of Indian foot-and-mouth disease virus serotype O. Unpublished.

Table 2. Primers for RT-PCR and sequencing of FMDV O RNA.

Designation	Sequence (5' to 3')	Genome location	
		Gene	Position within the gene
O-1C244F	GCAGCAAAACACATGTCAAACACCTT	VP3	244-269
O-1C272F	TBGCRGGNCTYGCCCAGTACTAC	VP3	272-294
O-1C283F	GCCCAGTACTACACAGTACAG	VP3	283-305
NK61	GACATGTCCTCCTGCATCTG	2B	58-77
NK72	GAAGGGCCCAGGGTTGGACTC	2A/2B	34-48; 1-6
O-1C499F	TACGCGTACACCGCGTC	VP3	499-515
O-1C583F	GACGGYGAYGCICTGGTCGT	VP3	583-602

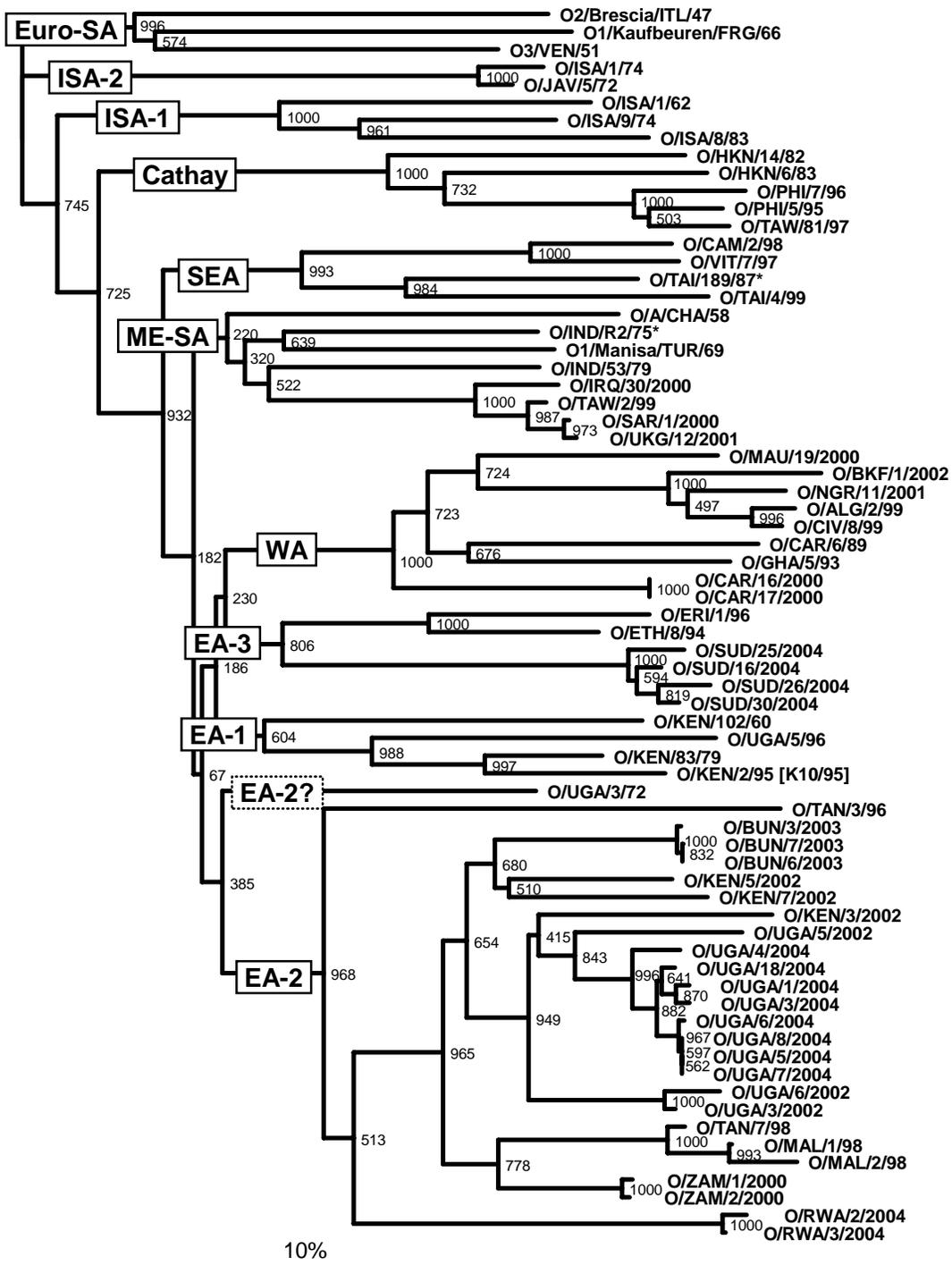


Fig. 1. Neighbor-joining tree showing the relationships between the complete VP1 gene sequences (length = 639 or 642 nt) of FMDV-O isolates from Africa, reference strains and other field isolates. The numbers shown at the nodes refer to the number of times (out of 1000) the branch to the left of the number was represented in the bootstrap analysis. Isolate names marked with an asterisk are not WRLFMD reference numbers.

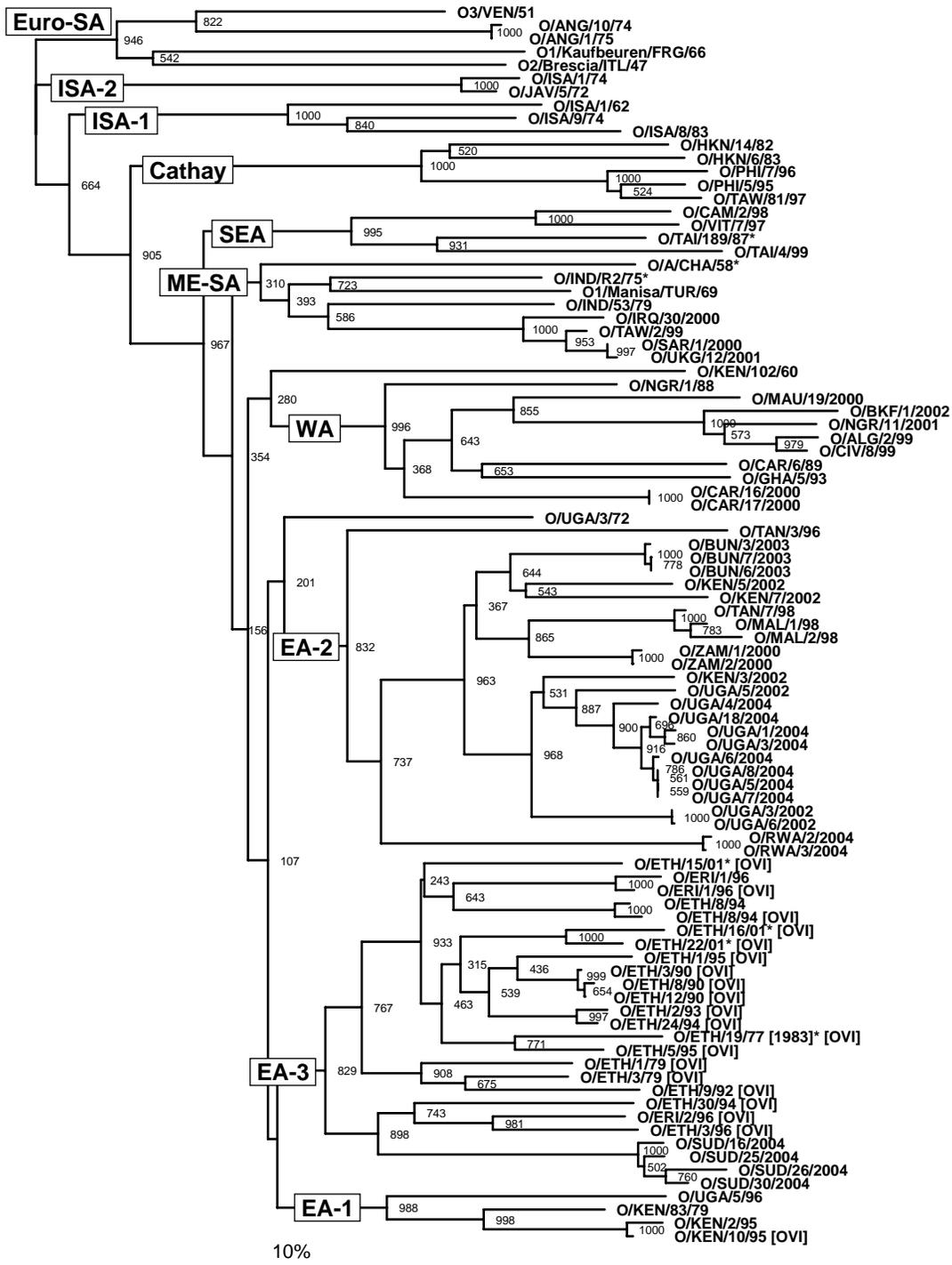


Fig. 2. Neighbor-joining tree showing the relationships between the partial VP1 gene sequences (nt 145 to 642) of FMDV-O isolates from Africa, reference strains and other field isolates. The numbers shown at the nodes refer to the number of times (out of 1000) the branch to the left of the number was represented in the bootstrap analysis. Isolate names marked with an asterisk are not WRLFMD reference numbers. OVI = Sequences determined at the Onderstepoort Veterinary Institute; for details of isolates see Sangare *et al.* (2001) and Sahle *et al.* (2004).

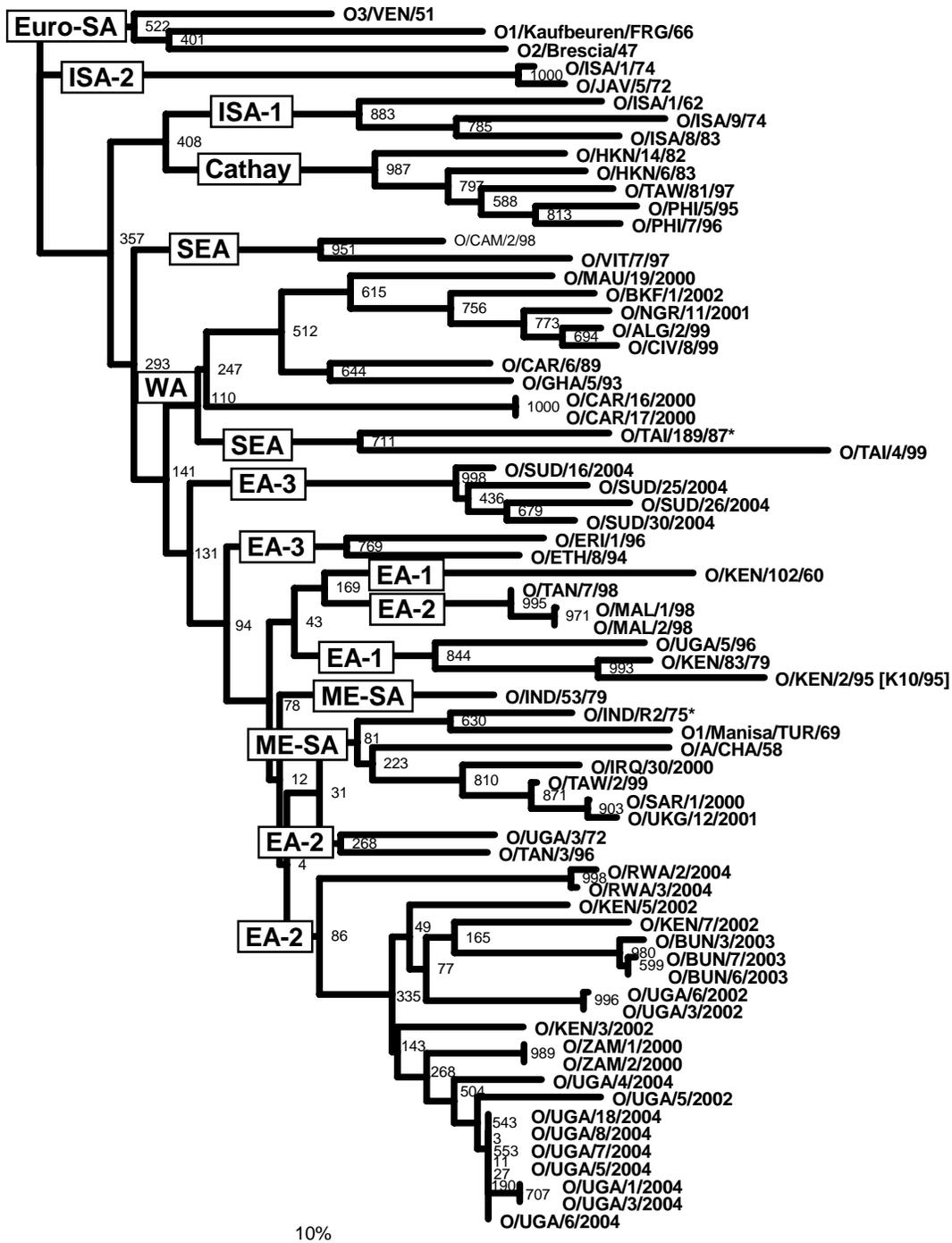


Fig. 3. Neighbor-joining tree showing the relationships between the partial VP1 gene sequences (nt 478 to 642) of FMDV-O isolates from Africa, reference strains and other field isolates. The numbers shown at the nodes refer to the number of times (out of 1000) the branch to the left of the number was represented in the bootstrap analysis. Isolate names marked with an asterisk are not WRLFMD reference numbers.

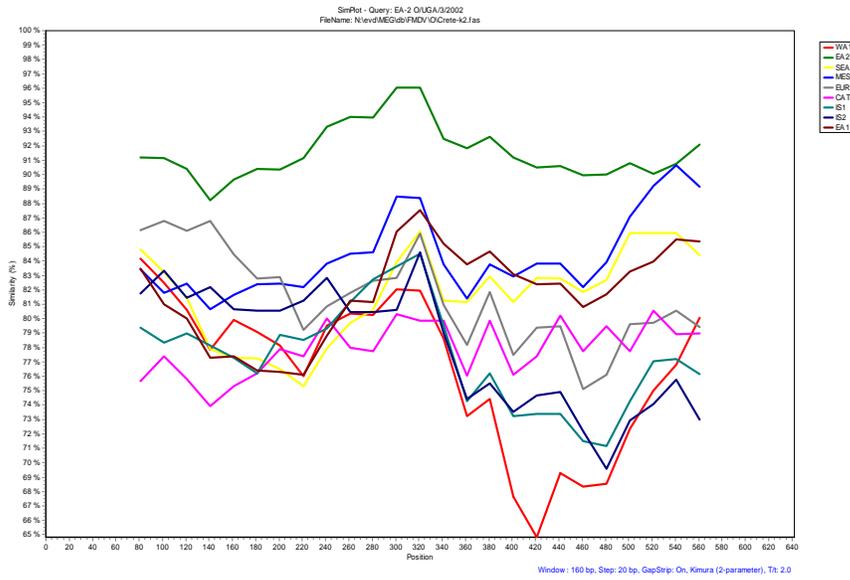


Fig. 4. Scanning plot of the percentage similarity of the VP1 gene of representatives of the nine FMDV O topotypes compared to O/UGA/3/2002 (EA-2). The topotypes are labelled as follows: EUR, Europe-South America; CAT, Cathay; SEA, Southeast Asia; MES, Middle East-South Asia; IS1, Indonesia 1; IS2, Indonesia 2; EA1, East Africa 1; EA2, East Africa 2; and WA1, West Africa.

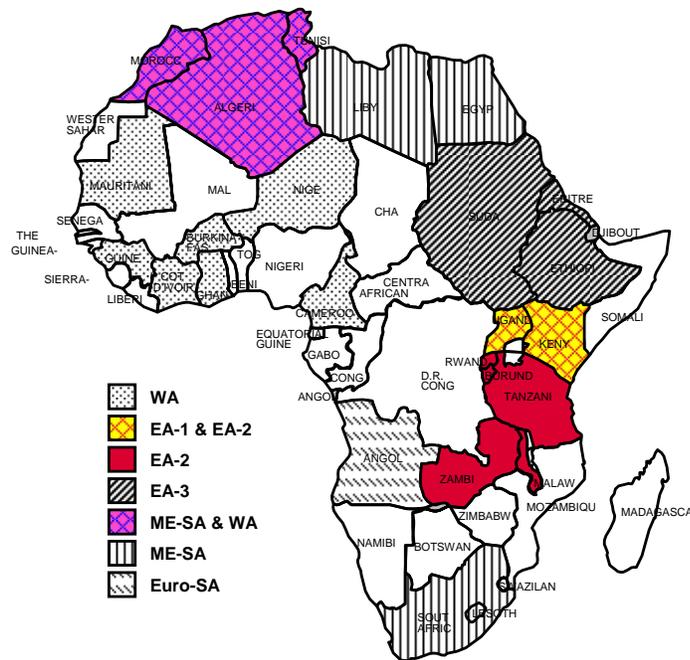


Fig. 5 The occurrence of FMDV type O topotypes in Africa.

High-resolution molecular analysis of the 1982-3 FMD epidemic in Denmark

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Abstract

The present study was conducted as a contingency exercise in molecular epidemiology, in particular exploiting the quasi-species structure of FMDV strains, with the additional purpose of gaining knowledge of epidemiological scenarios after introduction to a disease-free, non-vaccinated area. Seventeen isolates collected during the epidemic involving 23 outbreaks - 22 outbreaks on the island of Funen and one outbreak on the island of Zealand - were analysed by full-length sequencing of VP1 and aligned with available VP1 sequences using ClustalX.

The Denmark 1982-3 isolates cluster together with O1 strains but differs significantly from vaccine strain sequences available. The isolates can be clustered in three groups (strains) that correspond perfectly with the patterns of putative contact transmissions as revealed during the epidemic. The distribution of sequence markers is indicative of common recombination events in the history of the 3 strains. One outbreak on the island of Zealand was due to a virus strain identical to a strain emerging in 4 outbreaks on a location of Funen and meteorological data revealed conditions allowing an airborne transmission over a distance of 70 km to have taken place.

The data complies with a model where emerging epidemics most often is due to the appearance of a multiplicity of strains. Three strains were introduced to Funen, most likely by airborne transmission, from the former German Democratic Republic (GDR), and one case was introduced to the island of Zealand by airborne transmission from Funen. The indication of common recombination events in the history of the 3 strains suggest a source in an area with endemic FMD and this complies with a hypothesis that a preceding epidemic in the former GDR was due to an introduction from Ukraine.

Introduction

After absence of FMD in Denmark since 1970, vaccination against FMD was prohibited from January 1977. However, during March 14 to May 4, 1982, FMD was diagnosed in 20 cattle herds and 2 pig herds in Denmark (Westergaard, 1982). Twentyone farms were located on the island of Funen and one was located at the south-west coast of the island of Zealand. Still adopting the policies of non-vaccination and depopulation of FMDV-infected premises, a single outbreak of FMD was diagnosed in a cattle herd on the island of Funen in January 1983 (Anonymous, 1983). Thus, this epidemic in Denmark resembles the one in the UK by appearing in an FMDV-free, highly susceptible population of cloven-hoofed animals but differs significantly in terms of dissemination of the infection after introduction. The present study was conducted as a contingency exercise in molecular epidemiology with the additional purpose of gaining knowledge of epidemiological scenarios after introduction to a disease-free, non-vaccinated area. The study, in particular was an attempt to exploit the quasi-species nature of FMDV strains for high-resolution molecular analysis of epidemiological dynamics.

Materials and Methods

Virus samples and cultivation

A total of 21 of the 23 outbreaks were confirmed during the epidemic by virus isolation on primary cell cultures, or by inoculation in baby mice. Seventeen of these isolates collected from different farms were available and included in the present study. All isolates were propagated on monolayers of primary calf kidney cells grown to confluency according to standard procedures.

Sequencing and phylogenetic analyses

RNA was purified from cell culture supernatants and sequenced using a Type O VP1 full-length sequencing method to be published elsewhere. According to this method sequences are edited by reading in both directions of the PCR products using the PCR primers. Overlapping PCR products result in an additional 2-fold sequencing to be included as raw data in the sequence editing process. Sequences most identical to the VP1 coding regions of the Denmark 82-3 epidemic was identified using Blast (Altschul et al., 1997). Multiple alignments and phylograms of the sequences generated during the present study and sequences retrieved from the Genbank sequence database were done with ClustalX (EMBL, Heidelberg, Germany, May 1994) (Thompson et al., 1997) using the default parameters and 1000 bootstrap replications. The aligned sequences were presented with GeneDoc (Nicholas et al., 1997) and dendrograms were visualized with TREEVIEW (Page, 1996), version 25.

Meteorological data

Meteorological data were retrieved from weather charts recorded 6 times daily by the Danish Meteorological Institute.

Results

The Denmark 1982-3 isolates were found to cluster together with the O1 field isolates collected from Germany and the vaccine strain O1/FRG/Kaufbeuren/66 used as vaccine in FRG at that time (Beck and Strohmeyer, 1987). The isolates could be referred to three distinct populations as indicated by ellipses in Fig. 1 by a number of nucleotide differences consistently found between isolates of the three populations. The three populations are defined as distinct strains designated O/DEN/Funen/A/82, O/DEN/Funen/B/82 and O/DEN/Funen/83 and are suggested to represent individual introductions to Denmark (the O/DEN section of the names is omitted from the results and discussion of the present report). Within each of the strains a number of nucleotide differences are observed but these differences on several occasions could be identified among plaque clones of the same isolate and are considered to represent strain inherent diversities.

In Fig. 1 is also shown the epidemiological links as revealed by interviews of farmers conducted by the Danish Veterinary Service during the epidemic (Westergaard, 1982; Anonymous, 1983).

The relationship of Skelskor/22/82 with strain Funen/B/82 identified in northern Funen as indicated in Fig. 2 led to a hypothesis of airborne transmission from the herds in northern Funen. The meteorological conditions were reviewed for April 1982 on days with wind directions allowing such a transmission as shown in Table 1.

Discussion

The number of isolates of the present study is sub-optimal to define quasi-species structures of strains as they are to be composed of isolates from different farms based on the assumption that these farms were infected by the same strain. Yet, the revealing of epidemiological links by the Veterinary Service during the epidemic (Westergaard, 1982, Anonymous, 1983) and the sequence information as it presents itself makes the approach feasible in this case.

The identification of three populations representing three strains introduced to Denmark complies perfectly with the epidemiological links revealed during the epidemic (Westergaard, 1982; Anonymous, 1983) as shown in Fig. 1. However, the possibility that concurrent introductions to more than one farm of virus referred to any of the three strains cannot be excluded.

Strain Funen/83 was collected from an outbreak 9 months after the last outbreak on Funen in spring 1982. Based on a number of observations and a potential reservoir being sheep introduced to the barn in the winter of 1982-3 it was anticipated that this outbreak was due to an introduction in spring 1982 (Anonymous, 1983). The farm was located in an area where two outbreaks were diagnosed in April 82 in pig herds from which no virus was isolated. Whether these three outbreaks represent separate introductions or only one remains an open question.

The questions if any of the three strains could have evolved from each other during a spread in Denmark should be addressed. Mutations emerging in single virus particles and accumulating in a population would expand the diversity of the population unless a bottleneck appeared and provided there was no selective advantage of such mutations. A Dn/Ds ratio of 0.5 found for the nucleotide differences between the variants do neither indicate any restraints nor a selective advantage of the differences and given a bottleneck appeared, the manifestation of a mutation in a major sub-population of a strain therefore would be relatively rare. Given the unique markers of each strain found among all isolates of that strain successive incidences of bottlenecks resulting in the manifestation of minority variants as a majority population of the resulting strain and mutations would have to take place and this renders a linkage of any of the three strains by mutational evolution unlikely in such a short time span. The other question is if any of the three strains could have evolved from another strain by bottleneck transmission of a pre-existing minority variant. Given that the three strains were introduced from epidemiologically related sources (to be discussed below) any variant observed in any of the three strains could be present in trace amounts in both of the other two strains. However, based on the striking coincidence that no such hypothetical minority transmission is seen but in those cases where no transmission routes were indicated (Fig. 1) such an explanation remains hypothetical.

Although the three strains could hardly have evolved from each other, the isolates share the highly specific markers of an inherent diversity in two nucleotide positions. This strongly indicates a common source or epidemiologically related sources. Furthermore, the combination of an apparent strict linkage in isolates of some markers different for each of the three strains and the sharing of hyper-

variable markers by the three strains suggest that they share in their evolutionary history common recombination events which implies that the source(s) of the strains is located in an area with endemic FMD, i.e. an area where animals incidentally become co-infected or super-infected with different strains. Contacts between the national veterinary services at that time revealed that an epidemic had emerged at the north-west coastal area of GDR approximately 3 weeks before the outbreak in Denmark. This epidemic presumably emerged after the importation of large amounts of meat from Ukraine to the city of Anklam, GDR (Dr. E. Stougaard, former Chief Veterinary Officer of Denmark, Personal Communication). A spread from northern GDR to Denmark of FMDV strains originating in Ukraine complies perfectly with the deductions of the present study.

Vaccines against FMD used at that time were often inadequately inactivated and cases of FMD in Central Europe at the time were concluded to be sero-type O1 vaccine-related (Beck and Strohmayer, 1987). However, the characteristics and in particular the distinct differences between the three strains emerging in Denmark do not support a hypothesis of a common vaccine ancestry.

Long distance airborne transmission has previously been shown to incidentally play a significant epidemiological role in the introductions of Aujeszky's disease virus to the island of Funen as well as to other border areas of Denmark (Christensen et al., 1990; Christensen et al., 1993) and it is straightforward to suggest that the FMD epidemic in 1982-3 was also due to airborne transmission. We have no data to positively support an explanation of airborne transmission of FMDV to Funen but the conclusion that three strains were introduced almost concurrently render a number of alternative explanations less likely. An airborne transmission from northern parts of DDR would imply transportations over a distance of 125 - 160 km mostly over the sea, which is not unprecedented (Gloster et al., 1982; Donaldson et al., 1982). The suggestion that the outbreak at the south-west coast of Zealand was caused by airborne transmission from the northern part of Funen is based not only on strain identity but also on meteorological data allowing airborne transmission to have taken place on two days where the putative source was shedding virus.

The present report basically confirms the transmission routes as suggested by the investigations carried out by the Danish Veterinary Service during the epidemic in Denmark in 1982-3 (Westergaard, 1982; Anonymous, 1983). Thus, contact transmission by a practicing veterinarian during the initial phase attributed to a respiratory infection with FMDV and by truck drivers collecting dairy milk or pigs for slaughter could explain all cases of secondary spread of the infection. Strict measures were imposed on veterinarians and truck drivers in Denmark during the epidemic in the UK in 2001 to avoid similar contact transmissions. Had such measures been implemented after the first suspicion of FMD during the epidemic in Denmark in 1982-3 it is assessed, based on the epidemiological dynamics as now revealed, that no transmission from foci of primary introductions would have taken place.

Authors conclusions

- The 1982-3 FMD-epidemic in Denmark was due to three introductions and one case of airborne transmission between two islands in Denmark
- The source was from an area with endemic FMD most likely transmitted via East Germany

Authors Recommendation

- A European contingency in molecular epidemiology is elaborated allowing high-resolution analysis within 1-2 days

References

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W. & Lipman, D.J. 1997.

"Gapped BLAST and PSI-BLAST: a new generation of protein database search programs", *Nucleic Acids Res* 25:3389-3402.

Anonymous. 1983. Report on a single case of foot-and-mouth disease on the island of Funen, Denmark, January 1983. The Danish Veterinary Service and State Veterinary Institute for Virus Research, Denmark.

Beck, E. & Strohmaier, K. (1987) Subtyping of European foot-and-mouth disease virus strains by nucleotide sequence determination. *J Virol* 61: 1621 - 1629.

Christensen, L. S., Mousing, J., Mortensen, S. Soerensen, K. J., Strandbygaard, S. B., Henriksen, C. A. & Andersen, J. B. 1990. Evidence of long distance airborne transmission of Aujeszky's disease (pseudorabies) virus. *Vet Rec* 127 (19): 471 - 474.

Christensen, L. S.; Mortensen, S.; Boetner, A.; Strandbygaard, B. S.; Roensholt, L.; Henriksen, C. A. and Andersen, J. B. 1993. Further evidence of long distance airborne transmission of Aujeszky's disease (pseudorabies) virus. *Vet Rec* 132: 317 - 321.

Donaldson, A.I., Gloster, J., Harvey, L.D.J. & Deans, D.H. 1982. Use of prediction models to forecast and analyse airborne spread during the foot-and-mouth disease outbreaks in Brittany, Jersey and the Isle of Wight in 1981. *Vet Rec* 110: 53-57.

Gloster, J., Sellers, R.F. & Donaldson, A.I. 1982. Long distance transport of foot-and-mouth disease virus over the sea. *Vet Rec* 110: 47-52.

Nicholas, K.B., Nicholas, H.B.Jr. & Deerfield, D.W. 1997. GeneDoc: Analysis and Visualization of Genetic Variation, *EMBNEWS* 4:14

Page, R.D. 1996. Tree view: an application to display phylogenetic trees on personal computers. *Comp Appl Biosci* 12: 357-8

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin & F., Higgins, D.J. 1997. The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl Acids Res* 24: 4875-82.

Westergaard, J.M. (Ed.). 1982.. Report on the eradication of foot-and-mouth disease on the islands of Funen and Zealand, Denmark 1982. The Danish Veterinary Service, Copenhagen, Denmark.

Table 1: Meteorological conditions reported every 3 hours at the airport of Funen on days with wind directions along the putative route of transmission

Date	Wind velocity	Temperature	Relative humidity	Remarks
April 12	5-10 m/s	4-8°C	48-73%	Clouded, unsteady wind velocity and direction
April 15	4-6 m/s	12-13°C	48-62%	Clouded, unsteady wind velocity and direction
April 16	5-9 m/s	5,7-12,6	53-81%	Clear sky, unsteady wind velocity and direction
April 17	3-9 m/s		28-92	Partly clouded to clear sky, Steady wind, variable humidity
April 19	1-2 m/s	2,7-8,8	65-84%	Clouded, no wind
April 21	1-4 m/s	4,2-6,4°C	70-90%	Clouded, steady wind velocity and direction
April 24	1-4 m/s	3,1-4,8°C	89-92%	Rain, no wind
April 25	1-7 m/s	3,5-11,9°C	57-95%	Variable conditions
April 26	7-8 m/s	6,2-8,1°C	75-87%	Windy
April 27	2-9 m/s	2,3-15,8°C	50-91	Clear sky and variable humidity - otherwise periods of steady wind velocity and direction
April 28-30	5-13 m/s	2,3-10,8	41-91%	Windy

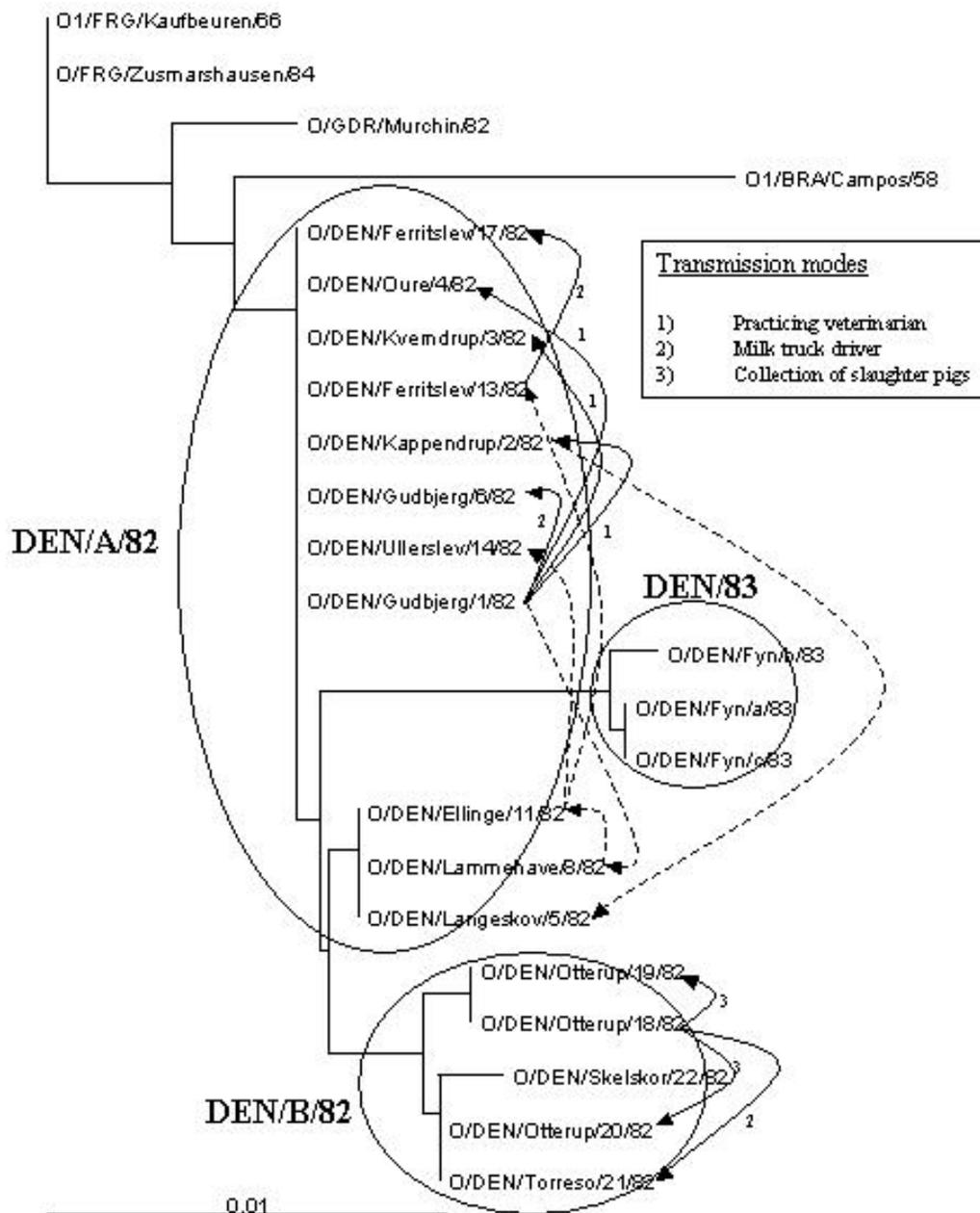


Figure 1. High-resolution dendrogram based on the alignment of VP1 sequences of all isolates from the outbreaks in Denmark in 1982-3 with VP1 sequences of other O1 FMDV strains. Putative strains are indicated by ellipses and putative transmission links as revealed by the Danish Veterinary Service during the epidemic (Westergaard, 1982; Anonymous, 1983) are also shown. Full lines indicate links of high validity and dashed lines indicate links of lower validity.

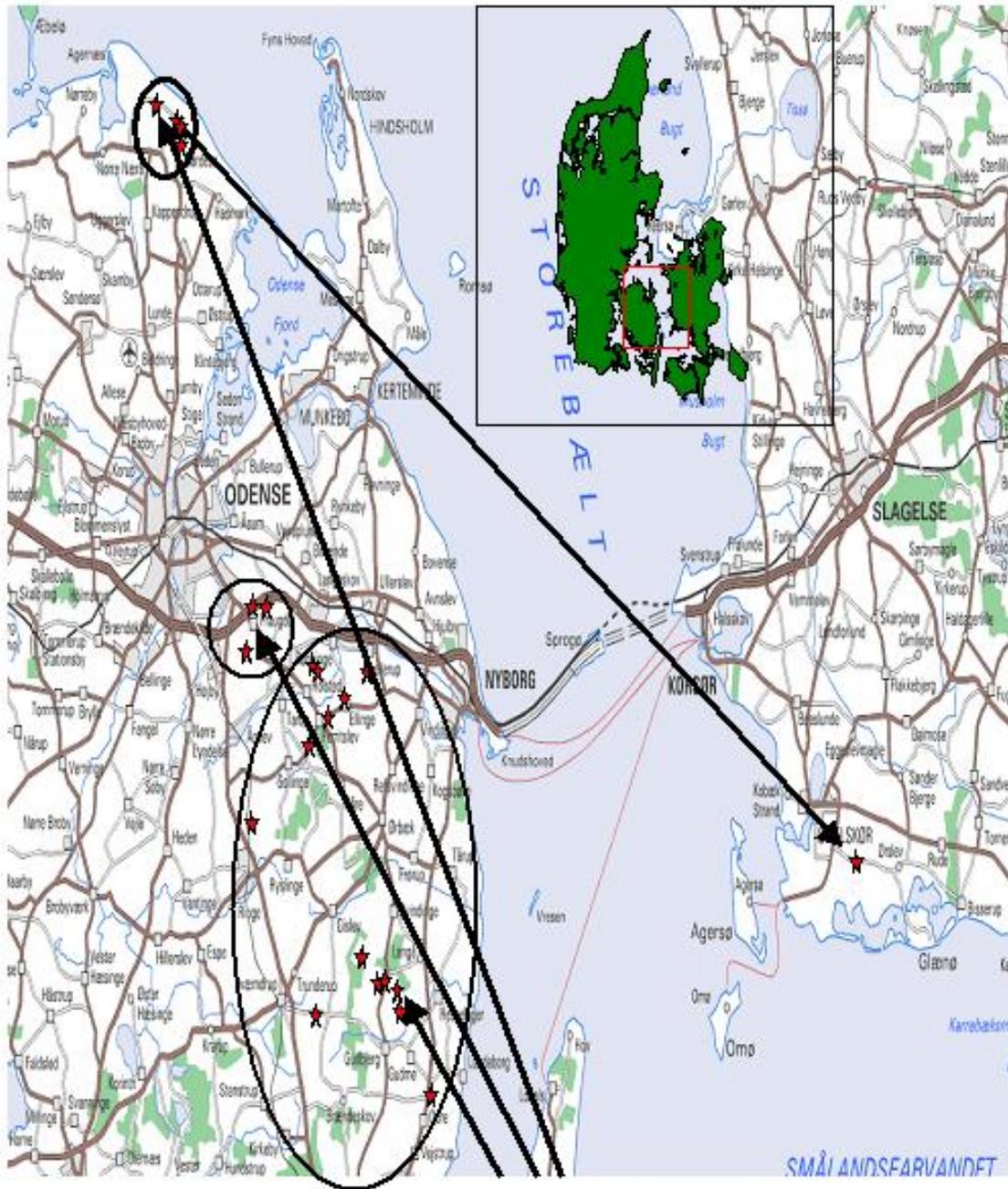


Figure 2. Localisation of the outbreaks of foot- and mouth disease in Denmark in 1982-3. Putative primary introductions and the case of airborne transmission from the island of Funen to the island of Zealand are indicated by arrows.

Genetic and antigenic analysis of Italian 1993 FMDV isolates

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Abstract:

The origin and evolution of foot-and-mouth disease type O viruses that caused the outbreak occurred in Italy in 1993, the first episode of the disease in the EU after adoption of a non-vaccination policy that started in 1991, have been studied by the analysis of sequences encoding three main antigenic sites on viral capsid proteins. The phylogenetic tree derived from sequences spanning the carboxyterminal end of VP1 showed that the Italian viruses were closely related to previous Middle East isolates. For some of the isolates analyzed values of fixation of nucleotide substitutions per year were observed in the three regions analyzed, ranging from 1.7 to 2×10^{-2} . The use of a panel of new monoclonal antibodies rose against an isolate from this outbreak, as well as monoclonal antibodies raised against FMDV O1-Switz, showed differences in the reactivity pattern among some of the Italian isolates analyzed. These results support the potential for viral diversification in a limited period of time and under epidemiological conditions in which no vaccination campaigns were being implemented.

Introduction:

Foot-and-mouth disease (FMD) was eradicated in the EU in 1991, after more than two decades of regular vaccination with chemically inactivated viruses (Barteling and Vreeswijk, 1992). Since then, a non-vaccination, total stamping out policy was adopted, following demonstration of the vaccine origin of the most of the latest outbreaks (Beck and Strohmaier, 1987; Carrillo et al., 1990). However, there is an evident risk of reintroduction of the disease not only from border areas but also from far locations as took place in U.K. and other European countries in 2001 (Knowles 2001), associated to the growing globalisation of livestock trade (Sobrino and Domingo, 2001).

Protection against FMD is associated to the induction of FMDV neutralizing antibodies in vaccinated and convalescent animals (Van Bekkum, 1969; Martin and Chapman 1961). FMDV capsid proteins contain B cell antigenic sites that constitute the targets of the neutralizing antibodies elicited in natural hosts (reviewed in Brown, 1995; Mateu et al., 1995). The main B cell antigenic site in capsid proteins has been identified for serotypes A, O and C (reviewed in Brown, 1995; Mateu, 1995).

The high potential for genetic and antigenic variation is one of the most important FMDV features (Domingo et al., 1990; 2003), which derives from the quasispecies structure of its viral populations (Domingo et al., 1992). This structure poses important implications on its biology and control: among them, the high antigenic diversity of viral populations that is reflected in seven serotypes and a multitude of variants (Arrowsmith 1977; Pereira, 1981). This antigenic variability affects vaccination strategies because of the need to adequate the vaccines to the antigenic properties of field viruses (Kitching, 1989). As complement of classical serological methods, nucleotide sequencing and phylogenetic analyses have become valuable tools for the characterization and epidemiological tracing of FMD outbreaks (Armstrong et al., 1994; Kitching et al., 1989; Samuel et al., 1988).

Phylogenetic analyses have been mostly based on sequences from the VP1-coding region (Beck and Strohmaier, 1987; Dopazo et al., 1988; Martinez et al., 1992; Sangare et al., 2003), particularly on its 3' end 250 nt (Pattnaik et al., 1998; Samuel et al., 1999; Vosloo et al., 1992), which contains one of the main B cell antigenic site, corresponding to VP1 residues 140-160 (Bittle et al., 1982; Strohmaier et al., 1992). Phylogenetic trees constructed from these 3' end region reproduce those derived from the whole VP1 sequence (Núñez et al., 2001).

The use of panels of monoclonal antibodies (MAbs) has proven useful to study the antigenic variation of type C viruses in the field (reviewed in Mateu, 1996). Thus, selection of antigenic variants, with amino acid replacements at neutralizing antigenic sites, has been reported during type C FMDV epizootics in epidemiological situations in which part of the susceptible animals were vaccinated (Mateu et al., 1987; 1988). For type O FMDV, a main neutralizing antigenic site has been identified on the capsid protein VP1 (site 1), being composed by residues 140-160 located at the G-H loop and residues 200-213 at the carboxyterminal end (Kitson et al., 1990). In addition, residues 70 to 77 and position 131 in VP2 and 56 to 58 in VP3 have been reported to contribute to the discontinuous neutralizing antigenic sites 2 and 4, respectively (Kitson et al., 1990).

During 1993 an important epizootic caused by a type O FMDV occurred in Italy. This was the first episode of the disease in the EU after adoption of a non-vaccination policy that started in 1991. Therefore, this epizootic mostly affected susceptible animals with low or null levels of anti-FMDV antibodies elicited by previous vaccination. The disease affected different species as cattle, buffalo, swine and sheep. It started in February in the South of Italy, and the last outbreaks were reported in June. The epizootic also affected localities in the North of the country.

To trace the origin and evolution of the viruses causing this epizootic we have determined and compared the sequences from different viral isolates corresponding to i) the 228 nt from the 3' end of VP1 gene, that define the antigenic site A, and ii) the regions of capsid proteins VP2 and VP3 previously reported to be part of the discontinuous antigenic sites 2 and 4, respectively (Kitson et al., 1990). A detailed antigenic characterization of the Italian isolates has been performed by using a panel of MAbs, which was raised against a representative Italian isolate.

Materials and Methods:

Viruses.

The Italian FMDV isolates studied are detailed in Table 1. The samples from which these viruses were isolated were collected from infected animals at different dates and locations. In some instances, the virus present in the lesion was grown in IBRS-2 cells, as indicated in Table 1.

Extraction, amplification and sequencing of viral RNA.

RNAs were directly extracted from epithelium of lesions or from supernatants of infected cell cultures with phenol-chloroform (Chomzynski and Sacchi, 1987). RT-PCR amplification of viral RNA was performed as previously described (Taboga et al., 1997). The following pairs of oligonucleotide primers (numbering as in Forss et al, 1984) were used to amplify RNA sequences from three of the major antigenic sites described in type O capsid proteins (Kitson et al., 1990). Site 1, primers d (antisense), GAAGGGCCCAGGGTTGGACT, nucleotide positions 3559 to 3578, V-27 (sense) GATTTGTGAAGGTGACACC (2998 to 3017); site 2 primers D2-I (antisense) GGCACCTCCGTTGAACTG, (1914 to 1932) D2-V (sense) ACCACTCTCCTCGAAGACCGC, (1590 to 1610); site 4 primers D3-I (antisense) GTACTGTGTGTAGTACTG, (2511 to 2528); D3-V (sense) GACCCGAAGAGGCTGACC, (2277 to 2294);. cDNAs were sequenced, as described (Taboga et al., 1997), using the "fmol DNA sequencing system" (Promega), with the same primers used to amplify and the following primers for site 1: I-39 GACTCAACGTCACCCGCCAAC (reverse and complementary to positions 3542-3562), V-8 TGGACAACACCACCAACCCA (3178-3197). In some instances, PCR products were cloned using pGEM vector system (Promega) and their sequences were determined, using flanking vector primers, as described by the manufacturer.

Phylogenetic analyses

Nucleotide sequences were aligned with those of FMDV isolates representative of serotype O (Table 2), using the CLUSTALW program (Higgins et al., 1994). The genetic distances were calculated using the Jukes and Cantor method (Jukes T.H. and Cantor C.R., 1969), and phylogenetic trees were constructed by means of the Neighbour-Joining method (Saitou et al., 1987). The trees were a consensus of 1000 bootstrap replicates (Felsenstein, 1985). The analyses were performed with MEGA 2.1 software (Kumar et al., 2001).

Monoclonal Antibodies

A panel of MAbs against FMDV Italian isolate 1111 Avellino 13/3 was produced by immunization of Balb/c mice with 2 to 4 doses of 30 µg each of purified virus at one-month intervals. Cell fusion and cloning of positive hybridomas were performed following standard procedures (Brocchi et al., 1993; Galfrè and Milstein, 1981). Selection was based on results of a trapping ELISA against the homologous virus. MAbs B2, 1C6 and 3C8 which recognise sites 1, 2 and 3, respectively, raised against FMDV isolate O1 Switzerland 1965 (McCahon et al., 1990) were also included in the analyses

ELISA

A trapping ELISA (Brocchi *et al.*, 1997) was used for both the screening of hybridomas and for the antigenic characterisation of FMDV isolates. Briefly, ELISA microplates were coated with a saturating concentration of anti-O-FMDV rabbit serum and viruses, as supernatant of infected IBRS-2 cells, were trapped. Each MAb (at a previously titrated dilution) was then incubated, followed by anti-mouse immunoglobulines labelled with peroxidase. The reactivity of isolates with each MAb was expressed as a percentage of the corresponding reaction with the parental strain, assumed to be 100%.

Results:

Comparison of VP1 sequences from Italian isolates and other type O FMDVs

The RNA region corresponding to the carboxiterminal half of VP1 protein, including the antigenic site 1, was amplified by RT-PCR from viral RNA directly extracted from 15 Italian viral samples included in

Table 1. The sequence of the 3' 228 bp was determined, as described in Materials and Methods. To address the genetic relatedness between the Italian isolates and other type O FMDVs, a phylogenetic tree was constructed (Fig.1) including sequences from the first (1096 Potenza 22//2) and the last (2010 Lecce 10/6) viruses isolated during the epizootic, as well as those from viruses representative of serotype O (accession numbers in Núñez et al., 2001 and Samuel and Knowles 2003). The topology of this tree maintained, with high levels of confidence, the main topotype groups clusters previously reported for complete type O VP1 sequences (Samuel and Knowles 2003). The Italian isolates appeared clustered in the tree with viruses from the ME-SA topotype, according to Samuel and Knowles (2003), which includes isolates from Middle East, as well as viruses related to the Pan Asia strain like those that produced epizootics in Japan and South Africa in 2000, and the virus causing the UK epizootic in 2001.

The comparison of the deduced amino acid sequences at the antigenic site 1 in VP1, among the Italian isolates and other type O FMDVs is shown in Fig.2. In this alignment, Italian isolates also grouped with the Middle-East and Asian FMDVs.

Comparison of the sequences corresponding to three main antigenic sites among Italian isolates

The alignment of the site 1 sequences in VP1 among the Italian viruses showed the presence of a silent nucleotide substitution T (3353)→G in 6 of 15 samples analysed (Table 2). Thus, all the viruses studied exhibited the same amino acid sequence at the site 1 region, including the hypervariable residues 140-160 and the carboxiterminal residues 200-213. Considering the substitution found in the 228 nucleotides sequenced, the rates of fixation of mutations estimated ranged from $1.8-2.0 \times 10^{-2}$ substitutions per site and year (s/s/y), between the last isolate 2008 Lecce 10/6 and 1115 Avellino 16/3 or 1121 Cosenza 18/3, respectively.

To further study the sequence diversity generated during the Italian epizootic, the nucleotide regions spanning the main residues involved in antigenic site 2 in VP2 (nucleotide positions 1655 to 1879) and site 4 in VP3 (nucleotide positions 2318 to 2483) were determined from viral RNA of those lesions in which sufficient yield of cDNA was recovered. The alignment of the site 2 sequences obtained revealed a silent nucleotide substitution A(1868)→G in isolate 2008 Lecce 10/6 (Table 2). Likewise, two nucleotide substitutions were found in the site 4 sequences: Transition C (2354)→T was observed in virus 840 Verona 16/3, which was isolated in the North part of the country. An additional transition, T (2433)→C was found in viruses 2008 Lecce 10/06 and 2010 Lecce 10/06 that were isolated in the same locality at the end of the epizootic (2-3 months latter than the remaining viruses analyzed). This substitution led to the amino acid replacement P(70)→S in VP3.

The rate of fixation of mutations estimated ranged from 1.7 to 2×10^{-2} s/s/y between 1109 Matera 12/3 and 2008 Lecce 10/6 in VP2 and 1096 Potenza 22/2 and 2008 Lecce 10/6 in VP3, respectively. Overall, a 25% of transversions was observed in the three regions considered and resulted in 20% of amino acid substitutions.

Evidence of heterogeneity in viral isolates

To explore heterogeneity of the Italian viruses due to the cuasispecies structure of FMDV populations (Domingo et al., 1980), FMDV cDNAs directly derived from viral lesions were cloned in plasmid pGEM-T, and the viral sequences from independent clones were determined as described in Materials and Methods. No nucleotide substitutions were found neither in the site 1 region from 5 independent clones derived from isolate 1115 Avellino 16/3, nor in the site 4 region from 6 independent clones of isolate R2010. Interestingly, a nucleotide substitution was identified in the site 2 region, C(1874)→T leading to the amino acid replacement T(71)→I in VP2, in one of the 6 clones analyzed from isolate R2010. This result support the heterogeneity of type O FMDV populations in a single lesion from an infected animal.

Antigenic characterisation

A panel of 24 MAbs was raised against the isolate 1111 Avellino 13/3, representative of the Italian epizootic (see Material and methods). None of the MAbs obtained significantly neutralized the infectivity of the parental virus (data not shown). These MAbs were used to characterize the antigenic variation among the isolates within the outbreak, by means of a trapping ELISA. The Italian viruses analyzed showed the same pattern of ELISA reactivity against most of the MAbs tested (Fig. 3). This panel of MAbs, however, recognised different viral epitopes, since they revealed differences in reactivity against other type O viruses (Fig. 3), which allowed its classification in three groups: i) those recognising epitopes conserved in all the Italian isolates and present also in all or most of the other O-viruses tested; ii) MAbs reactive to all Italian isolates but specific (or nearly) of these viruses, and iii) MAbs 7B12, 5C12 and 1D8, which were able to distinguish some of the Italian isolates.

When neutralizing MAbs raised against FMDV O1-Switz (Crowther et al.1984) were included in the ELISA, no major binding changes were found with MAb B2 (directed against site 1 in VP1) and 3C8 (recognizing site 4 in VP3). Conversely, the results obtained with MAb 1C6, which binds to a conformational epitope in VP2, revealed reactivity differences among Italian isolates.

Thus, in spite of the considerable level of conservation of the reactivity against the MAbs used in these assay, antigenic changes are observed among the virus recovered from the Italian epizootic

Discussion:

The location of the VP1 sequences from viruses isolated during the epizootic occurred in Italy in 1993 in a type O phylogenetic tree indicates that Italian isolates belong to the ME-SA topotype and are related to those that circulated in the Middle East region. Previous FMD reintroductions in western Europe of viruses circulating in this region have been reported. In the early 80's viruses with this characteristic, O Thalheim Aus/81 and O Wupp Ger/82, were identified as responsible for an outbreak occurred in Germany and Austria (Beck and Strohmaier, 1987, Saiz et al., 1993). Thus, even when the epidemiological factors for these reintroductions remain to be determined, this seems to be a potential way for FMD spread into Western Europe.

The high level of sequence conservation among different Italian isolates support a common origin for the different FMD foci occurred in the south and North of the country. In spite of this homology, nucleotide substitutions were found in different isolates at the sequences corresponding to three major neutralizing antigenic sites reported for type O FMDV (Kitson et al., 1990). This observation extends the potential for FMDV diversification (Sobrino et al., 1986) to epidemiological conditions involving a short period of four months of viral circulation and the absence of previous vaccination in most of the affected animals, which were, therefore, free of FMDV antibodies.

Even when the number of substitutions detected at the antigenic sites 1, 2 and 4 were low, the rates of fixation of mutations that could be derived among different Italian isolates, were similar among the three antigenic sites studied, around 10^{-2} s/s/y. These values are close to those reported to occur during a type C epizootic in Spain from 1979 to 1981, which took place when the vaccination policy was still being implemented (Sobrino et al, 1986). A direct comparison between these two epizootics is complex, as they may be affected by multiple variables, like serotype involved, time of the epidemic. However, the absence of previous immune response in most of the susceptible animals is not likely to be associated with a reduction in the fixation of mutations during the Italian epizootic.

Of the four nucleotide substitutions found in the Italian isolates, only one led to amino acid replacement. Thus, the two viruses isolated in Lecce by the end of the epizootic, showed an amino acid substitution near antigenic site S4.

The data obtained in the analysis of molecular clones from viral populations in lesions of affected animals, although limited, reinforce previous description on the heterogeneity of viral populations in natural hosts (Domingo et al., 1980, Rowlands et al., 1983). Thus, one of the clones from isolate R2010 Lecce 16/6 showed a nonsynonymous substitution, T(71)→I in VP2, which affects site 2 and which is present neither in the parental population nor in the 5 clones additional clones sequenced. Replacements at position 71 in VP2 have been previously shown to confer resistance to neutralization by MAbs raised against antigenic site 2 (Kitson et al, 1990). Thus, the recovery of this variant could be reflecting the minority presence of antigenic variants in the population of isolate R2010 Lecce 16/6.

Antigenic variation among Italian isolates was also detected by ELISA against a panel of MAbs raised against isolate 1111 Avellino 13/3, indicating the co-circulation of viruses with differences in their recognition by different MAbs. Since no amino acid variation was found in any of the 3 main antigenic sites sequenced it was not possible to correlate amino acid replacements with changes in Mab binding. This result indicates that the epitopes recognised by the MAbs produced in this study are located in areas other than those included in our sequencing analyses. Work is in progress to define the epitopes recognised by these MAbs.

Differences in ELISA reactivity were also found when the neutralizing Mab 1C6 that recognize the site 2 in VP2 (Kitson et al., 1990) was used. This result confirms that the selection of variant during the epizootic also affects the recognition of the virus by neutralizing antibodies. No amino acid replacements were found at site 2 sequences among the Italian viruses showing an altered reactivity with Mab 1C6, suggesting that this differential reactivity could be due to changes at other residues as VP2 131, not included in our sequencing analysis, which have been also reported to contribute to site 2 structure (Kitson et al., 1990).

References:

- Armstrong, R.M., Samuel, A.R., Carpenter, W.C., Kant, R. & Knowles, N.J.** 1994. A comparative study of serological and biochemical methods for strain differentiation of foot-and-mouth disease type A virus, *Vet. Microbiol.* 39 285-298.
- Arrowsmith, A.E.M.** 1977. A survey of foot-and-mouth disease type O strains from the Far East, *Dev. Biol. Stand.* 35 221-230.
- Barteling, S.J. & Vreeswijk, J.** 1992. Development in foot-and-mouth disease vaccines, *Vaccine* 9 75-88.
- Beck, E. & Strohmaier, K.** 1987. Subtyping of European foot-and-mouth disease virus strains by nucleotide sequence determination, *J. Virol.* 61 1621-1629.

- Belsham, G.J.** 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure, *Prog. Biophys. Molec. Biol.* 60 241-260.
- Bittle, J.L., Houghten, R.A., Alexander, H., Shinnick, T.M., Sutcliffe, J.G., Lerner, R.A., Rowlands, D.J. & Brown, F.** 1982. Protection against foot-and-mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence, *Nature* 298 30-33.
- Brocchi, E., Gamba, D., Poumarat, F., Martel, J.L & De Simone, F.** 1993. Improvements in the diagnosis of contagious bovine pleuropneumoniae through the use of monoclonal antibodies. *Revue Scientifique et Technique Office International Des Epizooties* 12, 559-570.
- Brocchi, E., Zhang, G., Knowles, N., Wildsen, G., McCauley, J., Marquardt, O., Ohlinger, V. & De Simone F.** 1997. Molecular epidemiology of recent outbreaks of swine vesicular disease: two genetically and antigenically distinct variants in Europe, 1987-94. *Epidemiol. Infect.* 118 (1): 51-61.
- Brown F.** 1995. Antibody recognition and neutralization of foot-and-mouth disease virus, *Semin. Virol.* 6 243-248.
- Carrillo, C., Dopazo, J., Moya, A., González, M., Martínez, M.A., Sáiz, J.C. & Sobrino, F.** 1990. Comparison of vaccine strains and the virus causing the 1986 foot-and-mouth disease outbreak in Spain: an epizootiological analysis, *Virus Res.* 15 45-56.
- Chomzynski, P. & N. Sacchi.** 1987. Single-step methods of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* 162:156-159.
- Crowther, J.R., McCullough, K.C., De Simone, F. & Brocchi, E.** 1984. Monoclonal antibodies against FMDV: application and potential use. *Report to the Research Group of the Standing Technical Committee of the European Commission for the Control of FMD* (Brescia, Italy), pp. 40-51. Rome: F.A.O.
- Domingo, E., Davila, M.. & Ortin, J.** 1980. Nucleotide sequence heterogeneity of the RNA from a natural population of foot-and-mouth-disease virus. *Gene.* 11(3-4):333-46.
- Domingo, E., Mateu, M.G., Martínez, M.A., Dopazo, J., Moya, A. & Sobrino, F.** 1990. Genetic variability and antigenic diversity of foot-and-mouth disease virus, in: Kurstak E., Marusyk R.G., Murphy S.A., Van-Regenmortel M.H.V. (Eds.), *Applied virology research*, vol 2, Virus variation and epidemiology, Plenum Publishing Corp., New York, pp. 233-266.
- Domingo, E., Escarmis, C., Martínez, M.A., Martínez-Salas, E. & Mateu, M.G.** 1992. Foot-and-mouth disease virus populations are quasispecies, *Curr. Top. Microbiol. Immunol.* 176 33-47.
- Domingo, E., Escarmis, C., Baranowski, E., Ruíz-Jarabo, C., Núñez, J.I. & Sobrino, F.** 2003. Evolution of foot-and-mouth disease virus. *Virus Res.* 91, 47-63
- Dopazo, J., Sobrino, F., Palma, E.L., Domingo, E. & Moya, A.** 1988. Gene encoding capsid protein VP1 of foot-and-mouth disease virus: a quasispecies model of molecular evolution, *Proc. Natl. Acad. Sci. USA.* 85 6811-6815.
- Felsenstein, J.** 1985. Confidence limits on phylogenies: an approach using the bootstrap, *Evolution* 39 783-791
- Galfre, G. & Milstein, C.** 1981. Preparation of monoclonal antibodies: strategies and procedures. *Methods Enzymol.* 7:33-46.
- Higgins, D., Thompson, J., Gibson, T., Thompson, J.D., Higgins, D.G. & Gibson, T.J.** 1994. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. *Nucleic Acids Res.* 22:4673-4680.
- Kitching, R.P., Knowles, N.J., Samuel, A.R. & Donaldson, A.I.** 1989. Development of foot-and-mouth disease virus strain characterization - a review, *Trop. Anim. Health. Prod.* 21 153-166.
- Kitson, J.D.A., McCahon D. & Belsham G.J.** 1990. Sequence analysis of monoclonal antibody resistant mutants of type O foot-and-mouth disease virus: Evidence for the involvement of the three surface exposed capsid protein in four antigenic sites, *Virology* 179 26-34.
- Knowles, N.J., Samuel, A.R., Davies, P.R., Kitching, R.P. & Donaldson, A.I.** 2001. Outbreak of foot-and-mouth disease virus serotype O in the UK caused by a pandemic strain. *Vet Rec.* Mar 3;148(9):258-9
- Kumar, S., Tamura, K., Jakobsen, I.B. & Nei, M.** 2001. MEGA 2: Molecular Evolutionary Genetic Analysis software. *Bioinformatics* 17, 1244-1245.
- Martin, W.B. & Chapman, W.G.** 1961. The tissue culture colour test for assaying the virus and neutralizing antibody of foot-and-mouth disease and its application to the measurement of immunity in cattle. *Res. Vet. Sci.* 2, 53-61
- Martínez, M.A., Dopazo, J., Hernández, J., Mateu, M.G., Sobrino, F., Domingo, E. & Knowles N.J.** 1992. Evolution of capsid protein genes of foot-and-mouth disease virus: antigenic variation without accumulation of amino acid substitutions over six decades, *J. Virol.* 66 3557-3565.
- Mateu, M.G., Rocha, E., Vicente, O., Vayreda, F., Navalpotro, C., Andreu, D., Pedroso, E., Giralt, E., Enjuanes, L. & Domingo, E.** 1987. Reactivity with monoclonal antibodies of viruses from an episode of foot-and-mouth disease virus. *Virus Res.* 8, 261-274.
- Mateu, M.G., da Silva, J.L., Rocha, E., de Brum, D.L., Alonso, a., Enjuanes, L., Domingo, E. & Barahona, H.** 1988. Extensive antigenic heterogeneity of foot-and-mouth disease virus of serotype Cl. *Virology.* 167, 113-124.

- Mateu, M.G.** 1995. Antibody recognition of picornaviruses and scape from neutralization: a structural view. *Virus Res.* 38, 1-24.
- Mateu, M.G., Valero, M.L., Andreu, D. & Domingo, E.** 1996. Systematic replacement of amino acid residues within an Arg-Gly-Asp-containing loop of foot-and-mouth disease virus and effect on cell recognition. *J Biol Chem.* 31;271(22):12814-9.
- Meyer RF, Pacciarini M, Hilyard EJ, Ferrari S, Vakharia VN, Donini G, Brocchi E & Molitor TW.** 1994. Genetic variation of foot-and-mouth disease virus from field outbreaks to laboratory isolation. *Virus Res.* Jun;32(3):299-312.
- Núñez JI, Martín MJ, Piccone ME, Carrillo E, Palma EL, Dopazo J & Sobrino F.** 2001. Identification of optimal regions for phylogenetic studies on VP1 gene of foot-and-mouth disease virus: analysis of types A and O Argentinean viruses. *Vet Res.* Jan-Feb;32(1):31-45.
- Pattnaik B., Venkataraman R., Tosh C., Sanyal A., Hemadri D., Samuel A.R., Knowles N.J. & Kitching R.P.** 1998. Genetic heterogeneity of Indian field isolates of foot-and-mouth disease virus serotype O as revealed by partial sequencing of 1D gene, *Virus Res.* 55 115-127.
- Rowlands DJ, Clarke BE, Carroll AR, Brown F, Nicholson BH, Bittle JL, Houghton RA & Lerner RA.** 1983. Chemical basis of antigenic variation in foot-and-mouth disease virus. *Nature.* 15-21;306(5944):694-7
- Saitou N. & Nei M.** 1987. The neighbour-joining method: a new method for reconstructing phylogenetic trees, *Mol. Biol. Evol.* 241 425-436.
- Saiz JC, Sobrino F & Dopazo J.** 1993. Molecular epidemiology of foot-and-mouth disease virus type O. *J Gen Virol.* Oct;74:2281-5.
- Sangare O, Bastos AD, Venter EH & Vosloo W.** 2003. Retrospective genetic analysis of SAT-1 type foot-and-mouth disease outbreaks in West Africa (1975-1981). *Vet Microbiol.* 10;93(4):279-89.
- Samuel AR, Knowles NJ & Kitching RP.** 1988. Serological and biochemical analysis of some recent type A foot-and-mouth disease virus isolates from the Middle East. *Epidemiol Infect.* 101:577-90.
- Samuel A.R. & Knowles N.J** 2003. Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.* 91(1):65-80.
- Samuel A.R., Knowles N.J. & Mackay D.K.** 1999. Genetic analyses of type O viruses responsible for epidemics of foot-and-mouth disease in North Africa, *Epidemiol. Infect.* 122 529-538.
- Sobrino F, Palma EL, Beck E, Davila M, de la Torre JC, Negro P, Villanueva N, Ortin J & Domingo E.** 1986. Fixation of mutations in the viral genome during an outbreak of foot-and-mouth disease: heterogeneity and rate variations. *Gene* 50, 149-159,
- Sobrino, F., Sáiz, M., Jiménez-Clavero, M.A., Núñez, J.I., Rosas, M.F., Baranowski, E. & Ley, V.** 2001. Foot-and-mouth disease virus: a long known virus, but a current threat. *Vet. Res.* 32, 1-30
- Sobrino F & E. Domingo.** 2001. Foot-and-mouth disease in Europe. *EMBO reports* 2, 459-461.
- Strohmaier K., Franze R. & Adam K.H.** 1982. Location and characterization of the antigenic portion of the FMDV immunizing protein, *J. Gen. Virol.* 59 295-306.
- Taboga O., Tami C., Carrillo E., Núñez J.I., Rodríguez A., Sáiz J.C., Blanco E., Valero M.L., Roig X., Camarero J.A., Andreu D., Mateu M.G., Giral E., Domingo E., Sobrino F. & Palma E.L.** 1997. A large scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of scape mutants, *J. Virol.* 71 2606-2614.
- Van Bekkum, J.G.** 1969. Correlation between serum antibody level and protection against challenge with FMD. Session of Research Group of the standing Technical Committee of the European Commission for the Control of Foot and Mouth Disease, Brescia, Italy, FAO.
- Van Maanen, C.** 1990. A complex-trapping-blocking (CTB) ELISA, using monoclonal antibodies and detecting specifically antibodies directed against foot-and-mouth disease types A, O and C. I. Method and characteristics. *Vet Microbiol.* 24,171-8.
- Vosloo, W., Knowles, N.J. & Thomson, G.R.** 1992. Genetic relationships between southern African SAT-2 isolates of foot-and-mouth-disease virus. *Epidemiol Infect.* 109,547-58.

Table 1 FMDV isolates from Italian epizootic in 1993 analyzed in this study

Viral isolates ^a
1096 Potenza 22/2/1993
714 Potenza 7/3/1993
1106 Verona 11/3/1993
1109 Matera 12/3/1993
1111 Avellino 13/3/1993
1115 Avellino 16/3/1993
840 Verona 16/3/1993
1116 Cosenza 17/3/1993
1118 Avellino 17/3/1993
1121 Cosenza 18/3/1993
1140 Verona 22/3/1993
1144 Salerno 24/3/1993
1147 Matera 25/3/1993
1158 Caserta 10/4/1993 ^c
2008 Lecce 10/6/1993
2010 Lecce 10/6/1993

^a Except for isolates 1106 Verona 11/3, 1140 Verona 22/3 and 1144 Salerno 24/3 that required amplification in IBRS-2 cells, nucleotide sequencing was performed from viral RNA directly extracted from lesion of infected animals. ^c Isolated from buffalo. The reactivity to Mab was analyzed from virus recovered of the 2 to 4 passages in IBRS-2 cells.

Table 2. Nucleotide substitutions found in antigenic sites 1, 2 and 4 regions^a

Isolate	S1 (VP1)	S2 (VP2)	S4 (VP3)
1096 Potenza 22/2		n.d. ^b	
714 Potenza 7/3		n.d.	n.d.
1106-IBRS Verona 11/3	n.d.		n.d.
1109 Matera 12/3	T(3353)→G		
1111-IBRS Avellino 13/3		n.d.	n.d.
1115 Avellino 16/3	T(3353)→G	n.d.	
1116-IBRS Cosenza 17/3	T(3353)→G	n.d.	n.d.
1118-IBRS Avellino 17/3		n.d.	n.d.
840 Verona 16/3		n.d.	C(2354)→T
1121 Cosenza 18/3	T(3353)→G		n.d.
1140-IBRS Verona 22/3	T(3353)→G	n.d.	n.d.
1144-IBRS Salerno 24/3	T(3353)→G	n.d.	n.d.
1147 Matera 25/3			n.d.
1158 Caserta 10/4			
2008 Lecce 10/6		A(1868) →G	T(2433)→C
2010 Lecce 10/6			T(2433)→C

^aSubstitutions relative to the consensus sequence are indicated. Numbering as in Forss et al. (1984).^b Not done.

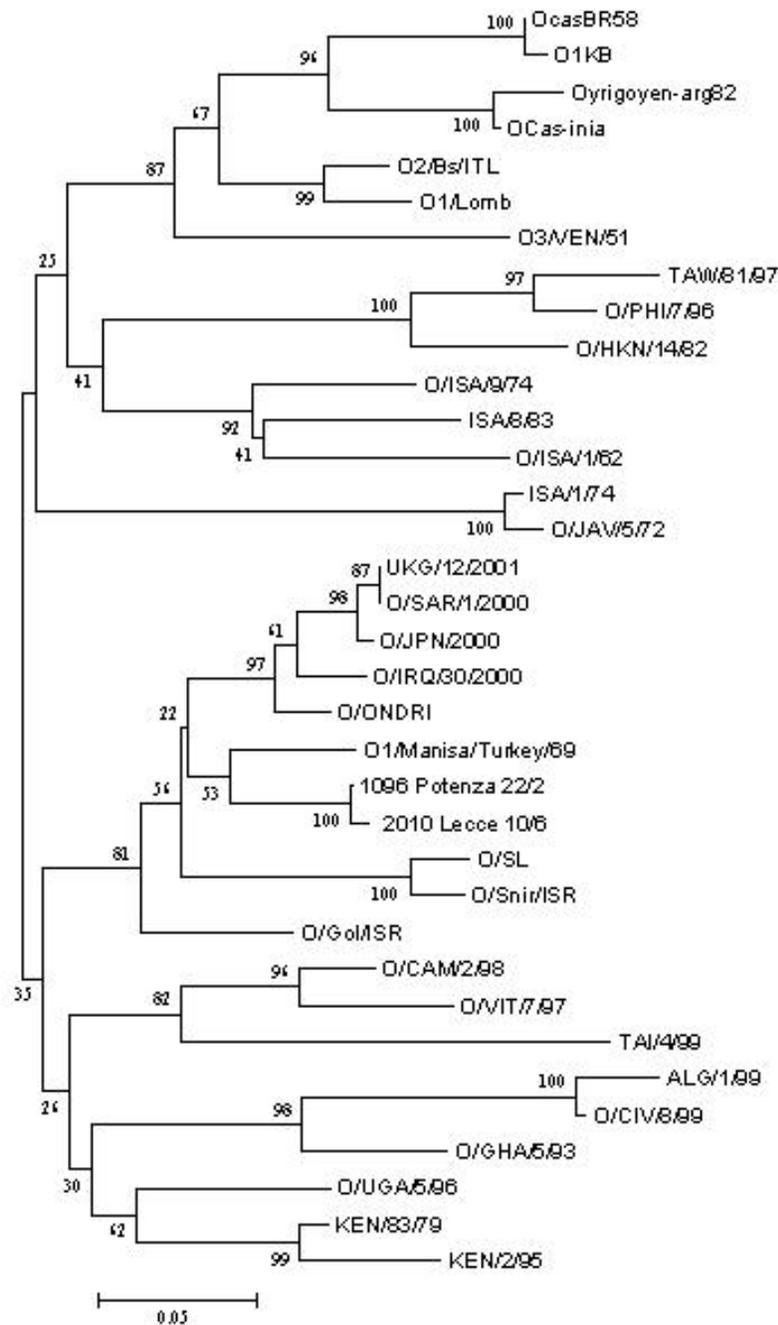


Fig 1. Phylogenetic tree derived from VP1 sequences of type O FMDVs. The sequences analyzed corresponded to residues 3297 to 3530, according to Forss et al., 1984. The sequence of the Italian isolates 1096 Potenza 22/2 and 2010 Lecce 10/6 was determined in this work. The origin of the remaining type O sequences viruses is indicated in Table 1.

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#TAW/81/97      DTSTNNVRGD LQVLAQKAER TLPTSFNFGA IKATRVTELL YRMKRAETYC PRPLLAIQPS DARHKQORIVA PAKOLL
#O/PHI/7/96     ...A.....R..K.....
#O/HKN/14/82   .ARAS.....A.....Y.....K.....
#O/Gol/ISR      NVAVT.....A.....Y.....?.....H..E.....K...V....
#O/SL           ES.VTA.....R..A.....Y.....?..H.N E.....KDCG R?----
#O/Snir/ISR     ES.VTA.....R..A.....Y.....H.N E.....NN-- -----
#UKG/12/2001    ESPVT.....A.....Y.....H..E.....K...V....
#O/SAR/1/2000   ESPVT.....A.....Y.....H..E.....K...V....
#O/JPN/2000     ESPVT.....A.....Y.....H..E.....K...V....
#O/IRQ/30/2000 ESPVT.....A.....Y.....H..E.....K...V....
#O/ONDRI        ES.VT.....A.....Y.....W.....H..E.....K--.E....
#O/it1115eo     .GAVA.....A..A.....Y.....H..E.....K.....
#O/it2008eo     .GAVA.....A..A.....Y.....H..E.....K.....
#O1/Manisa/Turkey/69 .GTVA.....A..A.....Y.....H.D Q.....K...V....
#O/OBAN         TNAV.T.I.....A..A.....Y.....H..E...N-K...-V.E..
#O/CAM/2/98     QG.PT.....A..P.....Y.....H..E.....K...Q....
#O/VIT/7/97     QGPLA.....A..P.....Y.....Q.....H.D E.....K...E....
#TAI/4/99       EG.LT.....A..P.....Y.....VH.D G...N.EL..?V..S.
#ALG/1/99       GAV.P.....RR.AP M.....?FH..E.G..?E?L..V....
#O/CIV/8/99     GAV.P.....RR.AP M.....FH..E.....K.....
#O/GHA/5/93     RVEVPK.....RR.A.....VH..E.T...K...M....
#KEN/83/79      .APVT.....A.....Y.....TH..E.....?..
#KEN/2/95       RAPVT.....A.....Y.....I.....TH..E.....I.....
#O/UGA/5/96     V.PVT.....V...A.....Y.....H..E.....K...R....
#ISA/1/74       SVAMA.E.....T..A..P.....Y.....V.....A.....E.....K...V..T.
#O/JAV/5/72     SVAMA.G.....T..A..P.....Y.....LV.....A.....E.....K...V..T.
#OcasBR58       RNAVP.....VA.....Y.....H.T E.....K...V..T.
#O1KB           RNAVP.L.....VA.....Y.....H.T E.....K...V..T.
#Oyrigoyen-arg82 SNAV.P.....PNL.E...A..M..AY..Y.....H.T .....RK...V.RT.
#OCas-inia      SNAV.P.....PN..E...A...??????? ???.....H.T .....K...V..T.
#O2/Bs/ITL      RNAVP?.....A...A.....Y...R.....H.T E.....K...T.
#O1/Lomb        RNTVP.....T..A.....Y...R.....H.T E.....K...V..T.
#O3/VEN/51     RDVVT.I...A..H..A..S.....Y.....H.T E.....K...V..T.
#ISA/8/83       THT.T.....A.....Y.....I.....I.....H..E.....K...T..T.
#O/ISA/9/74     AHTVT.....A.....Y.....I.....H.T E.....K...T..T.
#O/ISA/1/62     TDTAP.....A..P.....Y.....I.....R..E.....K...V..T.

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Fig. 2 Alignment of partial VP1 amino acid sequences of type O FMDV. Amino acid sequences were deduced from the nucleotide sequences indicated in the legend of Fig. 1.

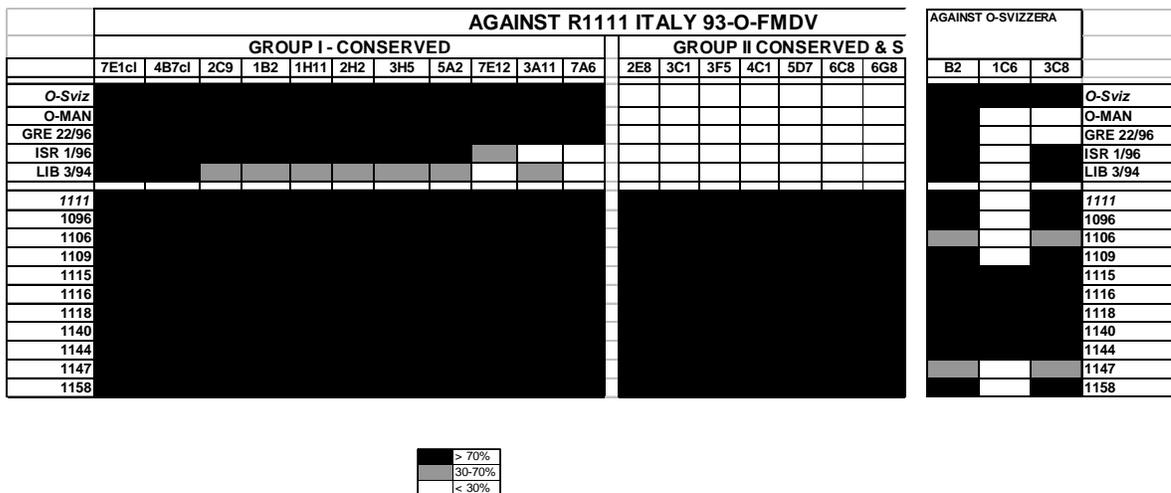


Figure 3. Reactivity patterns of FMDV with different panels of anti-FMDV monoclonal antibodies.

Outstanding but tractable questions regarding the micro-evolution of FMDV

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Our knowledge of fundamental processes underlying the population genetics of FMDV remains far from complete. Whether or not the dynamics of viral populations within individuals are governed by the principles of 'quasi-species' or those of more conventional 'Darwinian' selection depends critically on both the mutation rate and the intensity and scale of epistasis within the viral genome. Uncertainty in current estimates of RNA virus mutation rate are such that neither mode of evolution can be ruled out. High levels of epistasis will increase the role for quasi-species dynamics in the 'within-individual' evolution of FMDV but we know next to nothing about either the intensity or genomic scale of epistasis. Evidence is accumulating that recombination rates may be sufficiently high that recombinant genomes could pose a significant source of antigenic novelty – the threat of such recombinants arising will increase with the frequency that multiple strains and serotypes co-circulate within a region.

Simple and plausible models of FMDV population genetics suggest that virus excreted by an infected animal might on average differ by 1 nucleotide mutation to its capsid genes from the virus with which an individual was infected. If this were true – and there are many interesting reasons why it might not be – it suggests that genetic characterization could be used to trace transmission events at much higher resolutions than is routinely attempted, and there is increasing empirical evidence to suggest this to be the case.

Ultimately we need to link genetic diversity more explicitly and directly to antigenic characteristics. This will require advances in three areas: higher resolution, and more accurate and widespread comparisons of the antigenicity of the FMDV capsid, more complete capsid sequences from field and vaccine stains, and computational algorithms that can map between these two sources of information.

Role of European Food Safety Authority (EFSA) and current tasks related to FMD

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Objectives: to present the risk assessment tasks of EFSA and the FMD related activities.

Introduction: Following a series of food scares in the 1990s (eg BSE, dioxins... which under-mined consumer confidence in the safety of the food chain, the European Union concluded that it needed to establish a new scientific body charged with providing independent and objective advice on food safety issues associated with the food chain. Its primary objective as set out in the White Paper on Food Safety would be to: "contribute to a high level of consumer health protection in the area of food safety, through which consumer confidence can be restored and maintained. "The result was EFSA.

EFSA was legally born from the European Parliament and Council regulation (EC) No 178/2002 of 28 January 2002. The new Authority quickly found its feet, organising its first Management Board meeting 9 months later under the Chairmanship of S. Slorach. Shortly afterwards it nominated its first Executive Director, G. Podger, and created the Advisory Forum, made up of representatives from food safety bodies in the Member States.

EFSA truly opened for business in May 2003, with the establishment of its Scientific Committee and Panels. World-class scientists from all over Europe were appointed to eight Panels, covering everything from food additives to animal health, and to a Scientific Committee with oversight of these panels. Now as we approach the end of 2004, staff numbers have doubled and continue to increase, EFSA has published over a hundred opinions and steadily carries on with its push to establish itself permanently in Parma, Italy.

EFSA provides independent scientific advice on all matters linked to food and feed safety -including animal health and welfare and plant protection - and provides scientific advice on nutrition in relation to Community legislation. The Authority communicates to the public in an open and transparent way on all matters within its remit. EFSA's risk assessments provide risk managers (consisting of EU institutions with political accountability, i.e. European Commission, European Parliament and Council) with a sound scientific basis for defining policy driven legislative or regulatory measures required to ensure a high level of consumer protection with regards to food safety.

Overall capacity-building is set to continue, enabling the Authority to meet work programme commitments including the significant expansion of both its scientific and communications activities as well as the development of its institutional, stakeholder and international relations. EFSA is currently dealing principally with requests for risk assessments from the European Commission and plans to take on a wider brief from other European institutions in the near future. Notwithstanding the important needs of its key customers, EFSA is already undertaking its own work in order to look ahead and address broader issues of importance to its mandate. For example, through such "self-tasking", the Authority's Scientific Committee has initiated work in relation to the identification of emerging food safety issues.

Discussion on FMD activities: For many years now in Europe and worldwide, the most commonly accepted way of protecting disease-free areas from the risk of outbreaks of epizootic animal diseases has been to guard borders through strict controls on imports of live animals and products of animal.

Foot-and-Mouth Disease in Small Ruminants – An Issue of Concern

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Abstract

Introduction: Food and Agricultural Organisation has its long-standing interest in the global eradication of the foot and mouth disease. FAO recognized Pirbright Laboratory as the World Reference Laboratory (1958) for epidemiology and researches on FMD which paved the way for the eradication of FMD from most of the developing countries, but still the virus is circulating in about two third of the OIE member countries. If FMD control campaign is considered as an international public good, one of the issues that needs to be addressed is strategies and control of FMD in small ruminants.

Materials and Methods: The role of small ruminants in the epidemiology of FMD in India was elucidated as early as in 1972 (Uppal et al; 1972). The clinical sign of the disease in sheep and goat were hardly visible. The disease can easily be overlooked until and unless individual animals are carefully examined for disease lesion. With these peculiarities, sheep and goat may function as repository of the virus. Subsequently in India many workers came to the same observation. Besides India, Iraq, Iran there are reports where small ruminants have been responsible for the epidemic of FMD in cattle in Greece in 1994, South East Asia in 1999 and Turkey in 2001. Recently, in UK and Ireland (2001) the role of sheep for transmission of FMD cannot be ignored.

Discussion: Even with the growing evidence of small ruminants in the transmission of diseases very little attention has been paid either on the researches or control policy of FMD in this species. It is proposed that FAO takes visionary approach of regional and global nature for the control of FMD in small ruminants which function as a repository of viruses and play an important role as silent repository helping inter epidemic survival of FMD virus, the spill over of which in large or other susceptible animal population may cause severe outbreaks of FMD.

Introduction

Food and Agricultural Organisation has its long-standing interest in the global eradication of the foot and mouth disease (FMD). Therefore to meet this challenge FAO supported and recognized Pirbright Laboratory as the World Reference Laboratory (1958) for epidemiology and researches on FMD which paved the way for the eradication of FMD from most of the developing countries, but still the virus is circulating in about two third of the OIE member countries. The disease in small ruminants is generally silent and has not received much attention in European countries except when outbreaks numbering 4 in U.K. alarmed that it was the movement of sheep, which resulted into unprecedented outbreaks of FMD in United Kingdom. However the role of small ruminants in the epidemiology of FMD in Asian African and Arabian countries is well documented where the small ruminants contaminates river water, ponds, pastures, the shrubs and other environment. The source of infection to the other livestock occurs because the movement of ruminants in these countries is unrestricted and is of free-range nature. If FMD control campaign is considered as an international public good, one of the issues that needs to be addressed is to have strategies for the control of FMD in small ruminants.

Materials and Methods

The role of small ruminants in the epidemiology of FMD in India was elucidated as early as in 1972 (Uppal et al; 1972). Prior to this publication no attention was paid in India to the occurrence of this disease in small ruminants. FMD outbreak was investigated by the author in one of the organized cattle farm in northern India (Hisar) where FMD vaccination was followed regularly except in sheep, goats & pigs. All cross bred cattle were stall fed and were not allowed out of the premises and the movement of attendants was regulated except that Sheep & goats were allowed outside for grazing and returned back to the farm as a usual practice. Moreover these animals were unvaccinated. The disease at the farm was reported when 19 crossbred cattle out of 193 showed clinical evidence of FMD disease in spite of the fact that prior to this several

goats and sheep considered sick were housed separately. Extensive investigation by the author revealed that the sheep and goat had very tiny vesicles either on tongue, gums, hard palate and feet in the decreasing order. The clinical sign of the disease in sheep and goat were hardly visible. Therefore disease remained unnoticed in these animals. Most of the sheep look healthy except on careful examination of the tongue when rubbed gently with the muslin cloth the small vesicles ruptured. In all 100 adult Sheep were examined only 8 showed mouth lesions but no foot lesions. The disease can easily be overlooked until and unless individual animals are carefully examined for disease lesion. Interestingly no pig was involved with the disease. However, 27 lambs 2-8 days of age died at the farm without showing any clinical symptoms. Virus was isolated from the heart muscle of lambs. However compared to sheep the clinical syndrome in goats were more visible. The virus identified was type 'O'.

Experimental trials done in susceptible sheep, only small vesicles measuring 0.5 to 2 mm. in diameter appeared within 24 hours. None of the sheep in any experiment showed any secondary lesions in the feet. Thus, with these peculiarities it was considered that sheep and goat may function as repository of the virus. Subsequently, in India many workers came to the same observations that small ruminants shows subdued symptoms and lesions and the disease occurring in occult form without being noticed (Singh and Sharma 1980, Mishra and Ghei 1983, Datta et al; 1984). However, Shankar et al; 1998 in India reported severe form of disease in the goats with high fatality rate. Many FMD outbreaks in cattle in India are linked with the transmission of FMD virus from small ruminants. Besides India (P.K.Uppal, 1980 & 2003 unpublished observations), observed several severe outbreaks in cattle in Iraq of which many were traced to the silent nature of FMD in Sheep. Number of diseased sheep involved in the flock did not exceed more than 2%. Disease in sheep remained unnoticed till the time cattle were affected & productivity losses were noticed. The disease was mainly observed during the disease investigation work done in the region of Northern Iraq Mosul, which is bordering Turkey. Similarly the disease in sheep in Arbil, Kirkuk and Sulamanyia in the northern region of Iraq was noticed. In 2003, during my technical mission on food security through animal health care to Veterinary College Baghdad as well as to Veterinary College Mosul, the unapparent form of FMD in sheep at Namrud area as well as in sheep grazing along the coast of Tigris river were noticed. However, no isolation work was conducted. Besides Iraq, the author during his visit to Iran in 1996 was apprised that FMD in sheep is quite prevalent in mild nature in the different parts of Iran. During the OIE meeting at Paris in July 2003 the author questioned the epidemiological role of small ruminants in FMD control outside South America. During the meeting the Iranian delegate stated that FMD in sheep in Iran is prevalent and with their movement the disease spreads silently, therefore vaccination strategies for the control of disease in sheep has to be considered.

In Libya in 1986 mild nature of FMD in sheep & goat in the region of Jamahiriya due to type 'O' virus was considered responsible for the wide spread prevalence of the disease (Elmanjni & Hassan 1986). In 1989, Tunisia in Africa which was previously free from FMD got the disease in cattle with the importation of sheep and goat imported from the Middle East. Subsequently the disease spread into Algeria and Morocco (Kitching 1998).

The outbreak in Bulgaria in 1991 amongst cattle due to type 'O' was attributed to the alleged introduction of goat from the Turkish village, which was taken by the Bulgarian villagers due to rivalry, (Kitching 1998).

In the end of July 1994, Greece experienced an outbreak of type O in cattle and sheep population of Lesbos Island. The disease remained unnoticed until the time a consignment of infected sheep was transported to the mainland thereby contaminating the cattles. In all Greece noticed 95 outbreaks.

It was inferred that the illegal sheep trade from Turkey into Lesbos possibly in April was the most likely explanation for the origin of the disease. In 2000 Greece again experienced FMD in sheep and goats with Asia I (Leforban & Gerbier 2003). In Turkish Thrace 1996, there has been outbreak of serotype O FMD amongst cattle as well as in Evros prefecture of Greece. This perhaps happened because of illegal importation of live sheep by a farmer with close family connection in Turkey (Kitching 1998). Again in 2001 in Turkish Thrace in the District of Malakara of Tekirdag Province FMD type O in Goat flock was detected which seems to have occurred because of infected goat brought in from Asiatic Turkey by the dealers (Leforban & Gerbier

2003). The outbreak of 1999 due to type "O" virus in Southeast Asia was traced and its origin was considered to be Myanmar from where the goats were imported. (Gleeson et al; 2003).

In February 2001 FMD outbreak in United Kingdom lasted for about 32 weeks, where the role of sheep in the spread of FMD was realized. The spread of disease in Northumberland was attributed due to the movement of the infected sheep through a series of a market which finally resulted in extensive spread in the northwest and south west of England.

Additional movement of sheep resulted in the dissemination of the virus to Scotland, Wales, Northern Ireland, the Republic of Ireland and France. Calves that had been in contact with sheep imported into France from the UK spread the virus to the Netherlands. The clinical signs were very mild in the vast majority of outbreaks involving sheep. The average number of sheep displaying lesions within a single flock was less than 5%. The lesions occurred mainly in the mouths of sheep. A small minority of sheep exhibited foot lesions or lameness. The virus caused severe clinical disease in cattle and pigs, (Donaldson and Alexandersen 2003).

Discussion

In 1969, Bachrach in the First International Pilot Conference on Foot-and-Mouth Disease held in New York stated that lesions on feet in sheep and goats alone are symptomatic because mouth lesions usually go unnoticed. However, experimental and field investigation conducted in sheep showed that adult sheep under natural and experimental conditions did not show feet lesions (Uppal, 1972). Author happened to attend some outbreaks of concurrent infection of FMD with blue tongue or Sheep pox or mainly with Pasturellosis. It was observed that lameness in sheep appeared when other viral and bacterial ailments are involved especially when there was bacteriamia. Therefore, critical examination of small ruminants suffering from FMD is important. There are now publications that silent nature of FMD in small ruminants transmit virus and has caused outbreaks in Asia (India), Middle east (Iran, Iraq, Turkey), Africa (Libya, Algeria, Morocco, Tunisia), South east Asia (Cambodia, Laos and Thailand) and European Countries (Bulgaria, United Kingdom, Ireland and France). The author foresee that presently the bovine and porcine wealth of Europe is far more threatened to FMD than ever before, if the issue of FMD in small ruminants is not adequately addressed. This is suggested because of two major reasons: -

Firstly, the author believes from his experience in Iraq that the entry of FMD through the movement of small ruminants as salient carrier from Iran, Iraq, and turkey to the European countries could occur. It is stated on the assumption that the origin of disease in Bulgaria (1991), Greece (1994,1996), Turkish Thrace (1996,2001) was traced with the introduction of small ruminants from the Turkey. The country, which is in a close proximity to Mosul (Iraq) where FMD in sheep occurs quite often (P. K. Uppal 1980, 2003 unpublished observations). It was also noticed under field conditions that the disease in sheep was not self limiting at least for a period of one month. Since severe outbreaks was even noticed in vaccinated cattle in Iraq when recovered sheep came into contact with cattle after 4-6 weeks. Secondly, with no vaccination being carried out in most of the European countries against FMD the new livestock crop coming in these countries will be highly susceptible to the infection. Therefore the implications of sub clinically infected sheep in the transmission of FMD disease has to be considered as one of the risk factors.

No doubt the disease in most of the small ruminants is not discernable and could be responsible for perpetuating the epidemic in other livestock especially in those countries where there is a mixed farming and those countries where the movement of sheep is unrestricted. Moreover, small ruminants which function as a repository of viruses and play an important role as silent repository helping inter epidemic survival of FMD virus, the spill over of which in other susceptible animal population may cause severe outbreaks of FMD.

If one considers the threat of FMD in European countries from Turkey due to the movement of small ruminants from middle east which is in close proximately to turkey in north and to Asia in South east and south west to the African countries, then it would be pertinent that FAO takes visionary approach of regional or of international nature for the global eradication of FMD. If FAO considers regional approach for the safety of livestock of European Union countries against the incursion of FMD, then it would be pertinent that primary epidemic areas of FMD and the movement of animals especially of small ruminants is given due consideration in the control

programme. It is known that FMD virus has escaped through the buffer zone in Turkish Thrace from Asiatic Turkey.

There is sufficient knowledge about the FMD disease, epidemics in cattle and about their diagnostic tools and vaccination strategies. The control of FMD in small ruminants will require more emphasis on scientific work besides speedy reporting of FMD through fast track system and their wide coverage of vaccination. It will be required to study the distribution of Foot and Mouth virus strains in small ruminants in different agro climatic regions and to do the molecular epidemiological studies and as well as analysis on the genetic lineages of FMD isolates both from small ruminants and cattle from the same outbreak.

In Addition, the study on the factors responsible for the exaltation of virulence in cattle with strain showing sub clinical infection in small ruminants would be important to know the mechanism of pathogenicity in bovine. The binding capacity of Foot and Mouth virus in surface susceptible cell of sheep and goat and its comparison with susceptible cell of cattle and buffaloes with the isolates when the same isolates show unnoticed infection in small ruminants and frank disease in cattle and buffaloes need to be elucidated. The World Reference Lab (WRL) could examine these studies, which is the technical arm of OIE and FAO. In addition, regional units under the aegis of WRL could be created for diagnostic and epidemiological work and collate the information for speedily dissemination.

The detection of virus from silent infection in small ruminants requires rapid, reliable and specific sero-surveillance tests. The present solid phase immunochromatographic assay which can differentiate the infected from the vaccinated animals using 2C & 3ABC protein for capturing specific antibodies (anti NSP) can not detect early infection in sheep (Hyun et al; 2003). For early detection of virus in small ruminants and to ascertain that the virus is no longer circulating in the target population, the sero-surveillance diagnostic tests developed if any are evaluated.

In order to be more efficient in pilot testing programme for FMD diagnosis in small ruminants, there is a need for the availability of uniform standard sera raised in sheep & goat or obtained in large quantities from the naturally infected small ruminants. There is a possibility that immune reaction in small ruminants is different from that of cattle. Therefore the validity of sero-surveillance tests under various field conditions would be required. Besides it, it would be useful to study the role of IgA (Secretory antibodies), if any, in establishing a carrier status in small ruminants

For the control programme of FMD in small ruminants one of the options is the usage of large quantities of monovalent type O vaccine based on epidemiological data in target areas could be adopted. Before the implementation of the programme experimental trials on the antigen payloads for vaccines as well as in corporation of suitable adjuvant in small ruminants is worked out. Besides it the study on the genetic endowment character of sheep susceptibility to FMD virus strains for the better control programme of FMD may be helpful.

Conclusions

- FMD in small ruminants is generally silent and is not self-limiting and is responsible for the spread of disease in European, Middle East, Asia, South East Asia and African countries.
- Small ruminants from Iran and Iraq constitute one of the major risk factor for the transmission of virus to the susceptible livestock population of European countries.
- The speedy reporting of disease and wide coverage of vaccination would depend upon researches on the development of sero-surveillance tests for early detection of virus and its validation under field condition along with the development of potent vaccine.

Recommendations

- FAO may consider to develop FMD control programme for small ruminants in certain primary endemic target areas.
- FAO may set up regional diagnostic laboratories for sero-surveillance for small ruminants with the availability of uniform standard reagents.
- World Reference Laboratory may develop validated field based pen-side test for

early detection of virus in small ruminants and basic researches on FMD on these animals.

References

Bachrach, M.L. 1969. Proceedings of the First International Conference of Foot-and-Mouth Disease, New York. The Gustav Stern Foundation Inc., New York.

Dutta, P.K., Sharma, G. & Das S.K. 1984. FMD in sheep and goats. *Ind. Vet. J.* 61: 267-270.

Donaldson, A. I. & Alexandersen, S. 2003. The virological determinants of the epidemiology of foot-and-mouth disease: by B. Dodet & M.Vicari, eds. *Foot and Mouth Disease: Control Strategies* PP 174-188 Paris: Elsevier.

Elmanjani, A.A. & Hassan, N.K. 1986. An outbreak of FMD serotype 'O' in sheep and goats in Jarnahiriya (libya). *Vet. Med. Review*; 1: 100-106

Gleeson, L.J., Samuel, A.R. & Knowles, N.J. 2003. Epidemiology of foot-and-mouth disease in Southeast Asia. by B. Dodet & M.Vicari, eds. *Foot and Mouth Disease: Control Strategies* PP 85 - 102 Paris: Elsevier.

Hyun B.H., Lee K.N., Oem J.K., Cho I.S., Kye S.J., Park J.Y., Kim C.H., Shin N.G., Kang J.M., Joo Y.S., An S.H. 2003. Rapid serological immunochromatographic assay for differentiating infection from vaccination in foot-and-mouth disease using whole blood: by B. Dodet & M.Vicari, eds. *Foot and Mouth Disease: Control Strategies* PP 369-376 Paris: Elsevier.

Kitching, R.P. 1998. A recent history of Foot-and-Mouth Disease. *J. Comp. Path.* 118: 89-108.

Leforban, Y. & Gerbier, G. 2003. Recent history and epidemiology of foot-and-mouth disease in Europe. by B. Dodet & M.Vicari, eds. *Foot and Mouth Disease: Control Strategies* PP 153 - 171 Paris: Elsevier.

Mishra K.C., & Ghei J.C. 1983. Target sites of aphthovirus infection in Sikkim local Goats. *Ind. Vet. Med. J.* 7:227-228.

Singh, G.R. & S.K. Sharma 1980. FMD outbreak at a sheep farm. Some pizooriological observations. *Ind. Vet. Med. J.* 4:155-158.

Shankar H., S.K. Sharma, S.V. Singh, N. Singh & V.K. Gupta 1998. – FMD in small Ruminants; Some epidemiological observations. *Indian J.*, 14 (1); 21-25.

Singh, S.V. Shankar, H. Singh, N. Sharma S.K. & Sinha N.K. 1994. Outbreak of FMD causing heavy mortality in small ruminants. *Ind J Comp. Microbiol imm inf. Dis.*, 15 (1&2), 26-30.

Uppal, P.K., Singh I.P., & Kumar S. 1972. Role of sheep in the epidemiology of Foot and mouth disease. *Indian J. Anim. Prod.*, 3:123-125.

FMD in Turkey and Iran - trends and relationships

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Abstract

Introduction This study analysed spatial and temporal patterns of FMD occurrence in Turkey and Iran in the periods 1990-2002 and 1997-2002 respectively to explore factors associated to the disease persistence and spread.

Materials and Methods Annual records of FMD distribution, grouped by serotype (O, A & Asia 1), were analysed using a metapopulation model to test how disease status, expressed in terms of presence/absence, extinction, and colonisation, and measured at the province level could be predicted using province-level data on: ruminant livestock numbers; meat production-demand discrepancy; and the disease prevalence distribution as recorded for the previous year.

Results In Turkey, FMD occurrence was associated to host abundance, short distance contagion from adjacent provinces, and to meat production-demand discrepancies. FMD was more widespread in Iran with the maximum incidence of all separate type being higher than the most frequent type O from Turkey. FMD occurrence in Iran was associated to host abundance, and to meat production-demand discrepancies. The persistence of FMD in cattle was associated to sheep density in FMD type A and O.

Discussion The results of Turkey and Iran are compared and discussed in relation to main factors associated to FMD occurrences, and to the possible role of sheep in facilitating virus persistence.

Introduction

The area extending from central Asia to the eastern Mediterranean basin, and including the Arabian Peninsula, forms an area of particular interest as it presents an environment where disease spread frequently goes unchecked. In particular, the 'Eurasian ruminant street' is formed by a high ruminant livestock density area stretching from southern Asia to the Mediterranean basin, creating a narrow east-west connection just south of the Caspian Sea in Iran and acting as a corridor for the spread of pathogens such as FMD (Fig. 1). Despite the fact that FMD is endemic in both countries (Fig. 1 top), and the continuity in livestock distribution, clearly visible from the distribution of small ruminants densities in those two adjacent countries (Fig. 1, middle & bottom), the epidemiological context of FMD differs. Over the last decade, Turkey has been applying large vaccination programme in an attempt to eradicate the disease from the country, but the disease persists in several provinces, in large parts due to the periodic introduction of new strains from the Middle-East. As detailed in the previous study, the pattern of persistence is different for FMD type A and O, with type A going through occasional extinction, whereas type O remains present at a larger incidence (see Gilbert *et al.* submitted, and refs. therein). In contrast, very little control effort has yet been initiated in Iran, and the first efforts for implementation of animal disease surveillance systems started in 1996 and involved the recording of outbreaks, and serotyping of the most important ones. In a previous study (Gilbert *et al.* submitted), we examined the associations between province-level FMD status by type in Turkey over the period 1990-2002, and predictor variables including ruminant livestock numbers; meat production-demand discrepancy; and the disease prevalence distribution as recorded for the previous year. The aim of this analysis was to replicate the analysis using similar data available for Iran, and to compare the patterns, and associations between these two countries.

Material & Methods

Data

In Turkey, data on monthly numbers of FMD outbreaks per province (85 provinces in total) were compiled by serotype from the monthly summary reports on notifiable diseases produced by the General Directorate of Protection and Control (GDPC) under the Ministry of Agricultural and Rural Affairs (MARA, Turkey). Data on the province-level distribution of livestock (head of cattle, sheep and goats) and people was obtained from the State Institute of Statistics (Turkey). The five provinces of

the Thrace region were excluded from the analysis because of the very intensive vaccination scheme carried out in this area.

Two data sets were available for Iran. The first data set gives the number of FMD outbreaks and cases per province in sheep and cattle separately from 1997 to 2002. These outbreaks and cases are not typed and each individual case represented a individual herds from 1999 to 2000, whereas it represented an infected village or industrial farms from 2000 onwards. The numbers of cases from these two periods can thus not be treated on the same ground. The second data set provides the number of samples sent for serotyping (and the number of each type: A, O and Asia1) per province in sheep and cattle separately from 1997 to 2002. However, although there is a significant correlation between outbreaks and samples per province, the numbers do not always match (see details in Gilbert 2003). The variability between these two measures of FMD incidence have two consequences: i) the number of samples could not be used as a measure of incidence without an important risk of errors (especially for provinces where the FMD was reported but where no sample were taken), and ii) the number of outbreaks per province and host can not easily be broken down in different types according to the proportion observed in the samples (here again, provinces with low number of outbreaks would be at the highest risk of error using such method). The fact that different units were considered for outbreaks in 1999-2000 and 2001-2002 further highlighted the difficulties of using outbreak numbers as measures of incidence. It was thus decided to build a dataset of province-level FMD status (presence/absence) for cattle and sheep and for FMD type A, O and Asia1 to be used for further analyses. Provinces were set as infected by a given serotype if at least one sample of this serotype was found in the province. There were 29 provinces, each having three serotype status for two host types (cattle & sheep) over 6 years, thus a total of $29 \times 3 \times 2 \times 6 = 1044$ status. Some location with for example, outbreaks reported in cattle and no samples had an unknown serotype status. Such situation represented 219 status out of 1044, mostly in sheep that where much less sampled than cattle. The status of such situation was set as absent, which has the consequence of slightly underestimating each serotype distribution.

Meat production absolute deficit both for cattle and small ruminant meat were taken as a surrogate estimate of the disease risk arising from domestic trade in live animals, under the assumption that provinces with a high deficit (e.g. Ankara), or high surplus (e.g. Erzurum) have more movement of live animals. The absolute difference between meat production and demand was estimated for each province. Production was estimated by multiplying livestock numbers and the mean national meat output per animal. Meat demand was estimated by multiplying the number of people and the national demand per capita.

Analysis

The analysis procedure was similar as the one used in the analysis of Turkey data, where all details and formulation can be found (Gilbert *et al.* submitted). Basically, it involves pooling the data from the different years in a common data set, and test FMD presence/absence against predictors (including past status) using a multiple logistic regression analysis. A complementary spatial term is then added to the model account for the possible contagion from the neighbourhood provinces. The analysis is carried out to test variables associated to presence/absence at the province level (using all provinces), colonisation events (using the subset of provinces where FMD was absent in the previous year), and extinction events (using the subset of provinces where FMD was present in the previous year).

Results

The difference in FMD incidence and distribution between the two countries is presented in Fig. 2 along the temporal dimension, and in Fig. 3 in geographical terms. FMD type O is present in both countries much more widespread in Iran. FMD type A is stuttering to extinction in Turkey whereas it is still widely distributed in Iran, and the incursion of the exotic type Asia1 affected a much higher proportion of provinces in Iran. Data on FMD in Turkey were differentiated in serotype but not by host.

In Iran, data on FMD type and host were obtained and allowed comparing the dynamics in cattle and sheep. If we compare the dynamic of outbreaks (Fig. 4 left) with the dynamics of proportion of provinces infected by each FMD type for each host (Fig. 4 right), we can see that a rather complex dynamics explain the rather simple pattern of outbreaks. In 1998, the level of outbreaks was fairly low, but the proportion of provinces infected by FMD type A and O was high, and in close proportion in sheep and cattle (note the lower proportion for type A in sheep). In 1999, the burst of FMD outbreaks is mainly due to the entry of FMD type Asia1, that affected mostly cattle, and almost all provinces showed evidence of type A, O and Asia1 in cattle whereas the incidence of the three type in sheep was much lower. Then, disease incidence decreased in 2000 and then increased again in the three serotypes in 2001 which caused the high peak of outbreaks. One can note that the predominance of cattle FMD over sheep is the lowest for type O, intermediate for type A, and the highest for type Asia1 (Fig. 4, left). The spatial distribution of the cumulated number of years of infection of the three type is presented on Fig 5, and the higher host affinity of type O for sheep

already identified in Fig. 4 is also visible here, as the presence of FMD in sheep is the highest for type O, then type A, whereas only a few provinces showed evidence of FMD type Asia1 in sheep. Detailed statistical results of the metapopulation models can be found in Gilbert *et al.* (submitted), Gilbert (2003) and Gilbert *et al.* (*in prep.*), and the main results are summarised in Table 1. In general, new infection are related to the density of the host: this can be observed in Turkey (positive association with cattle density, negative association with goat density), in cattle in Iran (positive association with cattle density), and in sheep in Iran (positive association with sheep density). In Turkey, meat deficit in cattle and sheep is associated to persistence of type O, and no other relationship is found significant. In cattle in Iran, we found an significant association between persistence and sheep density in type A and O. In sheep, sheep density is found associated to the persistence of FMD type A in sheep. When looking at the overall association between the predictors and FMD presence/absence, we find frequent associations with the previous year status, with the main host density, with the meat deficit, and occasionally with the province area.

Discussion

Positive association with past status is expected, and simply reflects the persistence in previously infected provinces, and is found significant when the serotype is widespread. A positive association with the main host density is also expected as predicted by standard epidemiological models. In addition to these standard epidemiological variables, we find a frequent positive association with cattle and sheep meat deficit, which we introduced in the analysis as a surrogate estimate of meat trade. This association is found significant in predicting FMD status, and associated both to new infections or persistence patterns. It is believed that these relationships reflect the risk of FMD transmission relating to the trade of live animals or processed meat.

We also find in Iran a positive association between the persistence of FMD in cattle, and the density of sheep. Sheep numbers are related to persistence of FMD in cattle but the opposite is not true, as for sheep we do not observed a relationship with cattle abundance, but with the meat-deficit, i.e. a surrogate indicator for trade of live animals and animal products. These results support the hypothesis that cattle may participate in spreading the disease through trade, (cattle meat deficit is a significant variables for all types in Iran), but that sheep facilitate the persistence of the disease (sheep density, or previous status in sheep are also significant for all types). This result provides one of the first quantitative evidence suggesting that sheep contributes to maintaining FMD in cattle population, and that sheep may also possibly contribute to maintaining the disease in Turkey. This hypothesis could not be tested with the Turkey data because epidemiological data from cattle and sheep are pooled together, but a positive association was observed between type O status and sheep density, which suggest a similar type of association.

The question of host affinity between cattle, sheep and these three serotypes can be discussed by looking at their distribution patterns (Fig. 5). We see that there is clearly a lower overall incidence in sheep than in cattle, but that these differences are low for type O, intermediate for type A, and highest for type Asia1. Furthermore, we found that type O in sheep in Iran was associate to past disease status in sheep (and not in cattle) suggesting that FMD type O could persist in sheep alone. These elements are corroborative with some previous observations Turkey suggesting that the persistence of type O in Turkey, as compared to the very low incidence of type A, may be related to a higher affinity of type O for sheep, allowing the disease to circulate and maintain in the sheep reservoir.

Although the role of sheep in spreading the disease has been established on several occasions, their role in the persistence of the virus is a matter of debate. Recent results in isolation units indicated that FMD declined in viraemia level in a mixed sheep population (Hughes *et al.* 2002), and suggested that FMD could not persist in sheep alone. This results in confirmed by previous field observation of a decline of FMD in sheep in Greece during 1994, and the example of past successes in the control of FMD in Uruguay by targeting all vaccination efforts on cattle (see Donaldson 2000 and ref. therein). However, the level of virulence of FMD in sheep appears to be highly variable, and particularly high when animals are exposed to exotic strains of FMD and when the lambs are unlikely to be protected by maternal antibody. There are molecular ecology evidences indicating that Turkey is periodically exposed to new FMD strains entering the country from the middle-east (Aktas). The periodic introduction of new strains may thus allow maintaining FMD R_0 in sheep above 1 in Turkey, whereas FMD would simply go extinct if new FMD strains entry was prevented by movement control. Given that the role of sheep in this region is clearly not elucidated, that there are quantitative associations are found between sheep density and FMD persistence, and that their role may have important implications in terms of control and prevention, detailed surveillance should also focus on sheep, even if control is mostly directed toward cattle. For example, recording information such as the possibility of contact with sheep herds, or serological testing of sheep flocks in the neighbourhood, should be envisaged when FMD outbreaks are recorded in cattle.

Our maps also highlight the very high unbalance in FMD incidence level between the two sides of the Turkey/Iran border (Fig. 3). Given the spread ability of the FMD virus, and the difficulty in controlling trade in these geographical areas, the option of reducing this unbalance by moving a higher amount

of control effort within western Iran should be considered to reduce the frequency of FMD virus within Turkey.

References

- Aktas, S.** 1998. Molecular Epidemiology of Foot and Mouth Disease types O and A in Turkey PhD dissertation, University of Reading, United Kingdom.
- Donaldson, A.I.** 2000. The role of sheep in the epidemiology of foot-and-mouth disease and proposals for control and eradication in animal populations with a high density of sheep. Report of the Session of the Research Group of the Standing Technical Committee of EUFMD, Borovets, Bulgaria, 5 to 8 September 2000.
- Gilbert, M., Aktas, S., Alisafar, M., Otarod, V., Sumption, K., Tufan, M. & Slingenbergh, J.** In preparation. Comparing spatial and temporal patterns of FMD type A, O and Asia1 in Turkey and Iran.
- Gilbert, M., Sumption, K., Mohammed, H, Tufan, M., Aktas, S. & Slingenbergh, J.** Submitted. Patterns of spread and persistence of foot-and-mouth disease type A, O and Asia1 in Turkey: a metapopulation approach.
- Gilbert, M.** 2003 Animal health management: modelling disease spread. Part I : Modelling FMD spread along the ruminant street with province level data. Consultancy Report prepared for the Animal Health Service of the Animal Production and Health Division of the FAO (Rome, Italy), 28 pp.
- Grenfell, B. & Harwood, J.** 1997. (Meta)population ecology of infectious diseases. Trends Ecol. Evol. 12: 395 – 399.
- Hughes, G.J., Mioulet, V., Haydon, D.T., Kitching, R.P., Donaldson, A.I. & Woolhouse, M.E.J.** 2002. Serial passage of foot-and-mouth disease virus in sheep reveals declining levels of viraemia over time. J. Gen. Virol. 83: 1907-1914.

Table 1. Summary results of the logistic model of FMD type A, O and Asia-1 occurrence in Turkey and Iran (1997-2002)

		Type O	Type A	Type Asia1
Turkey	P/A	Py, A, Sd, Gd , CaMd, SrMd	A, Gd	Sd, Gd , CaMd
	Ext.	CaMd, SrMd	-	-
	Inf.	A, Cd	A, Gd	A, Cd
Iran Cattle	P/A	A, Cd, CaMd	PyCa, Cd, CaMd	A, Gd , CaMd
	Ext.	CaMd, Sd	Sd	PySh
	Inf.	Cd	Cd, CaMd	CaMd
Iran Sheep	P/A	Sd, PySh	Sd	-
	Ext.	-	Sd	-
	Inf.	Sd	Sd, CaMd	-

P/A: full data set using presence/absence; Ext: extinction (subset of provinces infected in the previous year); Inf: new infection (subset of provinces non infected in the previous year); Py: status in the previous year (0/1); A: province area (sq. km); Cd: cattle density (head / sq. km), Sd: sheep density (head / sq. km); Gd: goat density (head / sq. km); CaMd: cattle meat absolute deficit (kg); SrMd: small ruminants meat absolute deficit (kg); PyCa: : status in the previous year in cattle; PySh: : status in the previous year in sheep. * Variables with a negative coefficient are in boldface type.

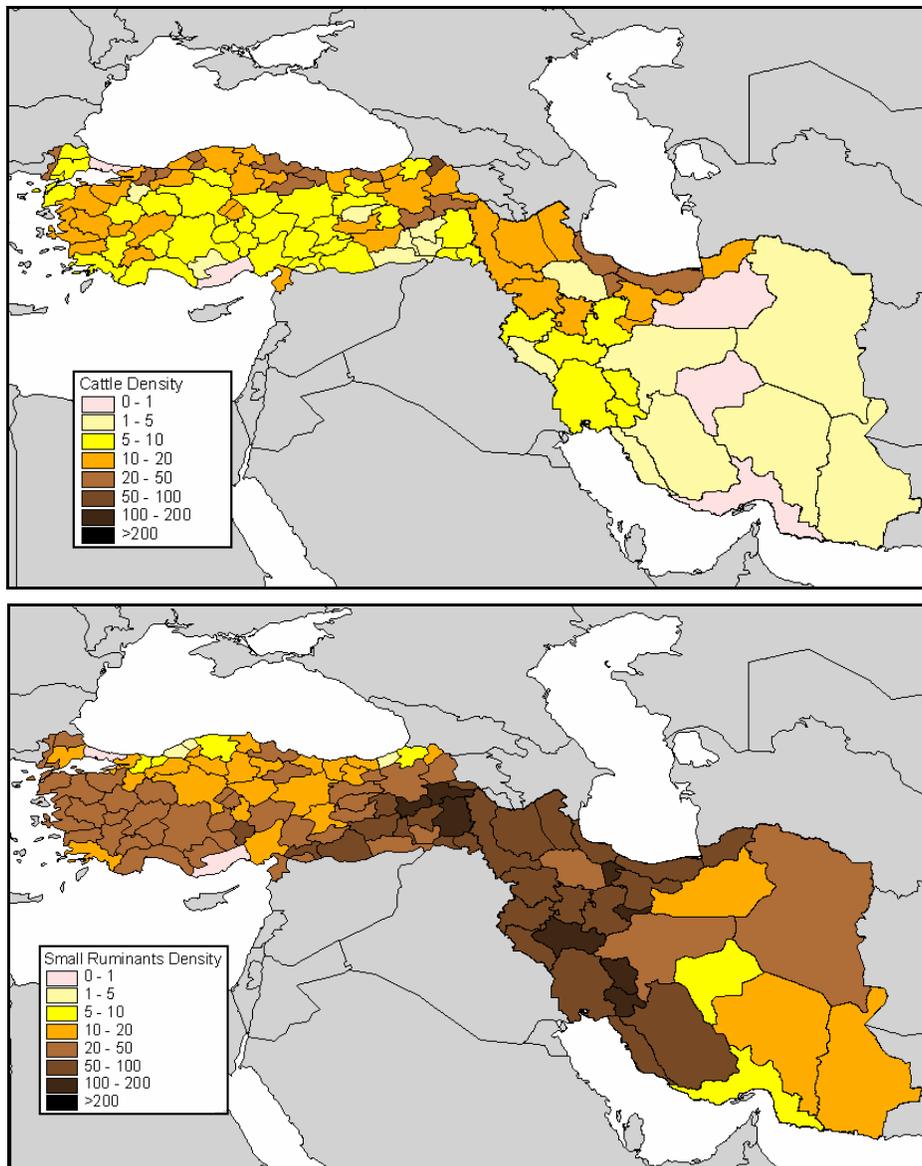
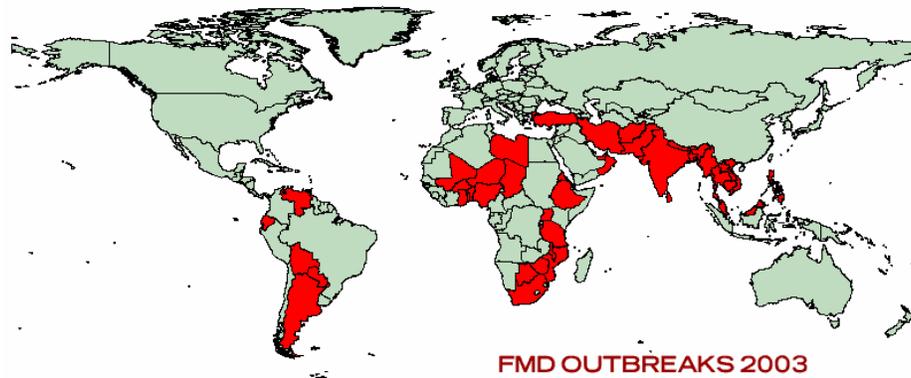


Figure 1. Distribution of countries with reported FMD outbreaks in 2003 (EUFMD 2003), showing a narrow band of FMD-affected countries stretching from Southeast Asia to Europe (top), and distribution of cattle and small ruminants densities in Turkey and Iran (animals / km²; bottom).

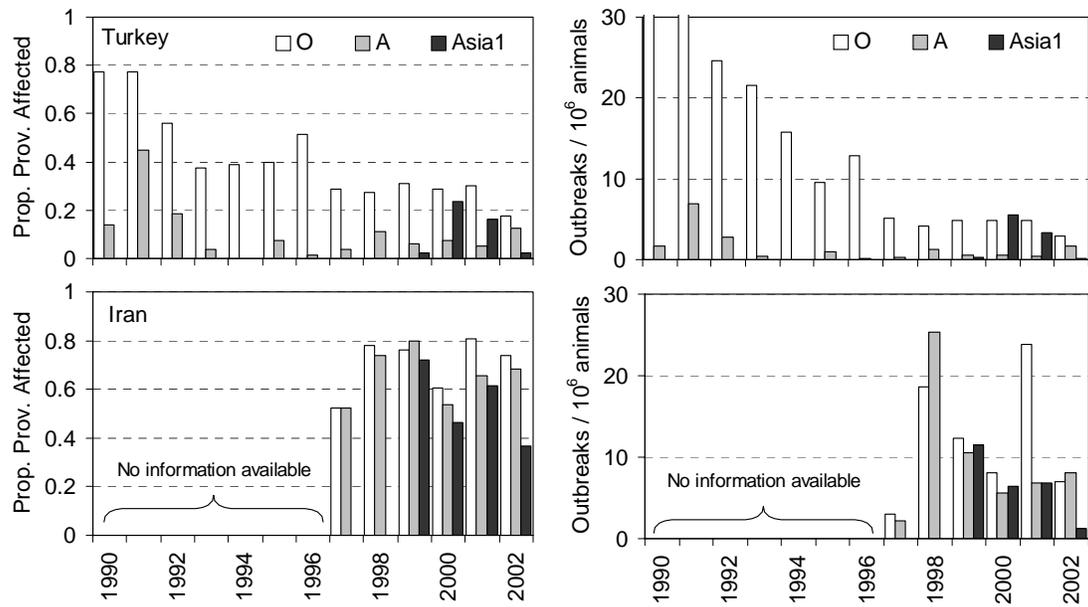


Figure 2. Proportion of provinces affected by FMD outbreaks in Turkey and Iran (left), showing that FMD was still widely distributed in Iran in the recent years whereas some types showed much lower distribution in Turkey, and number of outbreaks per 10⁶ cattle in the same period (right).

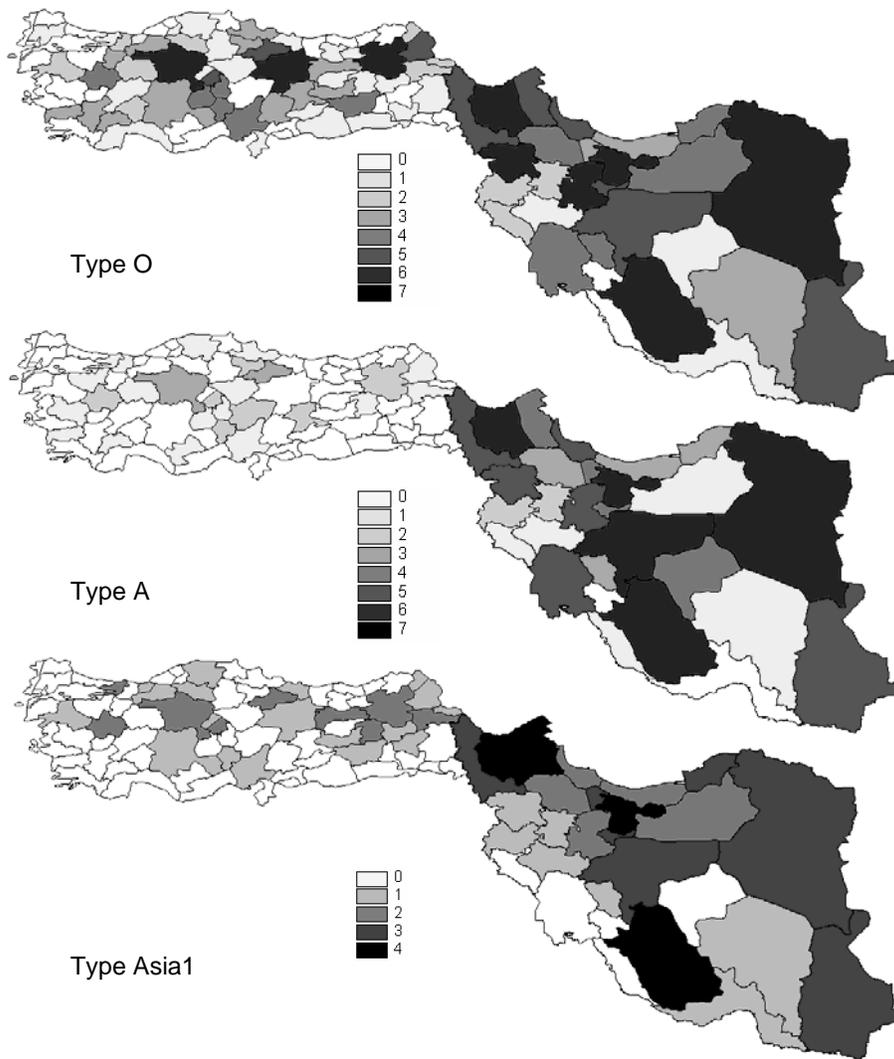


Figure 3. Number of years with reported FMD in Turkey and Iran provinces for Type A, O and Asia1 in the period 1997-2002.

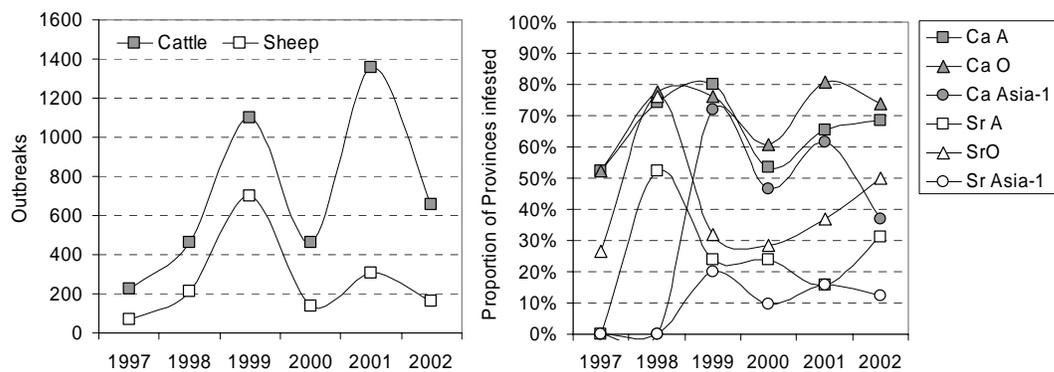


Figure 4. Temporal distribution of FMD in Iran. Outbreaks recorded in cattle and sheep (left), and proportion of provinces where the three types of FMD were reported on cattle and sheep.

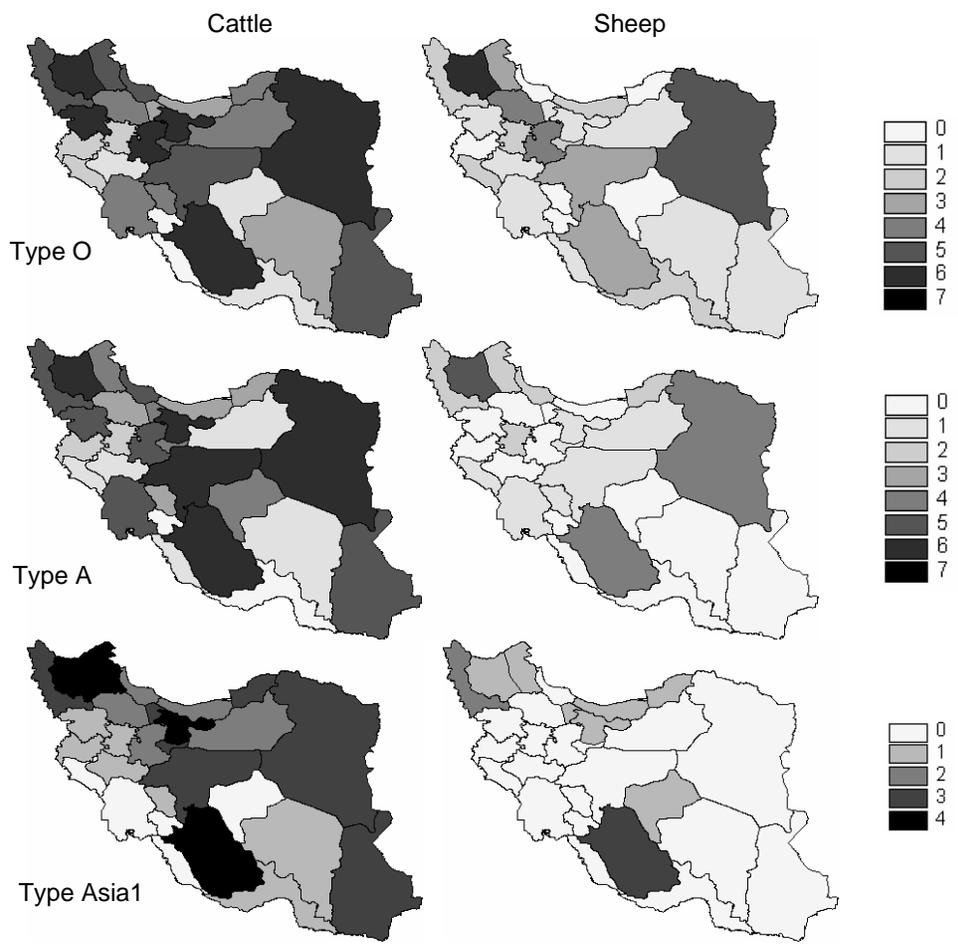


Figure 5. Number of years with FMD reported as serotype O, A and Asia1 in Cattle (left top to bottom) and sheep (right, top to bottom).

Epidemiological models for global surveillance of Foot-and-Mouth disease

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Abstract:

This paper summarizes some of the epidemiological models we have developed to describe the global temporal-spatial distribution and risk of FMD and to identify factors that predict changes in FMD status or movement, as applicable to real-time global FMD surveillance. Regional models focused on the time, space (geographic location), and time-space evolution of FMD in specific countries. Countries being studied include Iran, Pakistan, Afghanistan, Turkey, Nepal, Mongolia, Colombia, Bolivia, and Argentina. Country-specific models were validated using methods of cross-validation and expert opinion, as solicited from veterinarians in each country. The results of risk models are projected to assess when and where FMD can be expected and to forecast globally changing risks of FMD. These models could be applied via an FMD web-portal currently under development for real-time global risk surveillance of FMD to characterize changes in time, place, and transmission of FMD, and to identify new anomalous and unexpected FMD cases or precursor events. Real-time global risk surveillance for FMD, utilizing prediction, forecasting, and anomaly detection models, can improve our awareness and assessment of FMD globally and provide fundamental information to enhance biosecurity in free areas and control strategies in infected areas.

Introduction:

It is becoming increasingly apparent that, if we are to control and perhaps eradicate FMD on a global scale, a comprehensive understanding of the global distributions and changes in risk and movement of the disease must be developed. Currently, we lack of information relating to where FMD can be expected to be found and the new or emerging risks of FMD world wide. Key goals of our laboratory are to disseminate information on FMD relating to outbreaks and isolates, make available modeled distributions of FMD in regions where the information is uncertain, and project or predict risk of infection in FMD-free areas of the world. In this paper we summarize some of the models and approaches explored by our group in the past year.

Methods and Results:

BioPortal system: The FMD BioPortal is a web-based system designed for the real-time capture and dissemination of data, diagnostic results, and FMD-related risk information to and from countries, agencies, and laboratories. The aim of the portal is to offer a secure and confidential mechanism for rapid transfer of data, analyses, and maps needed to assess changes in FMD risk for specified geographic locations and times. FMD-related data may be obtained automatically or by 'hand' upload from websites of the international organizations, such as OIE, FAO, or the WRLFMD, as well as from individual country or agency databases. The database outputs are reformatted into a standardized structure via a data adapter specific to a particular messaging system. The data can be subjected to a variety of epidemiological and statistical analytic and mapping methods, including spatio-temporal clustering, anomaly detection, and spread or movement prediction.

Temporal models: The goal of temporal models is to provide an estimate, during some time i , of the expected number of FMD outbreaks in a given region of the world in time $i+1$. The mathematical process that best models the time series depends on the behavior of the infectious disease (trend, sporadic, seasonal, annual, and secular cycles). We are currently examining the potential application of different Bayesian models that alternatively use autoregressive, random mixed effects, stochastic processes, or the combination of these models to predict the number of FMD outbreaks in Iran. The number of outbreaks reported by Iran from January 1996 through December 2001 is used to select the model that best fit the distribution of the data, using estimates of the mean absolute prediction error and deviance information criterion. Then the selected model is used to predict the number of outbreaks in Iran for the next year, and the resulting prediction is compared with the actual observed number of outbreaks (as part of validation).

Spatial models: Spatial models were used to estimate the most likely distribution of FMD throughout an area under study, when incomplete information is available. We used a combination of smoothing techniques and elicitation of expert opinion to recover real or more realistic outbreak distributions, which were missed when data were grouped at a province level in Iran (actual outbreak locations were missing). A spatial scan statistic was run to estimate high FMD risk areas of Iran, and then compared estimates for the distribution of the susceptible population. The resulting relative risk estimates were validated through correlation tests with a subset of data with actual locations. The $R_s > 0.8$ indicates a very close fit of the smoothed spatial distribution model to the actual data of FMD cases, as described by experts in field. For Pakistan, which has not reported FMD outbreaks via OIE since 2001, the expected probability of having at least one outbreak was modeled by estimating the spatial correlation among reported outbreaks and the use of covariates hypothesized to be related with FMD prevalence (livestock and human population densities). The model was validated by comparing the expected probability with the true location of samples, from which FMDV was isolated, submitted by Pakistan to the world reference laboratory after 2001.

Time-space models: These types of models combine the characteristics of the previous temporal and spatial models and are an end goal of modeling and forecasting FMD spread in endemic countries. A spatiotemporal regression model was developed and applied using training data for the annual number of reported FMD outbreaks from 1996-2003 in each province of Turkey. Let Y_{ij} denote the number of reported FMD outbreaks in region i for year j , where $j=1$ corresponds to year 1996, and let μ_{ij} denote the mean number of FMD outbreaks and x_i denote a vector of covariates for region i , and let α be the corresponding vector of regression coefficients. The covariates included in the model were cattle and sheep density and whether the province bordered a body of water. The general Poisson regression model considered was of the form:

$$Y_{ij} | \mu_{ij} \sim \text{Poisson}(\mu_{ij}), \quad i=1,2,\dots,66; \quad j=1,2,\dots,8$$

$$\log(\mu_{ij}) = f(t_{ij}) + f_i(t_{ij}) + x_i^T \alpha + \theta_i$$

The functions $f(t)$ and $f_i(t)$ were used to model the time trend in FMD outbreaks for each province. To account for spatial correlation, the random effects (θ_i) were modeled using a conditionally autoregressive structure. A Bayesian approach was used to fit all models. The results of the model will be validated by comparison of the predictions obtained for year 2004 with the data actually observed in field in 2004.

Evaluation and design of eradication programs: Evaluation of past control strategies may lead to better design of future control programs in countries where FMD is endemic. To assess past control strategies in Colombia, we analyzed 20-years of data relating to FMD cases for both serotypes A and O. Smoothing of the time series using moving averages suggested a secular variation of large epidemics of each serotype, with large epidemic occurring every 4-6 years. The total number of FMD outbreaks caused by both serotypes decreased significantly ($P < 0.01$) throughout the 20-years period. However, the dramatic decrease in the number of cases that led to the control of FMD in Colombia occurred only after initiation of the eradication campaign in 1997, which involved international, national, and private organizations. The Colombian experience suggests that a successful eradication program must address a multitude of complex problems that extend beyond the technical issues involved in the selection of vaccines or the surveillance system.

Serotype and phylogenetic models: The probability of that an FMD outbreak is associated with a specific serotype will be modeled using Bayesian models that consider the time and space relation between isolates and information on specific covariates (density of roads, species affected). The models are currently being developed into prototypes using information on a viral infection of salmon in California as the training data. Further models will include assessment of the spatial distribution and factors associated with variation in the homology and divergence of FMDV strains. We expect to start working with FMD-specific information from Iran and Nepal before the end of the year.

Global models: The information collected for each country and the results obtained from national and regional models will be used to create comprehensive global models to predict FMD. The aims of these global models are 1) to quantify the association between economic, political, agricultural, and demographic factors and FMD status at the national and sub-national level and 2) to predict true national and sub-national FMD status globally, using publicly available data and controlling for suspected confounders. Surrogate variables describing political, agricultural, demographic, and economic status can be obtained from publicly available sources of information, such as national governments, the World Bank, and the United Nations. For example, the gross domestic product per capita was used as a surrogate for economic status in a country. Training data for the dependent variable (yearly FMD status) is based on the presence of FMD in each country, as estimated by expert opinion and OIE data. The association between FMD status and the predictor covariates described

above will be evaluated using a Bayesian logistic regression. Figure 1 shows a prototype of the expected outcome of the global models.

Discussion:

Development of reliable models is typically a very time-consuming process. The quality of the output is usually related with the quality of the input and therefore, improving the quality of the information used to design the models is key for the success of the predictions. Modeling infectious diseases in endemic countries allows for better opportunities for validation and improvement of the model design, a major advantage that compensates for the typical lack of information.

We expect during the next year to increase collaboration with national and international organizations to improve the quality and dissemination of our maps and models. We also expect to explore serotype-specific and phylogenetic models. The ultimate goal will be to make these models broadly available via the FMD web-based system (FMD BioPortal), currently under development for real-time global risk surveillance of FMD, to characterize changes in time, place, and transmission of FMD, and to identify anomalous and unexpected FMD cases or precursor events.

Conclusion:

Real-time global risk surveillance for FMD, utilizing prediction, forecasting, and anomaly detection models, can improve our awareness and assessment of FMD globally and provide fundamental information to enhance biosecurity in free areas and to develop control strategies in affected regions.

Recommendation:

Expand development of models to project changes in FMD distribution, risk and genomic variation.

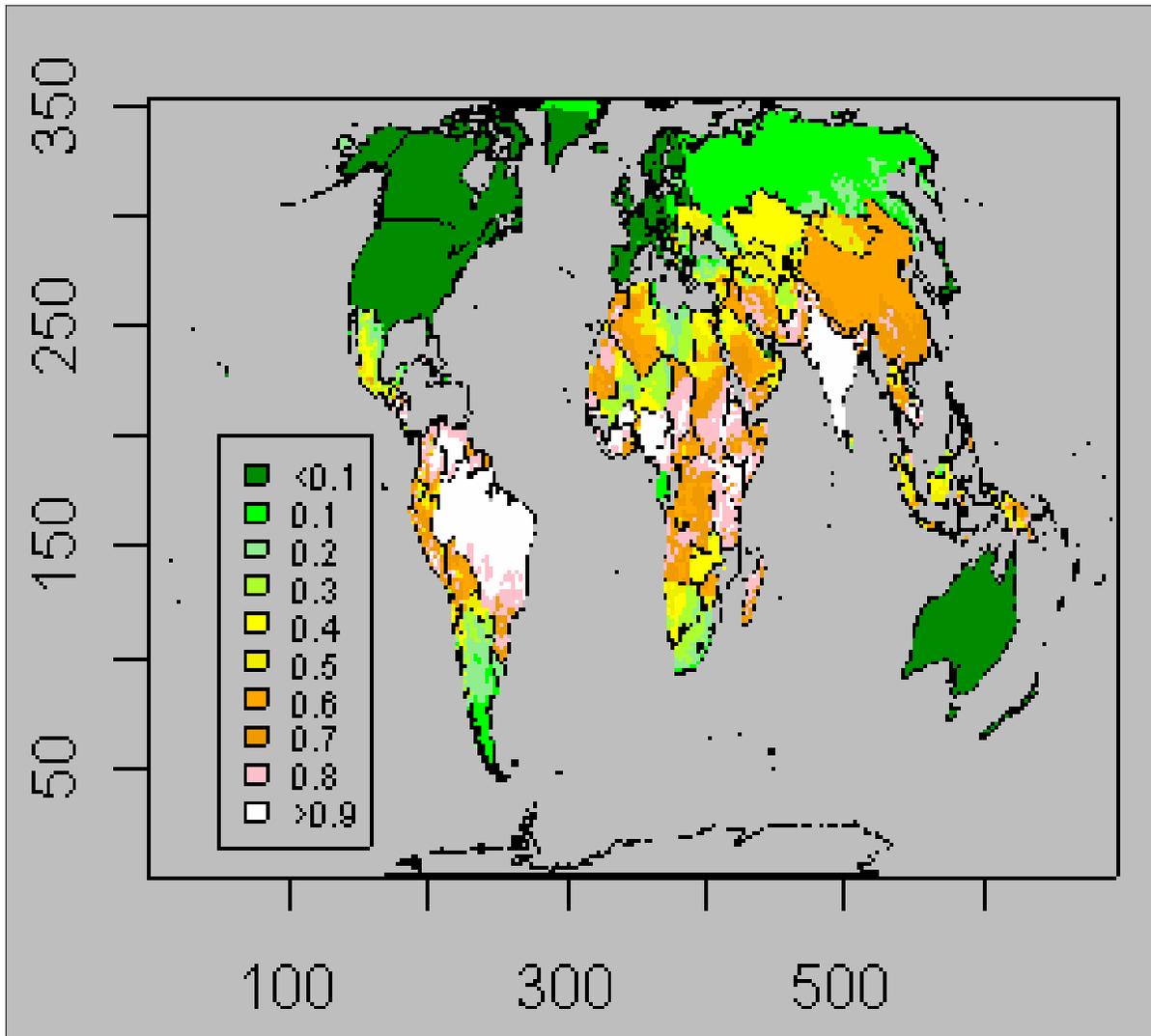
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Figure 1: Prototype of a system to display the results of the global models. Different shades indicate different risk for FMD. The predictions displayed here are presented using a partial set of covariates as predictors and at a relatively high scale of definition. The purpose of this figure is to demonstrate the outcomes that we are expecting to produce and not to present the actual outcomes of the model; model development is still in the design stage.



Concepts and considerations for global Foot-and-Mouth disease surveillance

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Abstract:

Introduction: This paper addresses some of the interest and discussion emerging about needs and prospects for a global surveillance system for foot-and-mouth disease (FMD). The purpose of this paper is to offer definitions, concepts, and considerations for global FMD surveillance function and operation, which hopefully will encourage surveillance research and promote discussions and dialogue necessary to move forward in formalizing an international surveillance effort.

Materials and Methods: A working definition of surveillance is proffered, along with presentation of surveillance system metrics and strategic surveillance designs. The definition is distinct from that of other terms often used synonymously with surveillance, including monitoring, passive surveillance, and survey. Metrics discussed include accuracy, precision, reporting time, efficiency, and value. General design features of surveillance systems should incorporate the biology and pathogenesis of FMD, dynamics of the animal populations being targeted, and the social and cultural aspects of the region or country. Regional and country-specific FMD surveillance systems nested within an over-arching global surveillance system would link operational elements with a standardized, translatable information and communication system that is compatible with real-time function. Other design concepts include intentional sample targeting and hierarchical surveillance intensities to maximize the probability of detection in high priority areas or time periods.

Conclusions: Creation of a global FMD surveillance system will require considerable international collaboration, partnerships, and understanding. Ideally, such a system would be developed and operated through a neutral and independent consortium, the main mission of which would be to provide high quality, accurate, real-time FMD surveillance service on a global scale.

Introduction:

There has been considerable discussion about the needs and prospects for a global surveillance system for foot-and-mouth disease (FMD). Unfortunately, no formal discipline exists specifically for surveillance or surveillance methodologies, and the 'science' of surveillance in general is very much in its infancy. The primary purpose of this paper is to offer some concepts, definitions, and considerations for global FMD surveillance function and operation, which hopefully will encourage surveillance research and promote (or provoke) appropriate discussions and dialogue necessary to move forward in formalizing an international surveillance effort. We also proffer strategies for organizing efforts and resources for a formal global FMD surveillance system or network.

Materials and Methods:

A working definition of surveillance and other competing terms

A fairly specific definition of surveillance offered here is "an active, ongoing, formal, and systematic process aimed at early detection of a specific disease or agent in a population or at the early prediction of an elevated risk of a population acquiring an infection or disease, with a pre-specified action that would follow detection of the disease, agent, or elevated risk".¹ Other definitions, including addressing serotype or detection of strain variation, also could apply. Surveillance is analogous to a diagnostic assay in that surveillance is a diagnostic system that is applied to a population for the purpose of detecting the targeted agent or disease, if it is truly present (surveillance sensitivity), and of verifying freedom from disease or infection, if it is truly absent (surveillance specificity).

This definition may differ considerably from those applied in common usage and from other terms often used in place of surveillance. "Passive" surveillance generally refers to *ad hoc* or *laissez faire* systems that rely on the good faith and competency of livestock owners or others to detect and report disease or to determine submission of samples for testing. Thus, "passive" surveillance typically is not systematic, and usually is not formally designed to detect disease early, if at all. The term "monitoring" is sometimes (inappropriately) used to mean surveillance because monitoring connotes an ongoing and formal process, and because it may be systematic in examining or studying disease or factors associated with disease. Monitoring differs from surveillance in that monitoring programs typically are intended to follow trends or progress made

to improve health; they are not designed for early detection of disease and they do not have a pre-defined event that would trigger action. If they did have these features, they should be referred to as surveillance systems. Monitoring systems, however, can operate within a surveillance system, where data or information obtained as part of a surveillance system also could serve a dual function in a system to monitor features of disease control or reporting, or some aspect of the surveillance system operation.

Another term often times used synonymously with surveillance is "survey". A survey is a process undertaken to obtain an estimate of the prevalence of disease or infection in a herd or population, or to identify possible risk factors associated with the disease or infection. Surveys rely heavily on use of sampling strategies that identify representative animals from the herd for testing. With respect to key risk factors for infection or disease, the sample of animals tested should mimic closely the animals in the herd. In contrast, surveillance sampling strategies preferably should be designed to target animals and herds at the highest risk of infection or at the highest risk of being the first to become infected, in order to seek out and detect the agent or disease very early. Consequently, surveys that utilize random sampling techniques to ensure representative animals are tested would not necessarily be appropriate in ongoing programs to assess freedom from disease or infection because high risk animals or groups may not be captured by random sampling techniques.

Surveillance system design and architecture

Local FMD surveillance design and architecture, such as that related to sampling schemes, should be directed by the biology underlying FMD, the population dynamics of species susceptible to FMDV infection, and the cultural and social features of the region or country that could influence risk or transmission of infection. The strain-and-host-specific pathogenesis, for example, influences duration of disease transition states, amount of virus shed, severity of clinical signs, and likelihood of transmission to other animals. Herd and flock management and husbandry practices can alter the chances for contact between infectious and susceptible animals and some animal trade networks can, more than others, promote spread of FMD to other regions or countries. Cultural or religious practices and events that involve animals also can affect transmission of the virus by bringing together infected and susceptible animals. Surveillance sampling schemes will need to vary depending on the likely location and timing of infected animals, in which high risk animals may be targeted for aggressive, frequent sampling. The design of local or regional surveillance, therefore, should take into account the biology of prevailing serotypes in the host species, as well as the cultural and husbandry practices that will affect changing geographical and temporal distributions of infected animals. The design of a global system would embrace all local, regional, and country-specific systems in a way that would interconnect and support each one, and that would identify in real time the FMD distributions and risk prevailing in the world.

Surveillance performance metrics

There are various measures that should be identified *a priori* to assess functional and operational performance of FMD surveillance systems. These metrics will depend on the specific objective, whether it be detection of the virus or of seropositive animals, or characterization of changes in the virus genome.¹

Accuracy: Accuracy relates to the sensitivity and specificity of the surveillance system, or how confident one can be that the system will identify correctly either truly FMD-free or truly FMDV-infected or exposed animals, herds, or regions. Sensitivity of a surveillance system is measurable directly in areas with endemic or epidemic FMD; however, in areas free of FMD, surveillance sensitivity can only be estimated indirectly, such as by use of models that predict sensitivity. Similarly, specificity of a surveillance system is directly estimable in areas or countries without FMD.

Precision: Precision is a measure of how repeatable and consistent a system would be in detecting FMD or evidence of infection. As for accuracy, precision can be estimated for surveillance systems in areas with FMD, whereas in areas free of FMD, estimates of precision could be obtained through use of models.

Time to detect and report: An important metric of surveillance is how quickly the system can communicate the true nature of FMD in a population to those who need to know. There are two components in this measure; one relates to the temporal sensitivity of the system and the other to the mechanism by which the system reports findings. Temporal sensitivity is a measure of how early the system would detect FMD or exposure in the course of disease progression in an individual animal, herd, or region. Detection very early in the course of population infection would constitute a high temporal sensitivity, and detection late would constitute low sensitivity. The estimated reporting period would be an indication of how long it took between identification of FMD,

or the lack of FMD, by the system and receipt of the report by those who need to know and to take action.

Efficiency: A general measure of efficiency of a surveillance system would be the ratio of its overall productivity to its overall cost. Surveillance productivity could be measured in a number of ways, including number of samples tested and evaluated, number of animals covered in the system, and number of regions or countries participating. An advantage of multi-purpose surveillance systems, in which several diseases would be embedded in one system, is that economies of scale can be expected to improve efficiency for any one disease.

Value: Value is a measure of a surveillance system's worth, utility, or importance, which usually are compared to competing needs for funding. The value would depend on direct and indirect political, economic, and social benefits derived in the short and in the long terms, including improved human nutrition and well-being, expanded trade, and political cooperation and good will. Other tangential benefits might accrue, including acquisition of new information or intelligence about factors contributing to transmission, susceptibility, or control. It is difficult to place a value on an investment made to detect a disease that may never appear. For regions or countries free of FMD, the value of surveillance investments would be measured in less tangible units, including what is the value in having peace of mind or of being able to assure trading partners that they will not acquire FMD.

Surveillance strategies

It is reasonable to assume that one approach to a global FMD surveillance system would involve a network of surveillance systems, whereby local or regional systems would be nested within a country or sub-continental system, which in turn would be nested within a global framework. The framework would provide the connectivity among the various layers of surveillance; the type or nature of surveillance for each sub-system would depend on whether FMD is epidemic or endemic, or if the area is considered free without vaccination or free with vaccination. An over-arching network connecting sub-systems would necessarily have to be able to communicate among the dissimilar aspects of each system, including disparate data and reporting formats, different assays and language, and unique aspects of other international reporting systems, such as those for OIE and FAO. Thus, design of a global system will need to address methodologies for standardization and translation in order to maximize communication compatibility with previously existing programs and systems.

Surveillance strategies also will need to be flexible in order to address new or expanded risks, as well as diminished risks, and to maximize efficient and effective allocation of resources. A system that is designed to accommodate a hierarchy of surveillance intensities will permit ramping up or cutting back on sampling numbers or frequency, depending on assessment of risk.² Thus, surveillance activities and relative allocation of resources should be guided by ongoing risk projections to predict modification of surveillance activities in specific times and geographic locations.

A critical strategic element in a global surveillance system will be real-time information transfer among the various operational groups, including laboratories, field units, and policy and decision makers. Web-based information technology will need to be developed and applied in ways that permit electronic access to and retrieval of data, information, maps, models, and analyses. The real-time sharing of information will be key to connecting and communicating with operational units, as well as to the early recognition and understanding of emerging risks or changes in the global FMD picture.

Discussion:

Creation of a global FMD surveillance system will require a true international collaboration and partnership that puts aside self interests and aspires to provide the best possible program for the early detection and diagnosis of FMD, identification of molecular changes in the virus, and projection of changes in global risk of FMD. Success will be contingent on removing knowledge barriers and sharing information, including data, reagents, and strains of the virus. In order to eventually develop a transparent surveillance system, requisite agreements must assure that there will be no retribution for sharing information.

Discussions of global surveillance can become preoccupied with questions relating to who should run such a program. Should surveillance simply be incorporated formally into FAO and/or OIE, or into the functions of world reference laboratories, or should development of a novel organization be considered that would be dedicated to surveillance and address gaps and pitfalls inherent in other organizations? Below are listed some criteria that may help guide what type of

administrative organization should be considered in providing global FMD surveillance. Ideally, the organization should be:

- dedicated primarily to providing service in surveillance to member agencies, laboratories, organizations, and countries.
- efficient, with minimal administrative overhead.
- able to provide information in real-time, which will require technology and expertise to access and submit information electronically via the web.
- able to employ a process by which decisions concerning surveillance issues are fair and equitable, and include all involved parties.
- able to develop an administrative culture that embraces new thinking, ideas, motivation, and initiative.
- able to support research and development necessary to improve surveillance and efficiency of operations.
- neutral and unencumbered by political or other outside influences.

If there truly is serious interest in some type of global FMD surveillance program or system, then efforts will need to be made to overcome inertia and to move forward in defining the depth and breadth of motivation and the types of actions to be taken. A possible next step would be creation of a consortium, representing key agencies, organizations, countries, and interested individuals to:

- Explore formally the feasibility of and issues relating to creation of global surveillance, including political barriers, international motivation, and alternative ideas.
- Solicit views on global surveillance from the 'FMD community'.
- Consider an international meeting to focus discussion on global FMD surveillance.
- Identify potential funding agencies to provide investment capital for formation of an international organization dedicated to FMD surveillance.

In summary, there are many aspects and issues of a global FMD surveillance system that were not addressed here and that deserve the benefit of broad debate and discussion. Careful consideration should be given to the prospects and likely chance of success for such an undertaking, including realistic expectations for true collaboration and partnerships, for long-term funding, and for control and eradication of FMD.

Conclusions:

- An FMD surveillance system developed and operated through a neutral and independent consortium could provide high quality, accurate, and real-time FMD surveillance service on a global scale.
- Prerequisite for such a global surveillance system is creation of an international partnership of agencies, countries, organizations, and individuals to initiate conceptual development of global surveillance.

Recommendation:

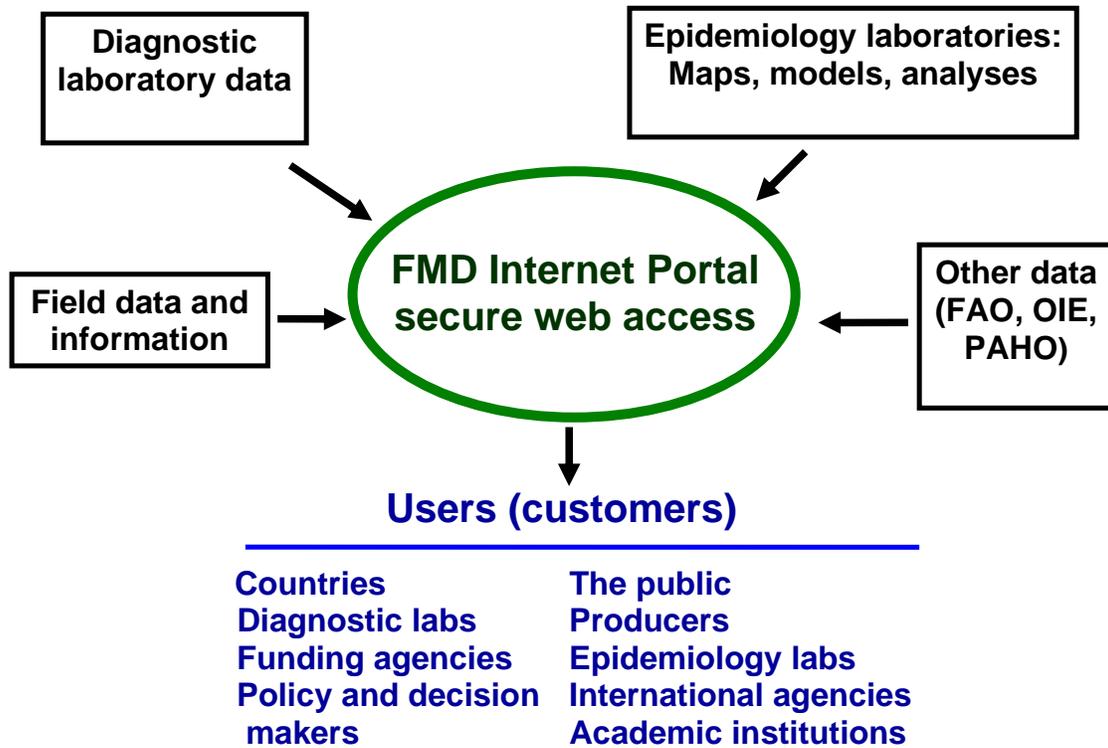
- An international consortium should explore formally the interest in, and funding and support for, developing a formal global FMD surveillance system and organization.

References:

1. **Thurmond, M.C.** 2003, Conceptual foundations for infectious disease surveillance. *J. Vet. Diagn. Invest.*, 15:501-514.
2. **Bates, T.W., Thurmond, M.C., Hietala, S.K.** 2003, Surveillance for detection of foot-and-mouth disease. *J. Amer. Vet. Med. Assoc.*, 223:609-614.

Figure 1

Surveillance real-time information links



GISVET project in Iran

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Introduction:

This project is started since 2001, in order to provide GIS information relevant to veterinary use. Animal diseases as health problems for animal welfare and public health are the topics which are considered to be controlled and eradicated in all of the countries. Implementation of the control measures without determining the specific risk factors associated with each disease can not lead to control of diseases and needs to use huge economical costs. On the other hand, needs of people to food developed international trade which in turn needs more attention to prevent global distribution of the diseases. Epidemiologically, the risk factors for diseases distribution are mainly associated with Agents, Hosts and environment. Parameters of this triad are a lot and the relationship between them determines the situation of the disease and makes their outbreaks conditional. In Iran there is different interaction & relationship between triad epidemiological determinants. There are about 70000 villages and most of rural families as animal husbandmen have less than 10 cows, and also there are a lot of Nomadic tribes & nomadic herds which their movement changes local and temporal distribution of both animals and diseases. Implementation of the control measures without determining the specific risk factors associated with each disease can not lead to control of diseases and needs to use huge economical costs. On the other hand, the complexity of investigation and control of multifactor diseases culminate in establishment of "GIS center" and "Animal diseases geographical information system" (GISVET) by Iran Veterinary Organization.

Identification of epidemiological units

Definition: Epidemiological unit is a group of animals kept in a specific geographical location with maximum contact among them which define the high risk transmission of diseases.

Types of units:

- Farms: cattle farms (beef farms & dairy cattle farms) and sheep farms.
- Villages: rural animal husbandmen & rural herds.
- Pastures: which can be used by rural herds or nomadic herds.
- Camel herds.
- Riding clubs.

Locations:

- Epidemiological units.
- Slaughter houses.
- Sale yards.
- Milk collecting centers.
- Veterinary offices.
- Veterinary-faculties.
- Stud-bull stations.

Registration of coordinate of locations

I.V.O has organized veterinary offices in all of the townships, so registration is performed by one of the official vet-Officers in township level.

In order to reduce the error of location registration, these vet-officers in all of the provinces are trained by Dr.V.Otarod & Dr.H.Wishte. They are trained to operating with GPS&to complete Registry forms.

Registry forms which are gathered specifically to fulfill the requirement of GISVET project and are not available from pre-existing data.

Controlling the random samples of registry papers is necessary to collecting "accurate registration".

Processing and finalization of data

Processing & finalization of attribute data and also transferring spatial data to central computers in order to convert Georeference database is performed by "national GIS" team.

Computerized reporting system

Computerized reporting system is designed in such a manner to be able to transfer data by a FTP server through internet. it is composed of three parts each one is installed in :

-Townships.

-provinces.

-IVO (national department of GISVET)

Report flow is from the townships to provinces and then to GIS center In IVO. The only reference for entering the data is veterinary government officers in townships level.

When the data entered and confirmed in the computer, it is impossible to be changed in provinces level or national level.

In the case of necessity for changing wrong data, it should be referred to Townships level, for any possible changes.

Data analysis

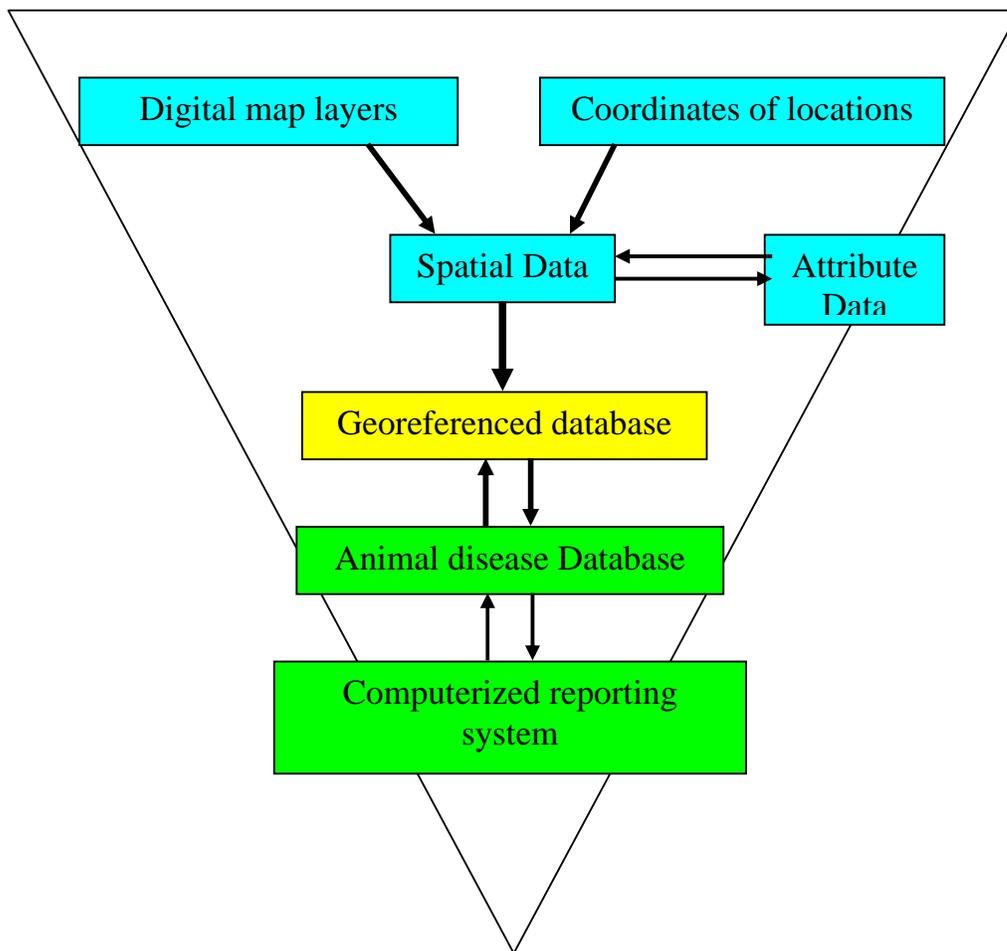
Beside the ability of reporting system to collect the data there is also facilities considered in the system to give statistical analysis of the data, means the epidemiological indexes: prevalence, incidence and daily, monthly, seasonal and annual reports based on the databases which are designed according to epidemiological units.

Therefore two databases, one which is produced by reporting system and the other produced by coordinates of units registered by GPS, will be linked to give the above calculations.

Visualization of data

Arcview software is used for developing the different image layers by using of GPS registered points and disease database. Therefore it would be possible to study the diseases with regard to available geographical information and layers.

Schematic Structure of Iran GIS-VET



Statistical and Imagery reports for decision-making

Influence of Exposure Intensity on Efficiency and Speed of FMD Transmission

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Abstract

FMDV can be spread by a variety of mechanisms including direct animal-to-animal contact, indirect contact facilitated by contaminated materials or airborne spread. The rate of spread, the incubation period until developing clinical signs of disease, as well as the severity of disease depends on many variables, including the dose received, the route of introduction, the virus strain, the animal species and the conditions under which the animals are kept. We previously presented data modelling infection in pigs directly inoculated with virus and also presented some experiments with varied intensity of contact among infected and susceptible pigs. Much more knowledge in regards to these variables is needed if model predictions are to be used in practical disease control. Here we extend our previous findings with more detailed studies in pigs exposed by contact to facilitate a better assessment of transmission risks.

We used groups of pigs inoculated with the FMDV O UKG 34/2001 strain and an experimental set-up that allowed the infectiousness and intensity of contact among pigs to be varied. Experiments with other strains of FMDV and experiments using sheep are also briefly mentioned. Virus replicated rapidly in inoculated pigs and when exposed by direct contact the mean incubation period was from 1-10 days for pig-to-pig transmission depending on the infectiousness of the source and the intensity of contact. These differences confirm our previous notion that a strong correlation exists between dose (i.e. infectiousness of source and intensity of contact) and length of incubation period, severity of clinical disease and efficiency of spread. Clearly, local conditions are of immense importance in determining efficiency and speed of FMD transmission and can be an important determinant for the outcome of an initial outbreak and a considerable source of inaccuracy if not truthfully accounted for in mathematical models of epidemiological spread.

Introduction

FMDV can be spread by a variety of mechanisms and the rate of spread and the incubation period as well as the severity of disease depends on many variables. These variables include the dose received, the route of introduction, the virus strain, the animal species and the conditions under which the animals are kept. We have previously presented data modelling infection in pigs directly inoculated with virus and also presented some experiments with varied intensity of contact among infected and susceptible pigs. Here we extend our previous findings with more detailed studies in pigs exposed by contact to facilitate a better assessment of transmission risks. Much more knowledge about these variables is needed if model predictions are to be used in practical disease control.

Materials and Methods

Animals and virus

One hundred and fifty four Landrace cross-bred Large White pigs weighing between 20 and 30 kg were used and inoculation was by heel pad injection or by contact as described previously (Alexandersen *et al.*, 2001; Alexandersen *et al.*, 2002a; Alexandersen *et al.*, 2003b; Alexandersen & Donaldson, 2002). These pigs were part of experiments conducted for several purposes and the descriptions and results described in the present paper are directed towards describing transmission aspects. Twelve sheep were housed and inoculated in the coronary band or kept as contacts as described previously (Alexandersen *et al.*, 2002b; Alexandersen *et al.*, 2003b; Alexandersen & Donaldson, 2002). The usual dose for inoculation was approximately 0.25-0.5 ml of a 1:10 dilution of virus stock containing $10^{5.6-5.9}$ TCID₅₀ (BTY) of O UK 34/2001 virus (first pig passage). Contact pigs were kept in direct contact with inoculated (donor) pigs for various periods of time and in various numbers as described for the individual experiments. The progression of disease were assessed daily using a subjective scoring system (Alexandersen *et al.*, 2003b; Quan *et al.*, 2004). Body temperatures were recorded daily and blood and swab samples collected as needed. Animals

with severe lesions were killed by euthanasia. The experiments were performed sequentially and further details are described below.

Pigs

Experiment 1 (min-17). 20 pigs. In this experiment, 4 pigs were inoculated with $10^{5.6}$ TCID₅₀ of FMDV and used to infect four sets of 4 pigs by contact. The direct contact pigs were infected by being placed into the box with the 4 inoculated (donor) pigs for a period of 2 hours during which the pigs were allowed to mix freely as a group of 8. The contact pigs were then moved back to a clean box. The exposure was done on post inoculation (PI) days 1, 2, 3 and 4.

Experiment 2 (min-18). 24 pigs. As above, 4 pigs were inoculated with $10^{5.6}$ TCID₅₀ of FMDV but in this experiment used to expose contact pigs in a one-on-one setup (i.e. one inoculated and one contact pig together). Four boxes were each divided into 3 cubicles (a total of 12 cubicles) in which 1 contact pig/cubicle was placed. In each box, the sizes of the cubicles were $\frac{1}{4}$ of the box for two of the cubicles and $\frac{1}{2}$ for the third cubicle in each group. On day 1 PI, three inoculated pigs were placed in a box for a period of 2 hours with three contact pigs in a one-on-one setup. This process was repeated on day 2, 3 and 4 PI using the same inoculated pigs placed with unexposed contact pigs in different boxes. In addition, a further group of 4 direct contact pigs were mixed with the four inoculated pigs for 2 hours as a group rather than one-on-one on day 4 right after the one-on-one exposures.

Experiment 3 (min-19). 16 pigs. In experiment 3, two sets of 2 pigs were inoculated with $10^{5.6}$ TCID₅₀. In addition, 2 pigs were inoculated with $10^{4.6}$ TCID₅₀, and 2 pigs with $10^{3.6}$ TCID₅₀ of FMDV, respectively. Each set of two inoculated pigs were placed in a cubicle in an individual box ($\frac{1}{2}$ the area of a box). Two pigs were housed with each set of inoculated pigs as continuous contacts. Inoculated and contact pigs were allowed to mix freely and continuously during the experiment. Detailed results for the inoculated pigs have been described elsewhere (Quan *et al.*, 2004).

Experiment 4 (min-20). 36 pigs. In this experiment, 18 pigs were inoculated with $10^{5.6}$ TCID₅₀. Another 18 pigs were housed with these as continuous contacts. The pigs were divided into 6 groups of 6 pigs in separate boxes, each group having $\frac{1}{2}$ the area of a box and each group containing 3 inoculated pigs and 3 direct continuous contacts. Inoculated and direct continuous contact pigs were allowed to mix freely during the course of the experiment. One box initially contained 2 groups of 6 pigs, but as these pigs were killed within 2-6 hours for other purposes, we will only describe the results of the other 4 groups of 6 pigs. Two inoculated and two contact pigs in each group were killed on days 1-4 allowing a variable intensity of contact for the 4 groups.

Experiment 5 (min-21). 36 pigs. Experiment 5 was an identical repeat of experiment 4.

Experiment 6 (Pig DEFRA 2). Eight pigs were kept in $\frac{3}{4}$ of a box (the other $\frac{1}{4}$ box had 3 sheep, see below), and four of these pigs directly inoculated and the four others as direct contacts. At 2 days pi fourteen pigs in a separate box were exposed by moving the four inoculated pigs into their box for 2 hours.

Sheep

Experiment 1 (DEFRA-1). 3 sheep infected by inoculation in the coronary band.

Experiment 2 (DEFRA-2). This experiment was done concurrently with the DEFRA 2 pig experiment mentioned above, and for this sub-experiment, 3 sheep was placed in $\frac{1}{4}$ of the box separated from the 4 inoculated and 4 contact pigs in the other part of the box by a metal gate which did not allow the animals to pass but freely allowed air to pass from one part of the box to the other. Direct contact was in principle possible through the metal gate, however, it appeared that the sheep and pigs did not try to have direct contact.

Experiment 3 (IE-JG-SA-Sheep-airb-1). The details of this experiment will be described in a separate presentation. Briefly, three sets of one recipient sheep were exposed to airborne virus from three sets of one inoculated donor sheep separated by wooden cubicles in 3 separate boxes.

Assay for virus, genome and antibodies

The infectivity in samples was assayed by inoculation of monolayer cultures of primary bovine thyroid (BTY) cells in roller tubes (Snowdon, 1966). The specificity of the cytopathic effect observed in cell cultures was confirmed by antigen ELISA (Ferris & Dawson, 1988; Hamblin *et al.*, 1984; Roeder & Le Blanc Smith, 1987). Quantitative real-time reverse transcription polymerase chain reaction (RT-PCR) was used to determine the amount of FMDV RNA in extracts of total nucleic acid from blood and swab samples as described in detail elsewhere (Alexandersen *et al.*, 2003b; Quan *et al.*, 2004; Reid *et al.*, 2003).

Serum samples were tested by an enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to FMD virus (Ferris, 1987; Hamblin *et al.*, 1986) and certain samples verified by virus neutralisation assay (as described in the Manual of Standards for Diagnostic Techniques and Vaccines, l'Office International des Epizooties, OIE, 2000).

Results

Pigs

Experiment 1 (min-17). This experiment was designed to test the efficiency and speed of spread of FMD in pigs exposed as a group of 4 inoculated (donor) and 4 contact pigs allowed to mix freely for a period of 2 hours. Exposure took place at PI days 1 to 4 for the individual groups. Inoculated pigs showed local lesions from 1 day pi and generalized lesions from 2 days pi and severe generalized disease from 3 days pi. For the group of contact pigs exposed at 1 day pi of the donor pigs, only 2 contact pigs showed any clinical signs of disease starting on days 7 and 9, respectively. These two pigs had a viraemia from days 4 and 6, respectively while the other two pigs in this group did not show any clinical signs nor a viraemia up to 11 days after exposure. For the other groups of contact pigs, all 4 contact pigs in each group showed clinical signs of disease starting on days 3, 5, 5 and 6, for the group of pigs exposed at 2 days pi; at days 4, 5, 5 and 9 for the pigs exposed at 3 days pi and on days 4, 5, 5 and 6 for the group of pigs exposed at 4 days pi. That the infectiousness of the donor pigs was very similar for the period from 2-4 days pi was further supported by the fact that the development of viraemia in these groups was virtually identical although perhaps with a slightly faster development in the contact pigs exposed at 3 days pi. This notion of slightly highest infectiousness of the day 3 pi donor pigs were further substantiated by testing of nasal swabs from either the donor or the contact pigs immediately after contact, which also showed the highest content of FMDV RNA on day 3 pi.

Experiment 2 (min-18). This experiment was essentially done as for experiment 1 with a 2 hour exposure on days 1, 2, 3 and 4 pi. The difference from experiment 1 was that pigs in experiment 2 were exposed one-on-one in cubicles with approximately the same space per pig as for the group exposure above. In addition, on day 4 pi a group of 4 contact pigs was also exposed to the donor pigs, i.e. in a group of 8 pigs. In this experiment the donor pigs showed generalized disease from 3 days and severe disease on day 4 pi. For the pigs exposed individually (one-on-one) none of the pigs exposed on days 1, 2 and 3 pi developed clinical signs of disease and none of them developed a viraemia although nasal swab results indicated some, perhaps abortive, local replication. For the pigs exposed one-on-one on 4 days pi, 2 out of 3 pigs showed clinical signs of disease starting on days 5 and 10, respectively and both had a viraemia starting on days 1 and 7, respectively. The third pig in this group did not develop clinical disease nor a viraemia. Although only a single observation, this pig was exposed in the "large cubicle", i.e. 1/2 box while the other two were exposed in "small cubicles", i.e. 1/4 box. Of the pigs exposed as a group on day 4 pi (4 donors plus 4 contacts) 3 out of 4 pigs developed clinical signs of disease starting on days 4, 5 and 5 and viraemia from days 1,2 and 3, respectively. The 4th pig in this group did not show any clinical signs but clearly had a subclinical infection manifested by a low grade viraemia lasting from day 5 to 11 post exposure. That the infectious dose for the pigs exposed as a group was higher than for the pigs exposed individually was supported by the fact that the development of viraemia and FMDV RNA load in nasal swab samples in the pigs exposed as a group was faster than in the pigs exposed individually. Based on the load of FMDV RNA in nasal swabs from the donor pigs and the contact pigs, the pigs exposed at 1 and 2 days pi received a very low dose while the pigs exposed at 3 and 4 days pi received a high dose (the dose the day 4 pi contact pigs received was slightly higher than the dose the day 3 pi contact pigs received). Interestingly, the pigs exposed individually on day 4 received an almost identical FMDV RNA load in the nasal swabs as the pigs exposed as a group, however, while the individually housed pigs appeared to initially almost clear their virus, the pigs housed as a group where more likely to show a rapid increase in virus load.

Experiment 3 (min-19). Detailed results for the inoculated pigs have been described elsewhere (Quan *et al.*, 2004). Out of the two times two pigs inoculated with $10^{5.6}$ TCID₅₀ of FMDV one showed lesions from day 1 pi and the other 3 pigs from day 2 pi with severe disease in all 4 pigs from day 2-3 pi. The 2 pigs inoculated with $10^{4.6}$ TCID₅₀ showed severe clinical disease from day 2 pi, essentially as seen for the higher dose pigs. Of 2 pigs inoculated with $10^{3.6}$ TCID₅₀, one had lesions from day 1 and the other from day 3. The impression from this experiment was that with intradermal (heel pad) inoculation within this dose interval the time to clinical disease or to viraemia did not differ significantly, however, at the low dose the outcome was less predictable with the possibility of a slower development of disease in some inoculated pigs, i.e. one pig showed a delayed development of viraemia (starting at 2 days as opposed to 1 day in the other pigs) and clinical disease (starting at 3 days as opposed to 1-2 days in the other pigs). Development of disease was very similar in the 3 groups of contact pigs, with clinical disease starting on days 3-4 and viraemia starting on days 1-2. Also, levels of FMDV in nasal swabs were very similar. It is of interest to note, that the disease

development in the two contact pigs exposed to the 2 donor pigs inoculated with the low dose were indistinguishable from the other groups.

Experiment 4 (min-20). The results described here cover the results for the 4 groups of contacts as the results for the inoculated pigs were similar for the 4 groups with viraemia starting at day 1 and clinical disease starting at days 1-2 pi (Quan *et al.*, 2004). The four groups of 6 pigs (3 inoculated and 3 contact pigs together) were housed in 4 individual boxes. Two of the inoculated pigs in each box were removed on day 1, 2, 3 and 4, respectively while the third inoculated pig in each box was kept in the box. In the box where all 3 donor pigs were kept until day 4 pi, all 3 contact pigs developed viraemia starting at day 2 and clinical disease starting on day 3 and becoming severe from day 4-5. In the box where 2 donor pigs were removed at 3 days pi, two pigs had a viraemia but not yet clinical disease on day 3 when they were killed, while the last contact pig in this box did not develop any clinical signs of disease when monitored up to 22 days after exposure. In the box where 2 donor pigs were removed at 2 days pi, two pigs had a viraemia but not yet clinical disease on day 2 when they were killed, while the last contact pig in this box developed clinical disease on day 5 pi. In the last group in this experiment, two of the donor pigs were removed at 1 day pi and none of 2 contact pigs killed at 1 day pi had viraemia, FMDV RNA in nasal swabs or clinical disease while the last contact pig in this group developed a viraemia and clinical disease from 5 days pi. Examination of nasal swabs showed that viral RNA loads were virtually identical in donor and contact pigs when kept under these conditions.

Experiment 5 (min-21). This experiment was a repeat of experiment 4 described above and involved four groups of 6 pigs. In this experiment the development of viraemia and clinical disease in the inoculated pigs were particularly fast and thus slightly faster than in experiment 4, above. In the box where all 3 donor pigs were kept until day 4 pi (NB, 1 donor pig killed at day 2 pi due to very severe clinical disease), all 3 contact pigs developed viraemia starting at day 2 and clinical disease starting and becoming severe on days 3-4. Similarly, in the box where 2 donor pigs were removed at 3 days pi, the 3 contact pigs had a viraemia and severe clinical disease on day 3. In the box where 2 donor pigs were removed at 2 days pi, two pigs had a viraemia but not yet clinical disease on day 2 when they were killed, while the last contact pig in this box developed clinical disease on day 3 pi. In the last group in this experiment, two of the donor pigs were removed at 1 day pi and none of 2 contact pigs killed at 1 day pi had viraemia or clinical disease but did have FMDV RNA in nasal swabs. The last contact pig in this group developed clinical disease from 3 days pi.

Experiment 6 (Pig DEFRA 2). The donor pigs developed viraemia and clinical disease within 1-2 days pi while the continuous contact pigs developed viraemia from 2 days and clinical disease from 3 days after inoculation of the donor pigs. The pigs kept in contact for 2 hours at 2 days pi developed a viraemia at 1-2 days and severe clinical disease at 2-3 days after exposure.

Sheep

Experiment 1 (DEFRA-1). One inoculated sheep developed clinical disease on day 1, another sheep on day 2 and the last inoculated sheep clinical disease on 3 days pi. All 3 sheep had increased body temperature from day 2-4 pi.

Experiment 2 (DEFRA-2). In this experiment the sheep started to develop a viraemia from 2-3 days after inoculation of the pigs and had peak viraemia and increased body temperature at day 4 and clinical disease from day 6.

Experiment 3 (IE-JG-SA-Sheep-airb-1). The inoculated donor sheep showed fever and typical clinical signs in the form of minor vesicular lesions along the coronary band, the interdigital space and the heel area within 1-2 days after inoculation. The recipient sheep were exposed to an estimated dose of airborne FMDV of around 300 TCID₅₀ over a 24 hour period and two out of the three recipient sheep developed antibodies against FMDV at days 14-35 p.i.. The same two sheep were also positive for virus by testing of probang samples on days 23, 25, 28 and 35 p.i.. The recipient sheep did not show any clinical signs (only subclinical infection) and only one recipient sheep had a fever 4 to 7 days after inoculation of the donor sheep.

Discussion

Experience from the field and from experimental infections indicates that the speed and efficiency of FMD transmission is highly variable depending on biological characteristics of the specific FMDV strain involved as well as the prevailing husbandry conditions (Alexandersen *et al.*, 2001; Alexandersen *et al.*, 2002b; Alexandersen *et al.*, 2003a; Alexandersen *et al.*, 2003b; Bouma *et al.*, 2004; Hughes *et al.*, 2002b; Hughes *et al.*, 2002a; Quan *et al.*, 2004). Consequently, apart from the intrinsic biological virus attributes as well as the stochastic uncertainty inherent in the early phases of an outbreak, the

development of an outbreak into a potential epidemic and the course of this epidemic, is extremely complex and difficult to accurately predict unless local factors are taken into account.

Pig experiment 1 described here clearly showed that when exposed as a group (4+4) the inoculated donor pigs were considerably less infectious at day 1 pi as compared to days 2-4 pi. On day 1 pi only two out of 4 contact pigs developed viraemia and clinical signs at day 7 and 9, respectively while the two other contact pigs did not show a viraemia nor clinical signs. In contrast, on days 2-4 pi all the contact pigs developed clinical disease starting around 5 days after exposure. Although no clear difference in development of the infection in the contact pigs exposed to inoculated pigs at days 2-4 pi was observed, a slightly higher infectiousness of the day 3 pi donor pigs was indicated by a slightly faster development of viraemia and a higher viral load in nasal swabs immediately after exposure of the contacts.

The results of the group exposure described above should be comparable with the one-on-one experiments described in pig experiment 2 although the development of infection and clinical disease in the donor pigs was slightly slower in experiment 2. Interestingly, in this one-on-one setup, transmission only occurred at 4 days pi but not on days 1-3 pi. Also, at 4 days pi the infection was only transmitted to 2 out of 3 contact pigs with clinical disease on days 5 and 10 after exposure, respectively and with no viraemia or signs of clinical disease in the third contact pig (followed for 14 days). In the pigs exposed as a group (4+4) at 4 days pi in this experiment, 3 out of 4 pigs developed viraemia from days 1-3 and clinical disease from days 4-5 while the fourth pig developed a subclinical infection with no clinical signs of disease but with a low level viraemia from days 5-11 after exposure. Interestingly, at 4 days pi the pigs exposed individually received an almost identical FMDV RNA load in the nasal swabs as the pigs exposed as a group, however, while the individually housed pigs appeared to partly clear their virus initially, the pigs housed as a group were more likely to show a rapid increase in virus load and to develop rapid disease reinforcing the influence of stocking density on both transmission efficiency but also on the development of clinical disease as previously hypothesized (Quan *et al.*, 2004). Interestingly, although the pigs exposed at days 1-3 pi did not develop clinical disease nor viraemia, they appeared to have some, perhaps abortive, viral replication as suggested by viral RNA in nasal swabs. Consequently, these two experiments clearly show the difference in infectiousness over time and also the difference between pigs being exposed as a group or as one-on-one. Pig experiment 3 looked at whether the dose for inoculation of the donor pigs had importance for the subsequent development of disease in the contacts. However, the result indicated that the inoculation dose is mainly of importance in determining the consistency of infection in the donor pigs, low doses being less consistent, but that transmission of infection is still highly efficient provided that the donor pigs develop fulminant infection and that the contacts are exposed as a group.

Pig experiment 4 and 5 explored whether minor differences in combined exposure level and intensity could be measured in the current experimental set-up. The results of experiment 4 were not clear but did indicate that lowering the density in a group of pigs during the early stages of infection may slightly prolong the incubation period (from around 3 days to around 5 days) and may result in the development of subclinical infection in some exposed pigs. However, this effect could not be reproduced in experiment 5, most likely due to the very rapid development of infection in the donor pigs in this experiment resulting in efficient spread of disease to the contact animals before reduced pig density was achieved.

Pig experiment 6 showed like in previous experiments, i.e. pig experiment 1 reported here and other experiments with a number of FMDV isolates reported previously (Alexandersen *et al.*, 2001; Alexandersen *et al.*, 2003b; Alexandersen & Donaldson, 2002) that transmission and development of FMD is very rapid and efficient when pigs are exposed as a group, even when only exposed for a short time provided the excretion levels of the donor pigs are high.

The sheep experiments essentially showed that development of infection and clinical disease may be variable. The experiments described previously indicated that the incubation period in sheep exposed by close direct contact at high density and indoors may be as short as 1-2 days (Alexandersen *et al.*, 2002b) while we here show that exposure by indirect contact to pigs result in an incubation period of around 6 days while exposure to a low dose of airborne FMDV (around 300 TCID₅₀ over a 24 hour period) resulted in no clinical signs of disease except for a slight increase in temperature at 4-7 days after exposure in 1 out of 3 exposed sheep although 2 of the 3 sheep became subclinically infected and carried the virus in probang fluid samples for at least 35 days after exposure.

Our studies together with previously published results and findings in the field confirm our previous notion that a strong correlation exists between dose (i.e. infectiousness of source and intensity of contact) and length of incubation period, severity of clinical disease and efficiency of spread. Furthermore, low intensity transmission may increase the risk of subclinical or abortive infection

which easily may escape clinical examination. Clearly, local conditions are of immense importance in determining efficiency and speed of FMD transmission and can be an important determinant for the outcome of an initial outbreak and a considerable source of inaccuracy if not truthfully accounted for in mathematical models of epidemiological spread. In effect, such model based disease control can at present only meaningfully be attempted by including detailed veterinary expertise and local knowledge.

Conclusions

- The number of animals kept together in direct contact and the timing and length of contact influence the incubation period, the efficiency of spread and the risk of subclinical or abortive infection.

Recommendations

- More studies should be done using varying conditions and different strains of virus to provide a better understanding of the epidemiology of FMD. Such data are urgently required for development of realistic simulation models of disease spread.

Acknowledgements

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References

Alexandersen, S., Brotherhood, I. & Donaldson, A. I. (2002a). Natural aerosol transmission of foot-and-mouth disease virus to pigs: minimal infectious dose for strain O1 Lausanne. *Epidemiol Infect* **128**, 301-312.

Alexandersen, S. & Donaldson, A. I. (2002). Further studies to quantify the dose of natural aerosols of foot-and-mouth disease virus for pigs. *Epidemiol Infect* **128**, 313-323.

Alexandersen, S., Kitching, R. P., Mansley, L. M. & Donaldson, A. I. (2003a). Clinical and laboratory investigations of five outbreaks of foot-and-mouth disease during the 2001 epidemic in the United Kingdom. *Vet Rec* **152**, 489-496.

Alexandersen, S., Oleksiewicz, M. B. & Donaldson, A. I. (2001). The Early Pathogenesis of Foot-and-Mouth Disease in Pigs Infected by Contact: A Quantitative Time Course Study using TaqMan RT-PCR. *J Gen Virol* **82**, 747-755.

Alexandersen, S., Quan, M., Murphy, C., Knight, J. & Zhang, Z. (2003b). Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *J Comp Pathol* **129**, 268-282.

Alexandersen, S., Zhang, Z., Reid, S. M., Hutchings, G. H. & Donaldson, A. I. (2002b). Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. *J Gen Virol* **83**, 1915-1923.

Bouma, A., Dekker, A. & de Jong, M. C. (2004). No foot-and-mouth disease virus transmission between individually housed calves. *Vet Microbiol* **98**, 29-36.

Ferris, N. P. (1987). Development and use of Elisa in the control of foot-and-mouth disease. *IAEA-Proceedings* **348**, 65-77.

Ferris, N. P. & Dawson, M. (1988). Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet Microbiol* **16**, 201-209.

Hamblin, C., Armstrong, R. M. & Hedger, R. S. (1984). A rapid enzyme-linked immunosorbent assay for the detection of foot-and-mouth disease virus in epithelial tissues. *Vet Microbiol* **9**, 435-443.

Hamblin, C., Barnett, I. T. & Hedger, R. S. (1986). A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. *J Immunol Methods* **93**, 115-121.

Hughes, G. J., Mioulet, V., Haydon, D. T., Kitching, R. P., Donaldson, A. I. & Woolhouse, M. E. (2002a). Serial passage of foot-and-mouth disease virus in sheep reveals declining levels of viraemia over time. *J Gen Virol* **83**, 1907-1914.

Hughes, G. J., Mioulet, V., Kitching, R. P., Woolhouse, M. E., Alexandersen, S. & Donaldson, A. I. (2002b). Foot-and-mouth disease virus infection of sheep: implications for diagnosis and control. *Vet Rec* **150**, 724-727.

Quan, M., Murphy, C., Zhang, Z., & Alexandersen, S. Determinants of early foot-and-mouth disease virus dynamics in pigs. *J.Comp Pathol.* In Press. 2004.

Reid, S. M., Grierson, S. S., Ferris, N. P., Hutchings, G. H. & Alexandersen, S. (2003). Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J Virol Methods* **107**, 129-139.

Roeder, P. L. & Le Blanc Smith, P. M. (1987). Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. *Res Vet Sci* **43**, 225-232.

Snowdon, W. A. (1966). Growth of foot-and mouth disease virus in monolayer cultures of calf thyroid cells. *Nature* **210**, 1079-1080.

Natural aerosol transmission of Foot-and-Mouth disease in sheep

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Abstract

An important parameter in estimating and predicting foot-and-mouth disease virus (FMDV) airborne spread is the amount of virus released in aerosols from infected animals and the dose needed to infect susceptible species. Such studies have primarily been done using high doses and short-term exposure but here we report an initial experiment designed to assess longer-term exposure of sheep to a low concentration of an FMDV containing aerosol.

We examined the airborne transmissibility of the FMDV isolate between infected "donor" and "recipient" sheep under experimental conditions, over a two week period. The donor and recipient sheep were in same room but in different cubicles in order to avoid any direct or indirect physical contact between animals. Different air samplers were used and samples were collected in the room and in a 610 litre sampling cabinet where the donor sheep was temporarily placed. The infectivity in the collection fluid was assayed by inoculating BTY cells and viral RNA was quantified by real time RT-PCR.

Airborne transmission occurred in two out of three recipient sheep, which although not showing any significant clinical disease developed antibodies against FMDV in blood samples and had infectious virus in probang samples collected between days 23 to 35. Infectivity in air samples was detected 1 and 4 days after the inoculation. The main source of virus detected in the room at day 4 seems to be due to excretion from the infected recipient sheep. The amount of airborne virus recovered from the room and the cabinet at day 1 was $10^{3.48-4.69}$ and $10^{4.11-4.84}$ TCID₅₀ per 24h, respectively. Viral RNA was detected between day 1 and day 4. A peak of RNA copies excreted per 24h was observed in the cabinet at day 3 ($10^{6.24-7.06}$) and in the room at day 4 ($10^{8.83-9.76}$). In summary, even at low dose, airborne transmission occurred after longer-term exposure of sheep to an FMDV containing aerosol. The next step will be to determine the minimal infectious dose by reducing the exposure time of the recipient sheep.

Introduction

Foot-and-mouth disease virus (FMDV) can be spread by a variety of mechanisms including airborne spread (Alexandersen *et al.*, 2003c). An important parameter in estimating and predicting airborne spread is the amount of virus released in aerosols from infected animals and the dose needed to infect susceptible species by the airborne route (Alexandersen and Gloster, 2004). Such studies have primarily been done using short-term exposure but here we report an initial experiment designed to assess longer-term exposure of sheep to a low concentration of an FMDV containing aerosol.

Materials and Methods

Animal inoculation and transmission experiment

We examined the transmissibility of the FMDV O UKG 34/2001 isolate (Alexandersen and Donaldson, 2002b; Alexandersen *et al.*, 2003a) between infected donor and recipient sheep under experimental conditions. Three isolation rooms in a biosecure building containing two cubicles per room were used (Alexandersen, S., Brotherhood, I. and Donaldson, A. I., 2002a; Alexandersen and Donaldson, 2002b). These cubicles prevent any direct or indirect physical contact between animals. The experiment was performed by placing one inoculated sheep (donor sheep) in each cubicle of the three rooms and one recipient sheep in the other cubicle in each of the rooms. One of the donor sheep had its lamb (1-2 weeks of age) in the same cubicle. Donor sheep were inoculated intradermally in the coronary band on the left fore foot (Alexandersen *et al.*, 2003c) with 0.5ml of a suspension of the UKG 34/2001 isolate of FMDV virus from the 2001 UK epidemic passaged once in pigs. The inoculum contained $10^{6.2}$ TCID₅₀/ml when assayed in bovine thyroid cells (BTY) (Snowdon, 1966) and thus each inoculated sheep received $10^{5.9}$ TCID₅₀.

Donor sheep were observed daily for signs of FMD over a two-week period, except for one infected sheep, which was killed on day 7 post infection (p.i.). This sheep belonged to another experiment and the reason why it was killed is explained in a separate presentation (Ryan, E). The recipient sheep

were only handled on day 14, 18, 23, 25 and 35 when blood samples were collected for testing for the presence of antibodies to FMD virus by ELISA (Hamblin *et al.*, 1986). Oesophageal/pharyngeal (probang) samples were also collected at day 23, 25, 28 and 35 to test for the presence of virus. We also examined the virus excretion of lambs infected by direct contact in a separate isolation room. This experiment was performed by infecting three ewes as above and having their 1-2 weeks old lambs in direct contact in the same box. The experimental detail of this experiment is described in a separate presentation (Ryan, E).

Air sampling methods

On days 0 (before inoculation), 1, 2, 3, 4 and 7 p.i. air samples were collected in one of the rooms containing one donor and one recipient sheep. The following air sampling equipment was used: Porton impinger (May and Harper, 1957), 3 stage liquid impinger (May, 1966), Cyclone sampler (Errington and Powell, 1969) as described previously (Alexandersen, S., Brotherhood, I. and Donaldson, A. I., 2002a; Alexandersen and Donaldson, 2002b; Alexandersen *et al.*, 2002c; Alexandersen *et al.*, 2003b) and a newly developed "AB" sampler (patent pending). Air samples were also taken with a Porton sampler connected in series after the AB sampler.

One donor sheep was also put temporarily in a 610 litre sampling cabinet on days 1, 2, 3 and 4 for air sampling (Alexandersen *et al.*, 2002c, Donaldson and Ferris, 1980); the May, Porton and AB sampler were used. Air sampling from lambs were also taken in the cabinet at day 1, 2, 3 and 4.

Air sample assays

The infectivity in the collection fluid (except for the AB sampler) was assayed by inoculating BTY cells (Alexandersen, S., Brotherhood, I. and Donaldson, A. I., 2002a; Snowdon, 1966). Viral RNA in all air samples was quantified by real time RT-PCR as described previously (Alexandersen *et al.*, 2002c; Alexandersen *et al.*, 2003b, Reid *et al.*, 2001; Reid *et al.*, 2002; Reid *et al.*, 2004). The results were expressed as the quantity of virus released in 24h (log TCID₅₀/24h). These values take into account the sample volume, the flow rate, the sampling time and the animal mean breathing volume (method A). The conventional way of calculating airborne excretion of FMD virus was also included for comparison to previous papers. This calculation included the sample volume and the sampling time (method B).

Results

Infection

All donor sheep presented typical clinical signs of FMD in sheep (lesions and fever) within 1-2 days after inoculation. At that time, viral RNA was also detected in serum and nasal swabs. Airborne transmission occurred in two out of three recipient sheep which although not showing any significant clinical disease developed antibodies against FMD virus in blood samples collected at days 14-35 p.i.. The same two sheep were also positive for virus by testing of probang samples taken on days 23-35 p.i.. Only one of the virus-positive recipient sheep had fever 4 to 7 days after the inoculation of the donor sheep.

Airborne virus recovery

The amount of airborne virus recovered from the room and the cabinet at day 1 was 10^{3.48-4.69} and 10^{4.11-4.84} TCID₅₀ per 24h, respectively (Table 1). In order to make the titres comparable between the room, which was ventilated (ten air changes per hour), and the cabinet, which was not ventilated, the quantity of virus recovered in room samples were adjusted upwards with 1 log₁₀ unit (10-fold). Day 4 data from the cabinet samples suggest that the main source of virus detected in the room likely was due to excretion from the recipient sheep (Table 1). Viral RNA was detected between day 1 and day 4. A peak of RNA copies excreted per 24h was observed in the room at day 4 (10^{8.83-9.76}) and in the cabinet at day 3 (10^{6.24-7.06}). The concentration of FMD virus aerosol in the room at the peak were approximately 0.02 TCID₅₀ per litre of air as estimated from the Cyclone sample which means that the recipient sheep were exposed to an accumulated dose by the airborne route of around 300 TCID₅₀ in a 24 hour period. As mentioned, this dose resulted in 2 out of 3 recipient sheep becoming (subclinically) infected.

Table 1- Estimated release of airborne virus in 24h (method A/method B)^a

Day p.i.	Infectivity (log TCID ₅₀)		Genome (log RNA copies) ^b	
	Room ^{c,d}	Cabinet ^{e,f}	Room ^d	Cabinet
1	3.48 / 4.69	4.11 / 4.84	7.11 / 7.85	5.69 / 6.41
2	Not detected	3.27 / 3.99	8.31 / 9.56	5.68 / 6.47
3	Not detected	Not detected	7.85 / 8.53	6.24 / 7.06
4	3.48 / 4.69	Not detected	8.83 / 9.76	5.57 / 6.08
7	Not detected	Not detected	Not detected	Not detected

^a Method A: calculation that take into account the sample volume, the flow rate, the sampling time and the animal mean breathing volume; Method B: calculation that take into account the sample volume and the sampling time.

^b Mean of all positive samples

^c Cyclone sampler

^d 1 log₁₀ has been added to compensate for the ventilation

^e May sampler

^f No AB samples were taken in the cabinet

Virus infectivity was also observed at day 2 in air samples taken from lambs in the cabinet (table 2). The amount of airborne virus recovered per lamb was 10^{3.33-4.51} TCID₅₀ per 24h. Viral RNA was detected between day 1 and day 4. A peak of RNA copies excreted per 24h per lamb was observed at day 4 (10^{6.78-7.49}).

Table 2- Estimated release of airborne virus per lamb in 24h (method A/method B)^a

Day p.i.	Infectivity (log TCID ₅₀) ^b	Genome (log RNA copies) ^c
1	Not detected	5.49 / 6.14
2	3.33 / 4.51	6.06 / 7.32
3	Not detected	6.77 / 7.16
4	Not detected	6.78 / 7.49

^a Method A: calculation that take into account the sample volume, the flow rate, the sampling time and the animal mean breathing volume; Method B: calculation that take into account the sample volume and the sampling time.

^b May sampler

^c Mean of all positive samples

The mean ratio between genome and infectivity for samples that were positive with both techniques was 300 +/- 30. This value is comparable to the ratio found in e.g. serum samples during the viraemic phase (Alexandersen *et al.*, 2003b).

In contrast to what was observed for infectivity, RNA copies were detected with all air samplers (Tables 1 and 2). The sampler that detected more RNA copies was the Cyclone (10^{8.85}), followed by the AB and the Porton samplers in series (10^{8.55}), the AB sampler (10^{8.17}), the Porton (10^{6.93}) and finally the May sampler (10^{4.83}).

Discussion

In the present study, FMD transmission by longer-term exposure of sheep to a low concentration of an FMDV containing natural aerosol was examined. Two out of three recipient sheep developed subclinical disease. The concentration of FMD virus aerosol in the room at the peak was approximately 0.02 TCID₅₀ per litre of air as estimated from the Cyclone sample. Assuming that an adult sheep inhales about 15 l/min (Alexandersen *et al.*, 2002c, Donaldson *et al.*, 2001) the accumulated dose received by the recipient sheep was around 300TCID₅₀ in a 24h period. In a previous experiment in which sheep were exposed for 2 h to airborne virus from pigs, almost all (3 out of 4) of the airborne infected sheep developed FMD clinical signs. But in this study the FMD virus concentration was much higher (about 1.5 TCID₅₀ per litre of air) and animals received an estimated accumulated dose of 4000TCID₅₀ (Aggarwal *et al.*, 2002, Donaldson *et al.*, 2001).

A previous experiment has shown that a dose of 10TCID₅₀ was enough to infect sheep (Gibson and Donaldson, 1986). In the present study, the recipient sheep are likely to have inhaled an infectious dose within approximately 1 hour. So the next step will be to determine the minimal infectious dose by reducing the exposure time.

The recipient sheep excreted airborne virus 4 days after infection of the inoculated sheep. In a previous experiment where sheep were infected by direct contact, excretion of airborne virus was maximal after 48h. The airborne accumulated dose received by the recipient sheep in that experiment was higher than in the present study and the sheep were continuously and closely confined so transmission to the in-contact could have occurred by several different routes explaining the speed of the transmission. This was confirmed with the lamb experiment in which virus excretion was observed 48h after infection by direct contact.

The quantity of virus excreted by the airborne infected sheep was the same as the needle infected sheep. Despite this same virus quantity, the airborne infected sheep did not develop any clinical symptoms except fever in contrast to what it was observed for the needle infected sheep. Another important finding was the quantity of airborne virus excreted by lambs which was as high as virus excreted by adult sheep. This is potentially of major epidemiological significance if subclinically infected sheep and lambs truly excrete as much virus as clinically affected sheep.

In the present study different air samplers were compared. Virus infectivity was detected with the Cyclone and May sampler. These samplers collected larger volume of air and thus may have greater sensitivity than the other sampler. In contrast, all samplers allowed genome detection and the best air samplers were the Cyclone and the AB sampler. A Porton sampler added in series after the AB sampler increased the quantity of viral RNA recovered. Generally, genome detection is more sensitive than infectivity detection and the ratio values between these two techniques are similar to what it was observed previously in serum samples during the viraemic phase (Alexandersen *et al.*, 2003b). This observation may be explained by the highest sensitivity of the PCR technique. The detection of genome without infectivity may be due to the violent impact of air into the impinger fluid that may destroy virus infectivity. It may also be due to the variability of the BTY cells infection assay that depends on the sensitivity of cells to virus infection. Also the manners in which the air samplers were operated and the assay system used were probably close to their limits of detection.

Conclusions

- Even at low concentration, airborne transmission occurred after longer-term exposure of sheep to an FMDV containing aerosol.
- Airborne virus excreted by lambs was as high as excreted by adult sheep.
- These data will increase the accuracy of predictive models that simulate airborne spread.

Recommendations

- Detailed studies of airborne transmission of FMD under controlled conditions should be intensified in order to improve the accuracy of simulation models

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References

- Aggarwal, N., Zhang, Z., Cox, S., Statham, R., Alexandersen, S., Kitching, R. P. & Barnett, P. V.** 2002. Experimental studies with foot-and-mouth disease virus, strain O, responsible for the 2001 epidemic in the United Kingdom. *Vaccine* 20, :2508-2515.
- Alexandersen, S., Brotherhood, I. & Donaldson, A. I.** 2002a. Natural aerosol transmission of foot-and-mouth disease virus to pigs: minimal infectious dose for strain O1 Lausanne. *Epidemiol Infect* 128, 301-12.
- Alexandersen, S. & Donaldson, A. I.** 2002b. Further studies to quantify the dose of natural aerosols of foot-and-mouth disease virus for pigs. *Epidemiol. Infect.* 128: 313-323.
- Alexandersen, S., Zhang, Z., Reid, S. M., Hutchings, G. H. & Donaldson, A. I.** 2002c. Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. *J. Gen. Virol.* 83: 1915-1923.
- Alexandersen, S., Kitching, R. P., Mansley, L. M. & Donaldson, A. I.** 2003a. Clinical and laboratory investigations of five outbreaks of foot-and-mouth disease during the 2001 epidemic in the United Kingdom. *Vet. Rec.* 152: 489-496.
- Alexandersen, S., Quan, M., Murphy, C., Knight, J. & Zhang, Z.** 2003b. Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *J. Comp. Pathol.* 129: 268-282.
- Alexandersen, S., Zhang, Z., Donaldson, A. I. & Garland, A. J.** 2003c. The pathogenesis and diagnosis of foot-and-mouth disease. *J. Comp. Pathol.* 129: 1-36.
- Alexandersen, S. & Gloster J.** 2004. Airborne transmission of Foot-and-Mouth Disease. *Aerosol Society*. In press.
- Donaldson, A. I. & Ferris, N. P.** 1980. Sites of release of airborne foot-and-mouth disease virus from infected pigs. *Res. Vet. Sci.* 29: 315-319.
- Donaldson, A. I., Alexandersen, S., Sorensen, J. H. & Mikkelsen, T.** 2001. Relative risks of the uncontrollable (airborne) spread of FMD by different species. *Vet. Rec.* 148: 602-604.

- Errington, F. P. & Powell, E. O.** 1969. A cyclone separator for aerosol sampling in the field. *J. Hyg. (Lond.)* 67: 387-399.
- Gibson, C. F. & Donaldson, A. I.** 1986. Exposure of sheep to natural aerosols of foot-and-mouth disease virus. *Res. Vet. Sci.* 41: 45-49.
- Hamblin, C., Barnett, I. T. & Crowther, J. R.** 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. II. Application. *J. Immunol. Methods* 93: 123-129.
- May, K. R. & Harper, G. J.** 1957. The efficiency of various liquid impinger samplers in bacterial aerosols. *Br. J. Ind. Med.* 14: 287-297.
- May, K. R.** 1966. Multistage liquid impinger. *Bacteriol. Rev.* 30: 559-570.
- Reid, S. M., Ferris, N. P., Hutchings, G. H., Zhang, Z., Belsham, G. J. & Alexandersen, S.** 2001. Diagnosis of foot-and-mouth disease by real-time fluorogenic PCR assay. *Vet. Rec.* 149: 621-623.
- Reid, S. M., Ferris, N. P., Hutchings, G. H., Zhang, Z., Belsham, G. J. & Alexandersen, S.** 2002. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *J. Virol. Methods* 105: 67-80.
- Reid, S. M., Ferris, N. P., Hutchings, G. H., King, D. P. & Alexandersen, S.** 2004. Evaluation of real-time reverse transcription polymerase chain reaction assays for the detection of swine vesicular disease virus. *J. Virol. Methods* 116: 169-176.
- Snowdon, W. A.** 1966. Growth of foot-and mouth disease virus in monolayer cultures of calf thyroid cells. *Nature* 210: 1079-1080.

Moving towards a better understanding of airborne transmission of FMD

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Abstract

In the event of an outbreak of FMD it is essential for those responsible for controlling and eradicating the disease to quickly assess how the initial animals became infected and its potential for further spread. Once this has been determined the appropriate control measures can be introduced. It has been established that airborne transmission of FMD virus is one of the mechanisms by which disease is transmitted and consequently it is important to develop a capability by which this can be rapidly assessed. This often involves the integration of epidemiological data collected in the field, laboratory investigations and the use of a transport and dispersion model.

Before an accurate prediction of airborne spread can be made it is of vital importance to understand each part of the disease chain together with its errors and uncertainties. How the model then represents the data is also key to obtaining a good estimate of disease spread. This paper identifies the key components in the models and identifies current errors and uncertainties.

Introduction

Laboratory and epidemiological studies have identified airborne transmission of FMD virus as one of the mechanisms by which disease is transmitted (reviewed by Alexandersen *et al.* 2003). A number of field studies have indicated that airborne virus was the most likely mechanism for disease transmission over many tens of km over land and several occasions involving a hundred or more km over the sea (Hugh-Jones and Wright 1970; Sellers and Foreman 1973; Donaldson *et al.* 1982; Sørensen *et al.* 2000; Gloster *et al.* 2003; Mikkelsen *et al.* 2003).

In principle the airborne disease cycle is easy to understand (emission, transport and infection). However, in practice the inter-relation is far more complex and requires the use of a computer model to assess the potential for disease spread. For example, virus emission rates depend critically on the species involved, the stage of disease and the meteorological conditions. The meteorological conditions may vary significantly throughout one day, let alone over a typical period of virus emission (often a number of days). Definition of areas of risk is often made more difficult in areas of complex topography.

The quality of an assessment of airborne transmission will only be as good as the input data and the assumptions and formulation of the model. Inaccurate model output may be misleading or wrong and any operational decisions taken on the strength of it flawed. Consequently it is important to have a detailed understanding of the quality and representivity model input data and how the model handles this information.

Materials and Methods

There are a number of atmospheric transport and dispersion models in use at centres around the world and potentially there are numerous combinations of inputs, outputs and degree of integration into decision support models (Sørensen 1998; Sanson, Morris and Stern 1999; Sørensen *et al.* 2000; Sørensen *et al.* 2001; Morris *et al.* 2002; Gloster *et al.* 2003). However, in general they all conform to a similar generic structure. This structure is used as the basis for assessing our current understanding and ability to predict airborne transmission of FMD.

Results - Disease transmission and model representation

Atmospheric transport and dispersion models are supplied with two major inputs; epidemiological to determine source terms and virus characteristics and meteorological data for virus transport. The output is typically presented in terms of virus concentration as a function of distance from the source. If a Graphical Interface System (GIS) is available other relevant data e.g. livestock distribution can also be presented simultaneously.

Epidemiological data

Epidemiological data is obtained from two sources; firstly, from the infected premises and secondly from the laboratory. The local State Veterinarian is responsible, amongst other duties, for establishing the precise status of disease on the premises and the development of disease from initial introduction. This involves a close examination of all of livestock which may have been moved off the premises and an accurate dating of lesions on infected animals. The local State Veterinarian may be assisted in these crucial examinations by an Epidemiological Team, including field epidemiologists and FMD experts from such organisations as the Institute for Animal Health (IAH). In the UK infected material is passed from the infected premises to the IAH laboratory for confirmation of the presence of disease together with its type/strain. Provided that an accurate pattern of disease on the farm can be established and that the aerosol characteristics can be quantified (Alexandersen and Donaldson 2002; Alexandersen *et al.* 2003; Alexandersen *et al.* 2002) an estimate can then be made on the amount of virus released into the atmosphere. Any errors introduced at this stage can have a significant impact on the output from a prediction model. The accuracy of the data and predictions can be improved by deployment of an experienced Epidemiological Team and be further enhanced by extended laboratory testing of serum samples for virus and for antibodies to substantiate the introduction, spread and time course of the infection on the infected premises (Alexandersen *et al.* 2003b).

Major sources of errors/accuracies

- Accurate lesion dating is often very difficult to perform, especially under field conditions. If the oldest lesion is missed or if the history of disease on the premises is not accurately assessed then the model output is likely to be in error, especially under rapidly changing meteorological conditions.
- Establishing virus characteristics in the laboratory is hard to characterise and involves making measurements at the limits of detection capability.
- Daily virus totals are typically calculated from short term measurements. How representative are these of a full 24 hour period?
- Detailed definition of source terms for example area and height of virus release, particle size distributions etc.

Meteorological data

Meteorological data is available from observing stations throughout the UK and abroad. There is a trend for the reduction of traditional observing stations and an increase in the introduction of a limited number of automated observing stations. For any given outbreak the location of an observing site may be many tens of km away and even then possibly in different terrain. The representivity of any data must be taken into consideration before it can be used in a transport and dispersion model. The presence of features such as mountains, hills, valleys and proximity of urban development and the coast can seriously influence the representivity of single site observations.

Whilst some transport and dispersion models require basic weather observations as input others, such as the longer distance models require data derived from Numerical Weather Prediction models (NWP). These take observed data and then calculate the conditions on a 3-D grid with points being representative of an area. Clearly the greater the distance between grid points the less accurate it is likely to be for a specific location, especially in areas of complex topography. Typically grid points may be tens of km apart, although with increased computing power this is being gradually reduced. It is possible that within the next ten years the resolution may be reduced to around 1 – 5km.

Some transport and dispersion models have a pre-processing stage which calculates a 3-D flow field in the light of topography. The wind speed and direction, and possibly some of the other inter-related atmospheric parameters, is then modified to allow for the effects of hills and valleys. Some models include the provision of using a number of schemes to reflect different flows under stable, neutral and unstable atmospheric conditions. Whilst these can be useful, few if any take into account locally generated drainage currents which may occur during periods of stable conditions (the highest risk of spread of virus in high concentrations). For example in a valley with steep sides the topographical routines may indicate that air is likely to flow up the valley, rather than flow over the hill to the side. However, local winds may in reality introduce an overall down valley flow, thus reversing the direction of the travel of the plume and consequent area of risk to livestock. In reality the situation will depend upon factors such as the orientation of the valley, its latitude and time of year. At this time there are no models which confidently predict these flows in all atmospheric conditions. It will not be until

model scales are reduced to around a few km, an improved understanding of local flows is established and improved physics is introduced into the meteorological models that this will be overcome.

Major sources of errors/accuracies

- Representivity of either the observed or model estimated data. This is especially important when the atmosphere is stable and the winds are light.
- The influence of topography, coasts, cities and local meteorological conditions.
- Meteorological forecast errors if the model is used to predict the future airborne emission pattern from infected premises.

Transport and dispersion models

There is large variety and considerable complexity in the operation of transport and dispersion models. However for simplicity we can break down the description into six aspects (the distance/range they work over, how they represent source emissions, how they represent turbulence, the surface properties which are treated, the loss processes which are calculated and finally their output).

Traditionally the models can be sub-divided into two types (long and short range); however there are an increasing number of models capable of representing a range of scales. The longer range models operate up to thousands of km and have often been developed for other purposes such as to provide guidance for an emergency response to nuclear accidents. These have been adapted to provide guidance on the long distance transport of FMD virus. They require gridded 3D met data, derived from NWP models and operate on either a lagrangian or eulerian reference frame. The former follows individual trajectories as they progress, the later work on a "box" principle and transport particles from one model grid box to another.

Short range models are valid for distances up to about 20km, although their accuracy at its outer limits will depend on the prevailing atmospheric conditions. These models are typically built around a Gaussian plume dispersion equation. They take a meteorological observation and assume that this remains constant for the length of the calculation, often one hour. In strong wind conditions 10msec^{-1} the outer limits are reached in just over half an hour. Under light wind conditions of 1 or 2msec^{-1} the particles will have only travelled 3.6 to 7.2km in one hour. In some instances meteorological data is only available at three hourly or greater intervals. Output from models using this information is likely to be considerably less accurate than those using hourly data.

Models differ on how they handle the virus source. Some treat the release as an instantaneous release, others as a continuous release over a defined period (typically 24 hours). In sophisticated models there is a capability to release particles as a function of time of day. The spatial release of particles is another variable with particles released from a point, along a line, over an area or into a volume. The height of release can also be input into some models. Some models do not calculate dispersion when calm winds are recorded. More sophisticated models may spread the particles in all directions for a limited distance. This is an important feature as it is under these conditions that high aerosol concentrations are maintained and as a consequence the risk of infection to animals near the source is particularly high. However, dispersion in such conditions is much less predictable and significant errors are likely.

Atmospheric transport and dispersion models represent the turbulent characteristics of the atmosphere. In general these can be divided into two groups; those which only take into consideration the conditions in the atmospheric boundary layer and those which take into account the overall conditions in the atmosphere. Some models derive these conditions internally, whilst others accept them from pre-processor packages including NWP.

Additional surface properties can also be included in the models and these can influence the representation of turbulence and airflow. For example the surface type and roughness can be included. The airflow over a city can increase the turbulence and as a result reduce the down-wind concentration of particles. Some models can include topography at this stage, but care has to be taken to ensure that this is not included more than once in the model.

FMD particles once airborne are subject to loss, either physical or biological. Larger particles ($10\mu\text{m}$ or greater) will, in the absence of turbulence, be deposited on to the ground by gravity within minutes. Smaller particle may remain airborne for many hours. Some models take this into account either by

assuming a standard or known size distribution. The formation of particles into water droplets or their capture by existing droplets increase their chances of removal from the atmosphere by wet deposition, although this capture process is likely to take some hours to reduce concentrations significantly. Biologically the virus may become inactivated if the RH of the air falls below 60% or the pH of the virus-containing aerosol particles water vapour becomes acidic or alkaline.

The output from the models can also vary considerably. Point or gridded data may be provided, with the size of the grid being selected by the modeller. For long distance models this may be in the order of 10km boxes and for shorter range be typically of the order of 1km or even less. The values output may be expressed as a maximum concentration for a period as short as one hour up to the entire emission period. Similarly mean concentrations over a range of times can be output. Dry and wet deposition maps and concentration fluctuations can be produced. In the future it may be possible to produce outputs of probability of infection for specific locations and numbers and type of animal. Whilst this is possible in theory there are too many uncertainties in the overall process to make this available at this time. A more realistic target would be to estimate animal virus challenge time (the time livestock at a given location are exposed to virus above a threshold concentration).

Major sources of errors/accuracies

- Accurate reflection of conditions experienced in the field e.g. virus release, its behaviour once airborne, its interaction with its surrounds.
- Correct choice of model.
- Model treatment of virus released during periods of calm winds.
- Understanding the link between airborne concentration of virus and infection.
- Production of appropriate output which mimics how animals become infected (time average conc. or instantaneous dosages).

Discussion

From the above it is clear that there are many variables in the disease cycle and this is compounded by the addition of others introduced at the modelling stage. Model output can look extremely convincing, especially if it is presented in GIS format. However decisions based on this alone may be severely flawed. For example if the model indicates a region, let us say in a sector 30° to 90° and out to a range of 5km where a pre-determined threshold virus concentration has been calculated it would be easy to identify those premises which were at risk and as a result introduce the appropriate control measures. However if the virus emission was underestimated by a factor of ten (possible) then the area at risk, assuming that the rest of the modelling process was without error (very unrealistic) could extend out to tens of km. The resultant containment strategy may be entirely different.

In view of the above it is essential to minimise or at least to quantify the individual errors through a detailed research and education campaign combined with validation against past FMD outbreaks. This is the overall rationale for a three year programme of work sponsored by Defra. In addition a thorough practical knowledge must be developed of the internal workings and sensitivities of the model. It is highly recommended that these activities should be conducted in the absence of an FMD outbreak. The resultant improved prediction models should be tested against a wide range of FMD outbreaks. It has been shown in the literature (see earlier) that individual models perform well in given particular conditions. However it is unwise, at this time, to assume that they will perform equally as well under all conditions.

Conclusions

- Airborne transmission of FMD is complex.
- FMD airborne prediction models can currently provide useful advice, but their input, internal formulation and output must be handled with considerable care if accurate advice concerning disease spread is required.
- There is considerable room for improvement before a definitive FMD transmission model is available. This may take 10 or more years to achieve.

Recommendations

- Work should be continued on determining each of the areas of uncertainty identified in this report and their inclusion in decision making tools.
- When model output is supplied to those responsible for disease control and eradication it should be accompanied by a verbal or written briefing concerning the confidence of the output.

- Meteorologists should include local flows in their transport and dispersion models at the earliest time possible. It is recognised that this requirement may be some years away.
- Further interdisciplinary collaboration between veterinarians, virologists, meteorologists, epidemiologists is required to fully resolve the contribution played in any outbreak of airborne transmission of FMD.

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References

Alexandersen, S. and Donaldson, A.I. 2002. Further studies to quantify the dose of natural aerosols of foot-and-mouth disease virus for pigs. *Epidem. Infect.*; 128: 313-323.

Alexandersen, S., Zhang, Z., Reid, S.M., Hutchings, G.H. & Donaldson, A.I. 2002. Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. *J. Gen. Virol.* 83:1915-1923.

Alexandersen, S., Zhang, Z., Donaldson, A.I. & Garland A.J.M. 2003. The pathogenesis and diagnosis of Foot-and-Mouth disease. *J. Comp. Path.*, 129: 1-36.

Alexandersen, S., Kitching, R.P., Mansley, L.M. & Donaldson, A.I. 2003b. Clinical and laboratory investigations of five outbreaks of foot-and-mouth disease during the 2001 epidemic in the United Kingdom. *Vet. Rec.* 152:489-496.

Donaldson, A.I., Gloster, J., Harvey, L.D. & Deans, D. H. 1982. Use of prediction models to forecast and analyse airborne spread during the foot-and-mouth disease outbreaks in Brittany, Jersey and the Isle of Wight in 1981. *Vet. Rec.*, 110: 53-57.

Gloster, J., Champion, H.J., Sørensen, J.H., Mikkelsen, T., Ryall, D.B., Astrup, P., Alexandersen, S. & Donaldson, A.I. 2003. Airborne transmission of foot-and-mouth disease virus from Burnside Farm, Heddon-on-the-Wall, Northumberland during the 2001 UK epidemic. *Vet. Rec.*, 152: 525-533.

Hugh-Jones, M.E. & Wright, P.B. 1970. Studies on the 1967-8 foot-and-mouth disease epidemic, the relation of weather to spread of disease. *J. Hyg. (Camb.)*, 68: 253-271.

Mikkelsen, T., Alexandersen, S., Astrup, P., Donaldson, A.I., Dunkerley, F.N., Gloster, J., Champion, H.J., Sørensen, J. & Thykier-Nielsen, S. 2003. Investigation of airborne foot-and-mouth disease virus transmission during low-wind conditions in the early phase of the UK 2001. *Epidem. Atmos. Chem. and Phys Discuss.*, 3: 677-703.

Morris, R.S., Sanson, R.S., Stern, M.W., Stevenson, M. & Wilesmith, J.W. 2002. Decision-support tools for foot-and-mouth disease control. *Rev. sci. tech. Off. Int. Epiz.* 21(3): 557-567.

Sanson, R.L., Morris, R.S. & Stern, M.W. 1999. EpiMAN-FMD: a decision support system for managing epidemics of vesicular disease. *Review Scientifique et Technique de l'O.I.E.* 18(3): 593-605.

Sellers, R.F. & Foreman, A.J. 1973. The Hampshire outbreak of Foot-and-Mouth disease (1967). *J. Hyg. (Camb.)*, 7: 15-34.

Sørensen, J.H. 1998. Sensitivity of the DERMA Long-Range Dispersion Model to Meteorological Input and Diffusion Parameters. *Atmos. Environ.*, 32: 4195-4206.

Sørensen, J.H., Mackay, D.K.J., Jensen, C.Ø. & Donaldson, A. I. 2000. An integrated model to predict the atmospheric spread of foot-and-mouth disease virus. *Epidemiol. Infect.*, 124: 577-590.

Sørensen, J.H., Jensen, C.Ø., Mikkelsen, T., Mackay D.K.J. & Donaldson A.I. 2001. Modelling the atmospheric spread of foot-and-mouth disease virus for emergency preparedness. *Phys. Chem. Earth* 26: 93-97.

Quantification of experimental transmission of FMDV O Taiwan in pigs

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Introduction

To quantify reduction of virus transmission, transmission experiments in which an SIR model is used to estimate reproduction ratio R with a final size method, have shown to be very suitable [1,2]. R is the average number of secondary cases per infectious individual during its entire infectious period [3]. This implies that an infection will fade out in a population when $R < 1$, but can spread on a large scale when $R > 1$. Besides R also the transmission rate β is an important parameter, because it can be used in models to optimise control strategies. A GLM method [4] in which information on the time span of the infectious process is used, allows for an accurate calculation of β . Moreover, point estimates of R can be made, using the results of β and the infectious period T [5]. In this study, we quantified the reproduction ratio R and transmission rate β of FMDV after several vaccination strategies.

Materials and Methods

We performed ten transmission experiments with groups of ten pigs. In each group, five randomly selected pigs were challenged by intra-dermal inoculation in the bulb of the heel of the left hind-foot with 0.1 ml of challenge virus containing 10^5 TCID₅₀/ml FMDV O Taiwan. The remaining 5 pigs of a group were contact exposed.

Four of the groups were non-vaccinated, two groups were vaccinated at 7 days before inoculation (-7dpi), one group was vaccinated with a 4-fold vaccine dose (4-FD) at -7dpi, two groups were vaccinated at -14dpi and one group was vaccinated with an (intra-type) heterologous vaccine at -14dpi. For vaccination we used a commercially available double-oil-in-water emulsion [DOE] containing 3 μ g of FMDV O Taiwan or O Manisa (heterologous vaccine) 146S antigen per 2 ml dose. Contact infections were determined by inspection of clinical signs, by virus isolation of oropharyngeal fluid (OPF) and plasma samples, (boost of) neutralising antibody titre (VN-titre) and response in an NSP-ELISA. OPF was collected daily after challenge, heparinised blood samples and serum samples were collected at days -14, -11, -7, -4, 0, 3, 7, 10 and 14 dpi.

OPF samples and plasma samples were assayed for the presence of virus by plaque titration on monolayers of secondary pig-kidney cells [6]. VN-titres to FMDV O Taiwan and VN-titres to FMDV O Manisa in serum samples were measured using a neutralisation assay [6]. Serum samples were also tested in a commercially available ELISA (UBI[®] FMDV NS EIA Swine) for detection of antibodies against non-structural proteins of FMDV (NSP-ELISA). Contact pigs were considered infected if FMDV was isolated in OPF or plasma samples or a statistically significant rise in VN-titre was detected or a response in the NSP-ELISA was detected.

In the final size method, the estimation of R was based on the final size of the outbreak observed in the experiments. For calculation of R , a stochastic susceptible-infectious-removed (SIR) model was used [7] in which R was calculated using a maximum likelihood estimator [1,8]. For the calculation of R , all inoculated pigs were considered infectious. To determine whether transmission differed significantly between groups, we tested the null hypothesis that there was no difference in transmission between the non-vaccinated and the vaccinated group.

In the GLM method, the information of the VI of OPF was used. Data for similar experiments were pooled and the results per day were entered in a GLM (Generalized Linear Model) analysis to estimate the transmission rate using the methodology of Velthuis et al. [4]. We assumed that animals were infectious only at the days that the VI was positive. If euthanasia was applied, the calculation was adjusted by reducing the total group size with one. In cases where the first contact animal became VI-positive simultaneously with the inoculated animals, we assumed that the inoculated group had become infectious half a day earlier.

To quantify the infectious period T we used a survival analyses for censored data, using a two-parameter Weibull distribution. Subsequently, R was calculated with the results of β and T as in Klinkenberg et al. [5]. For all three parameters (β , T and R) differences between the vaccinated and non-vaccinated groups were analysed with a T-test.

Results

In the four non-vaccinated groups, all contact pigs became infected. In the -7dpi vaccinated groups, also all contact pigs became infected. In the group that was vaccinated with a 4-fold vaccine dose at -7dpi, 3 of the 5 contact pigs became infected. In the -14dpi vaccinated groups, both homologous

and heterologous vaccinated, none of the contact pigs became infected. The results of the virus isolation of the OPF samples are shown in Table 1. In the -14dpi homologous and heterologous vaccinated groups, no pigs were determined that shed virus. The results of the clinical signs, virus isolation of plasma samples and antibody detection tests are not shown, but did not detect other contact infected pigs than the virus isolation test of OPF.

The results of the Maximum Likelihood Estimation of R with the Final Size Method are shown in Table 2. When testing $H_0: R_{vac} \geq 1$, p significantly < 0.01 for the -14dpi homologous vaccinated group. When testing $H_0: R_{non-vac} \leq R_{vac}$, p significantly < 0.01 for the -14dpi homologous vaccinated, the -14dpi heterologous vaccinated and the -7dpi vaccinated 4-fold vaccine dose groups.

The results of the estimation of the transmission rate β , the estimation of the infectious period T and the estimation of the reproduction ratio R with the GLM method are shown in Table 2. For all vaccinated groups, all three calculated parameters (β , T and R) differed significantly from the non-vaccinated group ($p < 0.01$).

Conclusions

We showed that vaccination significantly reduced transmission of FMDV. Using the final size method, we showed that homologous and heterologous vaccination at -14dpi and vaccination -7dpi with a 4-fold vaccine dose reduced R significantly, as compared to the non-vaccinated group. With the GLM method, we showed that vaccination significantly reduced β , T and R , also after vaccination at -7 dpi, even though R not < 1 . The estimates we made can be of importance in models to optimise control strategies.

References

Bouma, A., De Smit, A.J., De Jong, M.C.M., De Kluijver, E.P. & Moormann, R.J.M. 2000. Determination of the onset of the herd-immunity induced by the E2 sub-unit vaccine against classical swine fever virus. *Vaccine*, 18: 1374-1381.

De Jong, M.C.M. & Kimman, T.G. 1994. Experimental quantification of vaccine-induced reduction in virus transmission. *Vaccine*, 12: 761-766.

Diekmann, O., Heesterbeek, J.A.P. & Metz, J.A.J. 1990. On the definition of and computation of the basic reproduction ratio R_0 in models for infectious diseases in heterogeneous populations. *J. Math. Biol.*, 28: 365-382.

Velthuis, A.G.J., de jong, M.C.M., Kamp, E.M., Stockhofe & N., Verheijden, J.H.M. 2003. Design and analysis of an *Actinobacillus pleuropneumoniae* transmission experiment. *Prev. Vet. Med.*, 60(1): 53-68.

Klinkenberg, D., de Bree, J., Laevens, H. & de Jong, M.C.M. 2002. Within- and between-pen transmission of Classical Swine Fever Virus: a new method to estimate the basic reproduction ratio from transmission experiments. *Epidemiol and Infect*, 128(2): 293-299.

De Leeuw, P.W., Tiessink, J.W.A. & Frenkel, S. 1979. Vaccination of pigs with formaldehyde-inactivated aluminium hydroxide foot-and-mouth disease vaccines, potentiated with diethylaminoethyl-dextran (DEAE-D). *Zentralbl Veterinarmed B*, 26: 85-97.

Becker, N.G. 1989. Analysis of infectious data. London: Chapman and Hall.

Kroese, A.H. & de Jong, M.C.M. 2001. Design and analysis of transmission experiments. Society for veterinary epidemiology and preventive medicine; Proceedings Noordwijkerhout 28th-30th March., xxi-xxxvii.

Table 1: Results of the virus isolation of OPF samples as used in the GLM

	non-vaccinated								vac -7dpi				vac -7dpi, 4-FD	
	Exp. 1		Exp. 2		Exp. 3		Exp. 4		Exp. 1		Exp. 2		Exp. 1	
	I	C	I	C	I	C	I	C	I	C	I	C	I	C
0 dpi	0 ¹	0	0	0	0	0	0	0	0	0	0	0	0	0
1 dpi	5	3	5	4	1	0	2	0	4	2	0	0	2	0
2 dpi	5	5 ²	5	5 ²	5	5 ²	5	5 ²	5	5 ²	3	2	3 [#]	0
3 dpi											4 [#]	4	3 ^{##}	1
4 dpi											3 ^{*#}	4	2 ^{*##}	1
5 dpi											4	3 [*]	1 ^{###}	3
6 dpi											2 ^{##}	3 ^{#2}	0 ^{####}	1 ^{##}
7 dpi													0 ^{####}	0 ^{###}
8 dpi													0 ^{*###}	1 ^{*#}
9-14 dpi													0 ^{###}	0 ^{##3}
VI pos:	5	5	5	5	5	5	5	5	5	5	5	5	5	3

- ¹ = number of pigs found positive in virus isolation
- ² = end of input GLM (all contact pigs infected)
- ³ = end of input GLM (all infectious pigs stopped shedding virus)
- * = euthanasia pig, therefore number of pigs minus 1
- # = earlier infectious pig scores negative in VI

Table 2: Results Final Size Method and GLM Method

Groups	Final Size Method <i>R</i> (CI)	GLM Method		
		β (CI)	T (CI)	<i>R</i> (CI)
non-vac (4x)	∞ (2.1- ∞)	3.5 (2.1-5.9)	6.5 (5.7-7.3)	23 (14-39)
vac -7dpi (2x)	∞ (1.3- ∞)	1.6 (0.8-3.0)	5.3 (4.7-6.0)	8.4 (4.3-16)
vac -7dpi , 4-FD (1x)	1.2 (0.2-5.4)	0.4 (0.1-1.3)	2.3 (1.0-5.4)	0.95 (0.2-4.0)
vac -14dpi (2x)	0 (0-0.9)	*	0	0
vac -14dpi, het vac (1x)	0 (0-2.2)	*	0	0

*= none of the inoculated animals shed virus, therefore, the transmission rate cannot be quantified.

Comparison of transmission of FMDV in groups of vaccinated and non-vaccinated calves

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Abstract

To quantify the effect of a single vaccination on FMDV transmission in cattle, we performed two experiments with 26 calves each. In 12 groups of 4 calves each, two calves were inoculated intranasally with O/NET FMDV and two calves were contact exposed to the inoculated calves. And 2 groups of 2 calves were only vaccinated to serve as vaccine control group.

With the results of clinical inspection, virus isolation and RT-PCR in heparinised blood and mouth swab and sputum samples and the results from the NS ELISA, we determined whether contact-infections occurred. With the final size of the infection we estimated the maximum likelihood estimator MLE for the reproduction ratio R . We found that a single vaccination led to a $R_v < 1$. This was a significant reduction of virus transmission when compared to the R_c in non-vaccinated groups of calves.

Introduction

An outbreak of foot-and-mouth disease occurred in The Netherlands in 2001 (Bouma *et al.*, 2001). The capacity for pre-emptive culling and destruction appeared to be insufficient and therefore emergency vaccination was applied. Although in cattle it is known that vaccination prevents clinical signs of FMD, little is known about the effect on transmission (Donaldson *et al.*, 1989). Experiments with pigs showed that emergency vaccination reduces transmission of FMD virus (Salt *et al.*, 1998). Extrapolation of results from pigs to cattle may not be valid, because difference in susceptibility and clinical appearance are known (Kitching, 2002; Kitching and Alexandersen, 2002; Kitching and Hughes, 2002). Therefore the important question remains, whether single vaccination can reduce FMDV transmission sufficiently in cattle to stop an epidemic ($R < 1$).

The objective of the study was to quantify the effect of vaccination on the virus transmission within groups of calves.

Materials, methods and animals

For our experiments we used 12 groups of 4 calves each; of which 6 groups were vaccinated. In each group of 4 calves, 2 calves were intranasally inoculated (days post challenge = DPC 0) with 1500 CID_{50} (cow infectious dose 50%) of the first cattle passage of the FMD field isolate O/NET2001. Transmission of FMDV to contact exposed calves was observed by recording the clinical signs and by virus isolation and RT-PCR on OPF (oro pharyngeal fluid from mouth swabs) and plasma (from heparinised blood samples). Antibody response to the FMDV infection was detected by a virus neutralization assay on serum samples. At the end of the experiment (DPC 29-30-31), probang sputum samples and white blood cells were collected for virus isolation and RT-PCR.

Virus isolation on secondary ovine kidney cells was performed to measure virus titres in plasma, OPF and sputum samples (Terpstra *et al.*, 1990). The sera were tested in virus neutralisation assay and NS-ELISA (Dekker and Terpstra, 1996).

The calves were classified as S (=susceptible), I (= infectious) and R (= recovered) at the beginning and the end of the experiment. In both groups, a contact-exposed calf was classified as infectious at the end of the experiment when it showed clinical signs or when virus isolation or RT-PCR was positive for FMDV. The inoculated calves were all classified infectious by definition.

Transmission was quantified with the maximum likelihood estimator (MLE) for the reproduction ratio (R) in the S-I-R model.

Results

Clinical signs

In the vaccinated groups one inoculated calf had a small lesion in its mouth. Neither the other inoculated nor the contact-exposed vaccinated calves showed clinical signs.

In the non-vaccinated groups, vesicles in the mouth, on the coronary band or in the interdigital spaces were observed in 10 out of 12 inoculated calves, where 7 out of 12 contact-exposed calves showed clinical signs. The signs were very mild and did not have effect on behaviour or feed intake.

Virus Isolation and RT-PCR in OPF and plasma

In both the vaccinated and non-vaccinated groups 11 out of 12 inoculated calves tested positive in the OPF. In the vaccinated groups no positive samples were found in the OPF of contact-exposed calves. In the non-vaccinated groups 8 out of 12 tested positive. RT-PCR showed the same results, but in the vaccinated 2 and in the non-vaccinated 3 contact exposed calves also tested positive.

No VI or PCR positive samples were found in the plasma of the vaccinated calves. But VI in the non-vaccinated calves tested 2 out of 12 contact exposed and 6 out of 12 inoculated calves positive. RT-PCR tested 10 out of 12 inoculated and also 2 out of 12 contact exposed calves positive.

In the probang sputum samples with virus isolation and RT-PCR 11 animals tested positive. Of these, 3 animals were inoculated vaccinated calves, 5 were inoculated non-vaccinated calves. These calves are carriers because they were tested 28 DPC. Three contact-exposed non-vaccinated calves tested positive. All WBC samples at 28 DPC were tested negative.

Antibody tests

All vaccinated calves, except one, showed a rise in neutralizing antibodies in the virus neutralization test 14 days after vaccination. No fourfold increase in antibody titre was observed after inoculation. All inoculated non-vaccinated calves developed a VN titre higher than 0.3 ($^{10}\log$) TCID₅₀. Out of 12 contact-exposed calves 8 showed a positive antibody titre after contact with inoculated calves.

When testing in the NS ELISA in the vaccinated groups we found 6 out of 12 inoculated and 1 out of 12 contact exposed calves positive. In the control groups 10 out of 12 inoculated and 5 out of 12 contact exposed calves tested positive.

The animals that served as vaccine controls tested negative for all tests with exception of the virus neutralization assay. Neutralizing antibodies were found as a result of the vaccination, because they were not inoculated or contact exposed to FMDV, which was proved by negative test results in the NS-ELISA.

The test results were applied in the S-I-R model, and this resulted in a Maximum Likelihood Estimator for R in the vaccinated groups $R_V = 0.17$ ($p=0.04$). The MLE for R in the non-vaccinated groups was $R_0 = 3.30$ (0.032). When testing $H_0: R_0 \leq R_V$, $p = 0.004$.

Discussion and conclusion

From the estimators for R_0 and R_V we conclude that vaccination significantly reduced virus transmission in free-roaming calves.

The reproduction ratio in our experiment is estimated within the herd, but R can be estimated at different levels. The $R_{V, \text{within}}$ in our study <1 , it implicates that major outbreaks within the herd are not likely to occur (De Jong and Bouma, 2001). This also means that the probability of the infection being transmitted to other herds is very small, because an infected animal will probably infect fewer animals in other herds than in the same herd (Van Nes *et al.*, 1996).

Also less indirect contacts are likely between herds when compared to indirect contacts within herds. In the control groups a significant R_0 above 1 is estimated which implies a chance of a major outbreak within a herd. With increased numbers of infected animals and increased virus load a chance of a major outbreak between herds increases as well.

Single vaccination may therefore be an important tool in reducing virus transmission during an epidemic of FMD.

References

- Bouma, A., Elbers, A.R., Dekker, A. et al.** 2003. The foot-and-mouth disease epidemic in The Netherlands in 2001. *Prev Vet Med*, 57(3), 155-166.
- Donaldson, A.I. & Kitching, R.P.** 1989. Transmission of foot-and-mouth disease by vaccinated cattle following natural challenge. *Res Vet Sci*, 46(1), 9-14.
- Salt, J.S., Barnett, P.V., Dani, P. & Williams, L.** 1998. Emergency vaccination of pigs against foot-and-mouth disease: protection against disease and reduction in contact transmission. *Vaccine*, 16(7), 746-754.
- Kitching, R.P.** 2002. Clinical variation in foot and mouth disease: cattle. *Rev Sci Tech*, 21(3), 499-504.
- Kitching, R.P. & Alexandersen, S.** 2002. Clinical variation in foot and mouth disease: pigs. *Rev Sci Tech*, 21(3), 513-518.
- Kitching, R.P. & Hughes, G.J.** 2002. Clinical variation in foot and mouth disease: sheep and goats. *Rev Sci Tech* 21(3), 505-512.
- Terpstra, C., Van Maanen, C. & Van Bekkum, J.G.** 1990. Endurance of immunity against foot-and-mouth disease in cattle after three consecutive annual vaccinations. *Res Vet Sci*, 49(2), 236-242.
- Dekker, A. & Terpstra, C.** 1996. Prevalence of foot-and-mouth disease antibodies in dairy herds in The Netherlands four years after vaccination. *Res Vet Sci*, 61(1), 89-91.

De Jong, M.C. & Bouma, A. 2001. Herd immunity after vaccination: how to quantify it and how to use it to halt disease. *Vaccine*, 19(17-19), 2722-2728.

Van Nes, A., Stegeman, J.A., De Jong, M.C., Loeffen, W.L., Kimman, T.G. & Verheijden, J.H. 1996. No major outbreaks of pseudorabies virus in well-immunized sow herds. *Vaccine*, 14(11), 1042-1044.

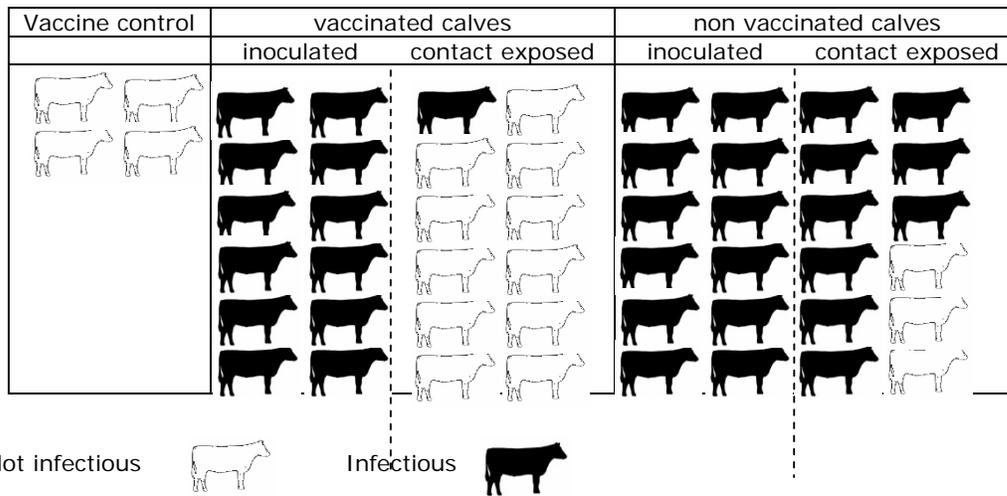


Table 1: Results of transmission in vaccinated, non-vaccinated and control groups of calves

Emergency FMD Vaccine: Effect of antigen payload on protection, sub-clinical infection and persistence following direct contact challenge of cattle

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Abstract:

Introduction: Previous work, in sheep vaccinated with emergency foot-and-mouth disease vaccine, indicated the benefits of antigen payload in inhibiting local virus replication and consequently persistence following homologous indirect aerosol challenge. The work presented investigates this possibility further using cattle and a more severe heterologous direct contact challenge. The quantitative dynamics of virus replication and excretion in both vaccinated and non-vaccinated cattle following challenge is examined. **Materials and Methods:** Two experiments were carried out each involving 20 vaccinated and 5 non-vaccinated cattle. An O₁ Manisa vaccine (18 PD₅₀) was used for the first experiment. The same vaccine was used for the second experiment except the antigen payload was increased 10-fold per bovine dose. Twenty-one days post vaccination the cattle received a 5 day direct contact challenge from 5 further cattle infected 24 hours earlier with O UKG 34/2001. Blood and probang samples for antibody and virus analyses were regularly taken following vaccination and challenge alongside frequent examination for clinical signs. **Results:** All vaccinated cattle regardless of antigen payload were protected against clinical disease. Localised sub-clinical infection at the oropharynx was detected in animals from both experiments but quantitative RT-PCR showed that the level of virus replication shortly after direct contact challenge was significantly reduced in vaccinated animals. Cattle immunised with the 10-fold antigen payload cleared the virus more readily and consequently at 28 days post challenge fewer animals were persistently infected compared to the single strength vaccine. Neutralising antibody titres were shown to be significantly higher for the 10-fold antigen payload (P<0.05). **Discussion:** Following an extremely severe challenge, the results show that use of emergency vaccine can prevent or decrease local virus replication and thereby dramatically reduce the amount of virus released into the environment, particularly during the early post-exposure period. Additionally, increasing the antigen payload of the vaccine may be a further means of reducing such sub-clinical infection, leading to less persistently infected and subsequent carrier animals.

Introduction:

Acceptance of FMD vaccines into the UK Defra strategic reserve is presently determined by a cattle potency test as described in the European Pharmacopoeia Monograph (1993) and OIE Manual of Standards (2000), from which a PD₅₀ value for a vaccine is calculated based on protection from clinical disease i.e prevention of generalisation of FMDV to the feet. For a vaccine to be acceptable as an 'emergency vaccine' it has to achieve a PD₅₀ of ≥6. It is well documented that despite being protected from disease some vaccinated ruminants can maintain replicating virus in the upper respiratory tract and become persistently infected carriers with consequential impact on control policies relating to vaccination. However, the potency test takes no account of how effective the vaccine is at reducing sub-clinical infection at the oropharynx.

A number of previous research studies have provided evidence that FMD vaccination, particularly emergency FMD vaccines, formulated to higher potency than conventional vaccines, can have some inhibitory influence on local virus replication and excretion in the oropharynx, thereby limiting transmission of disease to other susceptible animals (Barnett and Carabin, 2002, Donalson and Kitching, 1989). Although most of these studies assume the antigen payload used is higher than that incorporated in conventional vaccine with the same strain, they did not attempt to evaluate the effect of antigen payload directly, being primarily designed to investigate rapidity of protection. A more recent study in sheep, however, examined the ability of three similarly formulated vaccines, which differed only in antigen payload, to decrease or inhibit local virus replication (Barnett et al, 2004). This study indicated that higher payload vaccines were capable of inhibiting local virus replication and consequently persistence following homologous indirect aerosol challenge. The work presented investigates this possibility further using cattle and a more severe heterologous direct contact challenge. The quantitative dynamics of virus replication and excretion in both vaccinated and non-vaccinated cattle following challenge is examined.

Materials and Methods:

Animals

Two experiments, each using thirty Holstein/Friesian cattle (steers) aged 4-8 months were used for this study. All work was performed in a disease secure isolation unit at the Institute for Animal Health's Pirbright Laboratory. In both experiments cattle were housed in a group of twenty-five and a further five cattle were used as a source of virus for the direct contact challenge.

Vaccine and vaccination

In the first experiment (EXPT. 1) an O₁ Manisa vaccine was prepared from antigen concentrate stored over liquid nitrogen, which is being held at a commercial facility as part of a new UK strategic reserve. In accordance with the European Pharmacopoeia Monograph, this commercially produced oil adjuvanted vaccine had been shown to have a PD₅₀ value of 18. Twenty-one days prior to challenge, twenty cattle were vaccinated intramuscularly in the side of the neck with a full bovine dose (2ml volume). A further five animals remained unvaccinated as controls. The same vaccine was used for the second experiment (EXPT. 2) except the antigen payload was increased 10-fold per bovine dose.

Infection of donor cattle and challenge of vaccinates and controls

Five cattle in each experiment were used as a source of virus for direct contact challenge. These cattle were inoculated intradermally (into the tongue) with 10⁵ TCID₅₀/0.2ml live FMDV O UKG 34/2001 24 hours prior to the start of the direct contact challenge period, during which time they were housed separately. The direct contact challenge was carried out by allowing all cattle to mingle freely with each other for 5 days in a common holding area of 125m², after which the five needle challenged cattle and the control unvaccinated cattle were removed and housed separately from the vaccinates. All animals were examined regularly for clinical signs of FMD until 28 days post challenge and various samples were taken. Rectal temperatures were recorded daily until 10 days post challenge.

Sample collection

Clotted blood for serology and heparinised blood for virus isolation (whole blood) were collected at regular intervals prevaccination, post vaccination, at challenge, and up to 28 days post challenge. Additionally, oesophageal-pharyngeal fluid (probang) samples were collected at similar intervals for virus isolation by cell culture and for detection of viral RNA by reverse transcriptase polymerase chain reaction (RT-PCR). All samples were stored at -70° C except sera, which were stored at -20° C, until required for testing. The material for RT-PCR (200µl) was added to an 300µl of lysis/binding buffer (Roche, UK) before freezing.

Virus isolation and detection of FMD viral RNA by RT-PCR

Heparinised blood samples and probang samples were examined for the presence of virus by inoculation of monolayers of primary calf thyroid (BTY) cells (Snowdon, 1966). Four BTY tubes were each inoculated with 250 µl sample and incubated at 37 °C on roller drums. At 24, 48 and 72 hours post inoculation cell monolayers were examined for cytopathic effect (cpe). ELISA was used to confirm the presence of FMDV in cultures showing cpe (Ferris and Dawson, 1988). BTY cell culture supernatants from samples showing no sign of cpe after 72 hours were pooled and re-passaged once.

The probang samples were also tested by quantitative real-time RT-PCR using similar automated programmes for total nucleic acid extraction and RT and PCR set up, to those described by Cox et al. (2004, at press).

Serology

Serum samples were examined for both anti-FMDV neutralising antibodies (OIE, 2000) and for the presence of antibodies to the FMDV non-structural proteins (NSP) 3ABC (Ceditest FMDV-NS (Cedi-Diagnostics)).

Results:

Development of clinical FMD and viraemia

Within 3 days of needle challenge, all of the donor cattle, in both experiments, had developed typical FMD foot and tongue lesions. Although the amount of virus excreted from these animals was not measured, previous studies in cattle injected by the same route with the same challenge strain have shown that up to 10^{4.3} TCID₅₀ per animal per 24 hours may be excreted by aerosol from 1-3 days post infection (Alexandersen et al., 2002). The donor cattle were therefore a potent source of virus for the challenge, probably from day 1.

All of the vaccinated cattle in both experiments, regardless of antigen payload, were protected against clinical disease and none developed a detectable viraemia in samples collected at regular intervals up to 28 days after contact exposure with the needle challenged cattle. The five cattle in the

unvaccinated groups, however, all developed clinical signs of FMD including nasal discharge, with foot and tongue lesions appearing between 2-10 days and viraemia from 2-7 days after introduction of the needle challenged cattle.

Local virus replication, detection of FMD viral RNA and development of antibodies against non-structural FMDV polyprotein 3ABC

Following observation of BTY cells for signs of cpe and confirmation by ELISA, FMDV was recovered from probang samples collected from most vaccinated cattle and all unvaccinated cattle in both experiments, at various time points after the direct contact challenge, although detection, and therefore occurrence of local virus replication, varied greatly between individual animals (Tables 1 and 2). Likewise, FMDV RNA was variably detected by quantitative RT-PCR in the probang samples taken from individual animals of both groups and copy numbers per ml are also shown in Tables 1 and 2. Samples found positive on virus isolation were not necessarily positive by RT-PCR and vice versa. Combining the results of the two virological tests, virus or viral genome was confirmed in all but three animals from the vaccinated group in both experiments although an additional four vaccinated animals in EXPT. 1 and five in EXPT. 2 only had oropharyngeal virus or genome detected whilst the donor animals were still present. This might have been of environmental origin rather than virus replication. The total number of animals with some form of viral recovery at 28 days after challenge exposure, i.e. persistently infected, was 9 vaccinates and 1 unvaccinated control in EXPT.1 and 2 vaccinates and 2 unvaccinated controls in EXPT. 2. Samples from one vaccinated animal (UY83) and one unvaccinated animal (UY 97 - dead) were unavailable for testing at 28 days post challenge exposure.

NSP serology results at 28 days post challenge are also shown in tables 1 and 2. Thirteen of the 20 vaccinated animals in EXPT. 1 and 15 of the 20 in EXPT. 2 showed no evidence of non-structural antibody development, particularly those appearing to clear the virus. The unvaccinated cattle in both experiments all showed an NSP antibody response by 28 days post challenge exposure.

The mean quantity of FMD viral RNA [$\log_{10}(\text{copies ml}^{-1})$] in probang samples from both vaccinated and unvaccinated control cattle at different times after contact with the donor cattle, as measured by quantitative RT-PCR, are presented in Figure 1. During the period between 4 and 10 days post challenge exposure, the unvaccinated controls in both experiments, had highest average levels of detectable viral RNA in their oropharynxes, the levels being between 10^2 - 10^3 greater than that seen in the vaccinated animals at the same time points.

The unvaccinated animals demonstrated a very high initial load, peaking at 7 days, and then falling again. In contrast, some persistently infected animals, particularly in EXPT. 1 were unable to clear the virus as efficiently, and had their highest levels of detectable viral RNA at 28 days after the introduction of the needle challenged cattle.

In order to assess whether antigen payload was affecting the amount of sub-clinical infection and persistence (as measured by virus recovery either by virus isolation or RT-PCR), a comparison of how often virus was detected by either technique was made. The percentage number of animals positive at each time point is shown in Table 3. Results for the unvaccinated animals from both experiments were combined and are also included.

Virus neutralising antibody induction

Figure 2 shows mean serum neutralising antibody responses against O₁ Manisa in vaccinated and unvaccinated cattle, up to 14 days post challenge exposure, following vaccination and direct contact with infected donor cattle. Neutralising antibody responses were detected as early as 5 days post vaccination in some animals from both experiments. At 7 days post vaccination, nine animals in EXPT. 1 and twenty in EXPT. 2 had seroconverted. Neutralising antibody titres, at every time point, were significantly higher for the 10-fold antigen payload group ($P < 0.05$).

Discussion:

In order to assess how well emergency vaccines will protect from direct contact challenge a commercially prepared O₁ Manisa vaccine antigen, held as part of the new UK strategic reserve, was formulated to provide an oil vaccine with a calculated PD₅₀ of 18. Additionally, a similar vaccine was prepared which contained ten times more antigen. The vaccinated cattle of both groups were subjected to a severe heterologous direct contact challenge from 5 cattle infected with O UKG 34/2001, over a period of 5 days. All twenty cattle, regardless of antigen payload, were protected from clinical disease confirming that high potency emergency vaccines are capable of providing protection after a single application, even in the face of this severe direct challenge exposure. Further studies, however, utilizing shorter time periods between vaccination and challenge, are needed to identify how quickly such protection is achieved.

Protection from clinical disease did not always coincide with prevention of localised, sub-clinical infection. In the first 10 days after challenge exposure, FMDV was frequently recovered from the oropharynx of vaccinated cattle receiving both antigen payloads although to a lesser extent in animals receiving the higher antigen payload by 10 days post challenge exposure. There is a possibility that the virus recovered in the first five to seven days following start of the challenge exposure could have been directly inhaled or ingested rather than being an indication of oropharyngeal replication. During the first ten days after challenge exposure, the amount of virus produced was considerably less in the vaccinated groups due to the transient or intermittent extent of virus replication. Furthermore, even comparing the levels of viral RNA found in positive samples, the average levels were approximately 100-1000 times higher in the unvaccinated animals until 10 days after challenge exposure. These findings demonstrate the ability of the vaccines to either prevent or reduce viral replication shortly after direct contact challenge at the site of primary infection, thereby further limiting the amount of infectious material released to the environment from sub-clinically infected animals. The 10-fold antigen payload vaccine, however, did not add much in the way of additional benefit over the single antigen payload vaccine.

It is documented that conventionally vaccinated cattle may succumb to local infection and subsequently become persistently infected 'carriers'. This study shows that persistence can be observed following the administration of an emergency vaccine (X1) and severe direct contact exposure for 5 days. However, if the antigen payload of the vaccine is increased, the number of persistently infected animals is reduced. Additionally, virus was less frequently recovered on most other occasions from animals vaccinated with the higher antigen payload.

It is interesting that the levels of viral RNA found in probang samples collected from persistently infected animals receiving the lower antigen payload beyond ten days post challenge exposure was often higher than those in equivalent samples from unvaccinated animals. Although it cannot be excluded that unvaccinated animals with a similar response might have been identified if there had been a comparable number of unvaccinated to the vaccinated subjects it is possible that the local immune response to sub-clinical infection in vaccinated animals will be different from that of unvaccinated animals, resulting in a prolonged clearance of virus. However, it should be noted that a similar result was not seen in animals receiving the higher antigen payload which perhaps suggest an improved local immune response at the oropharynx following higher payload vaccination.

It is difficult to assess the relationship between RNA copy number and infectivity. Results presented here show that the correlation between isolation of live virus and detection of viral RNA from probang samples is variable. The most likely reason for this lack of correlation is the involvement of antibody, either from serum or local production at the mucosa following sub-clinical infection, which neutralises the virus effectively making it non-infectious. It is clear therefore that although detection of FMDV RNA may be useful for diagnostic purposes, as in determining whether an animal has had contact with the virus, RNA copy number alone may not be useful as an indicator for determining whether a persistently infected animal is likely to present a risk for disease transmission. Future studies will need to investigate more precisely the correlation between viral RNA recovery (copy number) and actual tissue culture infective dose at different time intervals following challenge in both vaccinated and unvaccinated animals.

The mean systemic neutralising antibody response for both groups of vaccinated cattle was examined to see whether any differences were evident as a result of antigen payload. Overall, the 10-fold higher antigen payload vaccine resulted in a quicker response, with titres at every time point being significantly higher than for the single strength vaccine. Although it is difficult to gauge the effect of such a systemic response on the local environment, the reduction seen in virus replication and viral persistence obtained with the 10-fold payload perhaps suggests that an improved immune response involving correlates of protection not investigated, as well as an improved neutralising antibody response, has been achieved.

Conclusions:

- High potency emergency vaccines are effective at preventing clinical disease and reducing local virus replication in the all important early post exposure period and therefore dramatically reduce the amount of virus released into the environment following severe direct contact challenge.
- Increasing antigen payload results in an improved immune response which has an effect on local virus replication and persistence.
- Emergency vaccines, as presently selected using potency tests based on development of clinical disease, may contain sub-optimal levels of antigen for combating sub-clinical infection.

Recommendations:

- Investigate further the effect of antigen payload on sub-clinical infection in order to optimise vaccines for emergency use.
- Investigate further the kinetics of protection in cattle under severe direct contact challenge conditions
- Rec. no. 3

Acknowledgements:

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References:

European Pharmacopoeia 1997. Foot-and-Mouth Disease (Ruminants) Vaccine, 63: 875-876

OIE. 2000. Section 2.1 List A Diseases, Chapter 2.1.1 Foot and mouth disease, *Manual of Standards for Diagnostic tests and Vaccines*, pp. 77-92.

Barnett, P.V. & Carabin, H. 2002. A review of emergency foot-and-mouth disease (FMD) vaccines. *Vaccine*, 20: 1505-1514.

Donaldson, A.I. & Kitching, R.P. 1989. Transmission of foot-and-mouth disease by vaccinated cattle following natural challenge. *Res. Vet. Sci.*, 46: 9-14.

Barnett, P.V., Keel, P., Reid, S., Armstrong, R.M., Statham, R.J., Voyce, C., Aggarwal, N. & Cox, S.J. 2004. Evidence that high potency foot-and-mouth disease vaccine inhibits local virus replication and prevents the 'carrier' state in sheep. *Vaccine*, 22(9-10): 1221-1232.

Snowdon, W.A. 1966. Growth of foot-and-mouth disease virus in monolayer cultures of calf thyroid cells. *Nature*, 210: 1079-1080

Ferris, N.P. & Dawson, M. 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet. Micro.*, 16: 201-209.

Cox, S.J., Voyce, C., Parida, S., Reid, S.M., Hamblin, P.A., Paton, D.J. & Barnett, P.V. 2004. Protection against direct contact challenge following emergency FMD vaccination of cattle and the effect on virus excretion from the oropharynx. *Vaccine* (at press)

Alexandersen, S., Zhang, Z., Reid, S.M., Hutchings, G.H. & Donaldson, A.I. 2002. Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. *J. Gen. Virol.*, 83: 1915-1923.

Animal Ref	Days post challenge																		NSAb
	2		4		7		10		12		14		16		21		28		
Vacc:	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	NSAb
UV2	-	4.09*	+	3.79	+	3.84	+	0	-	0	-	0	IS	4.78	-	0	-	4.39	-
UV3	-	0	-	0	-	0	-	0	-	0	-	0	IS	0	-	0	-	0	-
UV4	-	0	+	5.46	-	0	-	0	-	0	-	0	IS	0	-	0	-	0	-
UV5	-	0	+	5.18	-	4.86	+	3.66	+	4.1	+	3.73	IS	4.69	+	3.99	-	2.85	+
UV6	-	0	-	0	+	0	-	0	-	0	-	0	IS	0	-	0	-	0	-
UV7	-	0	-	0	-	0	-	0	IS	0	-	0	IS	0	-	0	-	0	-
UV8	-	0	-	0	-	0	-	0	-	0	-	0	IS	0	-	0	-	0	-
UV9	+	4.77	+	0	+	5.05	+	4.28	+	4.68	+	5.42	IS	5.75	+	4.35	-	6.22	+
UV10	+	5.58	-	0	+	5.26	+	5.52	+	5.11	+	5.46	IS	4.15	+	4.57	-	6.09	+
UV11	+	0	+	5.77	+	6.13	+	4.05	-	4.2	+	3.5	IS	3.64	+	4.38	+	6.06	+
UV12	-	3.06	+	3.71	+	0	+	0	-	0	-	0	IS	0	-	0	-	0	-
UV13	+	5.27	+	4.01	+	4.43	+	4.29	+	5.12	+	5.31	IS	4.28	+	5.34	-	5.59	+
UV14	+	0	+	3.54	+	4.78	+	0	+	3.41	+	0	IS	0	+	4.3	-	3.65	-
UV15	-	0	-	4.95	-	0	-	0	-	0	-	0	IS	0	-	0	-	0	-
UV16	+	0	-	3.29	-	0	-	0	-	0	-	0	IS	0	-	0	-	0	-
UV17	IS	IS	+	3.38	+	5.41	+	4.2	+	4.78	+	5.14	IS	5.27	+	5.32	-	5.63	-
UV18	IS	IS	-	0	-	0	-	0	+	0	-	0	IS	0	+	0	-	0	+
UV19	+	3.86	+	4.68	+	4.97	+	3.2	-	4.94	+	4.12	IS	4.8	+	5.19	-	5.96	-
UV20	+	6.66	+	6.2	-	0	-	0	-	4.25	-	0	IS	0	-	0	-	0	+
UV21	+	3.4	+	4.76	-	0	-	0	-	0	-	0	IS	0	-	0	-	0	-
Unvacc:																			
UV22	+	5.25	IS	6.05	+	6.71	-	3.27	-	0	-	0	IS	3.09	-	0	-	0	+
UV23	+	0	+	4.96	+	8.2	+	5.64	-	4.42	-	0	IS	0	-	3.41	-	0	+
UV24	-	3.27	+	5.51	+	6.84	-	5.06	-	4.2	-	0	IS	0	+	0	-	0	+
UV25	+	5.04	+	3.44	+	8.99	+	7.22	-	5.45	+	3.35	IS	0	+	3.36	-	3.25	+
UV26	+	7.29	+	8.52	+	5.97	+	4.38	-	3.86	+	3.43	IS	3.49	+	0	-	0	+

IS: Insufficient sample * Viral RNA levels [$\log_{10}(\text{copies ml}^{-1})$] 0: No viral RNA detected

+: Virus or nonstructural antibodies detected -: No virus or nonstructural antibodies detected

Table 1: Virus isolation (VI) and PCR results from probang samples and nonstructural antibody (NSAb) results from vaccinated and unvaccinated cattle (EXPT. 1)

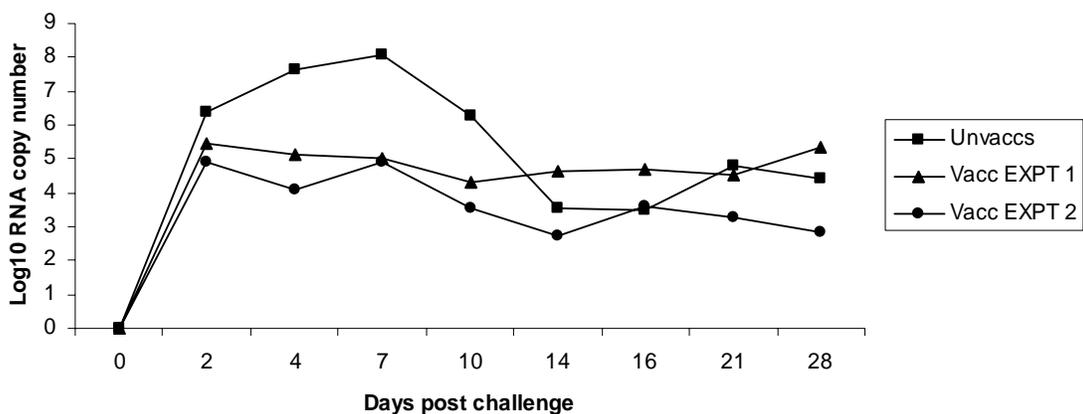
Animal Ref	Days post challenge																	
	2		4		7		10		14		17		21		28		NSAb	
Vacc:	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR	VI	PCR		NSAb
UY72	-	0	+	0	-	3.49*	IS	IS	-	0	IS	IS	-	0	-	0	-	
UY73	-	0	-	0	+	0	-	0	-	0	-	0	-	0	-	0	-	
UY74	-	0	+	4.07	-	0	-	0	-	0	-	0	-	0	-	0	-	
UY76	+	4.32	+	4.37	+	0	+	4.57	-	0	+	3.96	+	4.44	+	4.1	+	
UY77	-	0	+	0	-	IS	-	0	-	0	-	0	-	0	-	0	-	
UY78	-	0	-	0	-	0	IS	IS	-	0	IS	IS	-	0	-	0	-	
UY79	-	0	+	4.99	+	5.9	IS	IS	-	0	-	0	-	0	-	0	-	
UY80	-	0	+	0	+	5.71	+	3.96	-	0	-	0	+	0	-	0	+	
UY81	-	0	-	0	+	4.13	-	0	-	0	-	0	-	0	-	0	+	
UY82	+	3.86	-	0	+	3.58	-	0	-	0	-	0	-	0	-	0	+	
UY83	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	IS	-	3.33	IS	IS	-	
UY84	+	0	+	3.95	-	0	-	0	-	0	-	0	-	0	-	0	+	
UY85	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	
UY86	-	0	-	0	+	0	-	0	-	0	-	0	-	0	-	0	-	
UY87	+	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	
UY88	IS	IS	IS	IS	IS	IS	IS	IS	-	0	IS	IS	-	0	-	0	-	
UY89	-	0	-	0	-	0	-	0	-	0	-	0	-	0	-	0	+	
UY90	+	6.14	+	4.56	+	5.61	+	3.36	+	3.99	+	4.74	+	3.77	+	0	-	
UY91	+	0	-	0	+	5.56	+	3.88	-	0	+	3.6	-	0	-	0	+	
UY92	+	3.27	+	4.61	+	4.55	-	0	-	0	-	0	-	0	-	0	-	
Unvacc:																		
UY93	IS	IS	IS	IS	-	6.28	IS	IS	IS	IS	-	0	IS	IS	-	0	+	
UY94	+	5.79	+	7.6	+	5.98	-	4.16	-	0	-	0	-	0	-	0	+	
UY95	+	5.09	+	6.32	+	6.5	-	4.68	+	4.36	+	4.4	+	5.74	+	5.33	+	
UY96	+	0	+	4.23	+	5.97	-	5.16	-	0	+	0	+	0	+	4.46	+	
UY97	+	4.58	+	3.96	+	6.23	-	3.7	-	3.43	-	0	-	0	ND	ND	+	

IS: Insufficient sample * Viral RNA levels [$\log_{10}(\text{copies ml}^{-1})$] 0: No viral RNA detected

+: Virus or nonstructural antibodies detected -: No virus or nonstructural antibodies detected

Table 2: Virus isolation (VI) and PCR results from probang samples and nonstructural antibody (NSAb) results from vaccinated and unvaccinated cattle (EXPT. 1)

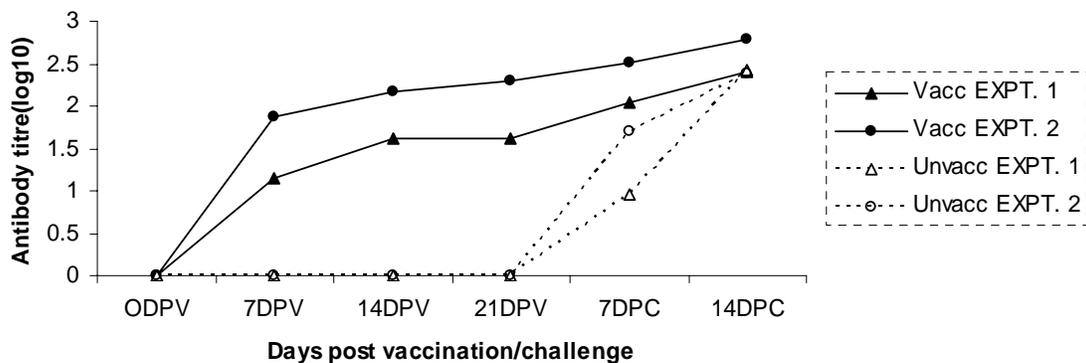
Figure 1: Mean RNA copy number detected over time in RT-PCR positive samples



Treatment	Days post Challenge							
	2	4	7	10	14	16/17	21	28
0 (Expt.1 & 2)	100	100	100	100	44	40	67	33
X1 (Expt. 1)	61	70	55	50	40	40	45	45
X10 (Expt. 2)	39	50	61	27	5	18	20	11

Table 3: Percentage of animals from which virus recovered at different time points post challenge exposure

Figure 2: Mean neutralising antibody titres over time



FMD and camelids: International relevance of current research

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Key words: Tylopoda, camelids, FMD

Abstract

Camelids regurgitate and re-chew their food and thus technically ruminate. In strict taxonomic terms, however, they are not recognized as belonging to the suborder *Ruminantia*. They belong to the suborder *Tylopoda*. Numerous differences in anatomy and physiology justify a separate classification of tylopods from ruminants. Many reports show that New World Camelids (NWC) and Old World Camelids (OWC) possess a low susceptibility to foot and mouth disease (FMD), and do not appear to be long-term carriers of the foot and mouth disease virus (FMDV). Recent preliminary results from Dubai have shown that two dromedaries infected subepidermolingually with FMD serotype 0 did not develop any clinical signs and failed to develop any lesions at the inoculation site. Infectious FMDV or FMDV RNA were not isolated and the two dromedaries failed to seroconvert.

It would, therefore, appear appropriate for OIE to refine the definition of NWC and OWC by clearly stating that these animal species are not members of the suborder *Ruminantia*. Furthermore, these recent results suggest that dromedaries (and most probably all camelid species), which are listed in the OIE Code chapter as being susceptible to FMD similar to cattle, sheep, goats and pigs, are much less susceptible or non-susceptible to FMD. Therefore, the importance of FMD in camelids should be re-assessed. The Central Veterinary Research Laboratory (CVRL) in Dubai, U.A.E., offers to become a reference laboratory for OWC. For more than a decade, CVRL has published in excess of 150 scientific papers and three reference books on camel diseases.

Classification, population and distribution

Although camelids ruminate, they are not modified ruminants in a taxonomic sense. A separate evolutionary history of 35 – 40 million years divides tylopods from ruminants. *Camelidae* belong to the suborder *Tylopoda* (Fowler, 1997; Table 1). Numerous anatomical and physiological differences justify the separate classification of *Tylopoda* from *Ruminantia*. The most important differences are shown in Table 2 and some are explained in several figures. The camelid stomach system differs from that of ruminants. There are only three distinct forestomachs compared to four in ruminants. In camelids they are called compartments (C) 1, 2 and 3. The rumen equivalent is C1, which possesses cranial and caudal glandular sacs. These were once considered to represent the water store of the animal; however they mainly function as absorption and fermentation areas as well as zones of enzymatic secretion (Wilson, 1989). The second, much smaller compartment C2 is the reticulum equivalent, and the elongated C3 is the combined omasum/abomasum equivalent, which might best be referred to as the tubular stomach due to its length. Compartments 1 and 2 are lined with non-papillary smooth epithelium (Figure 1). In camelids, the motility patterns are markedly different compared with ruminants. Another distinguished feature of all *Camelidae* is the unique structure of their feet (Fig. 2). The padded feet act like snowshoes allowing them to walk over soft, loose sand without sinking. Camelids walk on thick pads consisting primarily of fat. They possess two digits, and their second and third phalanges are horizontal. The reproductive physiology of camelids is of particular interest. Camels mate in a crouching position (Fig. 3) and while mating the bull exteriorises its "doula" (Fig. 4), a bright pink inflatable sac, to attract females. Camels are induced ovulators. Their gestation period lasted 13 months. A slippery surface of a third membrane surrounding the fetus eases its birth (Figure 5). Latest osteological investigations on post-cranial skeletons of *Camelus dromedarius* and *C. bactrianus* have shown that they derived from two different ancestors. Approximately twenty million OWC exist, of which two million are Bactrians (Table 3).

There are four different species of NWC which inhabit the high altitudes in South America. The estimated population of NWC is shown in Table 4. Llamas and alpacas were domesticated 7.000 years ago; the dromedary and the Bactrian around 5.000 years ago. Guanacos and vicuñas are wild and there are few wild Bactrians which roam in the Chinese and Gobi desert. There are no wild dromedaries anymore. The distribution of OWC is shown in Figure 6.

The knowledge of the susceptibility and resistance to infectious and parasitic diseases is of paramount importance in an area where tylopods mix with other livestock.

Review of findings on FMD in camelids

FMD remains the single most important animal disease, and OWC and NWC inhabit countries in North and East Africa, the Middle and Far East as well as in South America where FMD is endemic. It has been reported that dromedaries can contract the disease following experimental infection and via close contact with FMD diseased livestock, yet do not present a risk in transmitting FMD to susceptible animals (Kitching, 2002). Summarised results are presented in the following Tables 5 to 8 (Wernery and Kaaden, 2004). Only two reports exist of a natural infection. The execution of experimental infections is poor, and therefore conclusions are questionable. FMD serology and infection in Bactrian camels remains questionable, with FMD diagnosis only being made by means of clinical observations.

Results of recent FMD experiments in dromedaries in Dubai with serotype O

Two Holstein heifers of around 150 kg (6-8 months of age) and two castrated male dromedaries (*Camelus dromedarius*) around 400-450 kg (7-10 years of age) were each inoculated subepidermally with $10^{7.6}$ Tissue Culture Infectious Doses 50% (TCID₅₀) of foot-and-mouth disease virus (FMDV) type O UAE 7/99 in a volume of 0.5 ml (Fig. 7). While the heifers developed elevated body temperatures, were drooling saliva and had typical vesicular lesions (Fig. 8) on the tongue within 24 hours, the two dromedaries did not show any clinical signs of disease and had no vesicular lesions, even at the inoculation site. Infectious FMDV and FMDV RNA were detected at relatively high levels in sera and nasal and mouth swabs from the heifers, but no infectious FMDV or FMDV RNA were isolated in similar samples from the two dromedaries (Fig. 9). Furthermore, the two dromedaries did not develop any detectable antibodies to FMDV. Based on the overall results obtained, we conclude that dromedaries (*Camelus dromedarius*) are not susceptible to infection with this isolate of FMDV (Wernery et al., 2005).

Conclusion

Camelids belong to the suborder *Tylopoda*; they are not ruminants. Camelids possess a low flow susceptibility to FMD, and do not appear to be long-term carriers of the FMDV. These are the main two reasons to remove them from the OIE chapter as possessing the same degree of susceptibility as cattle, sheep and goats.

References

- Abou Zaid, A.A.**, 1991. *Studies on some diseases of camels*. PhD Thesis, Faculty of Veterinary Medicine Zagazig, Egypt
- Farag, M.A., Al-Sukayran, A., Mazlou, K.S, Al-Bokney, A.M.**, 1998. *The susceptibility of camels to natural infection with foot and mouth disease virus*. Assiut Veterinary Medical Journal 40, 201 – 211
- Fowler, M. E.** (1997), *Evolutionary history and differences between camelids and ruminants*, J. Camel Pract. and Research 4 (2), 99 – 105
- Hafez, S.M., Farag, M.A., Al-Mukayel, Al**, 1993. *Are camels susceptible to natural infection with foot and mouth disease virus?* Internal Paper: National Agriculture and Water Research, Center Riyadh, Saudi Arabia
- Hedger, R.S., Barnett, I.T.R., Gray, D.F.**, 1980. *Some virus diseases of domestic animals in the Sultanate of Oman*. Tropical Animal Health and Production 12, 107 –114
- Kitching, P.** (2002). *Identification of foot and mouth disease virus carrier and subclinically infected animals and differentiation from vaccinated animals*. Revue scientifique et technique. Foot and mouth disease: facing the new dilemmas. OIE 21 (3), 531 - 538
- Kumar, A., Prasad, S., Ahuja, K.L., Tewari, S.C., Dogra, S.C., Garb, D.N.**, 1983. *Distribution pattern of foot and mouth disease virus types in North-West India (1979 – 1981)*. Haryana Veterinarian 22, 28 – 30
- Metwally, M.A., Moussa, A.A., Reda, J., Wahba, S., Omar, A., Daoud, A., Tantawi, H.H.**, 1986. *Detection of antibodies against FMDV in camels by using fluorescent antibody technique*. Agricultural Research Review 64, 1079 – 1084
- Moussa, A.A., Daoud, A., Tawfik, S.**, 1979. *Susceptibility of camel and sheep to infection with foot and mouth disease virus*. Agricultural Research Revision Egypt 57, 1 –19
- Moussa, A., Nasser, M.I., Mowafi, L., Salah, A.**, 1986a. *Occurrence of foot and mouth disease in different species of mammals at Sharkia province*. Journal of Egypt Veterinary Medicine Association 40, 23 – 35

- Moussa, A.A., Tantawi, H.H., Metwally, N.A., Wahba, S., Hussein, K., Osman, O.A., Saber, M.S.,** 1986b. *Pathogenicity of foot and mouth disease virus isolated from experimentally infected camels to susceptible steers*. Agricultural Research Review 64, 1071 – 1077
- Moussa, A.A.M., Daoud, A., Omar, A., Meetwally, N., El-Nimr, M., McVicar, J.W.** 1987. *Isolation of foot and mouth disease virus from camels with ulcerative disease syndromes*. Journal of Egypt Veterinary Medicine Association 47, 219 – 229
- Moussa, A.A.M.,** 1988. *The role of camels in the epizootiology of FMD in Egypt*. In: FAO. The Camel: Development Research. Proceedings of Kuwait Camel Seminar, Kuwait, Oct. 20 – 23, 1986, pp. 162 – 173
- Moussa, H.A.A., Youssef, N.M.A.,** 1998. *Serological screening for some viral diseases antibodies in camel sera in Egypt*. Egypt Journal of Agricultural Research 76, 867 – 873
- Nasser, M., Moussa, A.A., Metwally, M.A., Saleh, R.EL.S.,** 1980. *Secretion and persistence of foot and mouth disease virus in faeces of experimental infected camels and ram*. Journal of Egypt Veterinary Medicine Association 40, 3 – 13
- Paling, R.W., Jerset, D.M., Heath, B.R.,** 1979. *The occurrence of infectious diseases and mixed farming of domesticated and wild herbivores and domesticated herbivores including camels, in Kenya I. Viral diseases: a serological survey with special reference to foot and mouth disease*. Journal of Wildlife Diseases 15, 351 – 359
- Richard, D.,** 1979. *Etude de la pathologie du dromedaire dans la souprovence du Borana (Ethiopie) (Study of the pathology of the dromedary in Borana Awraja, Ethiopia)*. These Doctorales Veterinaire, Paris No. 75, pp. 181 – 190
- Wernery, U. and O.-R. Kaaden** (2002). *Infectious diseases in camelids*, Blackwell Science, pp. 3 –17
- Wernery, U. and O.-R. Kaaden** (2004). *Foot-and-mouth disease in camelids: a review*, The Veterinary Journal
- Wernery, U., P. Nagy, C. M. Amaral-Doel, Z. Zhang and S. Alexandersen** (2005). *Dromedaries (Camelus dromedarius) appear not to be susceptible to infection with foot-and-mouth disease virus serotype O*, The Vet. Rec. (in press)
- Wilson, R. T.** (1989). *Ecophysiology of the camelidae and desert ruminants*, Springer Verlag, pp. 96 – 98

Table 1: Classification of camelids and other artiodactylids (Wernery and Kaaden, 2002)

Class	Mammalia	
Order	Artiodactyla	
Suborder	Suiformes	Hippopotamuses, swine, peccaries
Suborder	Tylopoda	Camelids
Old World		<i>Camelus dromedarius</i> – dromedary camel <i>Camelus bactrianus</i> – Bactrian camel <i>Lama glama</i> – llama
New World		<i>Lama glama</i> – llama <i>Lama pacos</i> – alpaca <i>Lama guanicoe</i> – guanaco <i>Vicugna vicugna</i> – vicuña
Suborder	Ruminantia	Cattle, sheep, goats, water buffalo, giraffe, deer, antelope, bison

Table 2: Differences between camelids and ruminants

Camelids	Ruminants
Evolutionary pathways diverged 40 million years ago	Evolutionary pathways diverged 40 million years ago
Blood	Blood
<ul style="list-style-type: none"> red blood cells elliptical and small (6.5 μ) predominant white blood cell is the neutrophil 	<ul style="list-style-type: none"> red blood cells round and larger (10 μ) predominant white blood cell is the lymphocyte
Foot	Foot
<ul style="list-style-type: none"> has toenails and soft solar pad second and third phalanges are horizontal 	<ul style="list-style-type: none"> has hooves and sole second and third phalanges are nearly vertical
Digestive System	Digestive System
<ul style="list-style-type: none"> foregut fermenter, with regurgitation, re-chewing and re-swallowing stomach – 3 compartments (C1-3), resistant to bloat compartment 1 and 2 have stratified squamous epithelium 2 glandular sacs in C1, act as “reserve water tanks” 	<ul style="list-style-type: none"> same (parallel evolution) stomach – 4 compartments, susceptible to bloat rumen has papillary epithelium no glandular sacs
Reproduction	Reproduction
<ul style="list-style-type: none"> induced ovulator no oestrus cycle follicular wave cycle copulation in prone position diffuse placentation epidermal membrane surrounding fetus cartilaginous projection on tip of penis ejaculation prolonged 	<ul style="list-style-type: none"> spontaneous ovulation oestrous cycle no follicular wave cycle copulation in standing position cotyledonary placentation no epidermal membrane surrounding fetus no cartilaginous projection on tip of penis ejaculation short and intense
Urinary	Urinary
<ul style="list-style-type: none"> smooth and elliptical kidney suburethral diverticulum in female at external urethral orifice dorsal urethral recess 	<ul style="list-style-type: none"> smooth or lobular kidney no suburethral diverticulum dorsal urethral recess in some species
Parasites	Parasites
<ul style="list-style-type: none"> unique lice and coccidia share some gastrointestinal nematodes with cattle, sheep and goats 	<ul style="list-style-type: none"> unique lice and coccidia share gastrointestinal nematodes
Infectious diseases	Infectious diseases
<ul style="list-style-type: none"> minimally susceptible to tuberculosis bovine brucellosis is rare mild susceptibility to foot-and-mouth disease rarely develop clinical disease following exposure/inoculation with other bovine and small ruminant viral diseases 	<ul style="list-style-type: none"> Highly susceptible to tuberculosis, bovine brucellosis and foot-and-mouth disease

Table 3: Old World camel population in Africa and Asia

Africa	Camel Population	Asia	Camel Population
Algeria	150,000	Afghanistan	270,000
Chad	446,000	India	1,150,000
Djibouti	60,000	Iran	27,000
Egypt	90,000	Iraq	250,000
Ethiopia	1,000,000	Israel	11,000
Kenya	610,000	Jordan	14,000
Libya	135,000	Kuwait	5,000
Mali	173,000	Mongolia	580,000
Mauritania	800,000	Oman	6,000
Morocco	230,000	Pakistan	880,000
Niger	410,000	Qatar	10,000
Nigeria	18,000	Saudi Arabia	780,000
Senegal	6,000	Syria	7,000
Somalia	6,000,000	Turkey	12,000
Sudan	2,600,000	United Arab Emirates	120,000
Tunisia	173,000	Yemen	210,000
Upper Volta	6,000	IPS*	200,000
Western Sahara	92,000	China	600,000
		Australia	120,000
		Canary Islands	4,000
Total	12,999,000	Total	5,256,000
	0		
Grand Total		18,255,000	

* Independent States of the Soviet Union

Table 4: Estimated population of South American camelids

Country	Llamas	Alpacas	Guanacos	Vicuñas
Argentina	75,000	2,000	550,000	23,000
Bolivia	2,500,000	300,000	?	12,000
Chile	85,000	5,000	20,000	28,000
Peru	900,000	3,020,000	1,400	98,000
Australia	< 5,000	> 5,000	A few in zoos	0
Canada	> 6,000	> 2,000	< 100 in zoos	> 10
Europe	< 2,000	< 1,000	< 100 in zoos	< 100 in zoos
United States	> 110,000	> 9,500	145, mostly in zoos	0
In registry zoos*	ISIS 343	303	397	100
Total	3,683,343	3,344,803	572,142	161,210
Grand Total	7,761,498			

* ISIS = International Species Inventory System

Table 5: FMD in New World Camelids

FMD Serology: **Field investigations. Reliable serological tests are available**
 So far all investigations are negative despite NWC mixing with FMD positive contact animals
Experimental investigations
 Antibodies have been produced to FMD using different routes and serotypes

FMD Infection: **Field investigations**
 One case in alpacas showing minor disease, but no virus isolated
Experimental investigations
 NWC can be infected with different serotypes and demonstrate mild to severe clinical signs. Virus can also be transmitted to other susceptible animals. FMDV was not isolated after 14 days. No carriers?

Table 6: Reports on dromedary FMD-antibodies from field surveys

Authors	Year	Country	Serotypes	Sera tested	Positive %	Test	Endemic
Richard	1979	Kenya	A,O,SAT _{1,2}	87	2.6	VNT*	yes
Hedger et al.	1980	Oman	A,O,C,SAT ₁ ,Asia ₁	203	nil	VNT	yes
Moussa et al.	1986a	Egypt	O	1755	5.4	VNT	yes
Paling et al.	1979	Nigeria	C,O,SAT ₂	88	nil	VNT	yes
Abou-Zaid	1991	Egypt	O	536	nil	AGID**	yes
			O	536	10.6	ICFT***	
			O	536	23.5	ELISA	
Hafez et al.	1993	Egypt	O	364	nil	VNT	yes
Hafez et al.	1993	Saudi Arabia	O	650	nil	VNT	yes
Moussa+Youssef	1998	Egypt	O	169	24.3	ELISA	yes
Farag et al.	1998	Saudi Arabia	A,O	25	nil	VNT, AGID	yes
Wernery pers. comm.	2003	U.A.E.	O	374	nil	ELISA	yes
Younan pers. comm.	2003	Kenya	O	324	nil	ELISA	yes

- * Virus Neutralisation Test
- ** Agargel Immunodiffusion Test
- *** Indirect Complement Fixation Test

Table 7: Seroconversion in dromedaries after inoculation with FMDV

Author	Year	Country	Dromedaries tested	Serotype	Inoculation Route	Method	Result	Duration of antibodies
Moussa et al.	1979	Egypt	5	O ₁ /2/72 Egypt	intranasal	SNT* AGID	5/5 0/5	nil
Nasser et al.	1980	Egypt	2	O ₁ /2/72 Egypt	intransal	Not done	?	?
Metwally et al.	1986	Egypt	2	O ₁ /2/72 Egypt	intranasal	FAT**	2/2 low titres	6 weeks
Moussa	1988	Egypt	?	O	intranasal	SNT	positive low	3 months
Abou-Zaid	1991	Saudi Arabia	?	O ₁ /2/72 Egypt	intranasal	?	Nil	nil
Hafez et al.	1993	Egypt	3	O ₁ /3/87 Egypt	intrader-molingual	SNT	3/3	10 weeks
						ICFT***	3/3	6 weeks
						AGID	0/3	nil
						ELISA	3/3	11 weeks
			1	O ₁ /3/87 Egypt	footpad	SNT	0/1	nil
						ICFT	0/1	nil
						AGID	0/1	nil
						ELISA	0/1	6 weeks

- * Serum Neutralisation Test
- ** Fluorescence Antibody Test
- *** Indirect Complement Fixation Test

Table 8: Reports on experimental FMD infection and virus isolation from field cases

Authors	Year	Country	Mode of infection	Dromedaries tested	Serotype	Clinical signs	Virus re-isolation
Moussa et al.	1979	Egypt	Intranasal	5	O ₁ /2/72 Egypt	nil	1-4 weeks OPF
Nasser et al.	1980	Egypt	Intranasal	2	O ₁ /2/72 Egypt	nil	1-6 days faeces
Metwally et al.	1986	Egypt	i.v.	2	O ₁ /2/72 Egypt	nil	1-3 weeks
Moussa et al.	1986	Egypt	Intradermolingual	5	O ₁ /2/72 Egypt	nil	Blood
Hafez et al.	1993	Saudi Arabia	Intranasal	?	O ₁ /2/72 Egypt	nil	?
Abou-Zaid	1991	Egypt	Intradermolingual	3	O ₁ /2/87 Egypt	yes	Blood, OPF, faeces
			Footpad	1		nil	Negative
Kumar et al.	1983	India	Natural	2	0	?	Tongue/gum from one
Moussa et al.	1987	Egypt	Natural	4	0	Vesicles ulcers swelling of limbs	Ulcers
Farag et al.	1998	Saudi Arabia	Natural	30	nil	nil	Probang

Figure 1: The forestomach system of *Tylopoda*

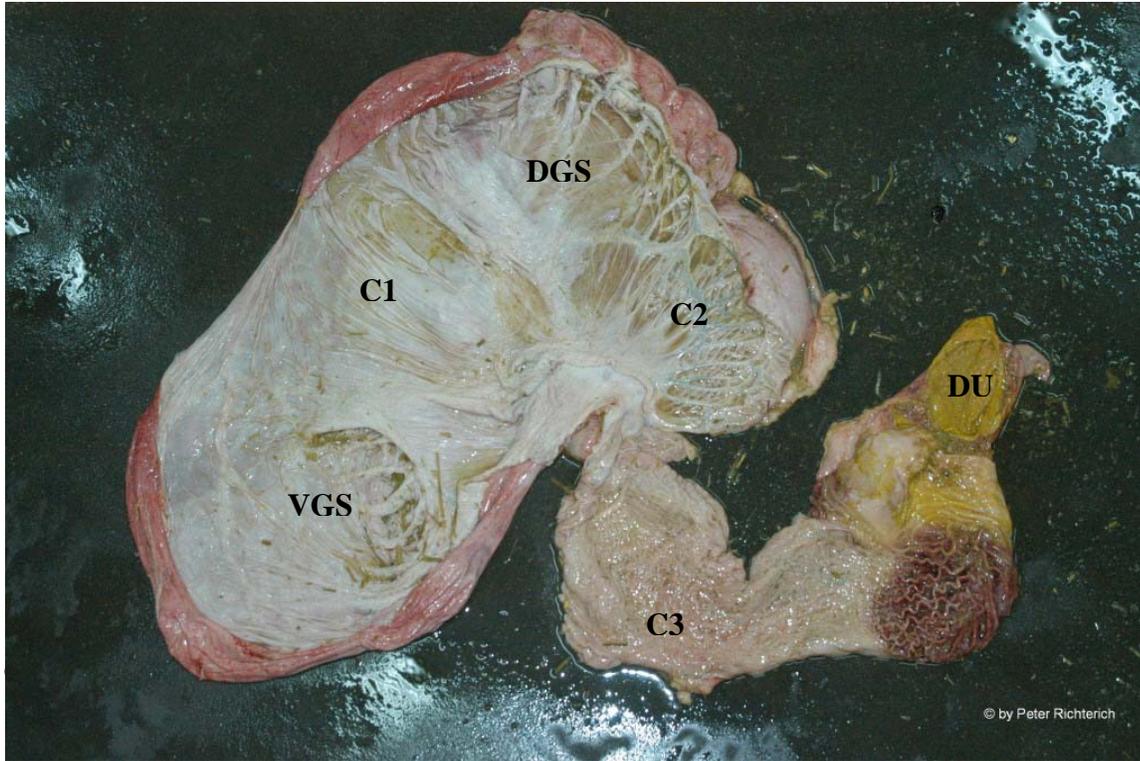


Figure 2: Feet of a llama and a dromedary



Figure 3: Mating camels



Figure 4: The rutting bull inflates and exteriorises its "doula"



Figure 5: A slippery third membrane surrounds the newborn calf



Figure 6: Distribution of *C. dromedarius* and *C. bactrianus*

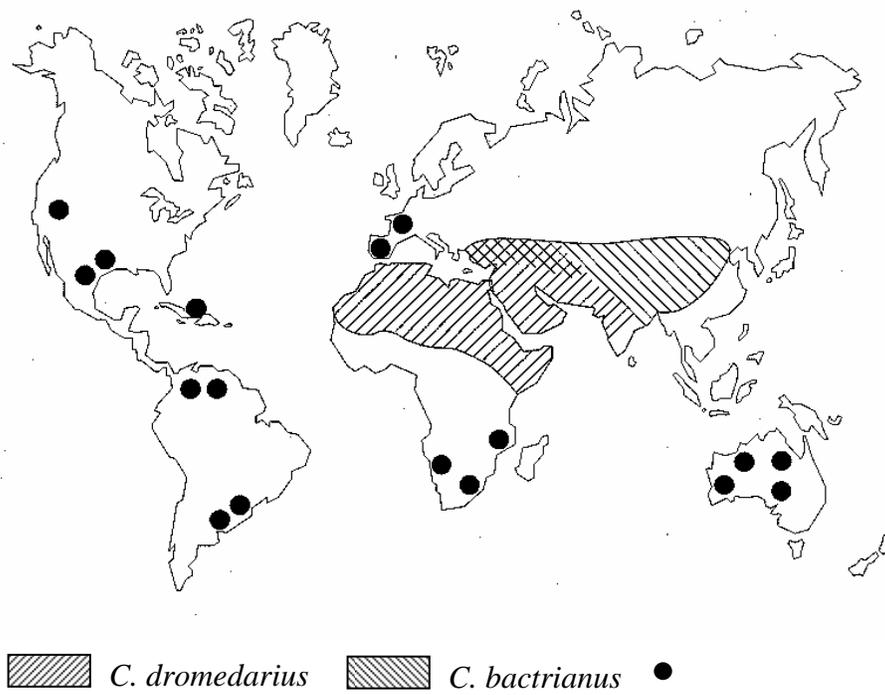


Figure 7: Subepidermolingual FMDV-inoculation of a dromedary

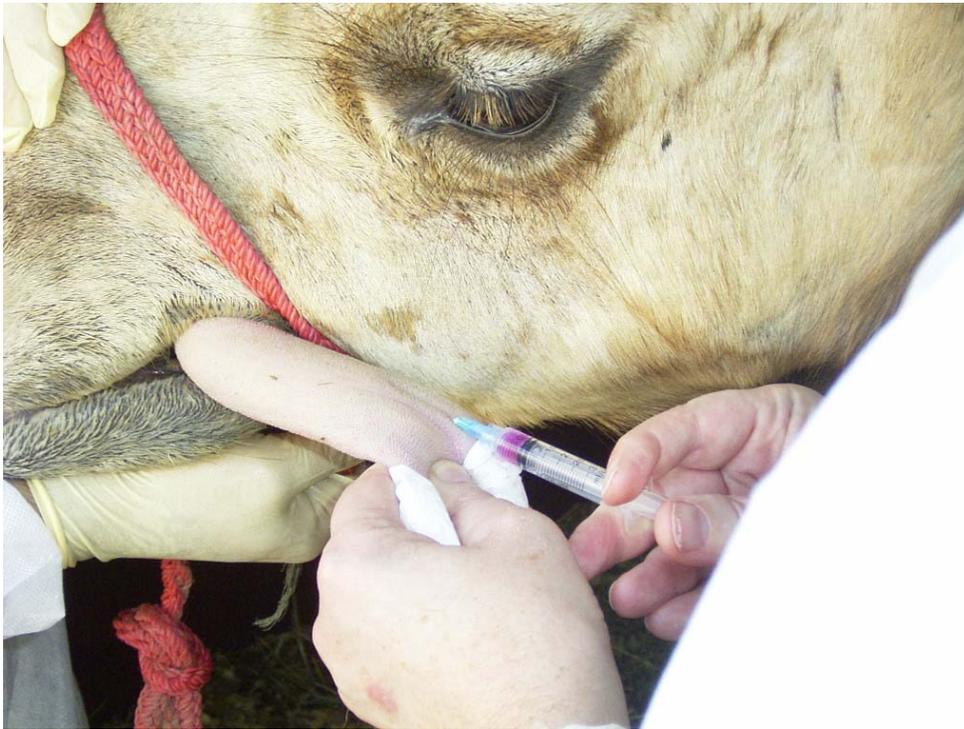
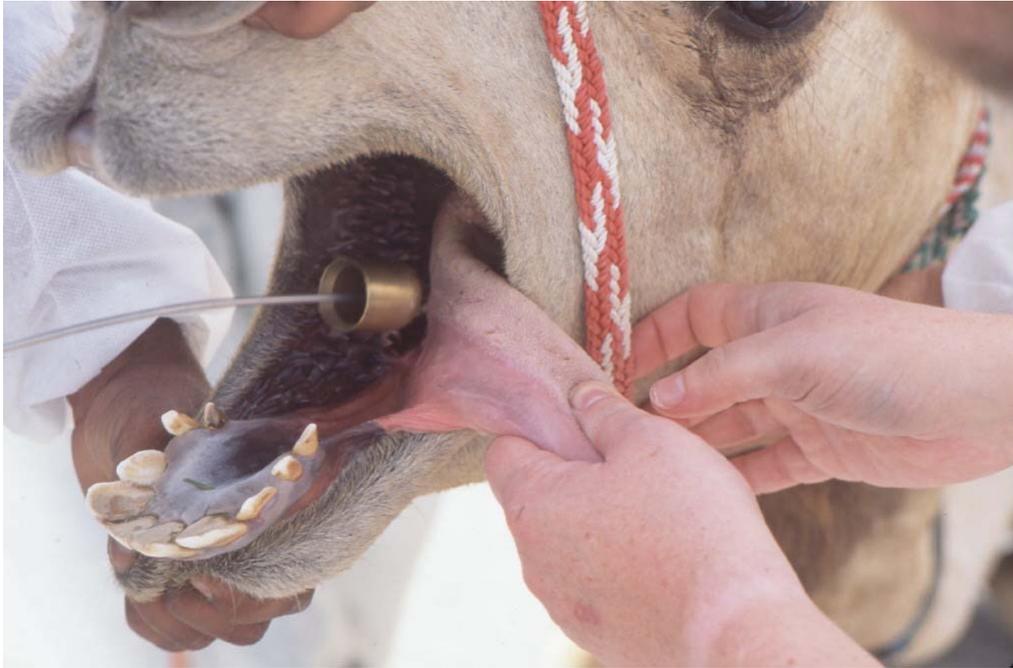


Figure 8: Typical FMD lesions on the tongue of a heifer three days after subepidermolingual inoculation



Figure 9: Probang sampling of a dromedary



Laboratory Surge Capacity - Australian Approach

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Introduction: Australia has been free of foot-and-mouth disease (FMD) since 1872, and has strong quarantine measures in place to ensure continued freedom. However, the 2001 outbreak in the United Kingdom and European Union highlighted the need to be well prepared for the threat of a major disease outbreak. In September 2002, the Council of Australian Governments held a major simulation of an FMD outbreak (exercise Minotaur) to test Australia's whole of government framework of preparedness, response and recovery in the face of this scenario. In June 2004, a second exercise (Crucible) was carried out to test the readiness of the National laboratory network and its ability to interact with the Australian Animal Health Laboratory (AAHL), which has a primary function to confirm a suspect case of FMD through exclusion diagnosis.

Materials and Methods: States or Territories were hypothetically 'infected' and responded at a decision-making level, including activating Local Disease Control Centres and State Disease Control Headquarters. 'Disease-free' States and Territories were also involved in response activities such as animal surveillance, responding to the economic and social consequences of closed international markets and cross-border transport and trade issues.

Results: As a result of these exercises a number of changes have been made to the way in which Australia will deal with an outbreak. AAHL has undergone some major changes and improvements in its platform capabilities to support FMD diagnosis and surveillance. The establishment of an emergency response plan, the introduction of a Laboratory Information Management System (LIMS) and robotic sample handling are all being instigated, along with a variety of testing options including high-throughput PCR screening.

The emergency response plan incorporates the preparation of job cards for key staff roles to be carried out during an outbreak, the training of staff in these roles and the establishment and maintenance of a staff skills database. In addition, the levels of on-site laboratory support services that will be required during an outbreak have also been reviewed and a laboratory supplies database is to be developed.

Continual improvements in the capacity of AAHL to rapidly diagnose FMD, such as, by the introduction of robot liquid handling devices to generate both high-throughput ELISA and molecular based real time PCR data linked to LIMS software, has added significantly to Australia's FMD preparedness capacity.

Further, as a direct outcome of the need for an immediate and comprehensive response to an outbreak, a new dedicated laboratory suite at AAHL has been designed and construction is soon to commence.

Discussion: Significant changes have been made to the way in which Australia will deal with an FMD outbreak. FMD outbreak simulation exercises helped to clarify those issues which would be of major importance during an outbreak. Alongside this, major progress in the ability of AAHL to respond rapidly and specifically to an FMD outbreak has significantly added to the level of Australia's FMD preparedness.

Prospects for improved laboratory diagnosis of FMD using real-time RT-PCR

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Abstract:

Definitive diagnosis of FMD requires the detection of virus, antigen or genome in clinical material. The aim was to evaluate the performance of an automated real-time RT-PCR procedure for this purpose.

Vesicular epithelia from eighteen countries were examined by ELISA, VI and RT-PCR. Retrospective analysis by RT-PCR was also performed on available material of two sample subsets collected from 'confirmed' cases during the 2001 UK FMD outbreak : firstly, samples which were negative by both ELISA and VI and secondly, others which were negative by ELISA on epithelial suspension but positive by VI. There was broad agreement between RT-PCR and VI for 79% and VI/ELISA combined for 82% of the overseas epithelial samples tested. There were no false negative results obtained with RT-PCR since all samples assigned negative by RT-PCR were also negative by VI/ELISA. However, the RT-PCR was able to detect FMDV in an additional 18% of the samples tested. Additionally, there was good agreement between the RT-PCR and ELISA/VI for the UK outbreak samples save for a group of related virus isolates from Wales. These viruses had evidently evolved during the epidemic and had a nucleotide substitution in the RT-PCR probe site, which prevented detection by RT-PCR using the routine diagnostic probe.

The ELISA and VI are deficient for specimens of poor quality where concentrations of infectious FMDV may either be low or absent. The features that influence sample quality appear to be less important for the RT-PCR as it can detect a small fragment of FMDV genomic RNA, not just live virus. Real-time RT-PCR provides an extremely sensitive and rapid procedure that contributes to improved laboratory diagnosis of FMD. However, the failure to detect the mutant FMDVs from the UK 2001 epidemic illustrates the importance of constant monitoring of representative field FMDV strains by nucleotide sequencing to ensure that the primers/probe set selected for the diagnostic RT-PCR is fit for purpose.

Introduction:

Control of outbreaks of foot-and-mouth disease (FMD) is dependent upon a system of monitoring and early detection, which requires basic familiarity with clinical signs and the ability to characterise the strain of virus responsible by laboratory tests. Definitive diagnosis of FMD requires the detection of virus, antigen or genome in clinical material. Ideally, the sample of choice should be vesicular epithelium from clinically affected animals since, during the acute stage of the disease, it is rich in virus. The World Reference Laboratory (WRL) for FMD typically receives between 400 and 700 samples annually from overseas countries (Ferris and Donaldson, 1992; Table I), including other sample types besides epithelia, e.g. epithelial suspensions, cell culture antigens, blood, throat swab (probang) and milk samples. For almost twenty years, the WRL for FMD has used an indirect, sandwich enzyme-linked immunosorbent assay (ELISA) (Roeder and Le Blanc Smith, 1987; Ferris and Dawson, 1988; OIE, 2004) to identify FMDV. However, the ELISA is not 100% sensitive. Consequently, suspensions of each specimen are also propagated in sensitive cell cultures (Ferris and Dawson, 1988) and the specificity of any isolated virus confirmed by the ELISA. Whilst such virus isolation (VI) methods are highly sensitive, they require four days before a negative result can be concluded (and reported as 'no virus detected' [NVD]). It is evident from Table I that FMDV antigen cannot be detected by ELISA and VI in approximately half the submitted samples. This has given cause for concern as to the efficiency of sample collection and dispatch and also with respect to the adequacy of the laboratory test procedures employed for their examination.

In emergencies, speed of diagnosis (clinical and laboratory confirmation) is of paramount importance to control spread and eradicate disease. Approximately, 90% of positive epithelial samples received during the 2001 UK FMD outbreak were so defined by the antigen ELISA on prepared suspensions, the remainder being serotyped after amplification and isolation of virus following cell culture passage. Negatives could only be classified following double passage in cell culture, which took 4 to 5 days. Consequently, the introduction by the UK Government towards the end of March of a 24/48 hour culling policy (all animals to be slaughtered on infected premises within 24 hours of diagnosis and those on neighbouring premises within 48 hours) meant that confirmation of disease was subsequently made by clinical judgement alone. This policy, although playing a critical role in controlling disease, caused huge controversy and provoked much debate on the likelihood of many animals being slaughtered unnecessarily, fuelled by the finding that neither virus nor antibody could be detected in samples received from many of the confirmed cases.

Recently, the development of a real-time reverse transcription polymerase chain reaction (RT-PCR) procedure has provided an additional tool which can be used for FMD diagnosis (Reid *et al.*, 2002). Furthermore, this real-time RT-PCR method can be automated allowing increased throughput of samples with fewer user-dependent steps (Reid *et al.*, 2003). The authors have compared the performance of a fully automated real-time RT-PCR (Reid *et al.*, 2003) with VI and ELISA for the detection of FMDV on the majority of epithelium samples received at the WRL for FMD from overseas during a recent eighteen-month period (August 2002 until January 2004) and on two subsets from confirmed cases from the recent UK outbreak : firstly, samples which were negative by both ELISA and VI and secondly, others which were negative by ELISA on epithelial suspension but positive by VI.

Materials and Methods:

Three hundred and thirty four samples of vesicular epithelium were received from eighteen countries during the period of the study and were examined by ELISA, VI and RT-PCR (Table II). Upon receipt, the pH of the transport buffer containing epithelial tissue was estimated using phenol red indicator. A suspension of the epithelium (ES) was made in 0.04 M phosphate buffer (ideally this should be done using a 10% concentration (w/v), but during this study a lower concentration was frequently used due to paucity of material). A 1.4 ml aliquot was taken for the antigen detection ELISA (Ferris and Dawson, 1988; OIE, 2004) whilst 0.2 ml aliquots were used to inoculate cell cultures of primary bovine thyroid (Snowdon, 1966) and IB-RS-2 cells (De Castro, 1964) (five tubes of each cell type per specimen). Additionally, 0.2 ml of ES were added to a 1 ml aliquot of Trizol solution and stored at -80°C until assay. Real-time RT-PCR was usually performed on the ES within one to two days of preparation, with a diagnostic result typically being obtained within a single working day.

Three hundred and eighty samples from 331 separate premises ('confirmed' cases) in the UK, which had been found to be negative by both ELISA and VI in 2001, and 199 samples from a further 188 separate premises, which had been found to be positive by VI but negative by ELISA on epithelial suspension, were re-evaluated by RT-PCR. In the majority of cases, the original epithelial suspension (which had been stored at -80°C) was used but new suspensions were prepared for others, while aliquots of the transport buffer were examined in a few cases (in the absence of both stored suspension and submitted epithelium).

The real-time RT-PCR assay used in this study has been described elsewhere (Reid *et al.*, 2003). Briefly, total nucleic acid was extracted from the solution of Trizol/ES using a fully automated robot system. This robot was then also used to pipette viral nucleic acid into a reverse transcription mix for reverse transcription and complementary deoxyribonucleic acid into PCR reaction mix (including an oligonucleotide primers/probe set targeting the internal ribosomal entry site of the FMDV genome). Thermal cycling and concurrent fluorescence detection were performed and a cycle detection threshold (i.e. the cycle at which a target sequence is detected [C_T]) was recorded for each test sample (Oleksiewicz, Donaldson and Sorensen, 2001). Sequencing of viruses was performed as described by Knowles and Samuel (2003).

Results:

Samples submitted to the WRL for FMD from overseas

The results achieved by ELISA, VI in cell culture and RT-PCR are summarised in Table II for comparison of the performance of the three assays. FMDV was detected in 195 samples by VI (58.4%), in 125 by antigen ELISA (37.4%) and in 204 samples by VI/ELISA combined (61.1%). These viruses represented four out of the seven FMDV serotypes (O, A, SAT 2 and Asia 1), although the majority (68.6%) were of serotype O.

There was broad agreement between RT-PCR and VI for 265/334 (79.3%) and VI/ELISA combined for 274 (82%) of the epithelial samples tested when a cut-off C_T value of '<39' was used to assign the samples as either FMDV positive or negative (results not shown). In general, VI positive samples corresponded to a real-time RT-PCR C_T value of <35, with only eight VI positive samples producing a C_T value in the range ≥ 35 -<39. Furthermore, there were no false negative results obtained with RT-PCR since all samples assigned negative by RT-PCR were also negative by VI/ELISA. Of particular interest was the finding that RT-PCR was able to detect FMDV in an additional sixty (18%) of the samples tested. Fresh ribonucleic acid (RNA) was prepared from these epithelial suspensions and the RT-PCR was repeated with similar results confirming the presence of FMDV genome in these samples. In addition, there were seventeen 'suspect positive' samples that generated C_T values in the range ≥ 39 to <50. Repeat procedures were also performed for these samples with similar results, suggesting that FMDV was also present in these samples although the FMDV copy number was lower than the diagnostic threshold. The majority (96.9%) of VI positive samples were detected on the first passage in cell cultures (within two days). However, in order to ensure detection of infectious FMDV

and to define NVD samples, a second cell culture passage was required (in total, up to four days). Indeed six samples required this second passage in cell culture before the presence of FMDV was demonstrated.

Calculations of the relative sensitivity and specificity of the RT-PCR at unit incremental increases in C_T value in comparison to either VI alone or to VI/ELISA combined are recorded in Table III. It shows that 100% relative sensitivity was achieved by the RT-PCR at a C_T value of <39, which justifies using this as the ideal diagnostic cut-off for the RT-PCR, since it is the lowest C_T value that achieves this.

2001 UK FMD outbreak samples – ELISA/VI negative

It can be seen from Table IV that 367 samples produced a C_T value of 50 (FMDV genome not detected by RT-PCR). However, 7 sample suspensions yielded C_T values of <35, and 2 in each of the ranges >35-<40, >40-<45 and >45-50. Subsequent to these findings, attempts were made to isolate virus from available epithelial suspensions, with success (i.e. positive type O FMDV) in 3 out of 5 samples from the first category (paucity of material precluded examination of 2 samples) and 0, 1 and 0 samples from the other categories, respectively.

2001 UK FMD outbreak samples – ELISA negative/VI positive

FMDV genome was detected in 179/199 samples by RT-PCR (for simplicity, using <40 as the C_T value cut-off point; Table V). A further 10 and 2 samples produced C_T values in the range >40-<45 and >45-<50, respectively, while the remaining 8 samples generated a C_T value of 50. FMDV failed to be isolated in 3/6, 0/2 and 4/5 of the samples which were available from each category for subsequent cell culture passage. The resulting cell culture grown antigens were tested by RT-PCR with positive results, except one virus (UKG 13795/2001, originating from Crickhowell, Powys, Wales), which consistently yielded a C_T value of 50 from replicate tests. This virus isolate was sequenced to see if the analysis might indicate why this virus was undetected by RT-PCR.

It was previously known that the Pan Asia type O FMDVs, whilst detectable by the real-time RT-PCR assay, contain a nucleotide substitution within the TaqMan® probe region (Fig.1). The causative FMDV strain of the UKG outbreak contains an additional substitution resulting in two mis-matches with the universal probe. The Welsh virus was found to have a further, additional nucleotide substitution resulting in three mis-matches within the probe region. This was evidently sufficient to make it unrecognised by the conventional diagnostic probe.

This prompted a mini-study to examine the reaction of other virus isolates from the Crickhowell region (all of which had originally typed as positive by ELISA on epithelial suspension) in RT-PCR procedures using either the conventional diagnostic probe or one (P282T) designed to match the Welsh mutant virus. The results of these tests are shown in Table V1 and indicate that all these isolates were related, failed to be detected by the RT-PCR using the conventional diagnostic probe but were recognised by the new probe.

Discussion:

The ELISA and VI have been recommended laboratory procedures for FMD diagnosis for nearly twenty years based on their suitability to detect the presence of FMDV antigen in tissue samples. It is evident from the present study that these procedures are deficient for certain specimens. The results for the overseas samples show that while all VI/ELISA positive FMDV samples were also positive by real-time RT-PCR (100% sensitivity at a C_T cut-off value of <39) (Table III), FMD viral genome was detected in a significant proportion of the samples examined in which FMDV antigen was not.

The low relative specificity values (a range in value from 63.1% down to 38.1% depending upon cut-off) of the RT-PCR result from the doubtful premise that the combination of VI and ELISA is 100% efficient for detection of FMDV. The authors are confident that this is not the case, that the RT-PCR procedure has specific reaction for FMDV genome and that the low value is actually related both to the performance of the comparative assays and to the quality of the samples submitted.

If one considers what the VI and ELISA procedures actually measure then it is evident that their effectiveness for diagnostic use is inherently compromised. Virus isolation is dependent upon the presence of infectious virus in sample submissions and while the ELISA can detect both infectious and non-infectious FMD viral antigen, it is dependent upon the antigen being present in sufficient concentration (1-2 ng/ml of antigen or 5-6 log₁₀/ml of live virus) to work (N.P. Ferris, unpublished results). If neither of these two conditions is met then FMDV will not be recognised.

Ideally, vesicular epithelium should be collected from an animal during the acute stage of FMD when the concentration of virus associated with the sample is high. Unfortunately, samples submitted to the WRL for FMD from overseas are very often collected late in the course of disease when the amount of virus may either be waning or indeed be absent after clearance. Delayed reporting of

disease and late sample collection can arise for a variety of reasons, e.g. a lack of resources, communication difficulties in areas of rugged terrain or a low perceived importance of FMD. Delays can be worse depending on who has responsibility for sample collection; in some countries it is local staff and in others countries staff from a specialised laboratory (often many miles away) carry out the collection (Ferris et al., 1992). Secondary bacterial infection is a common sequel to virus infection and can lead to a reduction in FMDV infectivity. Additionally, samples may be in transit for lengthy periods and subjected to physico-chemical stresses (e.g. elevated temperatures between collection and laboratory receipt) with the result that on arrival only small amounts of infectious virus, at best, may be present.

FMDV survival can be adversely affected by harsh environmental conditions, including excessive temperature, extremes of pH, disinfectants and desiccation. It is therefore advantageous to protect samples during the interval between collection and testing (especially for VI). Their dispatch to reference laboratories should follow specific guidelines to ensure their security (Kitching and Donaldson, 1987). These features, which influence sample quality, are likely to be less important for the RT-PCR as it can detect a small fragment of FMDV genomic RNA, not just live virus.

There was broad correlation between the results achieved by RT-PCR examination of the UK outbreak sample subsets with the original (2001) diagnostic results derived from ELISA/VI suggesting that definitive sample classification of both positivity and negativity by RT-PCR is achievable within a relatively short timescale. However, the failure to detect the group of related virus isolates from Wales (using the routine diagnostic probe), which had evidently evolved during the epidemic and had a further nucleotide substitution in the RT-PCR probe site, illustrates the importance of constant monitoring of representative field FMDV strains by nucleotide sequencing to ensure that the primers/probe set selected for the diagnostic RT-PCR is fit for purpose.

Conclusions:

- The real-time RT-PCR currently used at the WRL for FMD provides an extremely sensitive and rapid additional procedure for improved laboratory diagnosis of FMD

Recommendations:

- No specific recommendations

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References:

De Castro, M.P. 1964. Behaviour of the foot-and-mouth disease virus in cell cultures: susceptibility of the IB-RS-2 line. *Arch. Inst. Biol., São Paulo*, 31: 63-78.

Ferris, N.P. & Dawson, M. 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet. Microbiol.*, 16 (3): 201-209.

Ferris, N.P. & Donaldson, A.I. 1992. The world reference laboratory for foot and mouth disease: a review of thirty-three years of activity (1958-1991). *Rev. Sci. Tech. Off. Int. Epiz.*, 11 (3): 657-684.

Ferris, N.P., Donaldson, A.I., Shrestha, R.M. & Kitching, R.P. 1992. A review of foot and mouth disease in Nepal. *Rev. Sci. Tech. Off. Int. Epiz.*, 11 (3): 685-698.

Kitching, R.P. & Donaldson, A.I. 1987. Collection and transportation of specimens for vesicular virus investigation. *Rev. Sci. Tech. Off. Int. Epiz.*, 6 (1): 263-272.

Knowles, N.J. & Samuel, A.R. 2003. Molecular epidemiology of foot-and-mouth disease virus. *Virus Res.*, 91: 65-80.

OIE (World Organisation for Animal Health) 2004. Manual of diagnostic tests and vaccines for terrestrial animals. 5th Ed. Parts I and II. OIE, Paris, 1178 pp.

Oleksiewicz, M.B., Donaldson, A.I. & Alexandersen, S. 2001. Development of a novel real-time RT-PCR assay for quantitation of foot-and-mouth disease virus in diverse porcine tissues. *J. Virol. Meth.* 92 (1): 23-35.

Reid, S.M., Ferris, N.P., Hutchings, G.H., Zhang, Z., Belsham, G.J. & Alexandersen S. 2002. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *J. Virol. Meth.*, 105 (1): 67-80.

Reid, S.M., Grierson, S.S., Ferris, N.P., Hutchings, G.H. & Alexandersen, S. 2003. Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J. Virol. Meth.* 107 (2): 129-139.

Roeder, P.L. & Le Blanc Smith, P.M. 1987. Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. *Res. Vet. Sci.* 43: 225-232.

Snowdon, W.A. 1966. Growth of foot-and-mouth disease virus in monolayer cultures of calf thyroid cells. *Nature*, 210 (40): 1079-1080.

Table 1. Number of samples received by the WRL for FMD from overseas between 1994 and 2003 and the numbers (and percentages) found to be positive or negative for FMD virus by passage in cell culture and antigen detection ELISA

Year	Countries ^a	Number of samples		
		Total	Positive	Negative
1994	29	655	348 (53%)	307 (47%)
1995	29	697	421 (60%)	276 (40%)
1996	31	535	238 (44%)	297 (56%)
1997	29	526	334 (63%)	192 (37%)
1998	27	441	248 (56%)	193 (44%)
1999	43	595	357 (60%)	238 (40%)
2000	29	434	209 (48%)	225 (52%)
2001	32	619	197 (32%)	422 (68%)
2002	27	390	162 (42%)	228 (58%)
2003	19	475	262 (55%)	213 (45%)
Total		5,367	2,776 (52%)	2,591 (48%)

^a number of countries which submitted samples to the WRL for FMD

Fig. 1. Nucleotide sequence changes within the TaqMan® probe site of the Pan Asia type O FMD virus, the causative UKG 2001 FMD virus and the related Welsh viruses compared to the FMD virus consensus sequence.

TaqMan® Probe Site

FMD virus consensus sequence: 5'-GGATGCCCTTCAGGTACCCCGAGG-3'

Pan Asia Strain: 5'-GGATGCCCTTCAGGTACCC~~T~~GAGG-3'

UKG 2001 FMD virus: 5'-GGATGCCCTT~~T~~AGGTACCC~~T~~GAGG-3'

Related Welsh UKG 2001 FMD virus: 5'-GGATGCCCTT~~TAA~~GTACCC~~T~~GAGG-3'

Nucleotide substitutions are indicated **X**

Table II. Detection of FMD virus in suspensions of submitted epithelia achieved by ELISA, passage in cell culture (VI), ELISA and VI combined and by RT-PCR (August 2002-January 2004)

Country	No	ELISA		VI ^b		ELISA/VI		RT-PCR for FMD virus (C _T ^c value)				
		FMDV	NVD ^a	FMDV	NVD	FMDV	NVD	<35	>35	>39	>45	50
								-	-	-		
								<39	<45	<50		
Bhutan	58	2	56	17	41	17	41	36	4	6	-	12
Botswana	5	-	5	-	5	-	5	-	-	1	-	4
Burundi	7	4	3	5	2	5	2	1	4	-	1	1
Hong Kong	7	1	6	3	4	3	4	6	1	-	-	-
Iran	43	17	26	31	12	31	12	32	1	2	-	8
Iraq	18	1	17	6	12	7	11	11	1	1	-	5
Laos	35	18	17	33	2	33	2	33	2	-	-	-
Lebanon	5	3	2	3	2	5	-	5	-	-	-	-
Libya	1	1	-	1	-	1	-	-	1	-	-	-
Malaysia	12	8	4	12	-	12	-	12	-	-	-	-
Nepal	4	4	-	4	-	4	-	4	-	-	-	-
Pakistan	90	36	54	41	49	44	46	59	5	6	1	20
PAT ^d	1	-	1	1	-	1	-	1	-	-	-	-
Philippines	9	8	1	7	2	8	1	8	1	-	-	-
Thailand	7	6	1	7	-	7	-	7	-	-	-	-
Turkey	24	10	14	17	7	18	6	21	-	-	-	3
UAE ^e	3	1	2	3	-	3	-	3	-	-	-	-
Vietnam	5	5	-	4	1	5	-	5	-	-	1	-
Total	334	125	209	195	139	204	130	244	20	16	1	53

^a NVD, no virus detected

^b VI, serotype of FMD virus isolated by passage (VI) in cell culture characterised by ELISA

^c C_T, threshold cycle value

^d PAT, Palestinian Autonomous Territories

^e UAE, United Arab Emirates

Table III. Relative sensitivity of the real-time RT-PCR for FMD virus at successive threshold cycle value cut-off points in comparison with VI in cell culture or VI plus ELISA combined

Threshold cycle Value cut-off	VI ^a		VI/ELISA combined	
	Sensitivity	Specificity	Sensitivity	Specificity
<35	95.9	59.0	96.1	63.1
<36	97.0	56.1	98.0	60.0
<37	99.0	54.0	99.0	57.7
<38	99.5	51.1	99.5	54.6
<39	100.0	50.4	100.0	53.8
<40	100.0	46.0	100.0	49.2
<41	100.0	43.2	100.0	46.2
<42	100.0	42.4	100.0	45.4
<43	100.0	40.3	100.0	43.1
<44	100.0	40.3	100.0	43.1
<45	100.0	38.8	100.0	41.5
<46	100.0	38.8	100.0	41.5
<47	100.0	38.8	100.0	41.5
<48	100.0	38.1	100.0	40.8
<49	100.0	38.1	100.0	40.8
<50	100.0	38.1	100.0	40.8

^a VI, virus isolation in cell culture with specificity of cytopathic effect confirmed by ELISA

Table IV. Detection of FMD virus by RT-PCR in suspensions of submitted epithelia from clinically confirmed cases found to be ELISA/VI negative after sample receipt in 2001

Animal	No of samples	RT-PCR for FMD virus (C_T^a value)				50
		<35	>35-<40	>40-<45	>45-<50	
Sheep	253	2	2	2	2	245
Cattle	98	5	-	-	-	93
Pig	14	-	-	-	-	14
Cattle + Sheep	5	-	-	-	-	5
Deer	1	-	-	-	-	1
Not known	9	-	-	-	-	9
Total	380	7	2	2	2	367

^a C_T , threshold cycle value

Table V. Detection of FMD virus by RT-PCR in suspensions of submitted epithelia from clinically confirmed cases found to be ELISA negative/VI positive after sample receipt in 2001

Animal	No of samples	RT-PCR for FMD virus (C_T^a value)				50
		<35	>35-<40	>40-<45	>45-<50	
Sheep	131	87	33	6	1	4
Cattle	60	41	12	3	-	4
Cattle+Sheep	2	2	-	-	-	-
Goat	3	1	-	1	1	-
Not known	3	2	1	-	-	-
Total	199	133	46	10	2	8

^a C_T , threshold cycle value

Table VI. Detection of 'mutant' FMD virus by RT-PCR in suspensions of submitted epithelia from clinically confirmed cases in Powys, Wales found to be positive after sample receipt in 2001

Sample ref	Animal	Origin	FMD No.	RT-PCR for FMD virus (C_T^b value)	
				P269R ^c	P282T ^d
UKG 13795/2001 ^e	Sheep	Crickhowell, Powys	1861	50	35.81
UKG 13705/2001	Cattle	Crickhowell, Powys	1846	50	32.35
UKG 13708/2001	Cattle	Crickhowell, Powys	1848	50	31.52
UKG 13724/2001	Cattle	Powys	1852	50	29.75
UKG 13734/2001	Cattle	Powys	1857	50	27.6
UKG 13777/2001	Cattle	Powys	1860	50	40.91
UKG 13798/2001	NK ^f	Crickhowell, Powys	1868	50	31.06
UKG 13949/2001	Cattle	Abergavenny, Powys	1889	50	32.85
UKG 14004/2001	Cattle	Crickhowell, Powys	1896	50	31.46
UKG 14221/2001	Cattle	Abergavenny, Powys	1938	50	27.59
UKG 14339/2001	Cattle	Crickhowell, Powys	1945	50	31.08

^a OD, optical density value originally achieved on the epithelial suspension

^b C_T , threshold cycle value

^c P269R, the normal diagnostic probe

^d P282T, 'UK' probe

^e UKG 13795/2001 serotyped on first passage cell culture antigen, all other virus isolates listed serotyped on epithelial suspension

^f NK, not known

Use of automated RT-PCR to detect FMDV in milk

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Abstract:

Introduction: Foot-and mouth disease virus (FMDV) excreted in milk can play a role in the transmission of FMD. Using samples collected from experimentally infected cattle, the aim of this study was to assess the performance of automated RT-PCR as a diagnostic tool for the detection of FMDV in milk. **Materials and Methods:** Fore- and machine milk were collected over 4 weeks from 2 dairy cows inoculated with FMDV O UKG 34/2001, and from 2 in-contact cows. Clinical signs were closely monitored and correlated to the presence of FMDV in serum, "probangs", nasal and saliva swab samples. The whole, skim, cellular debris and cream components of the milks were tested by automated real-time RT-PCR and virus isolation (VI). Additional experiments investigating the effects of temperature stability and preservative treatment further evaluated RT-PCR for routine diagnosis of FMD in milk samples that might be submitted to the WRL. **Results:** The onset of severe clinical signs of FMD in all 4 cattle correlated with high levels of viraemia in the serum, and presence of FMDV in "probangs", saliva and nasal swabs. The RT-PCR results matched closely with those for VI in detecting FMDV in all milk components and generally coincided with, but did not precede, the onset of the clinical signs. RT-PCR was able to detect FMDV in milk up to day 23 post infection. The detection limit of FMDV in milk was greater by RT-PCR than VI. Furthermore, in contrast to VI, RT-PCR detected virus genome following heat treatment that mimicked pasteurisation. RT-PCR also detected FMDV in preservative (Bronopol/Kathon) treated milk. **Discussion:** This study shows that automated RT-PCR could be used for laboratory detection of FMDV in milk. The ability of RT-PCR to screen bulk milk tank samples and/or dairy herds may play a role in the control of FMD.

Introduction:

Foot-and-mouth disease (FMD) is characterised in dairy cows by fever, vesicular lesions, pyrexia and a reduction in milk yield. FMD virus (FMDV) excreted in milk from infected animals is thought to have played a role in the transmission of FMD in previous outbreaks (Dawson, 1970; Donaldson, 1997).

Milk represents an ideal medium for laboratory diagnosis of FMD that may be particularly appropriate for the surveillance of disease in dairy herds. Previous studies have also demonstrated that the FMD viroaemia can precede the presentation of clinical signs in experimentally infected animals (Burrows, 1968; Blackwell and Hyde, 1976; Blackwell et al., 1982) so that an assay detecting the presence of FMDV could potentially be used as a preclinical diagnostic tool.

Of the established diagnostic methods for FMDV, only virus isolation (VI) can be used to detect FMDV in milk samples successfully (Blackwell et al., 1982). The antigen-detection ELISA (Ferris and Dawson, 1988) currently used at the OIE/FAO World Reference Laboratory for FMD, Pirbright, is unsuitable for testing milk. Previous studies have demonstrated that automated RT-PCR is a valuable diagnostic procedure for the laboratory detection of FMDV in vesicular epithelial tissue (Reid et al. 2003; Shaw et al., 2004) and in serum, nasal swabs and oesophageal-pharyngeal scrapings ("probangs"; Zhang and Alexandersen, 2003). The aim of this study was to evaluate the performance of RT-PCR methods for the detection of FMDV in milk.

Materials and Methods:

Experimental infection and monitoring of cows with FMDV

Two adult milking cows held in separate pens were inoculated (day 0) by intra-dermolingual injection with 0.5 ml of an FMDV serotype O PanAsia strain at a titre of $10^{5.9}$ TCID₅₀/ml per animal. Two other cows (UQ59 and UQ61) were left in-contact with the inoculated cows (UQ58 and UQ60 respectively). All four cows were of Friesian-Holstein breed. Each cow was machine milked daily for the duration of the study (except on days 11 and 13) and fore-milk was also collected daily by hand from the quarters of each cow at morning milking. On each day the quarter milks were pooled and an aliquot of this 'whole' fore-milk, and of the whole machine milk, was separated by centrifugation into skim milk, cream and cellular components (cellular debris). In addition to milk samples, serum was collected at -3, 0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 18, 23 and 28 days post infection (dpi). Nasal swabs, mouth swabs (saliva) and "probang" samples were also collected from each cow on most days. Aliquots of these samples were also placed into buffer for RT-PCR. The clinical signs indicative of FMDV infection were monitored daily. Feet and teats were closely inspected, body temperatures were measured and milk yields recorded. One cow (UQ61) was euthanased on day 16

due to lameness while the other three cows were killed on day 28. A wide range of tissue samples were collected at post-mortem for subsequent analysis.

Optimisation of the automated RNA extraction protocol for milk samples

The optimal RNA extraction protocol for milk components was determined in preliminary experiments using uninfected whole milk spiked with another FMDV PanAsia isolate. QIAGEN robotic apparatus was used to process the virus dilutions added to the extraction buffers: TRIzol Reagent® (Invitrogen), Lysis/Binding Buffer (Roche) and Buffer AL Lysis buffer (QIAGEN). An automated programme on a QIAamp® Virus BioRobot® 9604 extracted nucleic acid from each sample and a BioRobot® 3000 performed all subsequent liquid handling steps for the RT and PCR procedures by transferring volumes similar to those described previously (Reid et al., 2003). Other details of the reverse transcription procedure and PCR reaction mixture were as described previously (Reid et al., 2003). PCR amplification was achieved with the programme previously used by Reid et al. (2002; 2003). As a comparison between different robotic equipment, RT-PCR was also carried out on the virus dilutions added to TRIzol Reagent® and to Lysis/Binding Buffer using automated programmes on a MagNA Pure LC robot (Roche) which were similar to those described previously (Reid et al., 2003).

Quantitative real-time RT-PCR testing of milk, serum and nasal swab samples

RNA extraction and subsequent RT and PCR set up were performed on the fore- and machine whole milk from each cow and on the corresponding components of skim milk, cells and cream using the QIAGEN robotic apparatus. Quantification of FMDV was achieved using a dilution series of an RNA standard (RNA transcribed *in vitro* [MegaScript, Ambion] from a plasmid clone containing an internal ribosomal entry site [IRES] fragment of the FMDV genome run in parallel in each RT-PCR assay plate [Zhang et al., 2004]).

Detection of FMDV in tissues collected at post-mortem

Small pieces of several tissues including soft palate, pharynx, tonsil, and mammary gland sinus were collected at post-mortem (at 28 dpi) from animals UV58, UV59 and UV60 and quantitative RT-PCR was performed on these tissues using reagents from a MagNA Pure LC mRNA Extraction Kit II ([Tissues], Roche) with automated programmes on a MagNA Pure LC robot which were similar to those described previously (Zhang and Alexandersen, 2004).

Virus isolation

Comparative titrations of the samples were performed in primary calf thyroid cells (CTY; Snowdon, 1966). The FMDV specificity of random samples producing a cytopathic effect (CPE) was confirmed by ELISA (Ferris and Dawson, 1988) and the titre of the virus stock was expressed as 50% tissue culture infective doses (TCID₅₀/ml; Kärber, 1979).

Dilution of FMDV in uninfected milk

Five samples of whole milk collected on days 3 and 8 of the experiment were diluted in log₁₀ steps from undiluted to 10⁻⁷ in uninfected milk. Each dilution plus a negative milk control was inoculated onto CTY cell culture monolayers and tested by the optimised real-time RT-PCR procedure in order to compare the end-point detection limit of FMDV in milk of the two procedures.

Effect of temperature upon the ability of RT-PCR and VI to detect FMDV in milk

The temperature stability of FMDV in milk was tested by RT-PCR and VI on whole and skim milk spiked with an FMDV isolate. Aliquots were stored at 4°C, room temperature and at 37°C for analysis by quantitative RT-PCR and VI. A thermal cycler also heated aliquots of the whole and skim milk at 72°C for 5 sec, 15 sec, 25 sec, 1 min and 5 min and at 95°C for 5 sec. The ability of the assays to detect FMDV in milk samples that had been treated with temperature conditions that mimic pasteurisation was also investigated. Aliquots of infected whole milk samples from the experiment (pre-pasteurisation) were subjected to a pasteurisation programme in a thermal cycler of 4°C for 5 min followed by 72°C for 25 sec and stored at -80°C. The pre- and post-pasteurisation samples were tested by the VI and optimised quantitative real-time RT-PCR procedures.

Effect of a common preservative solution on the performance of real-time RT-PCR

An experiment investigated whether addition of a milk preservative solution (PS) commonly used in the UK (c8.3% Bronopol/ c25.0% Kathon CG, Wychem Limited, UK) to FMDV-infected milk inhibited detection of viral genome by the real-time RT-PCR. The PS was added (0.25, 0.5 and 1.0 % v/v respectively) to three aliquots of 'spiked' whole milk (protocols recommend that 50 µl of PS is added per 10 ml of sample solution). No preservative was added to a fourth 'spiked' whole milk aliquot. The aliquots were separated by centrifugation into skim milk, cream and cell components and together with the whole milk component were tested by RT-PCR.

Results:

Experimental infection of dairy cows with FMDV

All four cows developed severe clinical signs consistent with FMD (Figure 1). Epithelial lesions first appeared on the feet and udder of the directly inoculated cows before the in-contact animals. Both inoculated animals had lesions on all feet by day 2 and lesions appeared on all teats of the donors by day 3 and 5 respectively. Lesions were observed on the feet of the in-contact cows by days 4 to 5 and 5 to 6 respectively (Figure 1). Body temperature was monitored daily. All cows showed evidence of short-lived pyrexia (temperature > 40°C) on 2, 1 and 5 dpi for cows UV58, UV60 and UV61 respectively. Animal UV59 had a maximum body temperature of 39.4°C on day 5 post infection. After these initial periods of post infection pyrexia, the temperature readings from all 4 cattle were consistently within 38.0 to 39.0°C. The daily milk yield was also affected by FMDV infection. Compared with the normal average milk yield (days -3 to -1), there was a maximum reduction of 20.5 % on 4 and 5 dpi and 65.3 % on 8 dpi for the inoculated cows UV58 and UV60, and 41.3 % on 8 dpi and 62.1 % on 10 dpi for the in-contact cows UV59 and UV61 respectively. The whole milk collected from cows UV60 and UV61 became noticeably clotted.

There was generally a close correlation between the results of the RT-PCR and VI on the serum and nasal swabs. Virus was first detected in the serum of both inoculated cows by RT-PCR on day 1 (days 1 and 2 by VI) and in the serum of both in-contact cows using both procedures on day 4 (Figure 1). FMDV was detected daily in serum for a period of 3 to 5 days in cows UV58, UV 59 and UV61. Cow UV60 similarly showed a daily positive response lasting 3 days but FMDV was subsequently detected in the serum of this cow on some days by RT-PCR and VI.

Nasal swabs from all cows were positive by RT-PCR on all days from 2 to 10 inclusive and positive by VI in at least two cows on days 2 through to 7. Virus was not isolated in the nasal swabs collected after day 7 but low copy numbers were detected by RT-PCR in the nasal swab of cow UV58 on day 23 and in the swab from cow UV60 on day 18. Mouth swabs (saliva) of cows UV58, UV59 and UV60 were positive by VI on day 2 and virus was isolated in cell culture from the swabs of all four cows on day 4 (data not shown). "Probang" samples of all four cows were positive on days 2 and 4 and virus was isolated from the "probangs" of cow UV58 through to day 28 (data not shown).

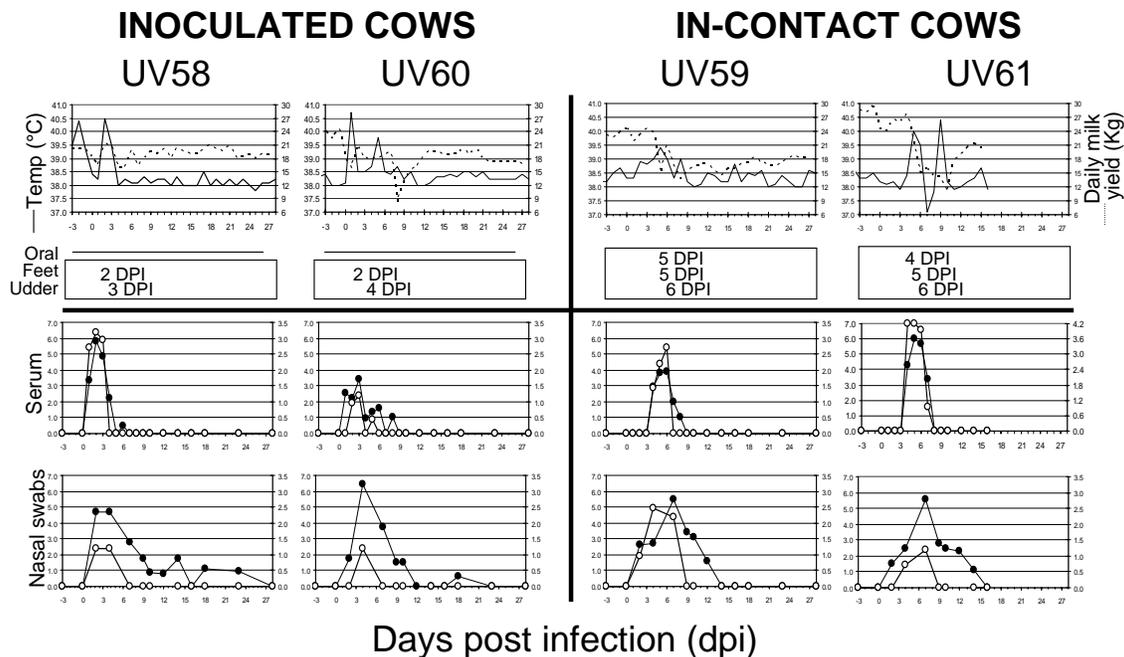


Figure 1: Infection of 4 dairy cows (UV58-61) with FMDV. Body temperature and daily milk yield were monitored over the duration of the study (-4 to 28 days post infection [dpi]). Animals were also observed daily for vesicular lesions indicative of FMDV infection: the timing (dpi) of the first occurrence of these lesions at three epithelial sites is indicated (oral lesions not shown for directly inoculated cattle). The presence of FMDV in serum and nasal swab samples was monitored by real time RT-PCR (●) and virus isolation (○). Values shown are log₁₀ FMDV copy number and log₁₀ FMD viral titre for RT-PCR and VI methods respectively.

Optimisation of automated real-time RT-PCR for detection of FMDV in milk

The performance of two robotic systems (QIAGEN QIAamp[®] Virus BioRobot[®] 9604: Figure 2A and MagNA Pure LC: Figure 2B) to prepare RNA template from milk samples was compared using 3 proprietary extraction/lysis buffers (QIAGEN AL buffer was not tested on the MagNA Pure LC robot). The best recovery of FMDV spiked into milk samples (Figure 2) was obtained using TRIzol Reagent[®] with the QIAGEN instrumentation. Interestingly, although the Roche Lysis/Binding buffer was able to recover RNA using the MagNA Pure LC (Figure 2B), this buffer failed to produce any amplifiable FMDV template on the QIAGEN robot (Figure 2A).

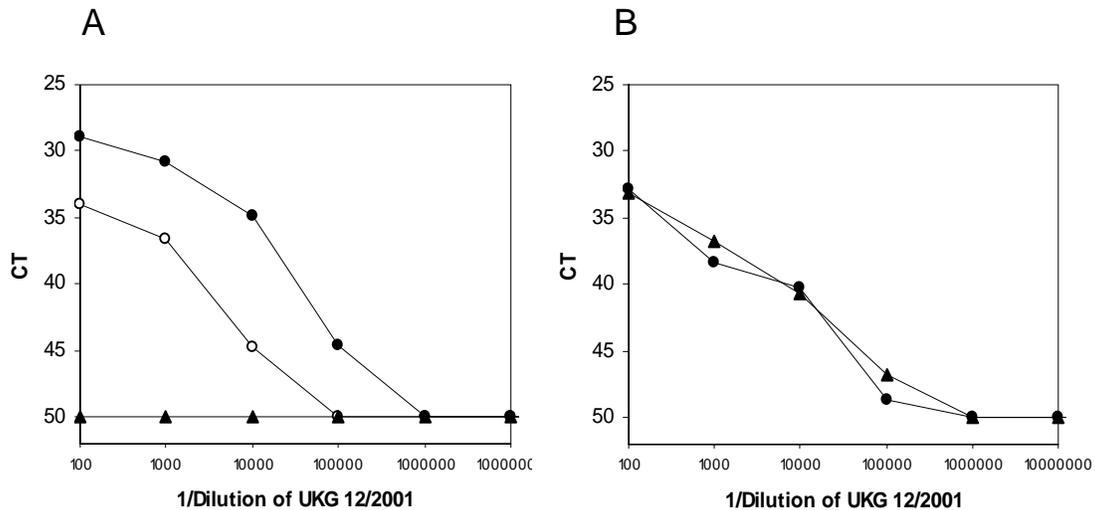


Figure 2: Optimisation of RNA template extraction methods for detection of FMDV in milk. The ability of real-time RT-PCR to detect a titration series of FMDV O/UKG 12/2001 in milk was compared using the QIAGEN robots (QIAamp[®] Virus BioRobot[®] 9604/3000: Figure A) or a MagNA Pure LC (Roche: Figure B). Samples were presented to the robots in Lysis/Binding Buffer (Roche: ▲), TRIzol[®] Reagent (Invitrogen: ●) or Buffer AL Lysis buffer (QIAGEN: ○). Values shown are the CT values obtained by RT-PCR.

Detection of FMDV in milk by quantitative real-time RT-PCR and VI

Temporal changes in FMDV copy number determined by RT-PCR and the VI titre are shown in Figure 3 for the fore- and machine whole milk and skim milk fractions in the four cows. The RT-PCR and VI results again generally correlated closely. The presence of FMDV in the milk coincided with the onset of, but did not precede, the expression of clinical signs in the cows. The earliest day that FMDV was consistently detected was day 2, day 3, day 5 and day 4 for UV58, UV60, UV59 and UV61 respectively. The peak amount of FMDV in the milk appears to correlate with FMDV loads detected in serum. Interestingly, UV60 had detectable FMDV in milk by RT-PCR but not VI. FMDV was detectable in milk for up to 23 dpi (in UV58) in contrast to the FMDV viraemia which only lasted 4-6 days. Figure 3 shows secondary peaks of FMDV detection particularly evident for RT-PCR in the whole milk and skim preparation of fore- and machine milk from cows UV58, UV59 and UV61. No significant secondary peaks of FMDV infectivity were obtained by VI. Virus was isolated in culture from the cell fraction of fore-milk from cows UV58, UV59 and UV61 on most of the days from 2 to 9 and from cow UV58 through to day 18 (data not shown). RT-PCR was positive on the cell fraction from all cows but with a reduced copy number in this fraction from cow UV60 compared to the other cows. The results from the machine milk cell fractions were similar to those of the fore-milk cell fraction although the cell fraction from cow UV60 produced a CPE in cell culture (data not shown). Virus was detected by both procedures in the cell fraction of the machine milk of cow UV58 up to day 18. Cream separated from fore- and machine milk from cows UV58, UV59 and UV61 was also positive by VI and RT-PCR but only cream separated from the machine whole milk of cow UV60 was positive. Virus was not found by either method in the cream component of the fore-milk from this cow (data not shown).

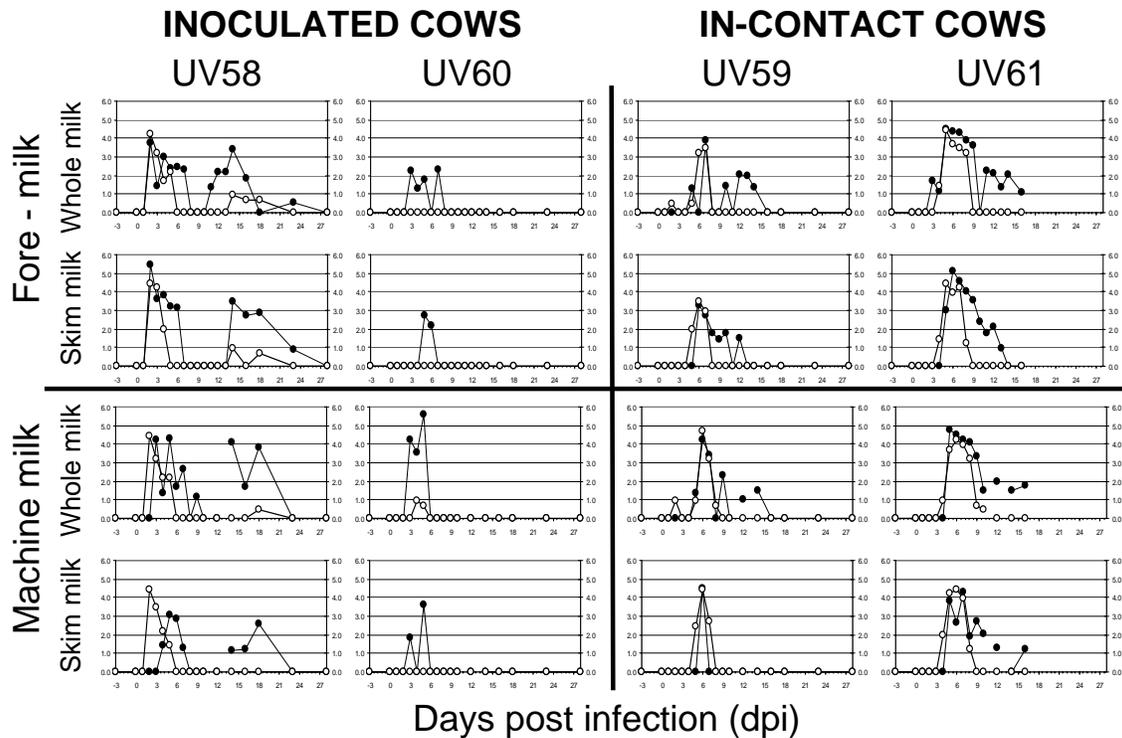


Figure 3: Detection of FMDV in milk samples collected from 4 experimentally infected cows (UV58-61). FMDV was detected by real-time RT-PCR (●) and virus isolation (○) in whole and skim milk fractions of fore-milk and machine milk collected at regular intervals after experimental infection (dpi). Values shown are \log_{10} FMDV copy number and \log_{10} TCID₅₀/ml FMD viral titre for RT-PCR and VI methods respectively.

In order to determine whether FMDV partitions into the different milk components, the ratio of FMDV recovered in the skim, cream and cellular fractions to that detected in the whole milk was calculated (Figure 4). This showed that a high percentage of virus was recovered in the cream component compared with the other components.

Detection of FMDV in tissues collected at post-mortem

Virus was detected in the soft plate of cows UV58 and UV59, in the pharynx of cow UV59 and in tonsil (UV58), medial lymph node (UV59 and UV60), mammary lateral lymph node (UV59) and retro pharyngeal lymph node (UV58). No virus was detected in the mammary glands or in the other tissues (data not shown).

Dilution of FMDV in uninfected milk

In two of the dilution series, RT-PCR was approximately 100-fold more sensitive than VI for detection of FMDV. RT-PCR was approximately 10-fold more sensitive than VI in two dilution series and the two procedures had an equivalent sensitivity for detection of FMDV in the fifth dilution series (data not shown).

Effect of temperature on the ability of RT-PCR and VI to detect FMDV in milk

The effect of incubating whole milk samples spiked with FMDV at different temperatures is shown in Figure 5. Similar RT-PCR and VI data was obtained for skim milk (data not shown). The presence of FMDV detected by both RT-PCR and VI declined rapidly at 95°C and 72°C, although in contrast to VI, RT-PCR was still able to detect FMDV in milk after treatment at 72°C for 5 min. Furthermore, RT-PCR was able to detect FMDV in milk incubated at 37°C and room temperature for a longer period of time. FMDV can be detected by both assays after two week incubation at 4°C, although a significantly reduced amount of virus is recovered after this period.

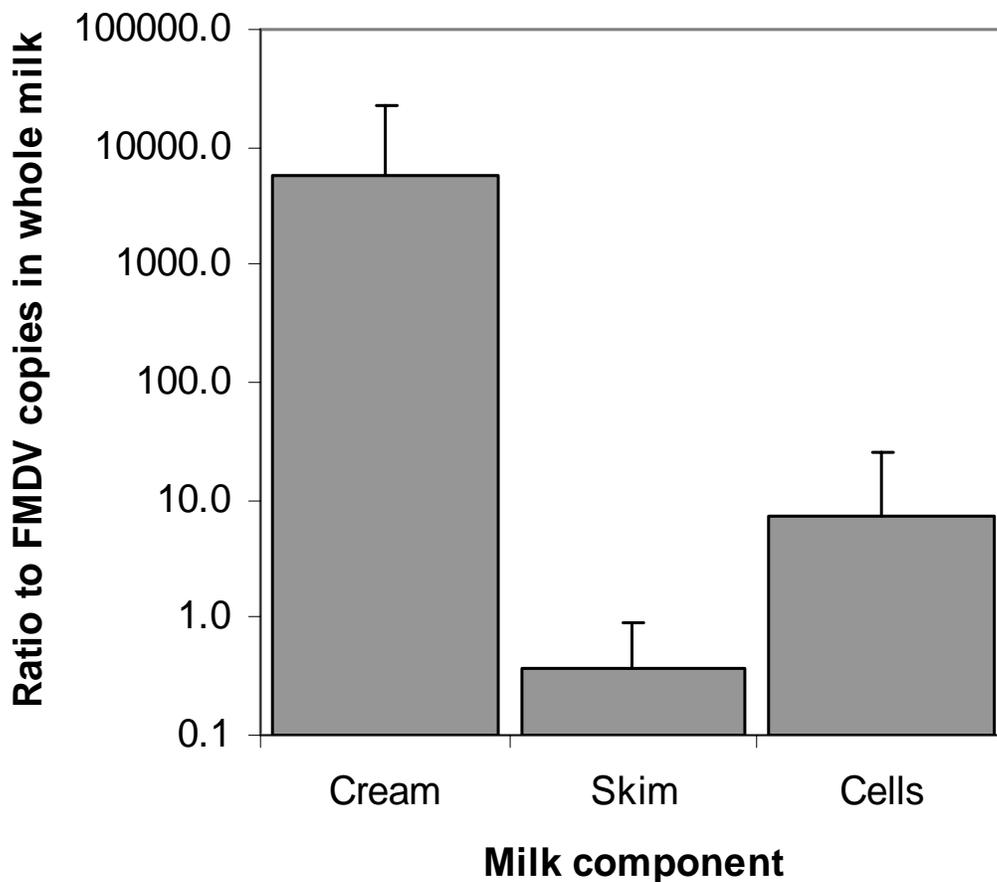


Figure 4: The detection of FMDV in milk components by real time RT-PCR. For each sample ($n = 8$) the ratio of FMDV present in the skim, cell pellet or cream to that present in whole milk was calculated. Error bars represent \pm SD.

The results for temperature stability were consistent with those obtained from an experiment which mimicked the effects of pasteurisation on milk samples obtained from infected cattle (data not shown). Heat treatment at 72°C for 15 sec, eliminated the ability of VI to detect FMDV but merely reduced the copy number obtained by RT-PCR compared with pre-pasteurisation determinations.

Effect of preservative treatment on the performance of real-time RT-PCR

Preservative treatment at concentrations ranging from 0.25% v/v to 1.0% v/v in infected milk did not have a significant inhibitory or deleterious effect on the ability of the RT-PCR to detect FMDV in any of the whole milk, skim, cell and cream fractions of that milk (data not shown).

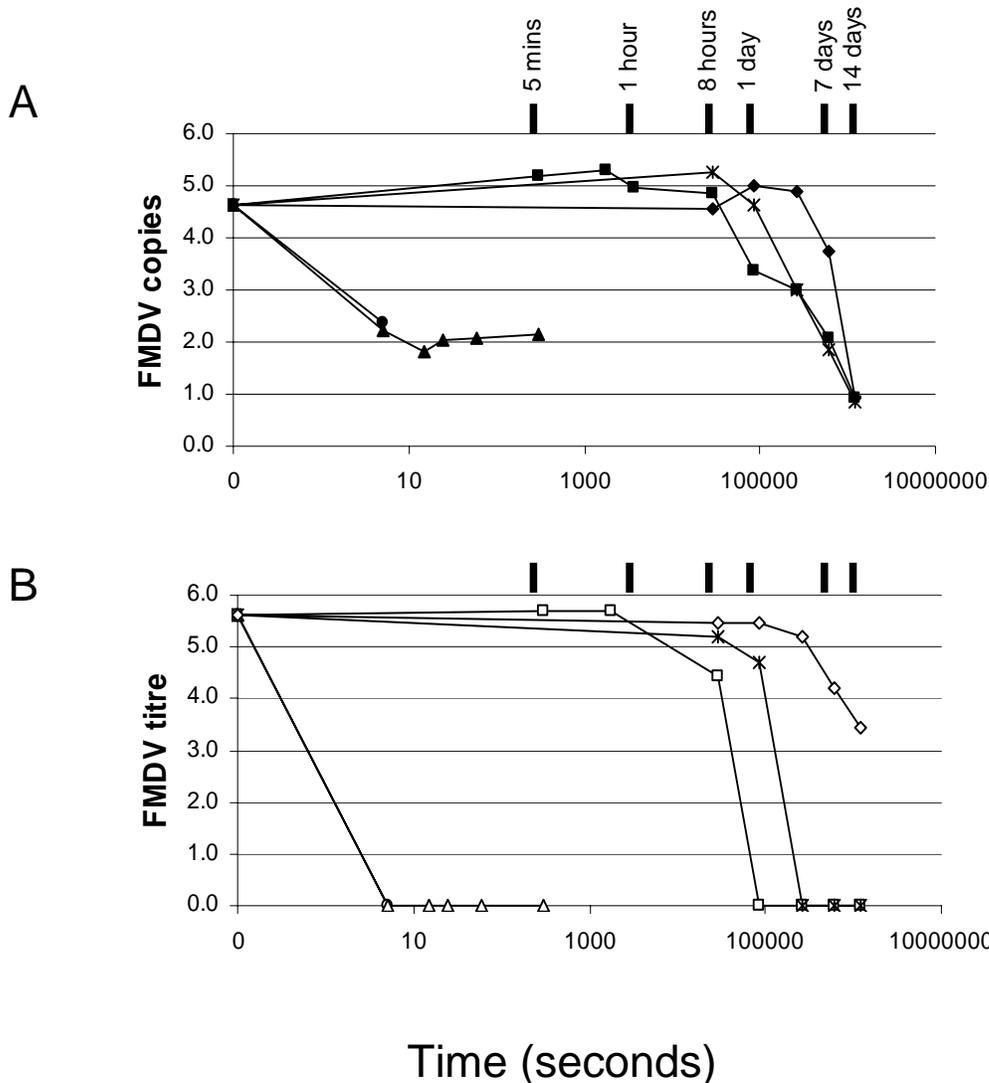


Figure 5: Effect of temperature on the ability of real-time RT-PCR (Figure A) or virus isolation (Figure B) to detect FMDV in milk. Samples of milk were spiked with O/UKG 12/2001 and were incubated at 95 °C (●), 72 °C (▲), 37 °C (■), room temperature ~ 22 °C (*) and 4 °C (◆).

Discussion:

The primary aim of this study was to assess the feasibility of using real-time RT-PCR to detect FMDV in milk. In order to select the most appropriate automated extraction protocol for use with milk samples, initial experiments were performed to compare the recovery of FMDV RNA template from milk using different robotic systems with 3 separate sample lysis buffers. Proprietary lysis buffers: Lysis/Binding Buffer and Buffer AL were able to recover RNA template successfully using their respective automated systems, although samples prepared in TRIzol Reagent® generated the highest signal with the QIAGEN robot. Therefore, the QIAGEN robotic apparatus was selected for the template extraction and subsequent liquid handling steps in this study. In addition to favourable FMDV RNA yields, this robot system benefits from a high sample through-put.

Suitable material for the evaluation of RT-PCR was generated by experimental infection of 4 dairy cows (2 by direct inoculation and 2 by contact) with FMDV type O UKG 34/2001. Fore- and machine milk were compared as both might be submitted for diagnostic evaluation, but there was little difference between the results obtained with these sample types. All four cows quickly developed multiple signs of clinical disease following inoculation. The clinical signs correlated with the high level of viraemia determined by real-time RT-PCR and VI and coincided with the presence of FMDV in the “probangs”, saliva and nasal swabs; results consistent with those achieved from previous

experimental infection of cattle with FMDV (Alexandersen et al., 2003). Milk samples (and milk fractions: skim, cream and cellular pellet) were tested by quantitative real-time RT-PCR assay, targeting the 5'untranslated region of FMDV (Reid et al., 2003). These milk samples were also tested in parallel by VI (Snowdon, 1966) in order to compare the performance of RT-PCR with an established diagnostic method with high sensitivity. Broad agreement was found between the ability of RT-PCR and VI to detect FMDV in samples of fore- and machine milk. The similarity between these two diagnostic approaches was particularly evident at early time-points after infection. At later time-points (typically >10 dpi), VI was less likely than RT-PCR to detect FMDV, probably due to the development of neutralising antibodies in these milk samples. A further benefit of RT-PCR over VI was the lower detection limit of the molecular assay on a dilution series of naturally infected milk. These data suggest that RT-PCR could potentially detect the presence of a single FMDV infected animal within an otherwise normal herd on the basis of testing an aliquot of milk from the combined bulk. RT-PCR was able to detect FMDV in milk collected from an individual cow that had been diluted 10,000-fold. To aid in the diagnosis of FMD further, RT-PCR could be used in parallel with assays to detect FMDV antibodies in milk (Armstrong and Mathew, 2001).

The excretion pattern of FMDV in milk as detected by VI paralleled results of previous studies (de Leeuw et al., 1978; Blackwell et al., 1982) and the presence of FMDV in the mammary gland up to 4 days after experimental infection has been documented by Blackwell and Yilma (1981). Interestingly, by RT-PCR the FMDV excretion profiles for one animal (UV58) showed a biphasic pattern: an initial period of FMDV excretion that lasted 6 days (2-7 dpi) followed by a subsequent peak (11-23 dpi). These data suggest that the mammary glands (or associated tissues) are a site of local replication of FMDV. However, there was no evidence for the persistence of FMDV in mammary tissue at 28 dpi despite the low copy numbers of RNA detected in the mammary lateral lymph node of cow UV59 during the post-mortem analysis.

In addition to whole skim milk, the RT-PCR was able to detect FMDV in cream and cellular fractions. In common with previous studies (Blackwell and Hyde, 1976), the RT-PCR data demonstrated that FMDV readily partitions into the cream fraction of milk. The ability of the real-time RT-PCR to detect FMDV in whole and skim milk after incubation at various temperatures was also investigated. As expected, and as reported in previous studies (Blackwell and Hyde, 1976; Tomasula and Konstance, 2004), VI was unable to detect viable virus after heat treatment at 95°C or 72°C. The results from this study show that although heat treatment reduces the amount of FMDV detected, RT-PCR was still able to detect FMDV genome in whole and skim milk that had been incubated at 95°C or under conditions that simulated pasteurisation. Therefore, RT-PCR may be an appropriate method for the detection of FMDV in dairy products made from pasteurised milk. In this study, no attempt was made to assess whether the pasteurised milk contained viable FMDV that could generate clinical disease in susceptible animals as shown previously (Blackwell and Hyde, 1976; Walker et al., 1984). These stability studies also showed that RT-PCR could successfully detect FMDV in milk after incubation at 37°C, room-temperature (approximately 22°C) and 4°C for extended periods. Addition of the preservative solution to FMD-infected whole milk also had no significant affect on the sensitivity of the RT-PCR when all components were tested, indicating that the ability of the RT-PCR to detect FMDV in bulk milk samples would not be impaired by this preservative.

Conclusions:

- Automated RT-PCR would be an excellent procedure for the laboratory detection of FMDV in milk.
- RT-PCR was more sensitive than VI and was able to detect FMDV in milk samples for longer periods after infection.
- The data from this limited number of animals did not provide any evidence that the presence of FMDV in milk could be used as a reliable preclinical indicator of FMD.

Recommendations:

- Automated RT-PCR could be used to test bulk tank milk samples and/or to screen herds for the presence of FMDV.
- Further experiments or field studies involving larger numbers of in-contact or naturally infected cattle are required in order to clearly define the relationship between the presence of FMDV in milk and the presentation of clinical signs.

Acknowledgements:

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References:

1. **Dawson, P.S.** 1970. The involvement of milk in the spread of foot-and-mouth disease: an epidemiological study. *Vet. Rec.* 87: 543-548.
2. **Donaldson, A.I.** 1997. Risks of spreading foot and mouth disease through milk and dairy products. *Rev. sci. tech. Off. Int. Epiz.* 16: 117-124.
3. **Burrows, R.** 1968. Excretion of foot-and-mouth disease virus prior to the development of lesions. *Vet. Rec.* 82: 387-388.
4. **Blackwell, J.H. & Hyde, J.L.** 1976. Effect of heat on foot-and-mouth disease virus (FMDV) in the components of milk from FMDV-infected cows. *J. Hyg. Camb.* 77: 77-83.
5. **Blackwell, J.H., McKercher, P.D., Kosikowski, F.V., Carmichael, L.E. & Gorewit, R.C.** 1982. Concentration of foot-and-mouth disease virus in milk of cows infected under simulated field conditions. *J. Dairy Sci.* 65(8): 1624-31.
6. **Ferris, N.P. & Dawson, M.** 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet. Microbiol.* 16: 201-209.
7. **Reid, S.M., Grierson, S.S., Ferris, N.P., Hutchings, G.H. & Alexandersen, S.** 2003. Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J. Virol. Methods* 107: 129-139.
8. **Shaw, A.E., Reid, S.M., King, D.P., Hutchings, G.H. & Ferris, N.P.** 2004. Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chain reaction. *Rev. sci. tech. Off. int. Epiz.* 23(3): in press.
9. **Zhang, Z. & Alexandersen, S.** 2003. Detection of carrier cattle and sheep persistently infected with foot-and-mouth disease virus by a rapid real-time RT-PCR assay. *J. Virol. Methods* 111(2): 95-100.
10. **Reid, S.M., Ferris, N.P., Hutchings, G.H., Zhang, Z., Belsham, G.J. & Alexandersen, S.** 2002. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic RT-PCR assay. *J. Virol. Methods* 105: 67-80.
11. **Zhang, Z., Murphy, C., Quan, M., Knight, J. & Alexandersen, S.** 2004. The extent of reduction of foot-and-mouth disease virus RNA load in bovine oesophageal-pharyngeal fluid after peak level may be a critical determinant of the outcome of virus persistence. *J.Gen.Virol.* 85: 415-421.
12. **Zhang, Z. & Alexandersen, S.** 2004. Distribution and quantification of foot-and-mouth disease virus type O RNA in bovine tissues: implications for the sites of viral replication. *J. Gen. Virol.* 85: 2567-2575.
13. **Snowdon, W.A.** 1966. Growth of foot-and-mouth disease virus in monolayer cultures of calf thyroid cells. *Nature* 210(40): 1079-1080.
14. **Kärber, G.** 1979. Calculation of the LD50 titer by the Kärber method. In E.H. Lennette & N.J. Schmidt, eds. *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*, 5th Edit., pp. 34-35. Washington, DC, American Public Health Association.
15. **Alexandersen, S., Quan, M., Murphy, C., Knight, J. & Zhang, Z.** 2003. Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally-infected with foot-and-mouth disease virus. *J. Comp. Path.* 129: 268-282.
16. **Armstrong, R.M. & Mathew, E.S.** 2001. Predicting herd protection against foot-and-mouth disease by testing individual and bulk tank milk samples *J. Virol. Methods* 97(1-2): 87-99.
17. **de Leeuw, P.W., van Bakkum, J.G. & Tiessink, J.W.** 1978. Excretion of foot-and-mouth disease virus in oesophageal-pharyngeal fluid and milk of cattle after intranasal infection. *J. Hyg. (Lond.)* 81(3): 415-25.
18. **Blackwell, J.H. & Yilma, T.** 1981. Localization of foot-and-mouth disease viral antigens in mammary gland of infected cows. *Am. J. Vet. Res.* 42(5): 770-3.
19. **Tomasula, P.M. & Konstance, R.P.** 2004. The survival of foot-and-mouth disease virus in raw and pasteurized milk and milk products. *J. Dairy Sci.* 87: 1115-1121.
20. **Walker, J.S., de Leeuw, P.W., Callis, J.J. & van Bakkum, J.G.** 1984. The thermal death time curve for foot-and-mouth disease virus contained in primarily infected milk. *J. Biol. Stand.* 12(2): 185-9.

Mapping of neutralising sites on FMD virus type Asia 1 and relationships with sites described in other serotypes

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Abstract

Knowledge on the antigenic structure of foot-and-mouth disease virus (FMDV) has, besides a scientific value, several practical applications: it may have relevance in the development of diagnostic assays, as well as the evaluation of the antigenic variability and the selection of appropriate vaccine strains. Antigenic sites have been studied only in FMDV type O, A and C, while it would be worthwhile to extend studies also to other serotypes. This paper reports on the identification of neutralising sites in FMDV type Asia 1 using a new panel of monoclonal antibodies (MAbs) and discuss their relation with sites described in other types.

MAbs specific for FMDV type Asia 1 were produced and characterised using different immunoassays. FMDV mutants resistant to MAbs neutralisation were selected and sequenced.

Out of 24 MAbs produced, 10 neutralise viral infectivity and 14 do not. The 10 neutralising MAbs, all type-specific, identify 4 independent antigenic sites on the basis of the reactivity profile with MAR-mutants. By comparing the amino acids sequence of the parental virus and of virus mutants, the amino acids crucial for the 4 sites were mapped at the following positions: VP1 142, VP2 67-79, VP3 58/59, VP3 218.

MAbs confirmed their powerful for making light insight the antigenic structure of FMDV. Three of the 4 neutralising sites identified and mapped on FMDV type Asia 1 correspond structurally and functionally to analogous sites described in FMDV type O, A and C, enforcing the evidence that these are dominant antigen sites in FMDV structure. One site, located at C-terminus of VP3, is a new independent site, described for the first time in FMDV.

Further sites, not involved in neutralisation, were detected by 14 more MAbs.

Introduction

Foot-and-mouth disease (FMD) viruses constitute one *genus* in the family *Picornaviridae*, but are classified in seven serotypes, each of which shows considerable intra-typic variation. This antigenic variation leads to continuing difficulties in controlling the disease.

Studies on the antigenicity of FMD virus, complemented with crystallographic analysis of the three-dimensional structure of the virus and of virus-antibody complexes, have improved understanding of the interaction between virus and the host immune system, including the mechanism of virus escape from neutralisation, which is also responsible for the high antigenic diversification of FMD viruses. Such studies may contribute to a better control of the disease.

The identification of antigenic sites in FMDV has mainly relied on the use of monoclonal antibodies (MAbs), and partly also on the evaluation of immunogenicity and antigenicity of viral peptides. However, synthetic peptides only allow the study and characterisation of sequential epitopes, while the use of MAbs, combined with detection of amino acids substitutions in virus mutants resistant to MAb-induced neutralisation, allows the identification of any kind of antigenic sites, either linear or dependent on conformation, provided their involvement in virus neutralisation processes.

Using this approach, antigenic sites have been identified and mapped on FMD viruses type O (Xie *et al.*, 1987; Pfaff *et al.*, 1988; Barnett *et al.*, 1989; Kitson *et al.*, 1990), type A (Thomas *et al.*, 1988; Baxt *et al.*, 1989; Bolwell *et al.*, 1989) and type C (Mateu *et al.*, 1990; Lea *et al.*, 1994). Results of these studies agree in the evidence that, besides the continuous epitope termed site A or site 1, located within the large and flexible G-H loop of VP1 and for a long time considered the main if not the single immunodominant site, other epitopes, not found in continuous sequences but dependent on capsid conformation, also exist in all the three serotypes studied (reviewed in Mateu, 1995). Amino acid residues in the three structural proteins VP1-3 exposing loops on the virus surface have been indicated as crucial elements for the site antigenicity.

These studies were conducted on isolates of FMDV types O, A, C which are extinct from the field. Furthermore, it would be worthwhile to extend investigations also to other serotypes of FMDV, either to improve basic knowledge on the virus structure and antigenicity, and for the benefits that this knowledge may reflect on the selection of appropriate strategies for the disease control. Further knowledge on the antigenic structure and the availability of well characterised MAbs have useful applications also in the design of functional diagnostic assays and in studies on the antigenic evolution of FMD viruses. In this report we describe the identification and characterisation of neutralising sites on FMDV type Asia 1 by a new panel of MAbs. Four independent sites have been demonstrated, three of which, located on VP1, VP2 and VP3 respectively, correspond to sites previously determined in FMDV serotypes O, A and C, while the fourth seems to be a new site described for the first time in FMDV.

Materials and Methods

Viruses

FMDV isolates used were received from the World Reference Laboratory, Pirbright, UK; they are listed in table 1. Viruses were propagated in IBRS-2 cells monolayers and harvested when cytopathic effect was maximum. The strain used for mice immunisation and SDS-polyacrilamide gel electrophoresis was preliminarily inactivated with binary ethylenimine and purified by ultracentrifugation through a 25% (w/w) sucrose cushion.

Monoclonal antibodies (MAbs)

The production of MAbs against FMDV Asia 1, strain Nepal 29/97 was described in Grazioli *et al.*, 2002.

Trapping ELISA

The assay used for MAbs titration and for the evaluation of their intra- and inter-types reactivity was a trapping ELISA (Samuel *et al.*, 1991). Essentially, each MAb was reacted with pre-titrated concentrations of viruses (supernatant of infected cells) which had been trapped using a polyclonal rabbit Asia 1 antiserum. Titres of MAbs were expressed as the reciprocal of the saturating dilution, while the reactivity of mutants and field isolates with each MAb was expressed as a percentage of the corresponding reaction with the parental strain, assumed to be 100%.

Virus Neutralization test (VNT)

VNT was carried out in microplates against 100 TCID₅₀ of the homologous FMDV Asia 1 or MAR-mutants and IBRS-2 as substrate. The final dilution required to neutralise 50% of the inoculated cultures was calculated.

MAB neutralization-resistant (MAR) mutants

The selection of mutants resistant to neutralization by MAbs was carried out as described previously (Borrego *et al.* 2002).

Immunoblotting analysis

Hybridoma supernatants were assayed against the purified homologous FMDV, resolved by SDS-polyacrilamide gel electrophoresis 10% or 12% and transferred to nitrocellulose filters, following standard procedures (Harlow & Lane, 1988; Towbin *et al.*, 1979).

Sequencing

RNA extracted from the supernatant of infected cultures, using a commercial kit (Qiagen RNeasy Mini kit, Qiagen, Inc.), was used as the template for RT-PCR. Reverse transcription was carried out using AMV reverse transcriptase with a hexanucleotide mixture of all possible sequences ("Random" primer) (Roche). The cDNA produced was used as the template for PCR amplification of the region encoding the structural proteins using the primers described in Table 1. The polyprotein P1 was amplified in five fragments which included also some flanking nucleotides from both the 5' non-translated region (NTR) and protein 2A. Sequencing of PCR fragments and sequence analysis were carried out using an ABI 310 Automatic Sequencer and LaserGene software (DNASTAR Inc., Madison, WI, USA).

Results

Preliminary characterisation of MAbs

A preliminary characterisation of 24 MAbs raised against FMDV type Asia1, strain Nepal 29/97, was achieved by analysing the immunoglobulin class, the capability to neutralise viral infectivity, the reactivity with separated, denatured viral proteins and the level of cross-reactivity with the other six heterologous FMDV types. Results of these preliminary analyses had been reported at the open session of the EUFMD Research Group held in Turkey, 2002 (Grazioli *et al.*, 2002); these results have now been updated with missing information and summarised in table 2.

According results of VNT, MAbs were divided in two main categories: one including 10 MAbs that neutralise virus infectivity, another composed by 14 MAbs that do not.

Although the major objective was focussed on mapping of neutralising MAbs, basic information were also collected for non-neutralising antibodies. Among them, 4 MAbs (4G6, 3D8, 4B1, 4B2) recognised only the homologous type Asia 1, while the other 10 MAbs showed different patterns of cross-reactivity with heterologous serotypes: the most common profile, shown by four MAbs (3G3, 2A4, 3B6, 5H5), was cross-reactivity with 4 serotypes, namely the homologous Asia 1 and the heterologous O, A, C types, but also 2 MAbs reacting with all the seven serotypes (4G2, 3H12) and another one recognising all serotypes but SAT 1 (5F10) were detected. Non-neutralizing MAbs displayed a broad reactivity also with 11 isolates of type Asia 1, representative of a thirty-year period, being the oldest virus of this panel originated in 1973 (Asia 1 Turkey 15/73) and the most recent one in the year 2000 (Asia 1 Greece 1/2000); the antigenic stability of the relevant epitopes was expected, particularly for those conserved among different serotypes.

Results of immunoblotting proved that one MAb (5F10) identifies a linear epitope in VP2 (figure 1), subjected to cleavage by trypsin treatment (not shown). In contrast, all other 13 non-neutralising MAbs were negative in immunoblotting, thus recognise conformation-dependent epitopes.

The 10 neutralising MAbs scored virus neutralising titres from 200 to more than 20000 (in ascitic fluids). Three of them reacted with denatured VP1 in immunoblotting (table 2, figure 1); trypsin treatment of the virus prevented this reactivity (not shown), suggesting that their target site should correspond to the linear amino acid sequence containing a trypsin-sensitive site and designing the flexible G-H loop of VP1,

well described in the FMDV structure. Interestingly, these three MAbs showed a high degree of cross-reactivity with the SAT 3 serotype, indicating a strong similarity of the target neutralising site between the Asia 1 and SAT 3 serotypes. However, the other 7 neutralising MAbs were all type-specific and all negative in immunoblotting with separated viral proteins: this implies they are likely directed against conformational sites, but does not allow to differentiate them.

MAb-resistant mutants (MAR-mutants)

The selection, characterisation and sequencing of viral mutants resistant to neutralisation by each of the neutralising MAbs was the system adopted to identify and then map the relevant target sites. From 1 to 9 MAR-mutants were independently selected for each MAb by serial passages of the parental virus in the presence of high concentration of MAb. After stabilisation, mutants were analysed by both ELISA and VNT against the 10 MAbs, in order to detect reciprocal relationships on the basis of similar patterns of reactivity.

The antigenic profile of MAR-mutants, determined by ELISA (figure 2) lead to the definition of 4 distinct neutralizing sites; sites were denominated I, II, IV, V to maintain a common denomination in relation with analogous sites previously described in other FMDV types; substantially the 4 sites are independent each other, as mutations in one site did not alter the reactivity of the other sites.

In particular, site I is defined by the 3 MAbs (5C12, 4E10, 4F10) reacting with a linear sequence of VP1: mutations induced in any of the corresponding escape mutants annulled the reaction of the three MAbs, without affecting the binding of the other seven MAbs. Another site, called site V, is defined by the unique MAb 5G4: it is clearly distinct in that the five mutants selected with this antibody were still recognised by all the other MAbs. Site IV includes three additional MAbs, 5E10, 3C6, 1F10: in fact, the 13 mutants selected with any of the three MAbs lost reactivity with all of them. Similarly, the remaining three MAbs, 2C3, 2G1, 4D8, correspond to a distinct site called site II. A structural relation could occur between site II and IV, since variations in one site may partially alter the reactivity of the other.

In order to evaluate the correlation between ELISA binding and neutralisation pattern, one or two representative mutants for each MAb were also tested for the susceptibility to neutralisation by MAbs; the neutralisation titres of the MAbs towards 100 TCID₅₀ of the parent virus and the mutant viruses were compared; it was arbitrarily assumed that differences in titres > 2 log₁₀ units indicate resistance, differences between 1 and 2 log₁₀ units indicate partial resistance and < 1 log₁₀ are indicative of sensitivity to MAb-induced neutralisation.

Results of these cross-neutralisation assays, shown in figure 3 fully confirmed the findings obtained by ELISA, providing further evidence of the four distinct sites, with a partial relation between sites II and IV.

Mapping of neutralising sites

The sequence of the capsid coding region of some mutants was also determined and compared to the parental sequence, in order to identify amino acid substitutions responsible for the antigenic variation (Figure 2 and Table 3).

The amino acids crucial for antigenic sites organization were determined, enabling us to map the 4 antigenic sites on the capsid proteins and identify the secondary structural element involved.

Sites I, IV and V could be related to a single amino acid change, while site II was associated to multiple simultaneous changes. According to results of mutants sequencing,

- Mutants selected with MAbs to site I repeatedly showed substitutions at residue 142 of VP1 (corresponding to residue 144 in type O), flanking the conserved RGD motif and included within the flexible G-H loop of VP1;
- site II is located in VP2 and involves multiple amino acid positions in the B-C loop: namely residues 67, 72, 74, 77, 79. Mutants in this site showed each three simultaneous changes; one of them presented also two further changes at amino acids 49 and 207 of VP1;
- site IV maps at the amino acid positions 58 or 59 of VP3, located within the secondary structural element B-B knob. However, a further mutation at amino acid 67 of VP2 was detected in two sequenced mutants: this second change is typical of site II and can justify the partially altered reactivity of some MAbs of site II, confirming the structural relationship between site II and IV;
- Site V maps on VP3, involving the amino acid 218, that corresponds to the VP3-carboxyl end.

Consistently with ELISA and neutralisation profiles, mutants selected with different MAbs defining the same site showed amino acid changes at the same or contiguous residues; however, residues involved in site IV may be substituted by different amino acids causing similar effects.

The profile of reactivity with MAbs of 11 FMDV isolates of type Asia 1, chronologically distant, proved evidence that site I and IV are stable in the majority of isolates, whilst sites II and V are subject to frequent antigenic variation: in fact MAbs to site I and IV broadly reacted with all but 1 isolate (Cambodia 3/93), while MAbs to site II and V reacted almost exclusively with the homologous strain (table 2).

Discussion

Mapping of antigenic sites by a new panel of MAbs, with focus on the sites involved in virus neutralisation processes, has provided a better understanding of the antigenic structure of FMDV type Asia 1, allowing to study the relationships with antigenic sites previously described in other FMDV types, more extensively studied.

Recently, the use of two panels of MAbs lead to the identification of independent antigenic sites also in the type Asia 1 of FMDV (Sanyal *et al.*, 1997, Marquardt *et al.*, 2000). The MAbs provided evidence of antigen variability among field isolates (Sanyal *et al.*, 1997; Sanyal *et al.*, 2003; Marquardt *et al.*, 2000), but mapping of the relevant sites was only attempted on the basis of the correspondence observed between MAbs-profiling and variations in the amino acid sequence of few isolates (Marquardt *et al.*, 2000). The characterisation of our new panel of MAbs, combined with sequencing of MAR-mutants, enabled us to identify and map four independent neutralising sites. Interestingly, three of the sites detected on type Asia 1 correspond structurally and functionally to analogous antigenic sites described in types O, A and C (as shown in figure 3).

In particular, site I, defined by three MAbs of our panel, was previously called site 1 or A in the other three serotypes; in all serotypes this site is located within the surface exposed G-H loop of VP1, that contains one trypsin cleavage site and the highly conserved RGD (Arg-Gly-Asp) amino acid triplet, corresponding to the presumed site of cell attachment (Fox *et al.*, 1989; Mason *et al.*, 1994). Several amino acid residues in the sequential segment 138-154 of the capsid protein are involved in determining site antigenicity. Substitutions detected either in MAR-mutants, or in field variants occur in residues flanking both sides of the RGD motif (Stave *et al.*, 1988; Bolwell *et al.*, 1989; Mateu *et al.*, 1990; Marquardt and Freiberg, 2000; Marquardt *et al.*, 2000). Consistently, also in the FMDV type Asia 1 the position 142, which was found repeatedly substituted in MAR-mutants, precedes the RGD motif. The G-H loop is easily accessible on the virus surface and is characterised by high variability (reviewed in Palmenberg, 1989 and Domingo *et al.*, 1990). The antigenic diversity in this region relies on its flexibility, as indicated by the disorder at G-H loop observed in crystal structures of both types O (Acharya *et al.*, 1989) and C (Lea *et al.*, 1994). In fact a flexible loop can accept different amino acid sequences while preserving a functional capsid structure.

In spite of this concept, and in contrast with the sequence variability observed in some field isolates of Asia 1 type in the corresponding region (Marquardt *et al.* 2000), our three MAbs to site I show a high level of conservation among the field isolates tested. Furthermore, unexpectedly, all of them recognise also the FMDV serotype SAT 3; the molecular bases that may explain cross-reactivity at this level should be further investigated.

It has been reported that VP1 C-terminus contributes to the formation of a discontinuous site together with site 1/A in the FMDV type O (Xie *et al.* 1987, Parry *et al.*, 1989), but represents a topologically independent site in type C (site C, Mateu *et al.*, 1990). In type A both situations have been observed in different strains (Baxt *et al.*, 1989, Thomas *et al.*, 1988). The epitope included in VP1 C-terminus in the sequence 200-213 of the serotypes O, A; C (figure 3). VP1 C-terminus was described as a trypsin-sensitive, linear epitope of minor importance, given the weak neutralising capability of MAbs towards it (Mateu *et al.* 1990, Thomas *et al.*, 1988), their failure to compete with sera from convalescent animals (Thomas *et al.*, 1988) and the poor capacity of VP1 C-terminus to generate protecting antibodies (Meloan and Barteling, 1986). Our MAb panel does not provide evidence of antigenicity of VP1 C-terminus in FMDV type Asia 1; however a substitution at residue 207 was detected in one MAR-mutant of site II; apparently it is not structurally related to site II itself, but rather indicates the occurrence of variability in this region.

Site II involves multiple amino acid positions in the structural protein VP2, ranging from 67 to 79; the same or contiguous residues were found crucial for the conformation of the analogous site in FMDV type O (site 2, Kitson *et al.*, 1990), A (Ag-site III, Thomas *et al.*, 1988) and C (site D2, Lea *et al.*, 1994). All concerned residues lie in the exposed B-C loop of VP2. Frequent amino acids substitutions were demonstrated in this region in field isolates of type Asia 1 (Marquardt *et al.*, 2000), indicating its susceptibility to variation. Consistently, our three MAbs to site II do not recognise most of the isolates examined.

Site IV maps in the type Asia 1 at positions 58/59 of VP3, located within the B-B knob structural element; in contrast to site II, this is a conserved region, as proved by the broad intra-typic reactivity of the three target MAbs and in agreement with the absence of substitutions observed in the amino acid sequence of field isolates (Marquardt *et al.*, 2000). The same epitope was found also in types O (site 4), A (described as part of Ag-site III) and C (site D3). While the same and unique residue 58 was found crucial for this site in the serotypes O and C (Kitson *et al.*, 1990; Lea *et al.*, 1994), in the serotype A several contiguous or close residues, namely 58 to 61, 69/70 and in addition two more distant positions 139 and 195, seem to be part of this site (Thomas *et al.*, 1988).

Evidence of structural relationship between site II and IV in the type Asia 1, based on the profile of reactivity and also on amino acids replacements of the relevant MAR-mutants, confirmed previous

findings. In fact, in type A, like in type Asia 1, mutants induced with MAb against the two sites showed a certain level of reciprocal cross-neutralisation (Thomas *et al.*, 1988), in type C they are considered parts of a unique complex antigenic site (Lea *et al.*, 1994). Furthermore, in the three dimensional structure of the capsid, residues involved in formation of the two sites lie close each other on the surface of the virion, around the three-fold axes of symmetry (Kitson *et al.*, 1990; Lea *et al.*, 1994).

The detection of equivalent sites in four different serotypes, detected through independent studies, enforces the evidence that these are dominant antigenic sites in the FMDV structure. However, there are further conformational sites, implicated in neutralisation processes, each described only in one FMDV serotype.

In type A a site of minor importance was found at position 169 (Baxt *et al.*, 1988) and 163 (Thomas *et al.*, 1998) of VP1; in type O the so called site 3 was mapped at the amino acid residues 43 to 45 and 48 of VP1 (Barnett *et al.*, 1989; Kitson *et al.*, 1990). It was supposed that these two sites could be related each other, as residues involved are located within two different loops (H-I and B-C loops respectively) of VP1 lying adjacent on the virus surface. We did not find these positions as part of any antigenic site in type Asia 1, however one mutant obtained with site II MAb presented, beside changes related to site II, a substitution in position 43, overlapping to site 3 of type O. This could suggest that the corresponding region is subjected to variability also in type Asia 1.

A further position in VP1, mapping at residues 193 was recognised as another neutralising site, named D1, only in type C (Lea *et al.*, 1994).

Finally the site called V, described here in type Asia 1, maps at position 218 of VP3 (VP3 C-terminus) and is reported for the first time in FMDV antigenic structure. The profile of reactivity of several isolates of type Asia 1 indicates variability in correspondence of this site, in accordance with the frequent substitution rate detected in position VP3-218 by sequencing field isolates (Marquardt *et al.*, 2000).

Further sites not involved in neutralisation were detected by another group of 14 MAb, reacting with all Asia 1 field isolates and presenting different degrees of even inter-types cross reactivity. One of these MAb (5F10) recognises a linear epitope in VP2, as proven by its profile of reactivity in immunoblotting; the epitope is susceptible to trypsin cleavage and is common to at least six serotypes. MAb with analogous reactivity were independently selected by other authors from mice immunised with either FMDV type O, SAT 1 or Asia 1. The epitope target of these antibodies resides at the intertypically conserved N-terminus of VP2, as demonstrated by the reactivity profile with synthetic peptides. (Freiberg *et al.*, 2001).

The remaining 13 not neutralising MAb recognise conformational epitopes, indistinguishable each other, except for different patterns of inter-types cross reactivity.

Also non neutralising MAb may have a potential value for the development of diagnostic assays, but they are more difficult to characterise than neutralising MAb. Therefore, there is a requirement to investigate alternative methods and strategies for their characterisation.

In conclusion, our results have improved understanding of the antigenic structure of FMDV, through the description of a new neutralising site and providing more evidence of the immunodominant character of three antigenic sites detected in 4 different serotypes. These data may have useful application in diagnostic and epidemiological investigations, as MAb directed against conserved epitopes provide universal reagents for FMDV detection systems, while MAb against known variable sites readily allow the identification of antigenic variants. The development of diagnostic immunoassays using these MAb is providing satisfactory perspectives.

Conclusions:

- 1) Three of the 4 neutralising sites identified and mapped on FMDV type Asia 1 correspond structurally and functionally to analogous sites described in FMDV type O, A and C, enforcing the evidence that these are dominant antigen sites in FMDV structure.
- 2) One site, located at C-terminus of VP3, is a new independent site, described for the first time in FMDV. Further antigenic sites, not involved in neutralisation, were detected by 14 more MAb.
- 3) MAb confirmed their powerful for a better understanding of the antigenic structure of FMDV.

Recommendations:

- 1) A detailed characterization of MAb should be achieved in order to select appropriate panels for different applications, such as diagnostic tests or antigen profiling, and to guide to a correct interpretation of results based on MAb use (it is important to predict which antigenic sites are being analyzed and their relevance for purpose).
- 2) In the antigenic structure of FMDV, sites not involved in neutralization are usually under evaluated, despite they could be useful target for diagnostic purposes. Few tools and systems that allow their study are known; more research should be encouraged also in these aspects.
- 3) Criteria and funding to create a bank of available MAb should be defined. Only well characterized MAb should be maintained within the MAb bank.

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References

- Acharya, R. Fry, E. Stuart, D. Fox, G. Rowlands, D. & Brown, F.** 1989. The three-dimensional structure of foot-and-mouth disease virus at 2.9 Å resolution. *Nature* 337:709-716.
- Barnett, P.V. Ouldrige, E.J. Rowlands, D.J. Brown, F. & Parry, N.R.** 1989. Neutralizing epitopes on type O foot-and-mouth disease virus. I. Identification and characterization of three functionally independent, conformational sites. *Journal of General Virology* 70:1483-1491.
- Baxt, B. Vakharia, V. Moore, D. M. Franke, A. J. & Morgan, D. O.** 1989. Analysis of neutralizing antigenic sites on the surface of type A₁₂ foot-and-mouth disease virus. *Journal of Virology*, 63(5): 2143-2151.
- Bolwell, C. Clarke, B.E. Parry, N.R. Ouldrige, E.J. Brown, F. & Rowlands, D.J.** 1989. Epitope mapping of foot-and-mouth disease virus with neutralizing monoclonal antibodies. *Journal of General Virology*, 70:59-68.
- Borrego, B. Carra, E. Garcia-Ranea, J. A. & Brocchi, E.** 2002. Characterization of neutralization sites on the circulating variant of swine vesicular disease virus (SVDV): a new site is shared by SVDV and the related coxsackie B5 virus. *Journal of General Virology*, 83: 35-44.
- Domingo, E. Mateu, M.G. Martinez, M.A. Dopazo, J. Moja, A. & Sobrino, F.** 1990. *Genetic variability and antigenic diversity in foot-and-mouth disease virus*. In Applied Virology Research, Volume 2, pp 233-266, Plenum Publishing, New York.
- Fox, G. Parry, N. Barnett, P.V. McGinn, B. Rowlands, D.J. & Brown, F.** 1989. The cell attachment site on foot-and-mouth disease virus includes the amino acid sequence RGD (arginine-glycine-aspartic acid). *Journal of General Virology* 70:625-637.
- Freiberg, B. Hohlich, B. Haas, B. Saalmuller, A. Pfaff, E. & Marquardt, O.** 2001. Type-independent detection of foot-and-mouth disease virus by monoclonal antibodies that bind to amino-terminal residues of capsid protein VP2. *Journal of Virological Methods*, 92: 199-205.
- Harlow, E. & Lane, D.** 1988. *Immunoblotting protocols*. In Antibodies: a Laboratory manual, p 479-510. Cold Spring Harbor, NY: Cold Spring Harbor laboratory.
- Kitson, J.D.A. McCahon, D. & Belsham, G. J.** 1990. Sequence analysis of monoclonal antibody resistant mutants of type O foot and mouth disease virus: evidence for the involvement of the three surface exposed capsid proteins in four antigenic sites. *Virology*, 179: 26-34
- Grazioli, S. Fallacara, F. & Brocchi, E.** *Monoclonal Antibodies against FMDV type Asia 1: preliminary characterization and potential use in diagnostic assays*. Report of the Sess. Res. Gr. St. Tech. Committee of the Europ. Comm. Control FMD, Izmir, Turkey 17-20 September 2002, p.194-202
- Lea, S. Hernández, J. Blakemore, W. Brocchi, E. Curry, S. Domingo, E. Fry, E. Abu-Ghazaleh, R. King, A. Newman, J. Stuart, D. & Mateu, M. G.** 1994. The structure and antigenicity of a type C foot-and-mouth disease virus. *Structure* 2: 123-139.
- Marquardt, O. & Freiberg, B.** 2000a. Antigenic variation among foot-and-mouth disease virus type A field isolates of 1997-1999 from Iran. *Veterinary Microbiology*, 74: 377-386
- Marquardt, O. Rahman, M.M. & Freiberg, B.** 2000b. Genetic and antigenic variance of foot-and-mouth disease virus type Asia 1. *Archives of Virology*, 145: 149-157.
- Mason, P.W. Rieder, E. & Baxt, B.** 1994. RGD sequence of foot-and-mouth disease virus is essential for infecting cells via the natural receptor but can be bypassed by an antibody-dependent enhancement pathway. *Proc. Natl. Acad. Sci. USA* 91:1932-1936.
- Mateu, M. G. Martínez, M. A. Capucci, L. Andreu, D. Giralt, E. Sobrino, F. Brocchi, E. & Domingo, E.** 1990. A single amino acid substitution affects multiple overlapping epitopes in the major antigenic site of foot-and-mouth disease virus of serotype C. *Journal of General Virology*, 71: 629-637.
- Mateu, M. G.** 1995. Antibody recognition of picornaviruses and escape from neutralization: a structural view. *Virus Research*, 38: 1-24.
- Meloen, R.H. & Barteling, S.J.** 1986. An epitope located at the C-terminus of isolated VP1 of foot-and-mouth disease virus type O induces neutralizing activity but poor protection. *Journal of General Virology* 67:289-294.
- Palmenberg, A.C.** 1989. *Molecular aspects of picornavirus infection and detection*. American Society for Microbiology, Washington D.C.
- Parry, N.R. Barnett, P.V. Ouldrige, E.J. Rowlands, D.J. & Brown, F.** 1989. Neutralizing epitopes of type O foot-and-mouth disease virus. II. Mapping three conformational sites with synthetic peptide reagents. *Journal of General Virology*, 70:1493-1503.
- Pfaff, E., Thiel, H.J., Beck, E., Strohmaier, K., & Schaller, H.** 1988. Analysis of neutralizing epitopes on foot-and-mouth disease virus. *Journal of Virology* 62:2033-2040.
- Samuel, A.R. Knowles, N.J. Samuel, G.D. & Crowther, J.R.** 1991. Evaluation of a trapping ELISA for the differentiation of FMDV using MAbs. *Biologicals* 19: 299-310.

Sanyal, A. Venkataramanan, R. & Pattnaik, B. 1997. Antigenic features of foot-and-mouth disease virus serotype Asia1 as revealed by monoclonal antibodies and neutralization-escape mutants. *Virus Research* 50:107-117.

Sanyal, A. Gurumurthy, C. B. Venkataramanan, R. Hemadri, D. & Tosh, C. 2003. Antigenic characterization of foot-and-mouth disease virus serotype Asia1 field isolates using polyclonal and monoclonal antibodies. *Veterinary Microbiology*, 93: 1-11.

Stave, J.W. Card, J.L. Morgan, D.O. & Vakharia, V.N. 1988. Neutralization sites of type O1 foot-and-mouth disease virus defined by monoclonal antibodies and neutralization-escape virus variants. *Virology*, 162: 21-29.

Stram, Y. Laor, O. Molad, T. Chai, D. Moore, D. Yadin, H. & Becker Y. 1994. Nucleotide sequence of the P1 region of serotype Asia1 foot-and-mouth disease virus. *Virus Genes* 8:275-278.

Thomas, A.A.M. Woortmeijer, R. J. Puijk, W. & Barteling, S. J. 1988. Antigenic sites on foot-and-mouth disease virus type A10. *Journal of Virology*, 62(8): 2782-2789.

Tjissen P. 1985. *Preparation of enzyme-antibody or other enzyme-macromolecule conjugates*. In Laboratory techniques in biochemistry and molecular biology. Practice and theory of enzyme immunoassays (R.H. Burdon & P.H. van Knippenberg, eds). Elsevier, Amsterdam, 221-277.

Table 1. Primers used for PCR amplification and sequencing of the capsid-coding region (polyprotein P1)

Primers	Position		Sequenze 5' → 3'
	Nucleotide	Gene	
1D	50-70	5' NTR	GATCAGAGACCACTCAACGGA
564D	564-587	VP2	CACCGAACTTGGCATTGGACACT
612D	665-685	VP2	GGGTGGGACATAGAGGTGACT
674R	674-696	VP2	TTTCCAACAGCAGTCACCTCTAT
1026D	1079-1098	VP3	CCAGTGTACGGGAAAGTGTT
1206R	1237-1256	VP3	GCCCCGAGCGAACGAGACA
1632D	1682-1702	VP1	TCAGCGACCCGGTGACAACC
1716R	1748-1766	VP1	CAAAGGCAACGTCAGTGTG
2313R	2313-2333	2A	ACTCAACGTCTCCTGCCAACT

(D): direct primers, sequence identical to the viral genome; (R): reverse sequence complementary to the viral genome. Nucleotide numbering is according to Stram Y. et al (1994). All primer sequences correspond to FMDV Nepal 29/97 determined in this work, except for primers 564D and 2313R, which were designed according to the sequence of serotype Asia 1, isolate L83 (Stram Y. et al, 1994, Accession N° U01207).

Table 3: Amino acid substitutions found in MAR-mutants

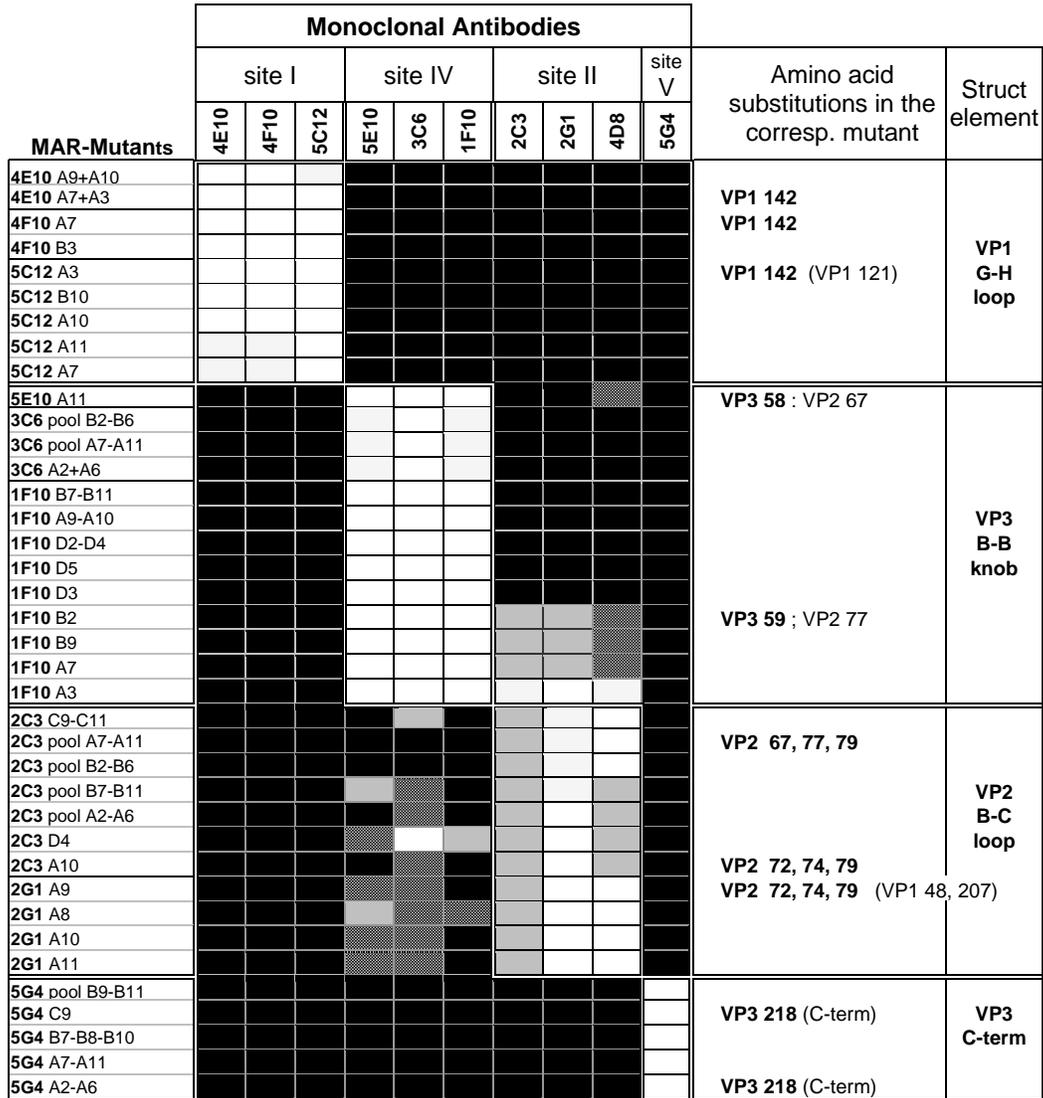
Ag site	MAR-mutant	Substituted amino acid
I	4E10/A7+A3	VP1 142 (R → Q)
	4F10/A7	VP1 142 (R → Q)
	5C12/A3	VP1 142 (R → Q); (VP1 121 A → G)
II	5E10/A11	VP3 58 (G → E); VP2 67 (F → L)
	1F10/B2	VP3 59 (E → D); VP2 77 (H → D)
IV	2C3/A7-A11	VP2 67 (F → L); VP2 77 (H → R); VP2 79 (Y → H)
	2C3/A10	VP2 72 (D → N); VP2 74 (A → T); VP2 79 (Y → N)
	2G1/A9	VP2 72 (D → G); VP2 77 (H → R); VP2 79 (Y → H) (VP1 48, VP1 207)
V	5G4/C9	VP3 218 (R → Q)
	5G4/A2-A6	VP3 218 (R → Q)

Table 2: Reactivity of MAbs raised against FMDV type Asia 1, strain Nepal 29/97

												MAbs reactivity against field isolates of type Asia 1															
Ig class	MAB	VNT titre		Immunoblotting	ELISA Trapping							Ag site	percentage reactivity (ELISA)														
		hybrid. culture	ascitic fluid		Asia 1 homologous	O1 Switz. 65	A 5 Italy 62	C1 Italy 64	SAT 1 Bot 1/68	SAT 2 Zim 5/81	SAT 3 Zim 4/81		Nepal 29/97 (homologous)	Cam 9/80	India 10/82	Pak 2/98	Pak 3/98	Iran 58/99	SAU 39/94	Gre 1/2000	Kuwait 2/81	Tur 15/73	Nepal 58/88	Cam 3/93			
NEUTRALIZING MAbs	IgM 4E10	12	5120	VP1	125 a)	-	+/-	-	-	-	-	+	1	100	100	100	100	100	100	100	100	100	100	100	70	100	0
	IgM 4F10	>32	20480	VP1	25	-	+/-	-	-	-	-	+		100	100	100	100	100	100	100	100	100	100	100	70	100	0
	IgG1 5C12	>32	>20480	VP1	625	-	-	-	-	-	-	+		100	75	100	100	100	100	100	100	100	100	100	25	20	0
	IgG1 5E10	24	3840	-	125	-	-	-	-	-	-	-		100	100	50	50	50	50	50	35	40	50	20	0		
	IgG1 3C6	3	3840	-	125	-	-	-	-	-	-	-		100	100	100	100	100	100	100	100	75	100	40	0		
	IgG1 1F10	>32	20480	-	625	-	-	-	-	-	-	-		100	100	100	100	100	100	100	100	100	100	100	0		
	IgG1 2C3	12	2560	-	375	-	-	-	-	-	-	-		100	50	10	10	10	10	10	10	10	10	10	10	5	
	IgG1 2G1	24	2560	-	375	-	-	-	-	-	-	-		100	50	0	0	0	0	0	0	0	0	0	0	0	0
	IgG2a 4D8	1	nd	-	5	-	-	-	-	-	-	-		100	0	0	0	0	0	0	0	0	0	0	0	0	0
	IgM 5G4	6	240	-	125	-	-	-	-	-	-	-		100	0	50	50	35	20	0	0	0	0	0	0	0	0
NON-NEUTRALISING MAbs	IgG1 4G6	-	-	-	125	-	-	-	-	-	-	-	100	100	100	100	100	100	100	100	100	100	100	100	100	0	
	IgG1 3D8	-	-	-	3000	-	-	-	-	-	-	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 4B1	-	nd	-	375	-/+	-	-/+	-	-	-	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 4B2	-	nd	-	625	-/+	-	-/+	-	-	-	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 2F7	-	nd	-	625	(+)	(+)	-	-/+	-/+	-/+	-/+	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 2G8	-	-	-	625	(+)	(+)	-	-/+	-/+	-/+	-/+	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 2B11	-	nd	-	625	+	+	-	-	-/+	-/+	-/+	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 3G3	-	nd	-	625	+	+	+	-	-	-	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 2A4	-	-	-	125	+	+	+	-	-	-	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 3B6	-	nd	-	625	+	+	+	-	-	-	-	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 5H5	-	nd	-	5	+	+	+	-	-	-/+	-/+	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 4G2	-	nd	-	2	+	+	+	+	+	+	+	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 3H12	-	nd	-	125	+	+	+	+	+	+	+	100	100	100	100	100	100	100	100	100	100	100	100	100	100	
	IgG1 5F10	-	-	VP2	75	+	+	+	-/+	+	+	+	lin. site	100	100	100	100	100	100	100	100	100	100	100	100	100	100

a) : highest saturating dilution of hybridoma supernatant; + : same reactivity as with the homologous virus type; (+): reduced signal with respect to the homologous virus type; -/+ : traces of reactivity; - : negative

Figure 2. ELISA reactivity profile of MAR-mutants with neutralizing anti-Asia 1 MAbs and location of amino acid substitutions



Percentage of reactivity related to the parental virus

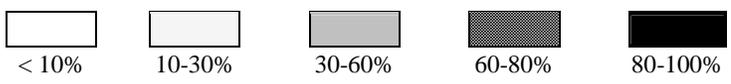


Figure 1. Profile of reactivity of anti-FMDV type Asia 1 MAbs in immunoblotting

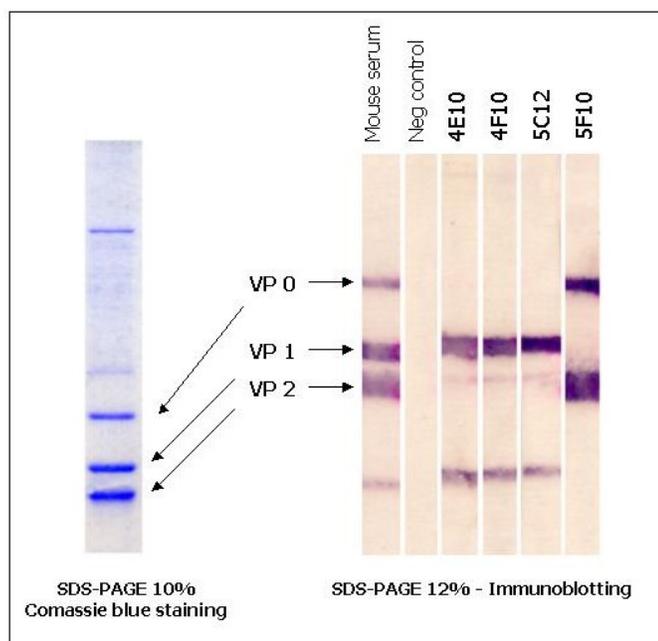
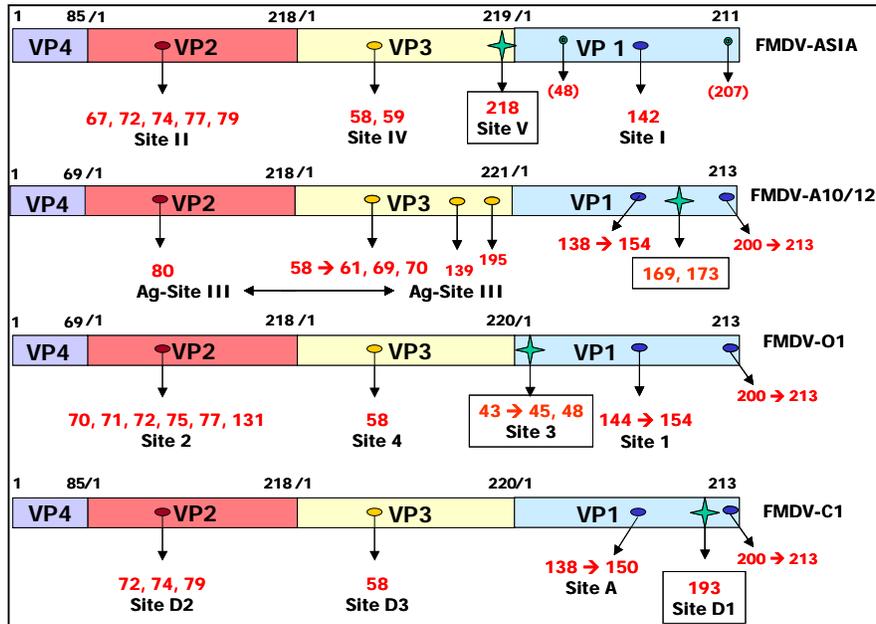


Figure 3. Neutralisation resistance pattern of MAR-mutants with MAbs

MAR-mutants	Monoclonal Antibodies								
	Site I			Site IV			Site II	Site V	
	4E10	4F10	5C12	5E10	3C6	1F10	2C3	2G1	5G4
Parental virus	4,186	4,01	4,311	3,885	3,885	4,612	2,38	2,681	2,204
4E10 A7-A3	>3	>3	>3	nd	0,78	0,3	0	0	0,3
4F10 A7	>3	>3	>3	0,61	0,47	0,11	0	0	0,42
5C12 A3	>3	>3	>3	0,31	0,3	0,42	0,48	1,2	0,72
5E10 A11	0	0,1	0	2,03	2,6	2,8	0	0	0
3C6 A2-A9	0,78	0,3	0,1	2	>3	3	0,18	0,6	0,6
1F10 B2	0	nd	0,1	2,3	2,6	>3	0,48	0,6	0,1
1F10 A3	0,48	nd	0,1	2,6	>3	>3	>2	>2	0
2C3 A7-A11	0,78	nd	0,6	1,5	1,1	0,7	>2	>2	0,1
2C3 D4	0,78	0,7	0,1	2	0,78	0,9	>2	>2	0,1
2G1 A8	0,6	nd	0,42	2	0,9	1,2	>2	>2	0,1
5G4 C9	1	0,89	0,2	0,78	0,3	0,42	0	0	>2

Numbers in cells express differences in VNT titres (\log_{10}) to the parental and mutants viruses; white cells: differences $> 2 \log_{10}$ indicate resistance to neutralisation; grey cells: differences between 1 and $2 \log_{10}$ indicate partial resistance; black cells: differences $< 1 \log_{10}$ indicate susceptibility to neutralisation.

Figure 4: Mapping of neutralising sites on FMDV structural proteins
 Correspondence between antigenic sites described in different FMD virus types



Validation of a Solid Phase Competitive ELISA (SPBE) based on the use a single neutralising monoclonal antibody for the measurement of antibodies to FMDV type Asia 1

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Abstract

There are many evidences that diagnostic ELISAs can benefit from the use of monoclonal antibodies (MAbs) to improve standardisation and specificity. Within the program to complete, update and/or improve the sets of reagents and diagnostics for Foot-and-Mouth Disease (FMD), we have developed a new Solid Phase Competitive ELISA (SPCE) for the assessment of antibody to FMDV type Asia 1. In this assay, specific antibodies in serum are detected due to their ability to compete with a peroxidase-labelled neutralising MAb. The paper describes the validation process of this assay.

A neutralising MAb (5E10), specific for FMDV type Asia 1 and directed against a conformation-dependent antigenic site including the amino acid position 58 of VP3, was selected either as catching and as labelled-competitor antibody.

Using a serum dilution 1/10 for test sera and a threshold of 70% inhibition, an overall diagnostic specificity of 99,3% was found by examining 983 cattle, 1709 pigs and 543 sheep from naive populations. Diagnostic sensitivity evaluated on a total of 666 positive cattle resulted of 99,7%. OIE reference sera (cut-off, weak and strong positive) were correctly detected. A high level of type-specificity was demonstrated examining sera strongly positive against other FMDV serotypes.

The MAb-based SPCE developed for the detection of antibodies to FMDV type Asia 1 proved to be a highly specific and sensitive assay. With respect to the polyclonal-based SPCE, carried out using rabbit and guinea-pig antisera, the MAb-based ELISA is simpler, faster and, above all, more standardised, thanks to the employment of a unique invariable MAb.

Introduction

Serosurveillance for Foot-and-Mouth Disease (FMD) plays an important role in the control of the disease: serosurveys must be implemented in countries that wish to attain or regain FMD-free status; furthermore, the assessment of antibodies is important for international trade of animals, for vaccine potency testing and for monitoring the vaccination coverage in the field.

Although a great innovation in FMD serology has been recently achieved thanks to immunoassays enabling the distinction between vaccinated and infected animals on the basis of antibody detection against non structural viral proteins (NSP), the traditional FMD serology, based on the measurement of antibodies to the capsid structural proteins (SP), is still valid in a number of applications: for example, to monitor virus circulation and to regain FMD free status in countries that have not vaccinated, to identify the virus serotypes responsible for antibodies detected in a population by an NSP-assay, and of course whenever the vaccine-induced immune response is the target of the research. Therefore, improvement of the internationally accepted tests for FMD-SP serology is a proper objective to pursue.

Currently, the OIE "prescribed" tests are the virus neutralisation test (VNT) and the recently introduced Solid Phase Competitive ELISA (SPCE). Whilst VNT represents the "gold standard" and it is indicated as a confirmatory test, SPCE is indicated for screening: it correlates well with VNT while having a number of advantages: it is quicker, simpler, more reproducible being independent on biological variability and safer, making use of inactivated antigens.

SPCE has replaced the Liquid Phase Blocking ELISA (LPBE) as prescribed test for screening, due to the evidence of a higher specificity combined with an equivalent sensitivity and a better robustness (Mackay *et al.*, 2001).

Either LPBE and SPCE are traditionally performed using polyclonal immune sera from rabbit and guinea pig (Hamblin *et al.*, 1986a; Hamblin *et al.*, 1986b; Van Maanen and Terpstra, 1989); differences in the source of antisera (batch to batch variability, immunisation protocols, procedures for the production of the immunogen, individual biological variability) may introduce variation between laboratories, so that standardisation of the assays may be affected.

Furthermore, the polyclonal-SPCE has been widely validated for the detection of the FMDV type O during the UK epidemic in 2001 (Paiba *et al.*, 2004), it has been positively evaluated for antibody detection to FMDV serotypes A and C (Mackay *et al.*, 2001; Anderson *et al.*, 2003), but little or none studies have been conducted for the other serotypes.

There are several evidences that immunoassays for the diagnosis of many infectious diseases including FMD can greatly benefit from the use of Monoclonal Antibodies (MAbs). MAbs are powerful tools which enable a more detailed understanding of the antibody response and which can characterise sera in respect to reactivity with specific viral epitopes. Moreover MAbs are not subject to variability or exhaustion of stocks as are polyclonal antisera. A complex-trapping-blocking ELISA and

a SPCE, both based on type specific MABs in the double function of catching and competitor antibody were described for the detection of antibodies to FMDV serotypes O, A, C (Van Maanen, 1990a; Van Maanen, 1990b; Brocchi *et al.*, 1990); the latter is being used in our National Reference Laboratory for almost 15 years. More recently a solid-phase blocking ELISA, based on a single MAB, was proven to be useful for type O mass serology (Chenard *et al.*, 2003).

Within the program to complete, update and improve the sets of reagents and diagnostics for FMD we have developed a new SPCE for the assessment of antibody to FMDV type Asia 1. In this test, specific antibodies in sera are detected due to their ability to compete with a peroxidase-labelled neutralising MAB. In this paper we describe the validation process of the assay.

Materials and Methods

Serum samples

Negative sera: a total of 3266 negative sera from healthy animals of different species (993 cattle, 1709 pigs, 574 sheep/goats) were randomly selected from those collected for other national serosurveillance programs.

OIE Reference sera: the bovine reference sera, including one universal negative sample and three positive sera, varying in strength from cut-off level to strong positive against FMDV types Asia 1, O1 Manisa, A22 Iraq, A Iran 96 and C1 Noville respectively, were tested to calibrate the assay and evaluate the serotype specificity.

Sera Positive to type Asia 1: a total of 666 cattle sera were classified as belonging to a positive population; they included:

- 436 samples originated from three herds in Israel, from cattle ranging in age from 4 to more than 46 months, which had received from 1 to more than 4 vaccinations with trivalent O, A, Asia 1 vaccine according the age; 30 samples originated from young animals (2-3 months old) not yet vaccinated but presumably maintaining maternally derived antibody;
- 200 samples originated from experimental cattle, provided by WRL, Pirbright, UK and by the three national reference laboratories of Germany, the Netherlands and Russia respectively. Of them,
 - a) 85 samples were from cattle used for Asia 1 vaccine potency testing; few of them were collected sequentially on two occasions;
 - b) 99 samples derived from cattle vaccinated and infected with the single FMDV serotype Asia 1; several of them were collected on two occasions from the same animal, within one month after challenge;
 - c) 16 samples were from infected cattle; of them 5 were obtained from convalescent cattle each infected with a different Asia 1 strain.

Sera positive to other FMDV serotypes: the type-specificity of the assay was evaluated using sera from animals infected against the heterologous FMDV serotypes O, A, C. In detail:

- Seventy-one sera positive to type O, including 65 sheep from a flock recently infected with type O, 3 convalescent cattle from experimental infections and the OIE reference sera for O1 Manisa;
- 8 cattle sera positive to type A, including the OIE reference sera for type A and experimental post-infection sera;
- 4 cattle sera positive to type C, including the OIE reference sera for type A and experimental post-infection sera.

Virus and antigen preparation

FMD virus type Asia 1, strain Nepal 29/97 was used as antigen in both VNT and SPCE. The virus was propagated in IB-RS2 cell monolayers and harvested when cytopathic effect was maximum (24h post-infection). The tissue culture harvest was clarified at 3000 g for 20 min. and stored at -70°C for use in the VNT. For ELISA, the virus was inactivated with 0.001 M binary ethyleneimine (BEI) for 48 h at 26°C before use.

Monoclonal antibody-based Solid Phase Competitive ELISA (SPCE)

The principle and the procedure of the MABs-based competitive ELISA, firstly described for the quantification of antibodies specific to the European subtypes O1, A5 and C1 of FMDV (Brocchi *et al.*, 1990), and later applied to other infectious diseases (Brocchi *et al.*, 1993; Brocchi *et al.*, 1995) was adapted for the detection of FMDV type Asia 1 specific antibodies. The MAB selected for this application was 5E10, used either as antigen catching or as competing, peroxidase-conjugated antibody.

The assay procedure is as follows: ELISA plates are coated with 50 µl per well of MAB 5E10 at a saturating dilution in carbonate/bicarbonate buffer pH 9.6 by overnight incubation at 4°C. The plates are washed three times with PBS containing 0.05% Tween 20 and 50 µl of FMDV antigen at a predetermined optimal dilution is added to each well. The plates are then incubated for 1h at 37°C and washed again.

Then 50 µl of test and control sera are incubated with the trapped antigen for 1h at 37°C, starting from the 1/10 dilution. Three-fold dilution series of sera are obtained directly in ELISA wells. After

incubation 25 µl per well of peroxidase-conjugated MAb 5E10 is added, without washing, to each well and plates are incubated at 37°C for a further 1h.

After a last series of washes the colorimetric reaction is developed by distributing 50 µl per well of the substrate solution (orthophenyldiamine (OPD) 0.5 mg/ml in phosphate-citrate buffer pH 5, containing 0.02% H₂O₂). The reaction is stopped after 10 min. by adding 50 µl of 2NH₂SO₄. Optical density values (OD) at 492 nm wavelength are read using a microplate reader.

Antigen, sera and conjugate are diluted in PBS pH 7.4 containing 0.05% Tween 20 and 1% yeast extract; an optimal dilution of antigen is determined by checkerboard titrations of antigen and HRPO-conjugated MAb which define working dilutions giving a spectrophotometric reading of 1.5 OD.

In SPCE used as single dilution screening assay, sera registering 70 % or more inhibition of the mean OD value recorded in 4 virus control wells, at the dilution 1/10, are considered positive; end point titres of positive sera are expressed as the reciprocal of the highest serum dilution giving a 50% inhibition. As a positive control, four standard dilutions of a secondary control serum are included in each plate: the third dilution is expected to produce 50% inhibition.

Virus Neutralisation Test (VNT)

The VNT was performed following the procedure described in the OIE Manual (Office International des Epizooties, 2004), Briefly 50 µl of tissue culture fluid containing estimated 100 TCID₅₀ of FMDV Asia 1, strain Nepal 29/97, were incubated for 1 h at 37°C with 50 µl of serial dilutions of inactivated test sera in flat bottom microtitre plates (2 wells/dilution). A volume of 100 µl of IB-RS2 cells at approximately 10⁵ cells/ml in medium containing 10% foetal calf serum was added to each well. After incubation for two-three days at 37°C, wells were scored for cytopathic effect and antibody titres were expressed as the reciprocal log₁₀ of the final dilution of serum required to neutralize 50% of the inoculated cultures.

Results

Selection of MABs and test design

Since one of the requirements is the possibility to use non purified and non-concentrated antigen, the strategy adopted was the use of a capture MAb coated to ELISA plates, able to immune-purify and concentrate the viral antigen from supernatants of infected cells and to present the trapped antigen to a second MAb, directly conjugated with peroxidase. Binding of the second antibody to the trapped virus must be competed by positive sera, i.e. it is essential that the conjugated MAb is directed against an immunogenic epitope. Further useful characteristics of the competitor MAb include a neutralising activity and an extended intratypic reactivity; this because epitopes involved in neutralisation of viral infectivity are surface-exposed and expected to be more immunogenic, while the antigenic stability of epitopes should prevent the risk to underestimate antibodies induced by strong antigenic variants.

Previous results showed that many combinations of MABs from the panel developed against FMDV type Asia 1 provide a strong and type-specific reaction when used as catching and conjugated MABs (Grazioli *et al.* 2002). Concerning the catching MAB, it appeared indifferent whether the virus was trapped by a type-specific or an inter-types reactive MAB, or by a neutralising or a non-neutralising antibody. In contrast, the conjugated (competitor) antibody must be type-specific. For the choice of the best competitor MAB, the capability of selected sera varying in antibody concentration to compete with individual MABs was preliminarily evaluated: different extents of competition were observed against all the tested MABs, however better performances were seen with the neutralising MABs directed to site I (G-H loop of VP1) or IV (VP3, B-B knob), which also satisfy the requirement of a broad intratypic reactivity (Grazioli *et al.*, 2004). The final choice for the competitor MAB fall on 5E10, representative of MABs defining the neutralising site IV. MABs to site IV are subject to cross-competitions, presumably due to steric hindrance, by a wide range of neutralising MABs, even if directed to different epitope (personal communication). This ability confers the potential to detect different sets and specificities of antibodies in animals.

Moreover, MAB 5E10 binds effectively to FMDV Asia 1 when used either as a capture antibody or as a peroxidase-labelled conjugate, so that a unique MAB was selected for both functions.

Cut-off definition and diagnostic performances

When used as single dilution tests, competitive immunoassays are more appropriate as qualitative or semi-quantitative rather than quantitative assays. Consequently, the selection of the screening dilution for test sera is a determinant step in the validation process. To this purpose, International Reference Sera for FMDV type Asia 1, selected according results of the FAO Phase XVII Standardisation exercise (Paton *et al.*, 2003), were titrated in a dilution series from 1/10 to 1/270. Results, expressed as percentage inhibition at each dilution, are shown in figure 1. Although the dilution that best discriminates sera varying in strength is 1/30, we preferred 1/10 as single screening dilution and as initial dilution for titration. The 1/10 dilution can be easily obtained directly into ELISA plates and is expected to provide a better sensitivity.

Figure 2 shows frequency distributions of percentage inhibitions generated by known negative and positive sera, examined at the screening dilution 1/10. Negative sera originated from 3266 different animals, including 983 cattle, 1709 pigs and 574 sheep; positive sera were from 666 vaccinated or infected cattle, as described in Materials and Methods. Seventy percent inhibition represented the threshold that best discriminated negative and positive population. Using this threshold, the test scored diagnostic specificity of 99,3%, combined with diagnostic sensitivity of 99,7%. No significant differences in the frequency distribution of naive cattle, pigs or sheep/goats were observed (figure 2, table 1).

Analytical sensitivity

Analytical sensitivity of the MAb-based SPCE was evaluated by testing a dilution series of four positive sera, 3 of them collected 23 days after challenge of vaccinated cattle and 1 from a mono-vaccinated bovine. As a term of comparison, the OIE prescribed test for screening, i.e. the polyclonal-based SPCE (Office International des Epizooties, 2004a), using reagents and protocols provided by the OIE/FAO World Reference Laboratory, was carried out in parallel (figure 3). The MAb-based SPCE exhibited an analytical sensitivity higher (3 to 9 fold) than that shown by the polyclonal-SPCE when evaluated on the 3 vaccinated and infected cattle; however, analytical sensitivities of the two assays were comparable with respect to the fourth serum sample from the only vaccinated cattle. The profile of dose-response curves also displayed some differences between the two tests: curves obtained using the MAb-based SPCE presented typical sigmoidal profiles, while percentage inhibition values generated by the polyclonal-SPCE are distributed according a linear profile. These differences presumably reflect the different populations of antibody measured in the two test.

Type specificity

Eighty-three sera, predominately from animals infected and a minority from animals vaccinated with FMDV serotypes O (n=71), A (n=8) and C (n=4), were titrated simultaneously by the MAb-based SPCE-Asia 1 (heterologous titres) and by the respective homologous ELISA (in-house assays used on a routine base) (figure 4).

A low degree of cross-reactivity in the SPCE-Asia 1 was observed in 35% of the heterologous sera: these sera scored cross reactive titres against Asia 1 ranging from 10 to 100 times lower (1-2 log₁₀) than against the homologous type. All the other (65%) were completely negative in SPCE-Asia 1, or scored percentages of cross reactivity lower than 1% (differences in homologous/heterologous titres higher than 2 log₁₀).

Correlation with Viral Neutralisation Test

A total of 108 serum samples, collected from experimental cattle either vaccinated with Asia 1 monovalent vaccine or vaccinated and then infected with FMDV type Asia 1, were titrated in the MAb-based SPCE and in VNT. The regression of ELISA /VNT end-point titres is shown in figure 5. A positive correlation was observed, with correlation coefficient between VNT and SPCE 0,66 and regression coefficient 0,77.

Applied to evaluate the 4 OIE definitive reference sera, which vary in strength from negative to strong positive, the SPCE recognised all of them correctly (table 2), with a sensitivity higher than it is required. In contrast, all OIE reference sera for types O1 Manisa, A22 Iraq, A Iran 96, C1 Noville scored negative (% inhibition < 70, titres < 10).

Discussion

The development and the evaluation of a novel test format for the measurement of antibodies to the FMDV type Asia 1, based on a solid phase competitive ELISA using a single MAb, is comprised within the objective to improve and complete where necessary the diagnostics available for FMD.

In this test, specific antibodies in serum are detected due to their ability to compete with a peroxidase-labelled neutralising MAb. The MAb selected is 5E10, that recognises the conformational site located within the B-B knob of VP3 (site IV), (Grazioli *et al.*, 2004).

The MAb-based SPCE described in this paper is suited either as a screening test, and also for antibody titration. The diagnostic sensitivity and specificity of the assay were studied on a large number of known positive and negative sera, adequate to the validation criteria established in the OIE guidelines (Office International des Epizooties, 2004). Taking 70% inhibition as the threshold level at the screening dilution 1/10, a specificity higher than 99% was demonstrated for the three species examined. A sensitivity close to 100% was detected by examining a positive population, predominantly composed by field vaccinated cattle and by experimental cattle, vaccinated and/or infected. The minimum time point of sampling after exposure to viral antigens was 8 days in five experimentally vaccinated animals: seroconversion was already detectable in these animals. Also 29 out of 30 calves, sampled before vaccination in herds regularly vaccinated, were scored positive, reasonably due to maternal immunity.

A high performance of the MAb-based SPCE was confirmed by the evaluation of internationally recognised reference sera: even the weak serum that should determine the cut-off of detection showed a level of positivity well above the threshold in the new ELISA.

The correlation observed between titres of 108 sera tested in ELISA and VNT suggests that the MAb-based SPCE actually detects the virus neutralising response of animals. Even neutralising MAbs which reacted to epitopes different from that recognised by 5E10 (Grazioli *et al.*, 2004) itself were able to block the binding of peroxidase-labelled 5E10, presumably due to steric hindrance. In contrast, parallel experiments demonstrated that the MAb 5E10 was not inhibited by non-neutralising MAbs (personal communication).

The observation that MAb 5E10 recognises a conserved site, as proven by its broad intra-typic reactivity (Grazioli *et al.*, 2004) and by the absence of amino acid substitutions in the corresponding region of field isolates (Marquardt *et al.*, 2000), ensures that the SPCE based on this MAb is able to detect antibodies regardless of the strain that induces them. Furthermore, the ability of a wide range of neutralising antibodies to block the binding of 5E10 confers to the SPCE the potential to identify different sets of neutralising antibodies, preventing the risk that herds infected with strains antigenically mutated are incorrectly diagnosed as negative.

When serology to FMDV structural proteins is used, it is convenient to apply an assay which is as serotype-specific as possible. Serotype-specificity of the SPCE was evaluated on a number of samples from animals infected with serotypes other than Asia 1: heterologous antibodies produced some cross-reactivity, but, when detectable, cross-reactive titres in Asia 1-SPCE were much lower than the homologous ones. This property was also reported for the polyclonal-SPCE (Mackay *et al.*, 2001), in contrast to a low type-specificity shown by another MAb-based ELISA developed for type O antibody (Chenard *et al.*, 2003). In the former assay, as well as in our SPCE, the virus is immune-captured before reacting with sera, whilst in the latter ELISA the antigen is directly coated to the solid-phase. Direct adsorption provokes a much greater alteration of the viral structure than immune-capture, causing the exposition of internal, common epitopes: cross-reactive antibodies present in sera can then bind to the antigen, enhancing the level of cross-reactivity due to steric hindrance. Differently, the immune-capture of the virus prevents degradation, so that mainly type-specific, exposed epitopes remained accessible for the reaction with sera.

The profile of titration curves of some sera, obtained with the MAb-based SPCE and the polyclonal-based SPCE in order to evaluate and compare the analytical sensitivities, provided evidence of differences between the two assays and between animals. The serum from a vaccinated cattle showed almost overlapping titration curves, suggesting that the quality of antibodies detected by the two assays in this animal are very similar; in contrast the curves obtained with three vaccinated and infected cattle differed considerably, regarding either the shape and the sensitivity: analytical sensitivity of the MAb-based ELISA appeared greatly increased in the challenged animals, presumably after a booster of subsets of antibody induced by infection, that better compete with the selected MAb 5E10. In addition, also differences in relative affinity and specificity of the antibody against which the competition is measured may be the cause of the observed divergences.

Besides a higher analytical sensitivity, the test developed has other advantages over the polyclonal-SPCE: i) firstly, the new test is based on MAbs rather than polyclonal antisera, with all the benefits of standardisation and continuity of supply that this implies; ii) the use of the MAb 5E10, either as the catching or as the detecting antibody, simplify the supply of reagents and the performance of tests; iii) the test is highly specific, without requiring considerable volumes of blocking agents, such as bovine and rabbit sera, to prevent non-specific reactions. The MAb-based ELISA uses synthetic buffer, easily reproducible, while the biological additives necessary in the polyclonal ELISA may represent a further source of variability.

In conclusion, the results suggest that the MAb-based SPCE developed for the detection of antibodies to FMDV type Asia 1 has potential applications as rapid, simple and inexpensive test in the serodiagnosis of FMDV and in serosurveillance programmes. Improvements of specificity and standardisation with respect to existing ELISA are warranted by the use of a selected MAb, that proved to confer also an excellent sensitivity. Further progress in the validation process of the test should include the organisation of inter-laboratories trials.

Conclusions

- 1) The MAb-based Solid Phase Competitive ELISA (SPCE) for the detection of antibodies to FMDV type Asia 1 has high diagnostic performances: specificity of 99,3%, sensitivity of 99,7%;
- 2) Advantages over polyclonal-based ELISAs are related to the use of a selected, neutralising MAb and include: continuity of supply, homogeneity of immunological ingredients, simpler buffers, a unique MAb replacing three antisera (rabbit, guinea pig and anti-species immunoglobulins)

Recommendations

- 1) The development and application of immunoassays making use of MAbs for FMD serology is recommended, with extension to all FMDV serotypes
- 2) The conversion of validated, simple tests in robust kit format should be encouraged

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References

Anderson, J., Corteyn, M., Hamblin, P. & Paton, D. 2003. Further validation of the solid-phase competitive ELISA for FMDV types a, C & Asia 1. *Report of Sess. Res. Gr. St. Tech. Committee of the European Commission for the Control of Foot-and-Mouth Disease*. Berne, Switzerland, September 16-19 2003, Appendix 24: 157-165.

Brocchi, E., De Simone, F., Bugnetti, M., Gamba, D. & Capucci, L. 1990. Application of a monoclonal antibody-based competition ELISA to the measurement of anti-FMDV antibodies in animal sera. *Report of Sess. Res. Gr. St. Tech. Committee of the European Commission for the Control of Foot-and-Mouth Disease*, Lindholm, Denmark, June 24-25 1990, Appendix 14.

Brocchi, E., Gamba, D., Poumarat, F., Martel, J.I. & De Simone, F. 1993. Improvements in the diagnosis of contagious bovine pleuropneumonia through the use of monoclonal antibodies. *Rev Sci Tech Off Int Epizoot.*, 12: 559-570.

Brocchi, E., Berlinzani, A., Gamba, D. & De Simone, F. 1995. Development of two novel monoclonal antibody-based ELISAs for the detection of antibodies and the identification of swine isotypes swine vesicular disease virus. *Journal of Virological Methods*, 52: 155-167

Chénard, G., Miedema, K., Moonen, P., Schrijver, R. S. & Dekker, A. 2003. A solid-phase blocking ELISA for detection of type O foot-and-mouth disease virus antibodies suitable for mass serology. *Journal of Virological Methods*, 107: 89-98

Grazioli, S., Fallacara, F. & Brocchi, E. 2002. Monoclonal antibodies against FMDV type Asia 1: preliminary characterization and potential use in diagnostic assay by European Commission for the Control of Foot-and-Mouth Disease. *Report of Sess. Res. Gr. St. Tech. Committee of the European Commission for the Control of Foot-and-Mouth Disease* Izmir, Turkey, September 17-20 2002, Appendix 24: 194-201.

Grazioli, S., Fallacara, F. & Brocchi, E. 2004. Mapping of neutralising sites on FMD virus type Asia 1 and relationships with sites described in other serotypes. *Report of Sess. Res. Gr. St. Tech. Committee of the European Commission for the Control of Foot-and-Mouth Disease*. Crete, Greece, October 12-15 2004. Appendix 44 (*This proceedings*).

Hamblin, C., Barnett, I. T.R. & Hedger, R.S. 1986a. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I Development and method of ELISA. *Journal of Immunological Methods*, 93: 115-121.

Hamblin, C., Barnett, I. T.R. & Crowter, J.R. 1986b. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. II Application. *Journal of Immunological Methods*, 93: 123-129.

MacKay, D.K.J., Naci Bulut, A., Rendle, T., Davidson, F. & Ferris, N.P. 2001. A solid-phase competition ELISA for measuring antibody to a foot-and-mouth disease virus. *Journal of Virological Methods*, 97: 33-48.

Marquardt, O., Rahman, M.M. & Freiberg, B. 2000. Genetic and antigenic variance of foot-and-mouth disease virus type Asia 1. *Archives of Virology*, 145: 149-157.

Office International des Epizooties. 2004a. Foot-and-Mouth Disease. In: Manual of Diagnostic tests and Vaccines for Terrestrial Animals. OIE, Paris, France, pp 111-128.

Office International des Epizooties. 2004b. Principles of validation of diagnostic assay for infectious disease. OIE, Paris, France, pp 21-29.

Paiba, G.A., Anderson, J., Paton, D.J., Soldan, A.W., Alexandersen, S., Corteyn, M., Wilsden, G., Hamblin, P., MacKay, D.K.J. & Donalson, A.I. 2004. Validation of a foot-and-mouth disease

antibody screening solid-phase competition ELISA (SPCE). *Journal of Immunological Methods*, 115: 145-158.

Paton, D., Armstrong, R. & Anderson, J. 2003. An update progress with the FAO Collaborative Studies for FMD Serology Standardisation, Phase XVII and XVIII. *Report of Sess. Res. Gr. St. Tech. Committee of the European Commission for the Control of Foot-and-Mouth Disease*. Berne, Switzerland, September 16-19 2003, Appendix 15: 102-104.

Van Maanen, C. 1990a. A complex-trapping-blocking (CTB) ELISA, using monoclonal antibodies and detecting specifically antibodies directed against foot-and-mouth disease types a, O, and C. I. Method and characteristics. *Veterinary Microbiology*, 24: 171-178.

Van Maanen, C. 1990b. A complex-trapping-blocking (CTB) ELISA, using monoclonal antibodies and detecting specifically antibodies directed against foot-and-mouth disease types a, O, and C. II. Application. *Veterinary Microbiology*, 24: 179-191.

Van Maanen, C. & Terpstra, C. 1989. Comparison of a liquid-phase blocking sandwich ELISA and a serum neutralization test to evaluated immunity in potency test of foot-and-mouth disease vaccines. *Journal of Immunological Methods*, 124: 111-119.

Table 1: SPCE specificity (SP) and sensitivity (SE) at the established cut-off

% inhibition	NEGATIVE ANIMALS						TOTAL NEGATIVE		POSITIVE CATTLE	
	cattle		pigs		sheep/goats					
< 70 %	975	99.18 %	1695	99.18 %	571	99.48 %	3243	99.3 % (SP)	2	0.3 %
≥ 70 %	8	0.82 %	14	0.88 %	23	0.52 %	3		0.7 %	664
Total n°	983	100 %	1709	100 %	574	100 %	3266	100 %	666	100 %

Table 2: Results, expressed either as percentage inhibitions and as end-point titres, of the OIE reference sera in the SPCE-Asia 1

OIE Ref. sera Asia 1	SPCE-Asia 1		VNT
	% inhib.	titre	
Strong positive	97	1/500	1/512
Weak pos 1	96	1/180	1/256
Weak pos 2 (cut-off)	91	1/60	1/45
Negative	21	<1/10	<1/4

Figure 1. Titration curves of OIE international reference sera in the MAb-based SPCE for antibody detection against FMDV type Asia 1

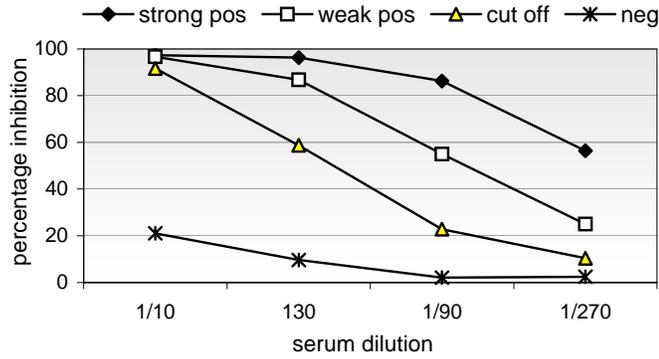


Figure 2: Frequency distribution of the percentage inhibitions recorded from the examination of known negative (n° 3266) and positive (n° 666) sera at the 1/10 dilution in the MAb-based SPCE for the detection of anti-Asia 1 antibodies

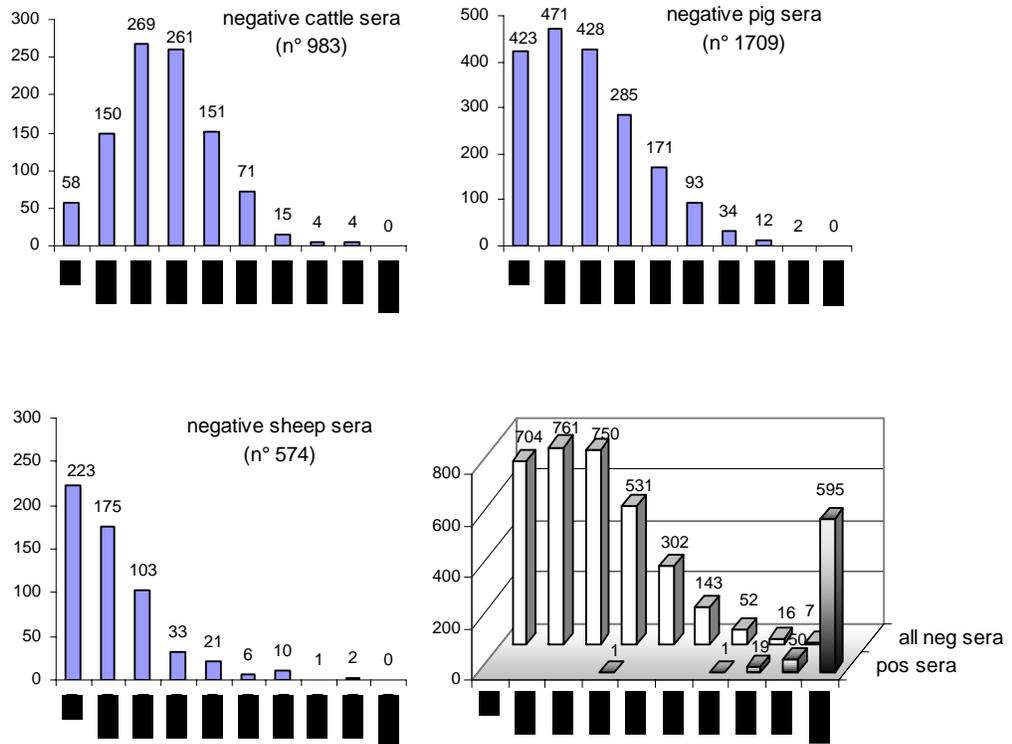
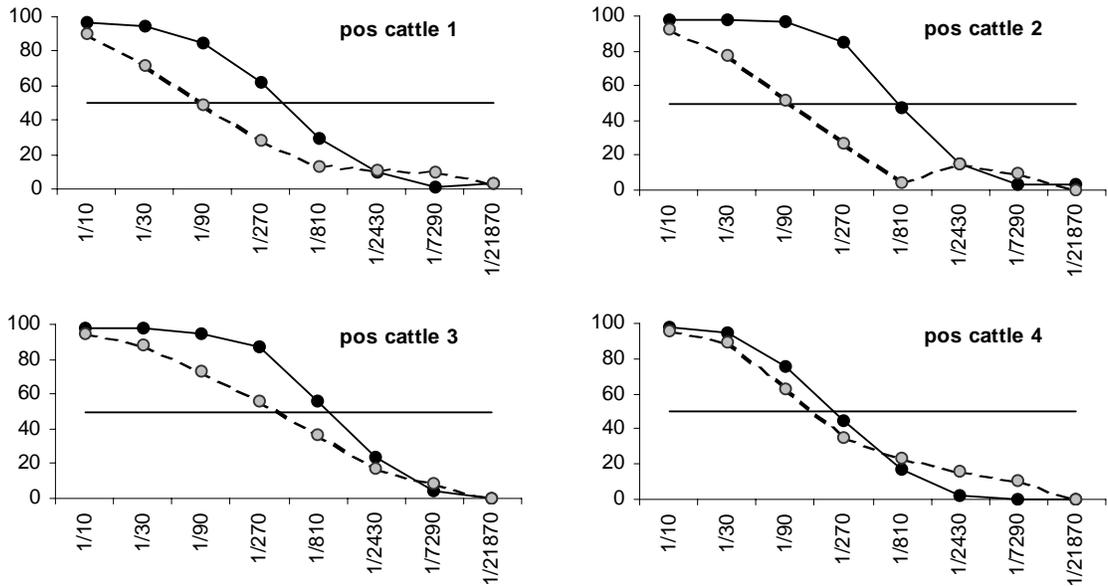


Figure 3: Analytical sensitivity of MAb-based SPCE and polyclonal-SPCE evaluated on 4 positive sera



Dose-response curves of 4 positive sera: cattle n°1, n°2, n°3, vaccinated and infected; cattle n°4 only vaccinated
 Continuous line: MAb-based SPCE; dotted line: polyclonal-SPCE
 Horizontal line indicates position of the cut-off, established at 50% inhibition for both tests, when used to calculate end-point titres of positive sera.

**Figure 4: Evaluation of the cross-reactivity generated by antibodies to heterologous serotype in the SPCE-Asia 1
 Comparison between homologous and heterologous titres**

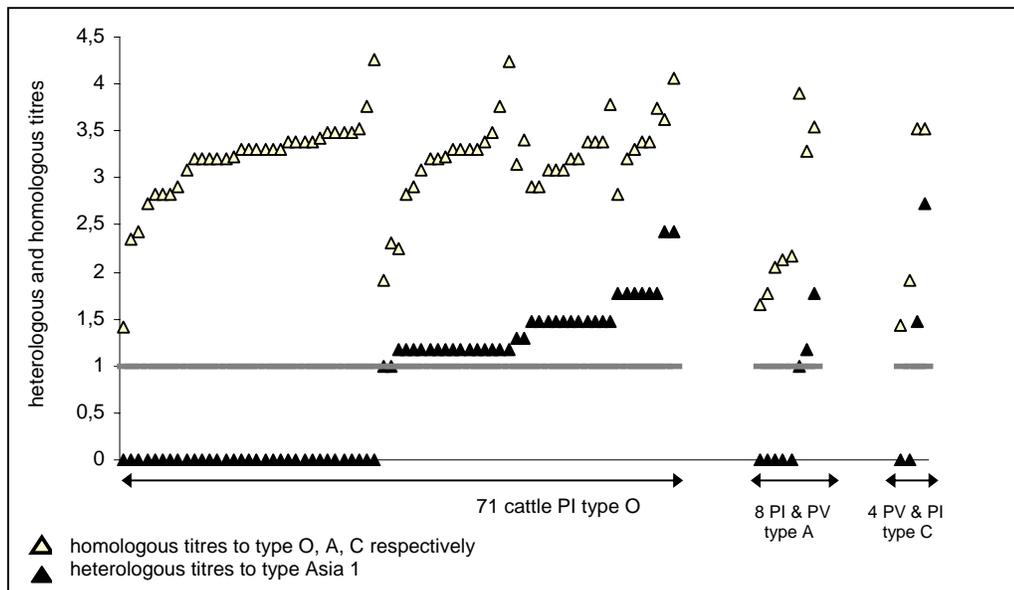
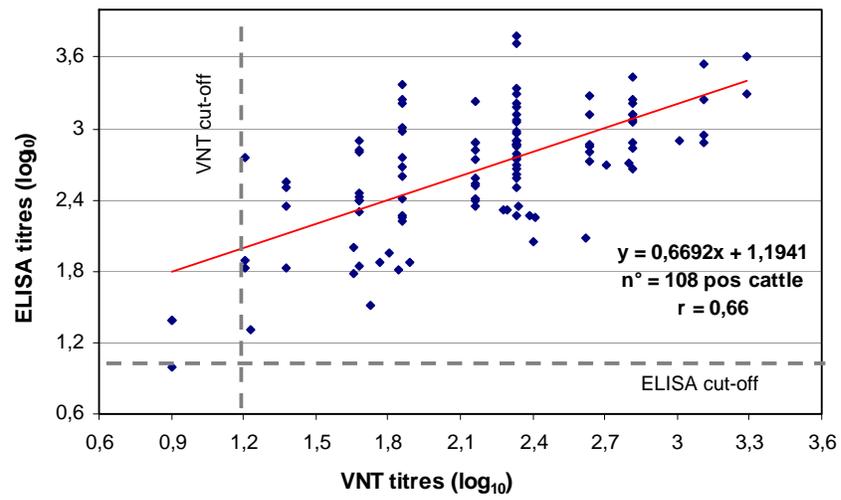


Figure 5: Regression and correlation of titres measured in VNT and MAb-based SPCE recorded for 108 experimentally vaccinated and/or infected cattle



Potential application of Bayesian probability diagnostic assignment (BPDA) method to predict FMDV infection from serologic results

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Introduction: In order to minimize the destruction of livestock and associated consequences of FMD, vaccination is now considered to be an acceptable strategy for the control and eradication of FMD. Some drawbacks exist, however, to use of vaccination. These problems include 1) the need for serologic testing following vaccination to identify infected animals and 2) the lack of accurate means to discriminate between infected-vaccinated animals and uninfected-vaccinated animals. The latter problem relates both to the failure of some infected animals to respond to vaccination, and thus not show any detectable antibodies to structural proteins (false negative), and to the presence in some uninfected-vaccinated animals of antibodies to non-structural proteins (false positive). Thus, there is likely some small, but as yet not well known, probability of a false negative result and of a false positive result. A general recommendation is that the issue of false positive and false negative responses would (somehow) be resolved on a herd basis, but we are not aware of any methodology proposed to accomplish this.

Materials and Methods: We have developed a statistical methodology that estimates the probability that an animal is infected with a specified agent given the specific antibody concentration (ELISA s/p value). The approach also permits estimation of the prevalence (and 95% prediction interval of the prevalence) of infection in a herd, based on serologic values for a representative sample of animals in the herd. The method is referred to as probability diagnostic assignment (PDA), and has been extended to a fully Bayesian format (BPDA). We have developed the method for *Neospora caninum* infection in cattle. The method utilizes two distributions of serologic values, one for animals that are infected and one for animals that are not infected. No cutoff values are used, thus there is no need for estimates of sensitivity or specificity, and the full scale of information inherent in the values of the assay is used. Consequently, information in the serologic values is not limited to two dichotomous representations of 'positive' or 'negative'; rather, the full range of serologic values is utilized. Several parameters are estimated, including the probability that a given animal is infected and the probability that the herd is infected (estimated prevalence of infection in the herd).

Results: In the absence of specific ELISA data for FMD-vaccinated, unvaccinated, infected, and uninfected animals, we have not yet had the opportunity to assess whether the Bayesian PDA might have application in predicting infection status of vaccinated animals.

Discussion: We will provide an illustration of the potential application of the BPDA to predict the probability of FMDV infection in an animal and in a herd, using serologic values and other covariate information.

Modelling early viral dynamics of FMDV *in vivo*

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Abstract:

To quantify and predict virus excretion and infectiousness of animals infected with foot-and-mouth disease virus (FMDV), an understanding of the virus dynamics in individual animals is needed. In this paper FMDV dynamics *in vivo* are described in mathematical terms and possible biological mechanisms suggested to explain the discrepancies between the mathematical model and the experimental data. The model described virus concentrations in the circulation and interstitial space, as well as uninfected and infected epithelial cells. The model was fitted to dose-response curves obtained from pigs inoculated with tenfold different doses of FMDV O UKG 34/2001 by intravenous injection (12 pigs in total, 4 pigs per treatment group, 3 treatments). The model qualitatively reproduced the decrease of FMDV in the circulation seen after intravenous inoculation, followed by an increase of FMDV due to replication of the virus in epithelial cells. The model output showed much smaller differences in the timing of viraemia curves between treatment groups than was indicated by the experimental data. The data were better described by a modified model, in which the rate of infection of epithelial cells at low FMDV concentrations was limited. The modified model was able to accurately describe early FMDV dynamics *in vivo*. Further theoretical and experimental work suggested by the modelling exercise is discussed.

Introduction:

The causative agent of foot-and-mouth disease (FMD) is the foot-and-mouth disease virus (FMDV), a positive strand virus that belongs to the *Aphthovirus* genus, family *Picornaviridae* (Belsham, 1993). An important characteristic of FMDV is the highly contagious nature of the virus. Cattle and sheep can be infected with as little as 10 TCID₅₀ (Gibson and Donaldson, 1986; Donaldson *et al.*, 1987) from as far as 270 km away from a source of FMDV under exceptional conditions (Gloster *et al.*, 1982). Considerable effort has been made to quantify the transmission of FMDV experimentally (Donaldson and Alexandersen, 2001; Aggarwal *et al.*, 2002; Alexandersen *et al.*, 2002a; Alexandersen and Donaldson, 2002; Alexandersen *et al.*, 2002b; Hughes *et al.*, 2002; Alexandersen *et al.*, 2003) and describe it in the form of mathematical models (Haydon *et al.*, 1997; Ferguson *et al.*, 2001b, 2001a; Keeling *et al.*, 2001; Morris *et al.*, 2001). Atmospheric models (Gloster *et al.*, 1981; Donaldson *et al.*, 1982; Baldock, 1993; Durand and Mahul, 1999; Sorensen *et al.*, 2000; Sorensen *et al.*, 2001; Gloster and Alexandersen, 2004) have been developed to describe the dissemination of FMDV in the atmosphere. All the models mentioned are influenced by the infectiousness and amount of virus excreted by individual animals, yet very little modelling has been undertaken to quantify and predict the infectiousness of an animal. The objective of this research was to quantify and describe in mathematical terms the early viral dynamics of FMDV *in vivo*, in order to predict the excretion of FMDV and infectiousness of individual animals.

Materials and Methods:

Model A

A model of FMDV replication *in vivo* (Model A) was considered (Figure 1).

Virus concentrations in the central compartment and interstitial space were represented by X [FMDV genomes per ml serum (ml⁻¹)] and Y [FMDV genomes per ml serum in the interstitial space (ml⁻¹)] respectively. FMDV was lost from the interstitial space through 3 routes: drained by the lymphatic system to re-enter the central compartment at rate k_{yx} , removed (k_{yo}) or used to infect epithelial cells (β_1). FMDV was added to the interstitial space from the central compartment (k_{xy}) or from burst infected epithelial cells (b).

The total numbers of uninfected and infected epithelial cells were represented by variables C and D respectively. Infected epithelial cells died at rate k_{do} and released virus back into the interstitial space or infected neighbouring uninfected epithelial cells (β_2).

This gave rise to the following ordinary differential equations:

$$\frac{dX}{dt} = \frac{1}{\lambda} k_{yx} Y - k_{xy} X \quad (1)$$

$$\frac{dY}{dt} = \lambda k_{xy} X - k_{yx} Y - k_{yo} Y + b k_{do} D \quad (2)$$

$$\frac{dC}{dt} = -\beta_1 Y C - \beta_2 k_{do} D C \quad (3)$$

$$\frac{dD}{dt} = \beta_1 Y C + \beta_2 k_{do} D C - k_{do} D \quad (4)$$

The dimensions and the calculation of the parameters used in the model are described in Table 1.

Model B

In Model B, we explored the effect of limiting the *per capita* infection rate of epithelial cells by FMDV (β_1) in Model A at low FMDV concentrations, by introducing a sigmoidal term $\left(\frac{Y^p}{m + Y^p} \right)$ to Equations

3 and 4:

$$\frac{dC}{dt} = -\beta_1 \frac{Y^p}{m + Y^p} Y C - \beta_2 k_{do} D C \quad (5)$$

$$\frac{dD}{dt} = \beta_1 \frac{Y^p}{m + Y^p} Y C + \beta_2 k_{do} D C - k_{do} D \quad (6)$$

The effect of the sigmoidal term on β_1 is illustrated in Figure 2.

Results:

A typical output from Model A is shown in Figure 3. A decrease in virus concentration in the central compartment was followed by an exponential increase. A maximum concentration was reached, before decreasing when the rate of removal of virus was greater than the rate of viral replication.

The output from Model A was compared to experimental data, as described in Quan *et.al.* (in press). In this experiment, blood samples were collected three times a day from pigs inoculated intravenously with different doses of FMDV ($10^{8.1}$, $10^{7.1}$ or $10^{6.1}$ FMDV genomes/animal) (Figure 4).

Model A reproduced the general shape (decrease, followed by an increase of virus concentration in the central compartment) of a viraemia curve, but showed much smaller differences in the timing of viraemia curves between treatment groups than was indicated by the experimental data. As a result, the model did not accurately reproduce the experimental data.

Model B differed from Model A in the modification of β_1 by the term $\frac{Y^p}{m + Y^p}$ (Equations 5 & 6). A

typical output from Model B (Figure 5) was similar to the output from Model A (Figure 3), with the exception that viral growth was delayed in the early stages of infection, an effect of modifying β_1 . Parameter m determined the virus concentration of Y β_1 reached maximum (Figure 2), the greater the value of m , the longer the delay in viral growth (results not shown).

The output from Model B was compared to experimental data (Quan *et.al.*, in press) in Figure 6 and a good overall fit was obtained.

Discussion:

A theoretical framework was developed to explore the early viral dynamics of FMDV *in vivo*. All the aspects of the intra-host life cycle of FMDV, from distribution, the infection of epithelial cells, replication within the cell, cell death with release of progeny virus, infection of neighbouring epithelial cells were included in Model A. The focus of the model was on viral dynamics in the early stages after infection and a full description of the adaptive immune response in this model was not included.

Virus concentrations in the central compartment and interstitial space were described. The interstitial space is an important compartment in the body as it the interface between the central compartment and the epithelial cells. This space contains water, small solutes, plasma proteins, collagen, elastin, hydrophilic polymers such as hyaluronate and proteoglycans, fat and cells (Geiger *et al.*, 1984). A large fraction of the plasma proteins and fluid reservoirs are found in the interstitium of connective tissues.

Experimental work had shown that FMDV in the central compartment decreased rapidly ($\frac{1}{2}$ life of 30 min) after intravenous inoculation (unpublished data). The loss of virus from the central compartment may have been due to the accumulation of the virus in the interstitial space, clearance of FMDV by the immune system and/or loss of FMDV to epithelial cells. Of the possibilities, the accumulation of FMDV in the interstitial space was considered the most likely mechanism for the decrease of the virus from the central compartment.

The immune system as the main mechanism for clearance of FMDV from the central compartment after an intravenous bolus of FMDV was unlikely based on experimental evidence that FMDV could not be detected in tissues of the mononuclear-phagocyte system, such as the liver, spleen and lymph nodes two hours after an intravenous inoculation of $10^{8.1}$ FMDV genomes (unpublished data). The detection threshold of the assay used to measure FMDV in tissue was approximately $10^{3.5-4.0}$ FMDV genomes/g tissue. If the same inoculation dose was used and we assumed 10% of circulating FMDV was removed by the liver, the approximate concentration was calculated to be $10^{4.3}$ FMDV genomes/g liver in a 25 kg pig [liver weight was calculated as 2.7% live weight (Collin *et al.*, 2001) and a homogenous concentration of virus in the liver was assumed], above the detection threshold of the assay. Virus should therefore have been detectable in these tissues if the mononuclear-phagocyte system was the main mechanism for clearance of FMDV from the central compartment.

The decrease of FMDV from the central compartment may also have been as a result of loss of virus to epithelial cells. Using this approach, it was not possible to reconcile Model A or other models (describing virus concentrations in epithelial cells; models and results not shown) with the delays seen in the experimental data between the time of inoculation and the onset of active viraemia. Where the uptake of virus by epithelial cells was the main mechanism for the loss of virus from the central compartment, the models predicted a very quick onset of viraemia (for a wide range of inoculation doses) and this mechanism was therefore not considered to be the main reason for the decrease of FMDV from the central compartment.

Model A qualitatively reproduced the general shape of a viraemia curve (Figure 3), but did not accurately reproduce the dose differences seen in the experimental data, nor capture the delay in time between inoculation and the start of an active viraemia when virus was undetectable in the central compartment (eclipse phase) (Figure 4).

A comparison between the output from Model A and the experimental data suggested a non-linear relationship between inoculation dose and onset of active viraemia. A non-linear relationship was explored by limiting the rate of infection of epithelial cells at low FMDV concentrations. *In vivo* experimental evidence to support this modification is lacking, but a limited infection rate at low FMDV concentration has been described *in vitro* (Thorne, 1962). The FMDV type O strain and pig kidney cells were used to show that the infection rate was proportional to virus concentration at low virus concentrations. At higher virus concentrations, the infection rate levelled off to a constant value, resulting in a curve similar to those seen in Figure 2.

Two biological interpretations of a limited rate of infection at low virus concentrations are a minimum infectious dose per cell hypothesis and virus-virus interactions.

The proportion of cells infected from a given virus concentration can be determined from a poisson distribution (multiplicity of infection) (Knipe *et al.*, 2001). From a hypothesis in which cells are able to support viral replication only if they contain a minimum number of viruses, the effective rate of infection (infection in cells that support replication) can be calculated and shown to be limited at low virus concentrations. There is no evidence to show that more than one FMDV is needed to initiate a successful infection in a cell, but the dose dependent inhibition of host protein synthesis and stimulation of viral protein synthesis by FMDV strongly suggests that a minimum infectious dose is likely.

Infection of cells with FMDV results in the inhibition of host protein synthesis. The mechanism involves the cleavage of the translation initiation factor eIF4G by the leader (or L) (Devaney *et al.*, 1988; Medina *et al.*, 1993) and 3C protease (Belsham *et al.*, 2000) of FMDV. eIF4G is part of the eIF4F complex that plays a critical role in the recognition of the cap structure of cellular mRNA by the translation machinery of the cell (Prevot *et al.*, 2003). Translation of FMDV RNA is cap-independent and depends instead on the presence of an internal ribosome entry site (IRES) element within the 5' noncoding region (Belsham and Brangwyn, 1990). FMDV infection therefore results in a switch of the cell's translational capacity from cellular to viral protein synthesis.

If a viral infection is unable to direct the cell's translational machinery towards viral protein synthesis, an abortive infection will be the result. Ohlmann *et al.*, (1995) has shown that the rate of translation

of uncapped mRNA *in vitro* is dose dependent and a determinant of this rate is the concentration of L protease. At low L protease concentration, the rate of uncapped mRNA translation was directly proportional to L protease concentration. The greater the number of viruses infecting a cell, the higher the L protease concentration within the cell and the greater the efficiency in directing the cell's translational machinery towards viral protein synthesis. An inability of FMDV to produce viral proteins at low virus numbers can therefore be expressed as a minimum infectious dose/cell.

It has not been shown for FMDV, but virus-virus interactions can enhance the uptake of virus by target cells. Non-receptor mediated murine mink cell focus-inducing virus infection could be triggered in *trans* by the ecotropic virus glycoprotein expressed on the cell surface in a complex with its receptor. In addition, *trans* activation increased the rate of spread of the virus through a population of target cells, indicating that receptor-dependent and -independent pathways functioned in parallel (Wensel *et al.*, 2003). The rate of infection of cells by this virus is therefore not a constant and would increase with increasing virus concentration up to a maximum rate.

The term $\frac{Y^p}{m + Y^p}$ was used to limit β_1 at low virus concentrations (Equations 6 & 7). The equation for calculating the rate of infection was first reported by Michaelis and Menten, (1913) to describe a saturating reaction rate in enzyme kinetics and has also been used by Holling (1959), to describe Type III functional response predator-prey interactions.

In the Michaelis-Menten equation, the equivalent to m is the Michaelis constant (K_m). The K_m value is the substrate concentration at which the rate of the reaction is half the maximum rate and indicates the affinity of an enzyme for substrate. The effect of m on the model was to shift curve a viraemia curve (solid lines curves in Figures 4 and 6) to the right with increasing values of m . In the context of Model B, m may be thought of as the affinity of FMDV for the its epithelial cell surface receptor.

The Hill equation is a modification of the Michaelis-Menten equation, in which the substrate concentration (in this case Y) is raised to a power (p). The modification been used to describe allosterism, a process in which conformational changes are induced in the binding sites of an enzyme when a ligand or substrate binds to that enzyme. The conformational change can either increase (positive cooperativity) or decrease (negative cooperativity) the activity or affinity of the enzyme. In Model B, increasing values of p amplified dose differences by increasing the separation in time of viraemia curves of different doses (results not shown).

The fit of Model B to the experimental data was an improvement on Model A as the former reproduced the dose differences, as well as the eclipse phase in the experimental data, which the latter model did not.

Conclusions:

- A model to describe the early viral dynamics of FMDV *in vivo* (Model A) did not accurately reproduce experimental data (Quan *et al.*, in press).
- Discrepancies between the model and the data could be resolved by limiting the rate of infection of epithelial cells at low FMDV concentration.

Recommendations:

- More *in vivo* quantitative and modelling work should be encouraged to allow for a better understanding of the detailed quantitative aspects of foot-and-mouth disease.

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References:

- Aggarwal, N., Zhang, Z., Cox, S., Statham, R., Alexandersen, S., Kitching, R.P. & Barnett, P.V. 2002. Experimental studies with foot-and-mouth disease virus, strain O, responsible for the 2001 epidemic in the United Kingdom. *Vaccine*, 19(19-20): 2508-2515.
- Alexandersen, S., Brotherhood, I. & Donaldson, A.I. 2002a. Natural aerosol transmission of foot-and-mouth disease virus to pigs: minimal infectious dose for strain O1 Lausanne. *Epidemiol. Infect.*, 128(2): 301-312.
- Alexandersen, S. & Donaldson, A.I. 2002. Further studies to quantify the dose of natural aerosols of foot-and-mouth disease virus for pigs. *Epidemiol. Infect.*, 128(2): 313-323.

- Alexandersen, S., Quan, M., Murphy, C., Knight, J., & Zhang, Z.** 2003. Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *Journal of Comparative Pathology*, 129(4): 268-282.
- Alexandersen, S., Zhang, Z., Reid, S.M., Hutchings, G.H. & Donaldson, A.I.** 2002b. Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. *J. Gen. Virol.*, 83(8): 1915-1923.
- Bacha, Jr., W. J. & Bacha, L.M.** 2000. *Color atlas of veterinary histology*, 2nd edn. Philadelphia, Lippincott Williams & Wilkins.
- Baldock, C.** 1993. The use of mathematical modelling in understanding foot-and-mouth disease. In M.J. Nunn & P.M. Thornber, eds. *Proceedings of the National Symposium on Foot-and-Mouth Disease: Canberra, 8-10 September 1992*, pp. 177-190. Canberra, Australian Government Publishing Service.
- Belsham, G.J.** 1993. Distinctive features of foot-and-mouth disease virus, a member of the picornavirus family; aspects of virus protein synthesis, protein processing and structure. *Prog. Biophys. Mol. Biol.*, 60(3): 241-260.
- Belsham, G.J. & Brangwyn, J.K.** 1990. A region of the 5' noncoding region of foot-and-mouth disease virus RNA directs efficient internal initiation of protein synthesis within cells: involvement with the role of L protease in translational control. *J. Virol.*, 64(11): 5389-5395.
- Belsham, G.J., McInerney, G.M. & Ross-Smith, N.** 2000. Foot-and-mouth disease virus 3C protease induces cleavage of translation initiation factors eIF4A and eIF4G within infected cells. *J. Virol.*, 74(1): 272-280.
- Cartwright, S.F., Pay, T.W.F. & Henderson, W.M.** 1957. Multiplication of the virus of foot and mouth disease in culture. *J. Gen. Microbiol.*, 16(3): 730-748.
- Collin, A., Lebreton, Y., Fillaut, M., Vincent, A., Thomas, F. & Herpin, P.** 2001. Effects of exposure to high temperature and feeding level on regional blood flow and oxidative capacity of tissues in piglets. *Exp. Physiol.*, 86(1): 83-91.
- Devaney, M.A., Vakharia, V.N., Lloyd, R.E., Ehrenfeld, E. & Grubman, M.J.** 1988. Leader protein of foot-and-mouth disease virus is required for cleavage of the p220 component of the cap-binding protein complex. *J. Virol.*, 62(11): 4407-4409.
- Donaldson, A.I. & Alexandersen, S.** 2001. Relative resistance of pigs to infection by natural aerosols of FMD virus. *Vet. Rec.*, 148(19): 600-602.
- Donaldson, A.I., Gibson, C.F., Oliver, R., Hamblin, C. & Kitching, R.P.** 1987. Infection of cattle by airborne foot-and-mouth disease virus: minimal doses with O1 and SAT 2 strains. *Res. Vet. Sci.*, 43(3): 339-346.
- Donaldson, A.I., Gloster, J., Harvey, L.D. & Deans, D.H.** 1982. Use of prediction models to forecast and analyse airborne spread during the foot-and-mouth disease outbreaks in Brittany, Jersey and the Isle of Wight in 1981. *Vet. Rec.*, 110(3): 53-57.
- Durand, B. & Mahul, O.** 1999. An extended state-transition model for foot-and-mouth disease epidemics in France. *Prev. Vet. Med.*, 47(1-2): 121-139.
- Ferguson, N.M., Donnelly, C.A. & Anderson, R.M.** 2001a. The foot-and-mouth epidemic in Great Britain: pattern of spread and impact of interventions. *Science*, 292(5519): 1155-1160.
- Ferguson, N.M., Donnelly, C.A. & Anderson, R.M.** 2001b. Transmission intensity and impact of control policies on the foot and mouth epidemic in Great Britain. *Nature*, 413(6855): 542-548.
- Geiger, S.R., Renkin, E.M. & Michel, C.C.** 1984. *The Cardiovascular system. Microcirculation*, 2nd edn. Bethesda, Md, American Physiological Society.
- Gibson, C.F. & Donaldson, A.I.** 1986. Exposure of sheep to natural aerosols of foot-and-mouth disease virus. *Res. Vet. Sci.*, 41(1): 45-49.
- Gloster, J. & Alexandersen, S.** 2004. New directions: airborne transmission of foot-and-mouth disease virus. *Atmospheric Environment*, 38(3): 503-505.
- Gloster, J., Blackall, R.M., Sellers, R.F. & Donaldson, A.I.** 1981. Forecasting the airborne spread of foot-and-mouth disease. *Vet. Rec.*, 108(17): 370-374.
- Gloster, J., Sellers, R.F. & Donaldson, A.I.** 1982. Long distance transport of foot-and-mouth disease virus over the sea. *Vet. Rec.*, 110(3): 47-52.
- Haydon, D.T., Woolhouse, M.E. & Kitching, R.P.** 1997. An analysis of foot-and-mouth-disease epidemics in the UK. *IMA J. Math. Appl. Med. Biol.*, 14(1): 1-9.
- Holling, C.S.** 1959. The components of predation as revealed by a study of small-mammal predation of the European pine sawfly. *Can. Entomol.*, 91: 293-320.
- Hughes, G.J., Mioulet, V., Haydon, D.T., Kitching, R.P., Donaldson, A.I. & Woolhouse, M.E.J.** 2002. Serial passage of foot-and-mouth disease virus in sheep reveals declining levels of viraemia over time. *J. Gen. Virol.*, 83(8): 1907-1914.
- Keeling, M.J., Woolhouse, M.E., Shaw, D.J., Matthews, L., Chase-Topping, M., Haydon, D.T., Cornell, S.J., Kappey, J., Wilesmith, J. & Grenfell, B.T.** 2001. Dynamics of the 2001 UK foot and mouth epidemic: stochastic dispersal in a heterogeneous landscape. *Science*, 294(5543): 813-817.
- Kelley, K.W., Curtis, S.E., Marzan, G.T., Karara, H.M. & Anderson, C.R.** 1973. Body surface area of female swine. *J. Anim. Sci.*, 36(5): 927-930.

- Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B. & Straus, S.E.** 2001. *Fields virology*. Philadelphia, Lippincott Williams & Wilkins.
- Medina, M., Domingo, E., Brangwyn, J.K. & Belsham, G.J.** 1993. The two species of the foot-and-mouth disease virus leader protein, expressed individually, exhibit the same activities. *Virology*, 194(1): 355-359.
- Michaelis, L. & Menten, M.L.** 1913. Die Kinetik der Invertinwirkung. *Biochem. Z.*, 49: 333.
- Morris, R.S., Wilesmith, J.W., Stern, M.W., Sanson, R.L. & Stevenson, M.A.** 2001. Predictive spatial modelling of alternative control strategies for the foot-and-mouth disease epidemic in Great Britain, 2001. *Vet. Rec.*, 149(5): 137-144.
- Ohlmann, T., Rau, M., Morley, S.J. & Pain, V.M.** 1995. Proteolytic cleavage of initiation factor eIF-4 gamma in the reticulocyte lysate inhibits translation of capped mRNAs but enhances that of uncapped mRNAs. *Nucleic Acids Res.*, 23(3): 334-340.
- Platt, H.** 1959. Renewal times of some squamous epithelia in the guinea pig. *Nature*, 184(Suppl. 21): 1654-1655.
- Pond, W.G. & Houpt, K.A.** 1978. *The biology of the pig*. Ithaca Cornell, University Press.
- Prevot, D., Darlix, J.L. & Ohlmann, T.** 2003. Conducting the initiation of protein synthesis: the role of eIF4G. *Biol. Cell*, 95(3-4): 141-156.
- Quan, M., Murphy, C.M., Zhang, Z. & Alexandersen, S.** Determinants of early foot-and-mouth disease virus dynamics in pigs. *J. Comp. Pathol.*
- Sorensen, J.H., Jensen, C.O., Mikkelsen, T., Mackay, D.K.J. & Donaldson, A.I.** 2001. Modelling the atmospheric dispersion of foot-and-mouth virus for emergency preparedness. *Phys. Chem. Earth (B)*, 26: 93-97.
- Sorensen, J.H., Mackay, D.K., Jensen, C.O. & Donaldson, A.I.** 2000. An integrated model to predict the atmospheric spread of foot-and-mouth disease virus. *Epidemiol. Infect.*, 124(3): 577-590.
- Thorne, H.V.** 1962. Kinetics of cell infection and penetration by the virus of foot-and-mouth disease. *J. Bacteriol.*, 84: 929-942.
- Wensel, D.L., Li, W. & Cunningham, J.M.** 2003. A virus-virus interaction circumvents the virus receptor requirement for infection by pathogenic retroviruses. *J. Virol.*, 77(6): 3460-9.

Table 1 Model parameter and variable definitions and calculations.

b	<p>Number of FMDV genomes released per ml of interstitial space per burst infected cell (ml^{-1}).</p> <p>A value of 30.6 was calculated from the burst size of FMDV per infected cell \div interstitial volume. The average burst size of an infected cell was taken to be 1×10^5 virus (S Alexandersen, personal communication, 2004). The interstitial volume was calculated at 13.09% of body weight (BW) (Pond and Houpt, 1978), giving a volume of 3 273 ml for a 25 kg pig.</p>
k_{do}	<p><i>Per capita</i> rate infected cells die per hour (hour^{-1}).</p> <p>This rate (0.33) was calculated as the inverse of the cell attached and intracellular life cycle of FMDV in vitro of between 2.5 and 3.5 h (Cartwright <i>et al.</i>, 1957).</p>
k_{xy}	<p><i>Per capita</i> rate FMDV genomes in central compartment lost to the interstitial space per hour (hour^{-1}).</p> <p>This rate (1.48) was determined from the mean rate of decrease of FMDV from the serum of pigs UU15 – UU19 and VC80 – VC83 in samples taken up to 2 h after intravenous inoculation of FMDV (unpublished data). The rate was calculated from log-linear regression equations fitted through the data.</p>
k_{yo}	<p><i>Per capita</i> rate FMDV genomes in interstitial space lost per hour (hour^{-1}).</p> <p>This rate was estimated by fitting the model visually to the experimental data.</p>
k_{yx}	<p><i>Per capita</i> rate FMDV genomes in interstitial space lost to the central compartment per hour (hour^{-1}).</p> <p>This rate (0.26) was determined from the mean rate of increase of FMDV in the serum of pigs UQ10 – UQ21 (Quan <i>et al.</i>, in press). Log-linear regression equations were fitted to the steepest part of the viraemia curves, using a minimum of three data points and over a minimum 24 hour period.</p>
β_1	<p><i>Per capita</i> rate uninfected cells infected per FMDV genome per ml serum in the interstitial space per hour ($\text{ml}^{-1} \cdot \text{hour}^{-1}$).</p> <p>This rate was estimated by fitting the model visually to the experimental data.</p>
β_2	<p><i>Per capita</i> rate uninfected cells infected per burst infected cell.</p> <p>This rate was estimated by fitting the model visually to the experimental data.</p>
λ	<p>Ratio of concentration of FMDV genomes in central compartment to concentration in the interstitial space.</p> <p>The ratio 0.53:1 was calculated from the volume of plasma (6.91% BW) to interstitial space (13.09% BW) (Pond and Houpt, 1978).</p>
C	<p>The maximum number of epithelial cells available for infection ($C_{(0)}$) was estimated from the total surface body area (BSA) of a pig, calculated from the formula $BSA \text{ cm}^2 = 734 \times (\text{body weight in kg})^{0.656}$ (Kelley <i>et al.</i>, 1973). We estimated an average cell size of approximately $20 \mu\text{m}^3$ (Bacha and Bacha, 2000) and a layer of epithelial cells 5 to 10 cells thick susceptible to viral infection. A figure of 1.25×10^{10} susceptible epithelial cells in a 25 kg pig was used in all models.</p>

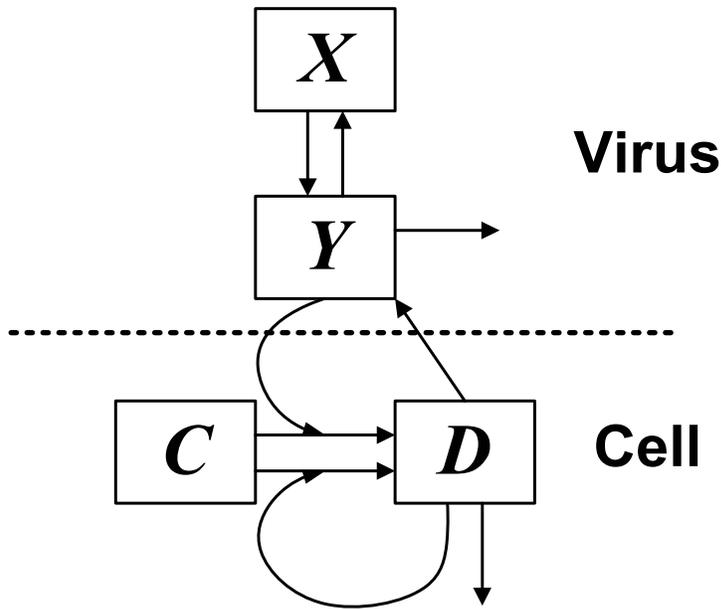


Figure 1 A model of FMDV replication *in vivo* (Model A). Variables X and Y represents the concentrations of FMDV in the central compartment and interstitial space; C and D represents uninfected and infected cells respectively. The parameters (e.g. k_{xz}) are constant *per capita* rates

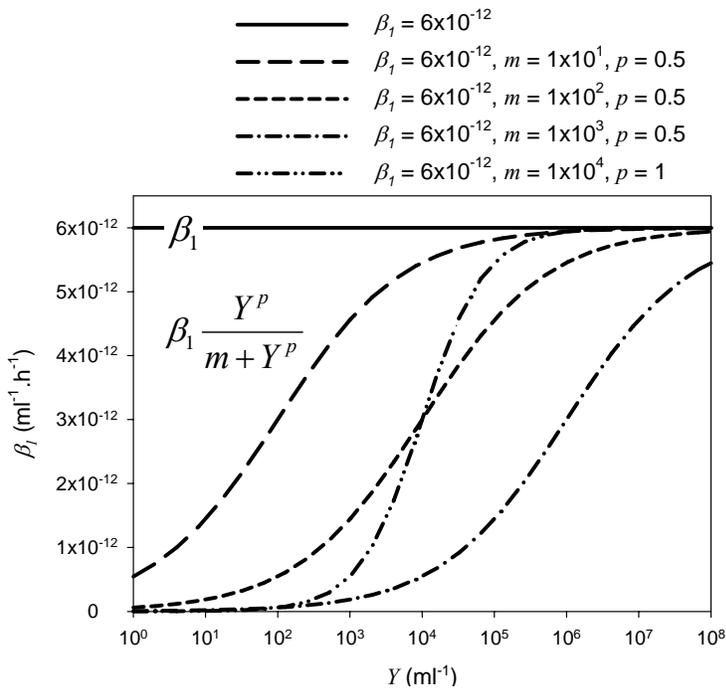


Figure 2 The effect of a sigmoidal term $\left(\frac{Y^p}{m + Y^p}\right)$ on β_1 (see Equations 5 and 6, Model B) where

β_1 = the *per capita* rate of infection of epithelial cells by FMDV and Y = FMDV genomes in the interstitial space.

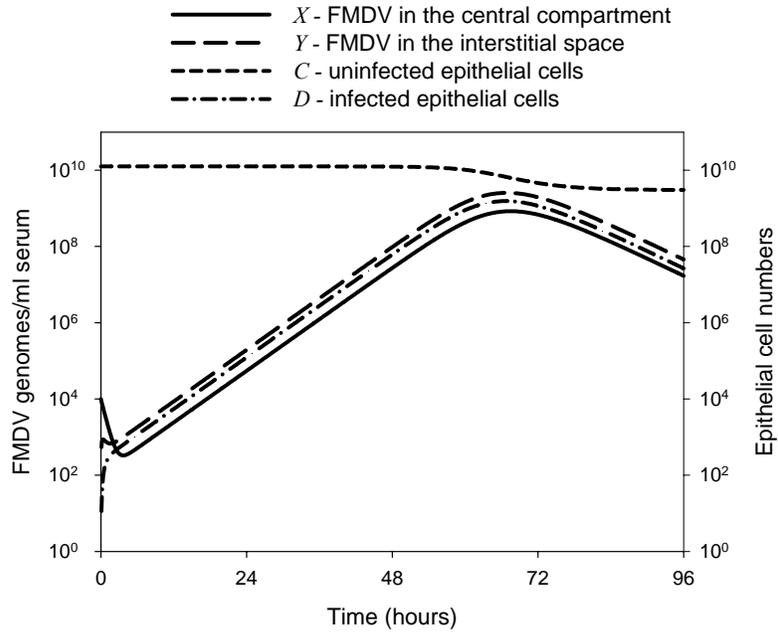


Figure 3 Model A ($X_{(0)} = 1 \times 10^4$, $k_{y0} = 6.0$, $\beta_1 = 3 \times 10^{-11}$, $\beta_2 = 0$ and other parameter values described in

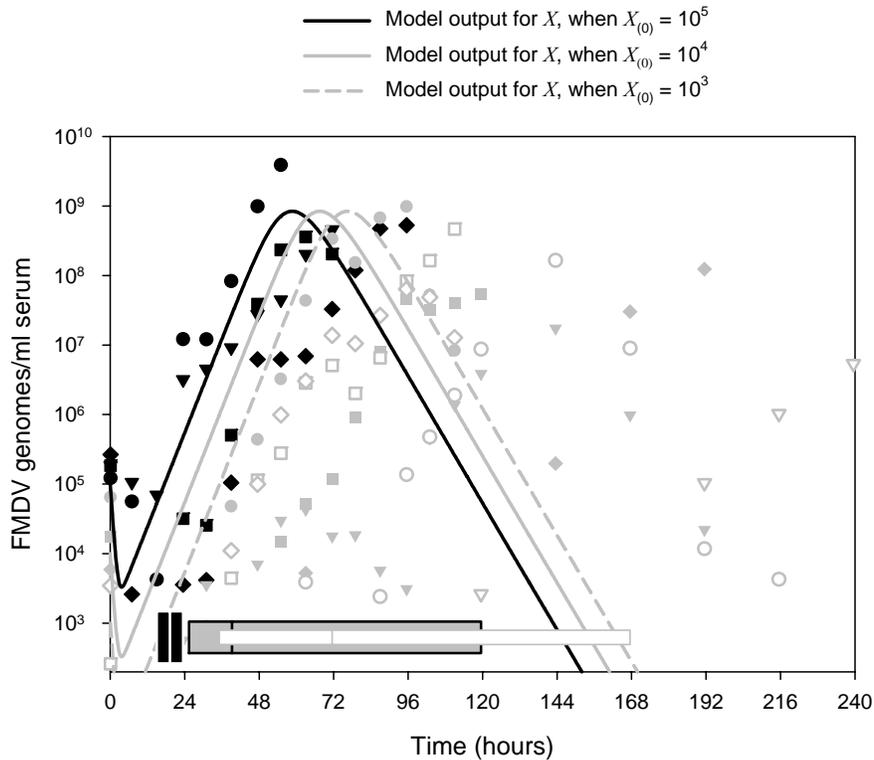


Figure 4 An illustrative parameter fit of Model A ($k_{y0} = 6.0$, $\beta_1 = 3 \times 10^{-11}$, $\beta_2 = 0$ and other parameter values are described in

Table 1) to experimental data (Quan *et. al.*, in press). Symbol colours indicate different treatments, i.e. $10^{8.1}$ (black), $10^{7.1}$ (grey) and $10^{6.1}$ (white) FMDV genomes/animal intravenous inoculation; shapes indicate individual animals. Box plots (using the same symbol colour scheme) show the median time (and 25th and 75th percentiles) active viraemia was first detected (detection

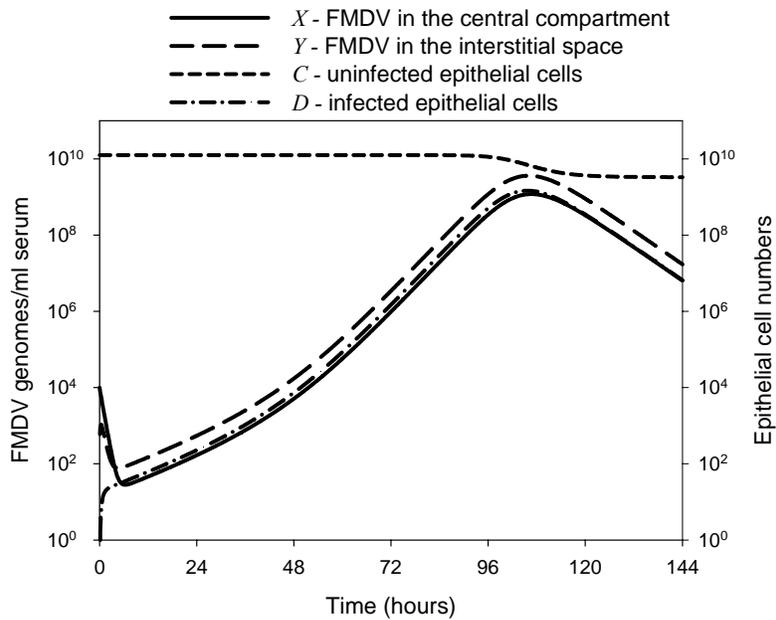


Figure 5 Model B ($X_{(0)} = 1 \times 10^4$, $k_{yo} = 4.0$, $\beta_1 = 6 \times 10^{-12}$, $\beta_2 = 1 \times 10^{-10}$, $m = 1 \times 10^2$, $p = 0.5$ and other parameter values described in

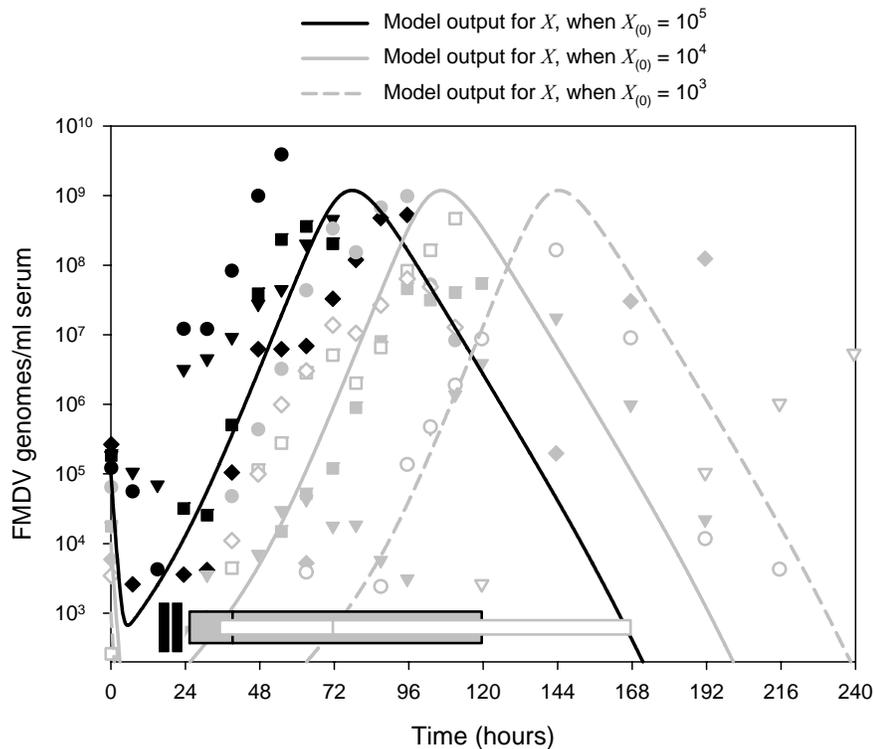


Figure 6 An illustrative parameter fit of Model B ($k_{yo} = 4.0$, $\beta_1 = 6 \times 10^{-12}$, $\beta_2 = 1 \times 10^{-10}$, $m = 1 \times 10^2$, $p = 0.5$ fitted visually to data; other parameter values are described in

Table 1) to experimental data (Quan *et al.*, in press). Symbol colours indicate different treatments, i.e. $10^{3.1}$ (black), $10^{7.1}$ (grey) and $10^{6.1}$ (white) FMDV genomes/animal intravenous inoculation; shapes indicate individual animals. Box plots (using the same symbol colour scheme) show the median time (and 25th and 75th percentile) active viraemia was first detected (detection of FMDV)

The pathogenesis of FMD in young lambs

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Abstract

Introduction: Outbreaks of foot-and-mouth disease (FMD) in lambs have been reported to cause collapse, fever, death and myocardial lesions. This is the first study we are aware of to experimentally investigate the pathogenesis of FMD in young lambs. **Materials and methods:** Four ewes, each with one lamb 10-14 days old, were inoculated with 5.9 logs TCID₅₀/ml FMDV O UKG 34/2001 strain. The lambs were infected by direct contact with their mothers. One ewe and her lamb were killed at two, three and four days after inoculation, and the remaining lamb was found dead on day five. The pathogenesis of FMD was characterised by measuring viral RNA levels in daily serum and nasal swab samples and in post-mortem tissue samples, measured by quantitative RT-PCR. In situ hybridisation and histopathology were performed on tissue sections. **Results:** The lamb killed on day 4 had cleared the viraemia and had low levels of virus in tissues, and none at all in the heart, when it was killed. In contrast, the lamb found dead on day 5 had a high viraemia throughout the disease and high levels of viral RNA in heart and muscle tissue. In situ hybridisation and histopathological lesions indicate viral myocarditis was the cause of death. Small lesions were seen on the lateral tongue and the coronary bands of the lambs. The different virus levels in tissues at various time-points characterised the development of the disease. **Discussion:** The infected lambs either cleared the viraemia rapidly and without myocarditis, or virus replication in the cardiac and skeletal muscle caused a high viraemia followed by myocarditis and death. The virus load in the blood was very high compared to infected adult sheep. Further studies are planned to characterise the pathogenesis in detail.

Introduction

Outbreaks of foot-and-mouth disease (FMD) in sheep flocks have been reported to cause death in lambs (Pay, 1988). Clinical signs in lambs previously reported include collapse, fever, tachycardia and marked abdominal respiration (Geering, 1967, Pay, 1988). Mortality is a feature of the disease in young lambs (Barnett *et al.*, 1999) and was first noted by Schrader in 1895. Deaths start to occur two to three days after the appearance of clinical signs in the ewes, and are usually the result of heart failure or starvation. Mortality rates vary widely, from 4.7% in an outbreak in India (Panisup *et al.*, 1979) to 94.5% in lambs of two to 25 days old in a Russian outbreak (Khankishiev *et al.*, 1958). A 40% mortality rate was observed in lambs in Kenya during a type O outbreak there (Geering, 1967). There are no estimates of clinical morbidity rates in lambs available. In type O outbreaks in Iran in 1971 and 1972, suckling lambs were particularly involved, with the disease occurring without warning in lambs aged 1 to 15 days and death generally occurring without any visible signs of distress. Post-mortem lesions in the lambs (reviewed by Littlejohn, 1970) include myocarditis, septicaemia, abomasitis and enteritis. Although outbreaks of FMD in lambs have been reported, the pathogenesis of the disease in lambs has not been previously investigated in an experimental setting. This is the first study we are aware of to experimentally examine the pathogenesis of FMD in lambs.

Materials and methods

Four Dorset down ewes, each with one lamb aged 10 to 14 days old, were inoculated in the coronary band with 5.9 logs TCID₅₀/ml FMDV O UKG 34/2001 strain (Alexandersen *et al.*, 2002a,b; 2003b,c). A fifth ewe and lamb were killed as negative controls prior to infection. The lambs were infected by direct contact with their ewes, most likely via the maternal milk.

Daily serum and nasal swab samples were taken and the temperatures and clinical signs of the lambs were recorded. Serum samples were stored at -70°C and nasal swabs were stored in Trizol at -70°C.

One ewe and her lamb were killed at two, three, and four days after inoculation. The remaining fifth lamb was found dead on day five. At post-mortem, tissue samples of liver, spleen, heart, lumbar muscle, cervical lymph node (CLN), mandibular lymph node (MLN), tonsil, tongue epithelium, coronary band epithelium and soft palate were taken and a portion of each preserved in 4% paraformaldehyde, in 30% sucrose in phosphate-buffered saline and in RNA*later* (Ambion).

Viral RNA levels in serum, nasal swab and tissue samples preserved in RNA*later* were measured by quantitative reverse-transcription real-time polymerase chain reaction (RT-PCR). RNA was

extracted using a MagNApure LC robot (Roche, UK). Reverse transcription and RT-PCR were carried out using TaqMan reagents (Applied Biosystems, UK as described by Reid *et al.*, 2003, and Alexandersen *et al.*, 2003c).

In situ hybridisation (ISH) was performed on formalin-fixed and sucrose-fixed tissue sections using the mRNA*locator*[™] In situ hybridisation kit (Ambion). The histopathology of the tissues was examined on haematoxylin-and-eosin-stained sections.

Results

Gross lesions: The lamb killed on day 2 (L39) had no gross lesions. L40 (killed day 3) had mild vesicular lesions on all four feet, an erosive vesicular lesion on the lateral side of the tongue at the level of the molars and enlarged tonsils and cervical lymph nodes. L41 (killed day 4) had mild vesicular lesions on all four feet and a small lesion on the lateral side of the tongue, again at the level of the molars. It also had CLN enlargement.

When found dead on day 5, L38 had vesicular lesions on both fore-feet and had three erosive lesions on the tongue, one on either lateral side at the level of the molars and one on the dorsal surface of the tongue.

Histopathology: Histological examination of sections of heart from the lamb found dead on day five (L38) revealed multi-focal, diffusely distributed areas of myocardial cell swelling with perivascular lymphocytic infiltration, and a localised larger area of cell swelling and hyalinisation (fig. 1). The heart sections of the other lambs showed no abnormalities.

In situ hybridisation: In situ hybridisation on heart sections from the lamb found dead on day five (L38) showed viral RNA distributed throughout the myocardium in a diffuse pattern, with positive signal found both in areas of cell swelling and in areas which appeared normal on H&E sections (fig. 2). The positive signal was found in the cytoplasm of the cells. No positive signal was found in the heart sections from the other lambs.

In situ hybridisation on lumbar muscle sections from the lamb found dead on day five (L38) showed viral RNA distributed throughout the sections in a pattern more multi-focal and less diffuse than in the myocardium (fig. 3). No positive signal was found in the lumbar muscle sections from the other lambs.

Viral RNA quantification: The lambs became viraemic on day 2 after the ewes had been inoculated (fig. 4, graphs 2, 4, 6, 8), whereas the ewes became viraemic on day 1. This indicates there is a one-day lag in the lambs becoming infected. L41 showed trace levels of viral RNA in a nasal swab on day 1 (fig. 4, graph 6), but this was most likely from virus shed by the ewe.

The lamb killed on day 2, L39, had viral RNA evenly distributed throughout its tissues, with the highest levels in the skin, tongue, soft palate, CLN, MLN and tonsil (fig. 4, graph 1). The lamb killed on day 3, L40, had the highest level of viral RNA in the skin, followed by the tongue, CLN, MLN and soft palate (fig. 4, graph 3).

The lamb killed on day 4 (L41) had cleared the viraemia by the time it was killed (fig. 4, graph 6), and had low levels of viral RNA in most of the tissue samples, and none at all in the heart (fig. 4, graph 5). Only the skin, tongue and tonsil had 8 logs of virus or more per gram. This is in marked contrast to the lamb found dead on day 5 (L38), which had a significant viraemia on days 2, 3 and 4 (fig. 4, graph 8) and had high levels of viral RNA in many tissue samples, particularly the heart and skeletal muscle, which had over 10 logs of viral RNA per gram (fig. 4, graph 7).

Discussion

The results from the first two lambs to be killed, L39 and L40, show the acute-phase distribution of the virus is primarily to the dermis and lymphoid organs draining the dermis. The absence of any virus-positive signal in the heart or muscle sections when ISH was performed suggests replication may not have occurred in those tissues and that the level of viral RNA detected there by PCR was principally due to the viraemia.

The results from L41 (killed at day 4) show this lamb became infected and cleared the viraemia rapidly. There was no myocarditis and no viral RNA was found in the heart, and little viral RNA was found in the muscle. The results from L38 show this lamb developed a very high viraemia, and this viraemia was maintained throughout the course of the disease. The level of this viraemia was very high compared to the viraemia in the ewes (11.13 logs per ml in the lamb vs. 9.4 logs per ml in its mother). The detection of abundant viral RNA by ISH in the heart and muscle suggest viral replication was occurring in these tissues and that the cell damage was due to FMD virus. The histopathological lesions in the heart indicate that viral myocarditis may have been a factor in its death.

The pattern of gross lesions on the tongue in lambs contrasts with that of older sheep and cattle, where lesions are more common at the rostral area of the tongue and the dental pad. It is thought that lesions are more likely to develop at sites of trauma or intensive physical stress

(Alexandersen *et al.*, 2003a). In young lambs, the actions of the tongue in suckling the ewe cause the tongue to rub against the molars and the dorsal aspect of the tongue rubs against both the lower teat of the ewe and the roof of the lamb's mouth. This may explain the difference in lesion distribution.

Conclusions

- The evidence suggests that FMDV in lambs is initially dermatrophic, but can become mycardiotrophic and myotrophic, with potentially fatal results.
- The infected lambs either cleared the virus and had no cardiac involvement, or a high viraemia was followed by myocardial involvement and death.
- Further studies are planned to characterise the pathogenesis of FMD in lambs in more detail.

Recommendations

- Studies on the importance of lambs in FMD transmission and disease control should be intensified.

Acknowledgements

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References

Alexandersen, S., Z. Zhang, S. M. Reid, G. H. Hutchings, & A. I. Donaldson. (2002a). Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. *Journal of General Virology*, **83**:1915-1923.

Alexandersen, S. & A. I. Donaldson. (2002b). Further studies to quantify the dose of natural aerosols of foot-and-mouth disease virus for pigs. *Epidemiology and Infection*, **128**:313-323.

Alexandersen, S., Zhang, Z., Donaldson, & A.I., Garland, A.J.M. (2003a). The pathogenesis and diagnosis of foot-and-mouth disease. *Journal of Comparative Pathology*, **129**, 1-36.

Alexandersen, S., M. Quan, C. Murphy, J. Knight, & Z. Zhang. (2003b). Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *Journal of Comparative Pathology*, **129**:268-282.

Alexandersen, S., R. P. Kitching, L. M. Mansley, & A. I. Donaldson. (2003c). Clinical and laboratory investigations of five outbreaks of foot-and-mouth disease during the 2001 epidemic in the United Kingdom. *Veterinary Record*, **152**:489-496.

Barnett, P.V., & Cox, S.J. (1999). The role of small ruminants in the epidemiology and transmission of foot-and-mouth disease. *Veterinary Journal*, **158**, 6-13.

Geering, W.A. (1967). Foot and mouth disease in sheep. *Australian Veterinary Journal*, **43**, 485-489.

Khankishiev, A.M., Gadzhiev, K.S., & Alekperov, J.G. (1958). *Veterinariya, Moscow*, **35** (9), 59.

Littlejohn, A.I. (1970). Foot-and-mouth disease in sheep. *State Veterinary Journal*, **25** (74), 3-12.

Panisup, A.S. (1979). *Haryana Agricultural University Journal of Research*. **9**, 111-114.

Pay, T.W.F. (1988). Foot and mouth disease in sheep and goats: a review. *Foot and mouth disease bulletin*, **26**, 3.

Reid, S. M., S. S. Grierson, N. P. Ferris, G. H. Hutchings, & S. Alexandersen. (2003). Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *Journal of Virological Methods*, **107**:129-139

Schrader. (1895). *Berliner Tieraerztliche Wochenschrift.*, 305.

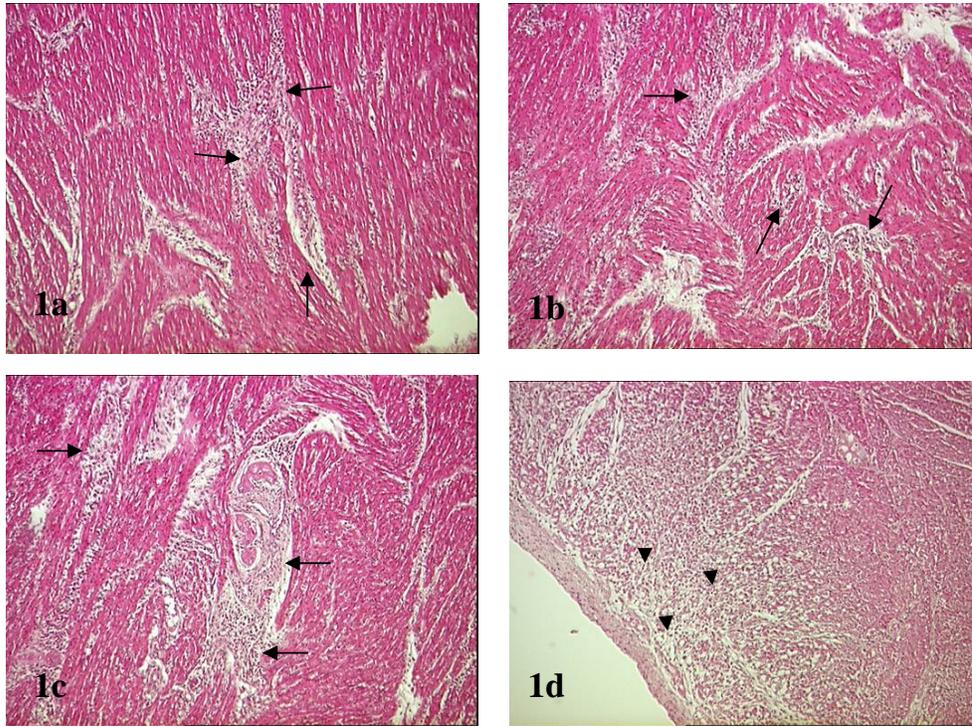


Figure 1: Haematoxylin and eosin stained sections of heart from lamb L38 showing multi-focal, diffusely distributed areas of myocardial cell swelling with perivascular lymphocytic infiltration (arrows), and a localised larger area of cell swelling and hyalinisation (Fig. 1d) (arrow head).

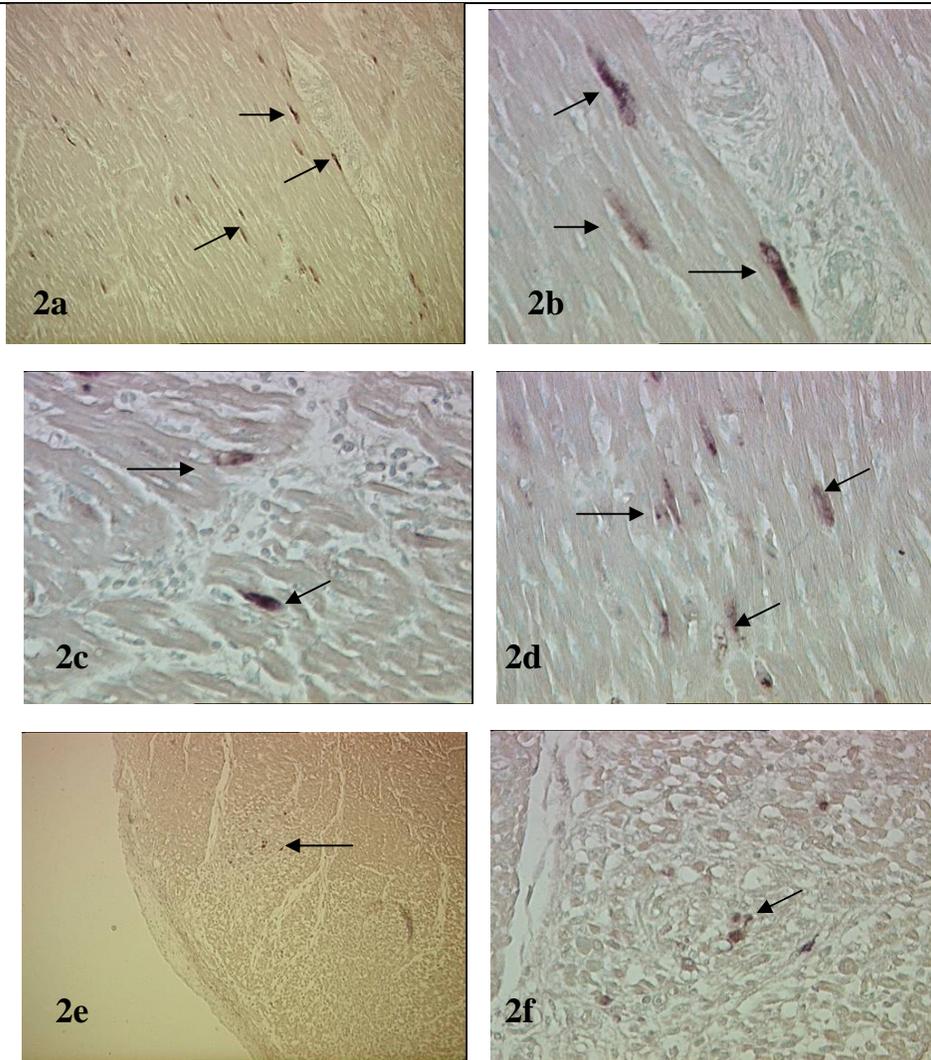


Figure 2: In situ hybridisation performed on sections of heart from L38 showing viral RNA (arrows) distributed throughout the myocardium in a diffuse pattern in the cytoplasm, with positive signal found both in areas of cell swelling (fig. 2c, 2d) and in areas which appeared normal on H&E sections (fig. 2a, 2b). Fig. 2e and 2f show the localised area of cell swelling and hyalinisation shown stained with H&E in fig. 1d.

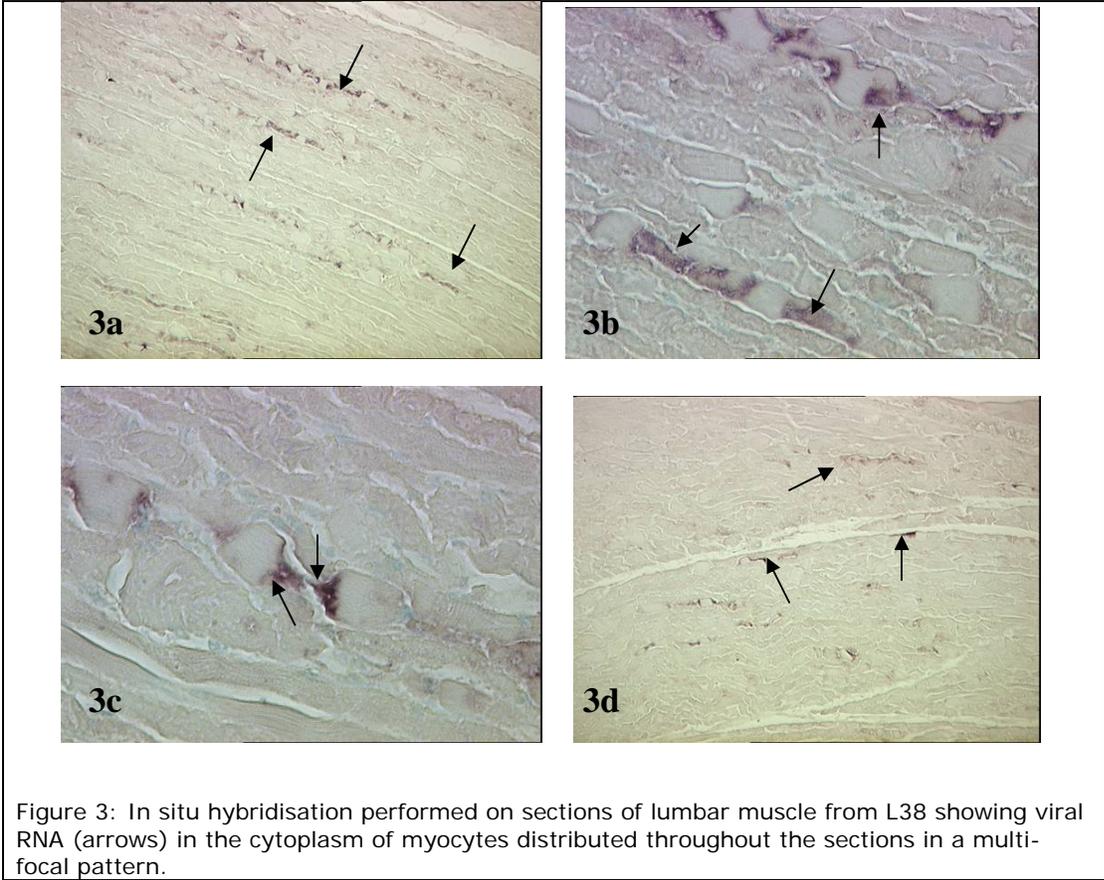


Figure 3: In situ hybridisation performed on sections of lumbar muscle from L38 showing viral RNA (arrows) in the cytoplasm of myocytes distributed throughout the sections in a multifocal pattern.

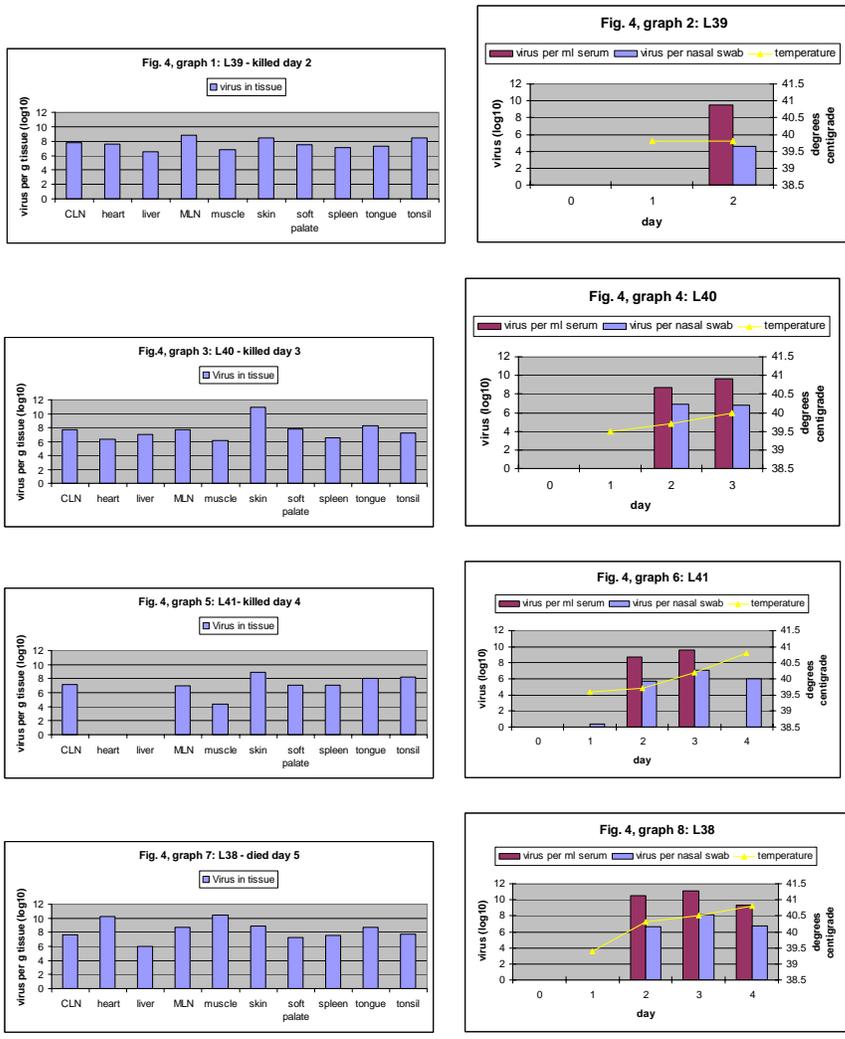


Figure 4: The pathogenesis of the disease in the lambs is shown in the graphs 1-8, showing the course of the viraemia, pyrexia, presence of viral RNA in nasal swabs, and quantifying the amount of viral RNA in various tissue samples.

Towards the development of engineered cell lines for FMDV diagnosis

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Abstract:

The ability to successfully propagate foot-and-mouth disease virus (FMDV) from field samples is a fundamental aspect of effective laboratory diagnosis. Unfortunately, established cell systems such as primary bovine thyroid cells and the IBRS2 cell line have disadvantages that limit their use. Therefore, the aim of this project is to engineer replacement cell lines suitable for routine detection of FMDV. Two separate strategies, reported here, have been employed to generate these cell lines:

(i) The integrin heterodimer $\alpha\nu\beta 6$ is an important cellular receptor for FMDV. Using sequences obtained by RT-PCR, mammalian expression constructs for porcine and bovine $\beta 6$ have been prepared in pcDNA6. Initial experiments have attempted to introduce the bovine $\beta 6$ construct into MDBK and Bovine Embryonic Kidney (BEK) cells. Using linearised plasmid containing the bovine $\beta 6$ sequence, a single cell line (1G8) demonstrated stable cell surface expression of $\alpha\nu\beta 6$. To date, all attempts to transfect BEK cells with bovine $\beta 6$ have been unsuccessful. (ii) The second approach used was to transfect cells to express the V-protein of the paramyxovirus, simian virus-5 (SV-5). SV-5v inhibits the response of type I and type II interferons due to interaction with the signalling protein, STAT1. After transfection with plasmid (pEF-IRES-SV-5v), cell lines derived from MDBK and BEK demonstrated stable intra-nuclear expression of SV-5v. In order to determine their suitability for FMDV diagnosis, the sensitivity of these engineered cells to FMDV was compared to the parental cell lines. Unfortunately, these experiments showed no increased sensitivity of any of these lines to a field isolate of FMDV, although binding of FMDV was enhanced with the 1G8 cell line compared with control MDBK cells. Future direction for this project will target transfection of other different cell lines with bovine/porcine $\beta 6$ and alternative integrin subunits.

Introduction:

Primary bovine thyroid cells (BTY: Snowdon, 1966) and the IBRS2 cell line (Castro, 1964) used for diagnosis are typically more sensitive than ELISA for FMDV antigen detection. Unfortunately, there are a number of drawbacks of these established cell systems (Ferris et al., 2002):

- limited life-span of primary cultures
- primary cultures can contain a mixture cell types. Slowly proliferating sensitive cell populations can be overgrown by cells (such as fibroblasts) that are insensitive to FMDV.
- The requirement to regularly source thyroid glands for the preparation of BTY cells is expensive, labour intensive and requires skill and experience to produce suitable BTY cell monolayers
- variability and inconsistency of the sensitivity of cultures and cell lines to FMDV
- presence of other viral agents such as Classical Swine Fever Virus or Bovine Viral Diarrhoea Virus that can interfere with FMDV growth

These factors place logistic limitations on the use of virus isolation methods for FMDV diagnosis. The objective of this project is to use molecular biology approaches to engineer stable cell lines with improved characteristics suitable for diagnostic use.

Materials and Methods:

Cell lines: Low passage cultures of Madin-Darby Bovine Kidney (MDBK) and bovine embryonic kidney (BEK) cells were used in the transfection experiments. Cells were cultured at 37 °C in 5% CO₂ using DMEM, 10% foetal calf serum (FCS) supplemented with L-Glutamine, penicillin and streptomycin.

Generation of Mammalian Expression constructs: The nucleotide sequence of porcine $\beta 6$ was determined by reverse-transcription polymerase chain reaction. Briefly, template RNA was extracted from porcine tongue tissue using Trizol[®] reagent (Invitrogen) according to manufacturer's instructions. cDNA was synthesised using annealed random hexamers (Promega), 10 μ l of the prepared RNA and M-MLV reverse transcriptase (Invitrogen). The design of two sets of PCR primers (5' fragment: 5'-ATG GGG ATT GAA CTG CTT TGC C-3' and 5'-CCT CAG ACC GCA GTT CTT CAT AAG-3' and 3' fragment: 5'-CAT TCT CCA GCT GAT CAT CTC AG-3' and 5'-CAT AAA GTA GTT CTA TCC ATC CGT GG-3' used for the amplification of the entire coding sequence for porcine $\beta 6$ was based on nucleotide similarities between the available sequences on GenBank. Fifty μ l PCR reactions contained 2 mM MgCl₂ (Invitrogen), 50 mM KCl, 10 mM Tris-HCl pH 8.0 (Thermo Buffer, Invitrogen), 0.2 mM of each dNTP, 50 pmol of each primer, and 2.5 U Taq DNA polymerase (Invitrogen). Amplification conditions (MJ, GRI, Braintree, UK) were 94 °C for 60 seconds, 61 °C for 60 seconds, 72 °C for 120 seconds for 35 cycles.

Chain elongation at 72 °C was extended to 7 minutes for the final cycle. PCR products were separated by electrophoresis using 1.2 % agarose gels visualised with ethidium bromide. Amplicons of the correct size were excised from the agarose gels and purified (QIAquick gel extraction kit). These amplicons were cloned (pGEM-T easy, Promega), after which three independent clones containing the PCR inserts were sequenced (Beckman CEQ8000) and were assembled (DNASTar) as overlapping fragments. Expression constructs for porcine $\beta 6$ and bovine $\beta 6$ (coding sequence in pcDNA3.1 obtained from T. Jackson) were prepared in pcDNA6 (Invitrogen: Figure 1). The presence of inserts encoding the correct amino acid sequences was confirmed by sequencing (Beckman CEQ8000).

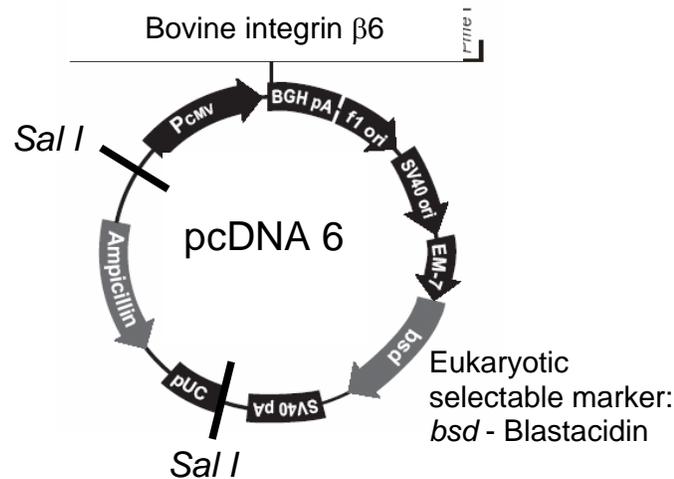


Figure 1: Schematic outline of the plasmid used to transfect cells with bovine integrin $\beta 6$. **NB:** The location of the *Sal I* sites used to generate linearised plasmid is indicated.

Expression of SV-5v protein was achieved using pEF-IRES - SV-5v (Didcock et al., 1999: provided as a kind gift from Prof. R. Randall, University of St Andrews) with G418 (Invitrogen) as the selectable marker.

Transfection: Plasmids (endo-toxin-free Maxi-prep kit, Qiagen) were linearised by restriction-enzyme digestion with *Sal I* and *Nde I* for the pcDNA- $\beta 6$ and pEF-IRES- SV-5v respectively. Adherent MDBK and BEK cell cultures were transfected with different concentrations (2 μ g - 500 ng) of plasmid using Lipofectamine 2000 (Invitrogen) in OPTI-MEM media (Gibco BRL). Double (bovine $\beta 6$ and SV-5v) expression using both plasmids was attempted for MDBK.1G8 cells. After overnight culture at 37 °C, cells were trypsinised and washed with fresh media (DMEM, 10 % FCS supplemented with L-Glutamine, Penicillin, Steptomycin and the relevant selectable marker: blastacidin for pcDNA6 or G418 for pEF-IRES). After 7-14 days growth, surviving cells were cloned twice by single cell cloning in the presence of the selectable marker to generate homogeneous cell populations.

Characterisation of cell lines:

(a) Expression of $\alpha v \beta 6$: After selection of cell clones with blasticidin, cell surface expression of the integrin heterodimer $\alpha v \beta 6$ was determined by flow cytometry. Briefly, cell suspensions were prepared by trypsinisation and incubated with the mouse anti- $\alpha v \beta 6$ monoclonal antibody 10D5 (Chemicon). After washing with FACS buffer (150 mM NaCl, 1 mM CaCl₂, 0.5 mM MgCl₂, 10 mM Tris pH 7.5, 2% bovine serum albumin, 2% goat serum), a secondary PE-conjugated antibody (goat anti-mouse IgG2A, Southern Biotech) was added and incubated with the cell on ice for 25 minutes. Cells were fixed in paraformaldehyde and level of $\alpha v \beta 6$ expression was determined (FACS-Calibur, Beckton-Dickinson).

(b) Expression of SV-5v: Cover slips of adherent cells from each of the engineered cell lines were prepared by overnight culture in 24-well plates. Cells were fixed in 4% paraformaldehyde and washed twice in PBS. After permeabilising the cells with 0.1% Triton-X, primary monoclonal antibody (anti-V5-tag, Serotech) was added for 60 minutes. After further washes in PBS, the presence of SV-5v was detected using an Alexa488 conjugated secondary antibody visualised on a confocal microscope (Leica TCS SP2 confocal microscope). DAPI stain was used to locate cell nuclei.

Assessment of cell lines for the detection of FMDV: In order to assess their suitability for FMDV diagnosis, the sensitivity of these engineered cells to FMDV was compared to the parental cell lines. The ability of each of the cell lines to detect a titration series of two O serotype FMDV viruses (UAE 2/2003 and BFS 1860) was determined. These comparative titrations of the samples were performed in primary calf thyroid cells (CTY; Snowdon, 1966). Tenfold dilution series were made in 0.04 M phosphate buffer and inoculated onto cell monolayers (pre-washed with phosphate buffered saline [PBS]) grown in plastic cell culture tubes (0.2 ml per tube, 3-5 tubes per serial tenfold dilution). After absorption at 37°C for 30 min, the cell monolayers were washed three times with PBS and overlaid with 2 ml of serum-free Eagle's maintenance medium. The tubes were subsequently rolled continuously at 37°C and the cell cultures examined microscopically for evidence of a cytopathic effect (CPE) daily for 3 days post-infection, and recorded as positive or negative. The relative titre of the virus detected by these different cell lines was expressed as 50% tissue culture infective doses (TCID₅₀/ml; Kärber, 1979).

Results:

αVβ6 expression: Using the bovine β6 plasmid, 25 MDBK-derivative cell lines were generated. However, only 2 of these contained the full-length β6 transcript (determined by PCR) and only one line (1G8) demonstrated stable cell surface expression of αvβ6 (Figure 2). A high level of αvβ6 expression was maintained for over 1 month with this cell line in the presence of 10 μg/ml blasticidin. Preliminary experiments showed that FMDV bound more readily to the 1G8 cell line compared with parental MBDK cells (data not shown). To date, all attempts to transfect BEK cells with bovine β6 have been unsuccessful.

SV-5v expression: 11 MDBK (cells derived from bovine β6 expressing line 1G8) and 3 BEK stable cell lines were characterised. The presence of SV-5v coding sequences was confirmed by PCR in 7/11 MDBK (1G8) and 3/3 BEK cell lines. Confocal microscopy performed on 2 selected MDBK and 2 BEK cells lines demonstrated predominately intra-nuclear expression of SV-5v (Figure 3), similar to that previously reported for other cell systems (Young et al., 2003)

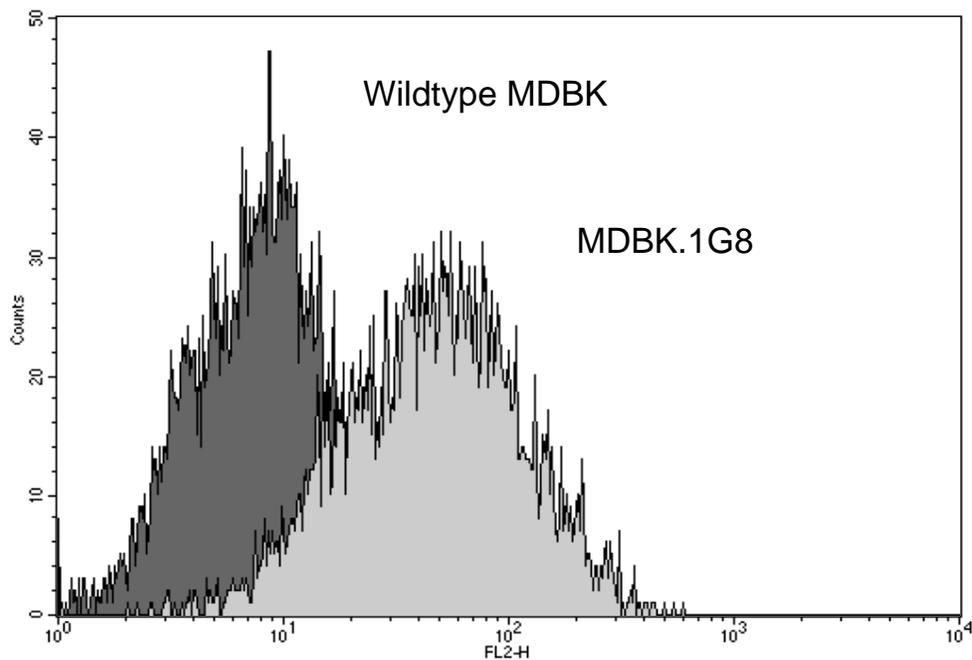


Figure 2: Cell surface expression of bovine αvβ6 integrin on the 1G8 MDBK cell line detected by flow-cytometry.

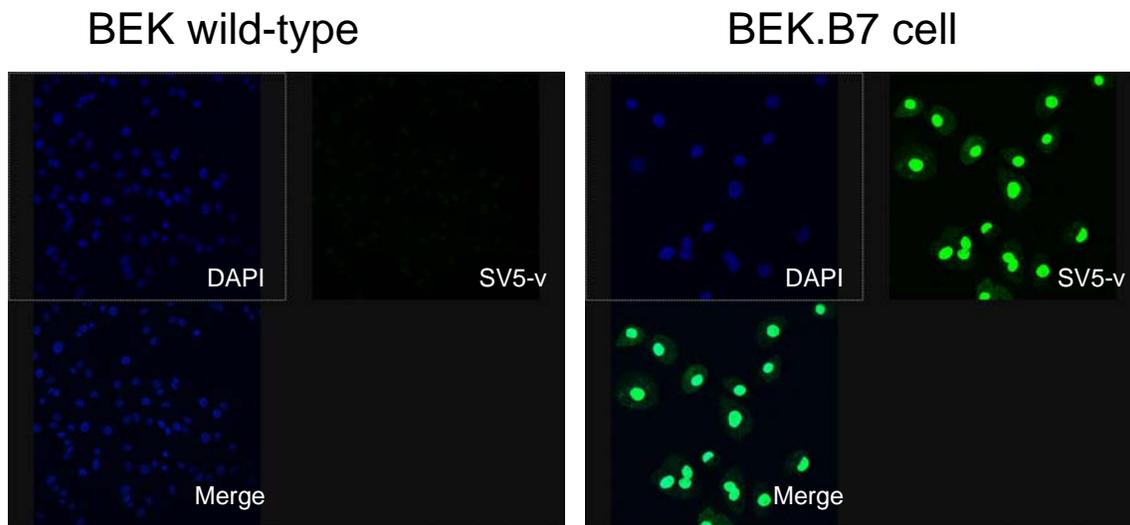


Figure 3: Intranuclear expression of SV-5v shown by confocal microscopy. Presence of SV-5v is predominantly located in the nucleus of cells (matches with DAPI staining; similar staining patterns were shown in a previous study Young et al., 2003).

Unfortunately, subsequent experiments showed no increased sensitivity with respect to the parental lines of any of these engineered cells to a field isolate of FMDV (O UAE 2/2003) shown in Table 1. All cell lines derived from MDBK cells were unable to replicate UAE 2/2003 whereas the sensitivity of the BEK lines to FMDV was not increased after introduction of the SV-5v transgene. The poor replication of UAE 2/2003 was in contrast to that obtained with FMDV isolate O₁-BFS 1860. This virus can enter cells through an integrin-independent pathway (Heparan sulphate), and was able to replicate in the MDBK parent and derivative cell cultures.

Discussion:

The aim of this project is to engineer cell lines with characteristics suitable for the routine propagation of field isolates of FMDV. In these preliminary experiments, two separate approaches involving the introduction of bovine $\beta 6$ or SV-5v transgenes into 2 bovine cell lines were investigated. Successful introduction of the plasmid coding sequences and stable expression of the targeted protein in derivative cell lines was achieved using pcDNA6 and pEF-IRES for bovine $\beta 6$ and SV-5v respectively.

The presence of $\alpha V\beta 6$ heterodimer molecules on the surface of MDBK cells was demonstrated by flow cytometry (these cells lines constitutively express the αV protein). Furthermore, the 1G8 cell line showed enhanced binding of FMDV compared with parent MDBK cells suggesting that the cell surface expression of $\alpha V\beta 6$ was functionally correct. Although this initial characterisation data was encouraging, subsequent experiments showed that the 1G8 clone did not show increased sensitivity to infection by field isolates of FMDV.

Expression of SV-5v protein was predominately localised to the nucleus of MDBK and BEK cells. Type I and Type II interferons are produced in response to virus infection and mediate the establishment of an antiviral state. The aim of this approach was to attempt to increase the sensitivity of the cell cultures to FMDV by abolishing any anti-viral activity of interferons (including any bystander affect in neighbouring cells). Similar approaches have been recently used to generate cell lines for the detection of a variety of slow-growing wild-type viruses and vaccine candidate viruses (Young et al., 2003). However, neither MDBK cells nor BEK cells expressing SV-5v showed any evidence of increased sensitivity to field isolates of FMDV. These findings are possibly not unexpected, since FMDV rapidly shuts off cap-dependent translation, thereby limiting the role of interferon-mediated activity in infected in-vitro culture systems.

Future direction for this on-going project will target transfection of a number of different cell lines with bovine/porcine $\beta 6$ and alternative integrin subunits such as $\beta 8$.

Acknowledgements:

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References:

De Castro MP. 1964. Comportamento do virus aftoso em cultura de células: susceptibilidade da linhagem de células suínas IB-RS-2. *Arch. Inst. Biol., São Paulo*; 31: 63–78.

Didcock L, Young DF, GoodBourn S & Randall RE 1999. The V protein of Simian Virus 5 inhibits interferon signalling by targeting STAT1 for proteasome-mediated degradation. *Journal of Virology*; 73: 9928-9933.

Ferris NP, Hutchings GH, Mouldsle HJ, Golding J & Clarke JB 2002 Sensitivity of primary cells immortalised by oncogene transfection for the detection and isolation of foot-and-mouth disease and swine vesicular disease viruses. *Veterinary Microbiology*; 84(4):307-16.

Kärber, G. 1979. Calculation of the LD₅₀ titer by the Kärber method. In: *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*, 5th Edit., E. H. Lennette and N. J. Schmidt, Eds, American Public Health Association, Washington, D. C., pp. 34-35.

Jackson T, Ellard F, Ghazaleh RA, Brookes SM, Blakemore WE, Corteyn AH, Stuart DI, Newman JW & King AMQ 1996. Efficient infection of cells in culture by type O foot-and-mouth disease virus requires binding to cell surface heparin sulphate. *Journal of Virology*; 70: 5282-5287.

Snowdon WA 1966. Growth of foot-and-mouth disease virus in monolayer cultures of calf thyroid cells. *Nature*; 210: 1079–1080.

Young DF, Andrejeva L, Livingstone A, Goodbourn S, Lamb RA, Collins PL, Elliott RM & Randall RE 2003. Virus replication in engineered human cells that do not respond to interferons *Journal of Virology*; 77(3): 2174-81.

Table 1: Comparative titrations of FMDV in the cell cultures

		FMDV isolate	
		O UAE 2/2003	O1 BFS 1860*
		Epith. suspension	Cell culture derived
	BTY	6.2	8.5
Wild-Type	Parent MDBK	<2.2	7.2
β6	MDBK.1G8	<2.2	8.0
β6	MDBK.1C6	<2.2	8.5
β6 and SV-5v	MDBK 1G8.D4	<2.2	6.2
β6 and SV-5v	MDBK 1G8.E5	<2.2	7.5
Wild-Type	Parent BEK	5.5	7.7
SV-5v	BEK.B7	5.2	8.2
SV-5v	BEK.3B11	<2.2	7.7

*Cell culture adapted isolate O₁ BFS 1860 can enter cells via an integrin-independent pathway (Jackson et al., 1996)

Recombinant integrin $\alpha\nu\beta 6$ as a capture reagent in immunoassays for the diagnosis of FMD

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Abstract:

It is thought that the integrin $\alpha\nu\beta 6$ is a principal cell receptor for binding wild-type FMDV. The aim was to exploit this knowledge by evaluating the performance of recombinant integrin $\alpha\nu\beta 6$ as a capture ligand in a sandwich ELISA for the detection and serotyping of FMDV. Soluble recombinant $\alpha\nu\beta 6$ protein was produced from Chinese hamster ovary cells transfected with truncated human $\alpha\nu$ and $\beta 6$ genes and used as the capture reagent in indirect sandwich ELISAs in combination with either guinea pig polyclonal or monoclonal antibodies (mabs) as detectors. These formats were evaluated for their ability to react with, and characterise the serotype of, suspensions of field epithelia and a range of cell culture antigens of all seven serotypes of FMDV plus SVDV. Their performance was also compared with our conventional assay, which uses polyclonal antisera as capture and detecting antibodies.

The recombinant $\alpha\nu\beta 6$ protein bound all antigens of FMDV irrespective of serotype but did not react with SVDV. Heterotypic cross-reactivity was evident with some of the test samples using rabbit/guinea pig polyclonal antisera raised against individual virus serotypes and was considerably exacerbated using the integrin/polyclonal antibody combination. Conversely, totally type-specific reactions resulted from the ELISA using integrin as capture and mabs as detectors.

The results illustrate that the use of the recombinant protein as a capture reagent in the ELISA in combination with appropriate mab(s) as the detector has the potential to improve upon conventional FMD diagnostic assays using rabbit and guinea pig polyclonal antisera. Additionally, the recombinant integrin could be usefully used in other immunoassays for FMD diagnosis (e.g. for characterizing the antigenicity of field virus strains and for detection of FMDV antibody) as well as in other FMDV test procedures such as immunocapture RT-PCR and pen-side chromatographic strip-test devices.

Introduction:

Diagnosis of FMD is dependent upon early clinical recognition of the disease in the field, followed by confirmation of the presence and type of FMD virus by objective tests that are usually carried out in specialised laboratories. The preferred specimen is vesicular epithelium and the most commonly used laboratory diagnostic tests are the antigen detection ELISA combined with virus isolation in cell culture (Roeder and Le Blanc Smith, 1987; Ferris and Dawson, 1988) and fluorogenic reverse transcription polymerase chain reaction (RT-PCR; Reid *et al.*, 2002, 2003). The type of ELISA, which has been found to be most sensitive is an indirect sandwich assay employing type-specific antibodies of polyclonal and/or monoclonal origin. The assay is rapid and identifies the serotype of FMD virus that is present. If necessary, the sensitivity of the method can be enhanced by prior amplification of virus in cell cultures.

Integrin molecules on cell surfaces are receptors for a number of viruses including field isolates of FMD virus (Jackson *et al.*, 1997). Integrins are heterodimers comprising α and β subunits, each of which can occur in a number of different forms. Six or seven different varieties of integrin are known to bind to the conserved RGD amino acid motif found on the VP1 capsid protein of FMD virus. However, it is thought that the integrin $\alpha\nu\beta 6$ is the principal receptor for binding wild-type FMD virus (Jackson *et al.*, 2000). This knowledge could be exploited in a number of ways to develop improved diagnostic tests for FMD. We have examined firstly the utility of a recombinant protein of $\alpha\nu\beta 6$ to function as a ligand for FMD virus in the antigen detection ELISA and present the results in this communication.

Material and Methods:

Preparation of recombinant $\alpha\nu\beta 6$. Recombinant $\alpha\nu\beta 6$ was produced from Chinese hamster ovary (CHO) cells stably transfected with truncated $\alpha\nu$ and $\beta 6$ genes of human origin (Weinacker *et al.*, 1994) to secrete $\alpha\nu\beta 6$ as a soluble protein in serum-free cell culture supernatant fluids. The supernatant was clarified by filtration and stored at -20°C .

Polyclonal antibodies. Polyclonal antisera to FMD virus types O₁ BFS 1860, A_{5/22/24} (Combination), C₃ Resende, SAT 1 BOT 1/68, SAT 2 ZIM 5/81, SAT 3 ZIM 4/81 and Asia 1 CAM 9/80 plus SVD virus UKG 27/72 which are routinely employed in the indirect sandwich ELISA for FMD/SVD virus antigen detection within the FAO World Reference Laboratory for Foot and Mouth Disease [WRL for FMD]) were used. These antisera had been raised in rabbits and guinea pigs according to the methods of Have and Schjerning-Thiesen, 1984 and Ferris and Donaldson, 1984, respectively.

Monoclonal antibodies. Mouse monoclonal antibodies (mabs) to strains of each serotype of FMD and SVD virus were selected for test. The mabs to FMD virus serotypes C (strain C₁ Oberbayern, mabs E2B4 and D7G2), SAT 1 (BOT 1/68, Clone 5), SAT 3 (ZIM 4/81, C14) and Asia 1 (PAK 1/54, C1) plus SVD virus (UKG 27/72, C70) were produced in-house. Other mabs against the remaining FMD virus serotypes were gifts from other FMD laboratories: O (O₁ Lausanne, C9) from Dr E Brocchi, Istituto Zooprofilattico Sperimentale Della Lombardia E Dell'Emilia, Via A. Bianchi, 9 - 25124 Brescia, Italy; A (A₂₂ IRQ 24/64, 18H11) from Bayer AG, BG Tiergesundheit, Biologische Produktion, Osteratherstr 1A, D-50739 Koln, Germany and SAT 2 (ZIM 07/83, 810) from Dr D Fargeaud, Botswana Veterinary Institute, Broadhurst Industrial Estate, Private Bag 0031, Gaborone, Botswana). All these mabs have been shown to be type-specific (N.P. Ferris, unpublished results). Two other FMD virus mabs (types C, 4A3 and Asia 1, 5F10) were also obtained from Dr E Brocchi, Italy, both of which react with FMD viruses belonging to all of the seven serotypes (E. Brocchi and N.P. Ferris, unpublished results).

Virus sample preparation. Inactivated, purified antigens to each of the seven serotypes of FMD virus plus SVD virus (Ferris *et al.*, 1984) were used. Epithelial suspensions (ES) of reference samples of all seven serotypes of FMD virus plus SVD virus (as indicated in the figures) had previously been prepared in phosphate buffer (Ferris and Dawson, 1988) and had been stored at -80°C. Supernatant fluids derived from cell cultures (primary calf thyroid, IB-RS-2 or baby hamster kidney cells) inoculated with ES or cell culture grown antigens of all seven serotypes of FMD virus plus SVD virus (strains as indicated in Table 1) were selected from a collection stored at -80°C.

Blocking Buffers. Three different blocking buffers were employed depending on the particular ELISA (capture/detecting) reagent combination : i) blocking buffer 1 - PBS (pH 7.6) with 0.05% Tween 20 containing 5% skimmed milk powder ("Marvel"); ii) blocking buffer 2 - 0.85% saline with 0.02 M Tris, 0.002 M CaCl₂ and 0.001 M MgCl₂, and 2% bovine serum albumen (pH 7.6) and iii) blocking buffer 3 - PBS (pH 7.4) with 0.05% Tween 20 and containing 10% normal bovine serum and 5% normal rabbit serum.

Indirect sandwich ELISA. The basic format for the indirect sandwich ELISA, unless otherwise stated, was as follows: 50 µl reagent volumes were used throughout; ELISA plates (Nunc Maxisorp immunoplates) were incubated for 1 h at 37°C on a rotary shaker and plates washed with phosphate buffered saline [PBS, pH 7.4] after each incubation step, except the final stage in which sulphuric acid was added to stop the substrate/chromogen reaction.

ELISA using polyclonal antisera as capture and detecting antibodies. Plates were coated with an optimal dilution of rabbit antiserum to FMD virus in 0.05 M carbonate/bicarbonate buffer, pH 9.6 and incubated in a fridge overnight at +4°C. Next, either purified virus antigen, suspensions of vesicular epithelia or cell culture supernatants were added to each well. Where applicable, antigen was diluted in PBS. After plate incubation, homologous guinea pig antiserum, diluted to the optimal concentration in blocking buffer 1 was added to each well. After plate incubation, an optimal dilution of rabbit anti-guinea pig immunoglobulins conjugated to horse radish peroxidase in blocking buffer 1 was added to each well. The plates were washed after incubation and plates blotted dry before substrate (0.05% H₂O₂)/chromogen (orthophenylene diamine) in citrate/phosphate buffer, pH 5.0 was added. After 15 min incubation at room temperature the reaction was stopped by adding 1.25 M sulphuric acid. The OD of each well was read by using a spectrophotometer with a 492 nm filter.

ELISA using recombinant αvβ6 as a capture ligand and polyclonal antibodies as detectors. Plates were coated with an optimal dilution of recombinant αvβ6 in a solution of 0.85% saline, 0.02 M Tris, 0.002 M CaCl₂ and 0.001 M MgCl₂, pH 7.6 and incubated in a fridge overnight at +4°C. The following day, the coating buffer was tipped off the plate without washing with PBS and 100 µl of blocking buffer 2 was added to every well. After plate incubation for 1 h at 37°C, the blocking buffer was simply tipped off the plate, test sample added and the ELISA completed as described in the previous section. Where applicable, antigen was diluted in blocking buffer 2.

ELISA using recombinant αvβ6 as a capture ligand and monoclonal antibodies as detectors. Plates were coated with recombinant αvβ6, wells blocked with blocking buffer 2 and test sample added. Where applicable, antigen was diluted in blocking buffer 2. After plate incubation, an optimum dilution of mab in blocking buffer 3 was added and the ELISA completed as described, except that

rabbit anti-mouse, instead of rabbit anti-guinea pig, immunoglobulins conjugated to horse radish peroxidase, and diluted in blocking buffer 3, was used.

Optimisation of recombinant $\alpha\beta 6$ dilution for use in the ELISA. The optimal dilution of recombinant $\alpha\beta 6$ for coating immunoplates was assessed as follows. Two-fold dilution series of the integrin in saline coating buffer were made (from columns 1 to 12 of immunoplates) from undiluted CHO cell culture supernatant fluid. A fixed dilution (1 $\mu\text{g/ml}$) of purified antigen of each FMD virus serotype plus SVD virus was then added, one virus serotype for each of the 8 rows, and the test run to completion. The optimal dilution of the recombinant preparation was then chosen from the titration plot of the reactivity of recombinant dilution against the virus antigens.

Evaluation of recombinant $\alpha\beta 6$ as a ligand for FMD virus. The ability of the recombinant $\alpha\beta 6$ to bind FMD virus was assessed by comparing a series of immunoplates which had been coated with an optimal concentration of recombinant integrin with others which had been left uncoated. The plates were blocked after 'incubation' with blocking buffer 2 prior to the addition of cell culture supernatants to 12 isolates of each FMD virus serotype plus SVD virus (Table 1). The two pan-reactive mabs (4A3 and 5F10) were used as detecting antibodies and the format of the ELISA was carried out as previously described for recombinant $\alpha\beta 6$ as a capture ligand and mabs as detectors. The results were also compared to the reactivity of the antigens in the described ELISA procedures using rabbit antiserum as a trapping reagent in combination with either guinea pig polyclonal or mouse monoclonal antibodies.

FMD virus diagnosis by ELISA. The three strategies for the ELISA (i.e. rabbit polyclonal antibody as trapper and guinea pig polyclonal antibody as detector, recombinant $\alpha\beta 6$ as trapper/guinea pig as detector and recombinant $\alpha\beta 6$ as trapper/type-specific mab as detector) were compared for their ability to serotypically discriminate samples of cell culture grown antigens and epithelial suspensions of each serotype of FMD and SVD virus (plus another suspension negative for FMD or SVD virus).

Results:

Optimal recombinant $\alpha\beta 6$ dilution for use in the ELISA. It was found that the unpurified CHO cell culture supernatant fluid containing the $\alpha\beta 6$ protein bound the inactivated, purified viruses of all seven FMD virus serotypes but did not react with SVD virus. The results of titrations of this recombinant preparation against purified virus preparations are shown in Fig. 1A. Too high an integrin concentration proved to be inhibitory for binding virus and reactivity rapidly tailed off past an integrin dilution of 1:512 but the mid-range of the dilution series (from dilutions 1:16 to 1:256) proved effective for trapping FMD virus. Consequently, a dilution of 1:100 of the CHO cell culture supernatant fluid was chosen to coat immunoplates for subsequent examination of test antigen preparations in the ELISA.

The conclusion that FMD virus was indeed bound by the recombinant integrin was supported by the demonstration that virus preparations were prevented from binding directly to the surface of immunoplate wells treated with blocking buffer 2 prior to the antigen step (Fig. 1B). However, this treatment did not interfere with the positive reaction between integrin and FMD virus on integrin coated plates.

Evaluation of recombinant $\alpha\beta 6$ as a ligand for FMD virus. The reaction of 12, cell culture grown antigens of each FMD virus serotype and SVD virus (Table 1) was evaluated in each ELISA format and the results are shown in Fig. 2 (each mini graph is entitled with the serotype of each group of 12 viruses used for the ELISA reaction). The results show that the integrin bound all FMD viruses irrespective of serotype (the plot profiles being similar between Fig. 2 sections A, B, C, E and F) but not SVD virus. The specificity of the reactions being confirmed by the inability of the viruses to bind directly to the surface of immunoplates uncoated with either rabbit antiserum or integrin but which had been first 'blocked' with blocking buffer 2 (Fig. 2D).

Serotypic characterisation of test samples. The results of comparisons between the three ELISA formats to classify the serotype of the test samples are shown in Fig. 3 (using epithelial suspensions). Each mini graph is entitled with the virus serotype and sample strain used for the ELISA reaction. The pattern of results which were achieved using cell culture grown antigens was essentially the same.

Although the signals for the homotypic reactions were strongest, there was evidence of a degree of heterotypic cross-reactivity with some of the test samples using rabbit/guinea pig polyclonal antiserum (Fig. 3A). This heterotypic cross-reactivity was further and considerably exacerbated using the integrin/guinea pig polyclonal antibody combination (Fig. 3B). Conversely, totally type-specific reactions resulted from the ELISA employing recombinant integrin as capture and mabs as detecting

reagents (Fig. 3C). No reactions were evident when utilising integrin as the trapping reagent with the addition of SVD virus or the negative epithelial suspension.

Discussion:

Molecular techniques, such as RT-PCR procedures, are certain to play an essential role in the future diagnosis FMD and other vesicular diseases. The real-time, fluorogenic RT-PCR is being shown to be more sensitive than the ELISA for FMD diagnosis (Reid *et al.*, 2003; Shaw *et al.*, 2004). It has the potential to examine certain diagnostic samples such as blood, milk and other fluids, which cannot be examined directly in the ELISA and to reduce the necessity for virus isolation and amplification in cell cultures. Although the ELISA has inferior sensitivity to real-time RT-PCR, the predictive value of the test can be high if suitable samples can be collected. For example, around 90% of positive epithelium samples received during the course of the 2001 FMD outbreak in the UK were so defined at the initial stage of testing epithelial suspensions by ELISA (N.P. Ferris, unpublished results). The ELISA is also quicker, easier and cheaper to perform than the PCR. It is an assay, which is readily transferable from a reference laboratory to other FMD laboratories, the majority of which already have the necessary equipment and reagents for its use and the expertise to perform it.

The main disadvantage of the ELISA as used by the WRL for FMD and the majority of other FMD laboratories (besides not having 100% sensitivity) is that it uses FMD virus type-specific polyclonal antisera: both to trap FMD virus onto the plate and for its detection. The disadvantages of polyclonal antisera include a continual need to ensure that reagents for diagnostic use have suitable affinity for new emerging field virus strains and that each stock is of finite supply, while the replacement stock often exhibits slightly different reaction characteristics. To counter the finite supply of polyclonal antiserum reagents, type-specific mabs can be used (Brocchi *et al.*, 1986) but the problem of ensuring that the selection of panels of either individual mabs, or cocktails of several, is suitable for the recognition and serotypic discrimination of new antigenic virus strains remains.

The objective of the work reported on here was to examine the utility of recombinant integrin $\alpha v\beta 6$ (arising from CHO cells, which have been transfected to secrete soluble integrin into the maintenance medium) for binding strains of all serotypes of FMD virus in the ELISA. This was demonstrated by testing purified FMD and SVD virus antigens in an ELISA using serum-free maintenance medium from the transfected CHO cells (as collected directly from the culture flasks, save for a clarification of the medium to remove cell debris by centrifugation) containing recombinant integrin protein as the capture reagent. This was followed by the demonstration that panels of cell culture grown antigens of each FMD virus serotype were bound by the recombinant protein while those of SVD virus were not. Although there were examples of variable optical density values for certain viruses between the different ELISA formats, most of the differences in signals were probably a reflection of the reactivity of the reagent used to detect the bound virus. For example, although the pan-reactive FMD mabs 4A3 and 5F10 react with FMD viruses of each serotype, mab 4A3 had a higher affinity for SAT 1 FMD viruses than mab 5F10 while the reverse was evident for some FMD viruses of the Asia 1 serotype.

A limitation of the conventional ELISA (using rabbit antisera as capture antibodies and polyclonal antibodies as detectors) is that of heterotypic cross-reactions which sometimes reduce the certainty of serotype definition. Such heterotypic reactions were evident from the described experiments on serotyping both cell culture grown viruses (results not shown) and epithelial suspensions of field strains of FMD viruses (as illustrated in Fig. 3). Homotypic signals were the highest for each of the viruses examined by the ELISA using rabbit/guinea pig antibody combination with a reasonably clear distinction from the heterotypic reaction (Figs 3A). This proves to be generally the case during the course of examining submitted diagnostic samples received under the auspices of the WRL for FMD. However, problems of test interpretation occur in two situations. Firstly, high concentrations of antigens in sample preparations that have a poor antigenic match with the typing reagent may give rise to a lower optical density value in the ELISA against the homotypic reagent than would normally be expected. Consequently, the differences between homotypic and heterotypic reaction signals are reduced, lowering the confidence with which the serotype of the FMD virus is defined. Secondly, it is normal to assume that heterotypic reactions are indeed non-specific cross-reactions. However, this can be a dangerous assumption. Multiple-infected samples are uncommon but they do occasionally occur and their classification by ELISA, with or without virus isolation in cell culture, is problematic (Ferris, Oxtoby and Hughes, 1995). The combination of integrin as capture and guinea pig polyclonal antibody as detector yielded even greater heterotypic cross-reactions (Figs 3B). This was disappointing and somewhat surprising but illustrates that the integrin is not solely specific for FMD virus and that the guinea pig anti-146S sera are not as FMD virus type-specific as previously thought or hoped for. However, replacing the polyclonal reagents with mabs eliminated heterotypic reactions and yielded totally FMD virus type-specific results of the correct definition. This is not to suggest that the particular mabs used in this study are necessarily the optimum ones for routine diagnostic use (some have too narrow a range of within serotype strain recognition for use on their own). Rather the results show that the use of the recombinant protein as a capture reagent in the ELISA in

combination with appropriate mab(s) as the detector has the potential to improve upon conventional diagnostic assays using rabbit and guinea pig polyclonal antisera.

There is further potential for use of the recombinant integrin $\alpha\beta 6$ protein in other FMD virus test procedures where the use of a single reagent to recognise FMD viruses of all serotypes could be advantageously exploited, e.g. in immunocapture RT-PCR, pen-side chromatographic strip-test devices and other biosensors. Additionally, the recombinant could be usefully employed in any immunoassays employed for FMD diagnosis. Those designed for characterizing the antigenicity of field virus strains (Kitching, Rendle and Ferris, 1988) and for detecting FMD virus antibody (Mackay *et al.*, 2001) come to mind. These assays commonly use type-specific rabbit polyclonal antibody to trap virus onto the immunoplate, the efficiency of which is limited by the antiserum selection.

Conclusions:

- The recombinant $\alpha\beta 6$ protein bound all antigens of FMD virus irrespective of serotype but did not react with SVD virus
- Totally type-specific reactions resulted from the ELISA using integrin as capture and mabs as detectors
- This format has the potential to improve upon conventional FMD diagnostic assays using polyclonal antisera and could be usefully used in other immunoassays for FMD diagnosis

Recommendations:

- No specific recommendations

Acknowledgements:

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References:

- Brocchi, E., Capucci, L., De Simone, F. & Panina, G.F.** 1986. Potential of monoclonal antibodies (Mabs) for FMD diagnosis and characterisation of the isolates. *Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Madrid, Spain, 14-17 October 1986*, Appendix 5: 30-31.
- Ferris, N.P., Donaldson, A.I., Barnett, I.T.R. & Osborne, R.W.** 1984. Inactivation, purification and stability of 146S antigens of foot and mouth disease virus for use as reagents in the complement fixation test. *Rev. Sci. Tech. Off. Int. Epiz.* 3: 339-350.
- Ferris, N.P., & Donaldson, A.I.** 1984. Serological response of guinea pigs to inactivated 146S antigens of foot and mouth disease virus after single or repeated inoculations. *Rev. Sci. Tech. Off. Int. Epiz.* 3: 563-574.
- Ferris, N. P., & Dawson, M.** 1988. Routine application of enzyme-linked immunosorbent assay in comparison with complement fixation for the diagnosis of foot-and-mouth and swine vesicular diseases. *Vet. Microbiol.* 16: 201-209.
- Ferris, N.P., Oxtoby, J.M. & Hughes, J.F.** 1995. Multiple-infected diagnostic specimens from foot and mouth disease endemic areas. *Rev. Sci. Tech. Off. Int. Epiz.* 14: 557-565.
- Have, P., Lei, J.C. & Schjerning-Thiesen, K..** 1984. An enzyme-linked immunosorbent assay (ELISA) for the primary diagnosis of foot-and-mouth disease. Characterization and comparison with complement fixation. *Acta Vet. Scand.* 25: 280-296.
- Jackson, T., Sharma, A., Abu-Ghazaleh, R., Blakemore, W.E., Ellard, F.M., Simmons, D.L., Newman, J.W.L., Stuart, D.I. & King, A.M.Q.** 1997. Arginine-glycine-aspartic acid-specific binding by foot-and-mouth disease virus to the purified integrin $\alpha\beta 3$ in vitro. *J. Virol.* 71: 8357-8361.
- Jackson, T., Sheppard, D., Denyer, M., Blakemore, W.E. & King, A.M.Q.** 2000. The epithelial integrin $\alpha\beta 6$ is a receptor for foot-and-mouth disease virus. *J. Virol.*, 74: 4949-4956.
- Kitching, R.P., Rendle, R. & Ferris, N.P.** 1988. Rapid correlation between field isolates and vaccine strains of foot-and-mouth disease virus. *Vaccine* 6: 403-408.

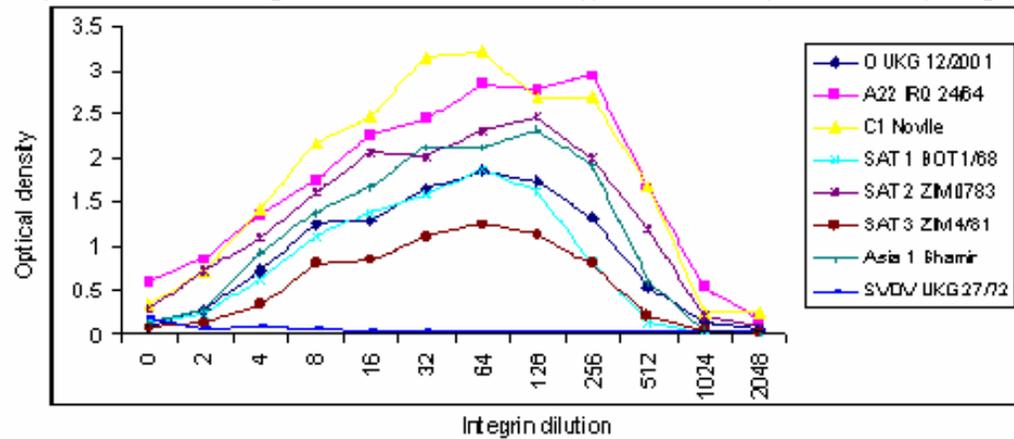
- Mackay, D.K.J., Naci Bulut, A., Rendle, T., Davidson, F. & Ferris, N.P.** 2001. A solid-phase competition ELISA for measuring antibody to foot-and-mouth disease virus. *J. Virol. Methods* 97: 33-48.
- Reid, S.M., Ferris, N.P., Hutchings, G.H., Zhang, Z. Belsham, G.J. & Alexandersen, S.** 2002. Detection of all seven serotypes of foot-and-mouth disease virus by real-time, fluorogenic reverse transcription polymerase chain reaction assay. *J. Virol. Methods* 105: 67-80.
- Reid, S.M., Grierson, S.S. Ferris, N.P., Hutchings, G.H. & Alexandersen, S.** 2003. Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *J. Virol. Methods* 107: 129-139.
- Roeder, P.L. & Le Blanc Smith, P.M.** 1987. Detection and typing of foot-and-mouth disease virus by enzyme-linked immunosorbent assay: a sensitive, rapid and reliable technique for primary diagnosis. *Res. Vet. Sci.* 43: 225-232.
- Samuel, A.R., N.J. Knowles, N.J., Samuel, G.D. & Crowther, J.R.** 1991. Evaluation of a trapping ELISA for the differentiation of foot-and-mouth disease virus strains using monoclonal antibodies. *Biologicals* 19: 299-310.
- Shaw, A.E., Reid, S.M., King, D.P., Hutchings, G.H. & Ferris, N.P.** (2004). Enhanced laboratory diagnosis of foot and mouth disease by real-time polymerase chain reaction. *Rev. Sci. Tech. Off. Int. Epiz.* 23 (in press).
- Weinacker, A., A. Chen, A.A., Agrez, M., Cone, R.I., Nishimura, S., Wayner, E., Pytela, R. & Sheppard, D.** 1994. Role of integrin $\alpha v\beta 6$ in cell attachment to fibronectin. *J. Biol. Chem.* 269: 6940-6948.

TABLE 1. FMD and SVD virus strains used to evaluate the capacity of recombinant $\alpha\beta 6$ to act as a ligand

Virus sample no.	FMD virus serotype							SVD virus
	O	A	C	SAT 1	SAT 2	SAT 3	Asia 1	
1	BUR 1/89	BHU 2/90	ITL 2/89	SA 13/61	IVY 2/90	BOT 109/66	ISR 3/89	UKG 314/73
2	KEN 102/60	BRA 1/58	C ₃ Indaial	ZAM 3/88	TAN 5/68	RHO 1/74	BUR 1/88	NET 3/92
3	BHU 1/90	TUR 1/90	NEP 35/96	ZAM 2/88	ZIM 19/89	RV 7/34	IND 5/89	MTA 22/75
4	SUD 3/89	BUN 4/90	BAN 1/92	TUR 323/62	GHA 2/90	KNP 1/86	OMN 21/89	AUR 1/73
5	ISR 1/90	KEN 46/65	PHI 9/94	ISR 4/62	ZAM 6/82	RHO 4/75	NEP 2/90	HKN 1/89
6	CAR 3/89	USSR 1/64	C 997	SWA 40/61	SEN 1/83	MAL 3/76	CAM 1/90	GRE 1/79
7	CAM 1/89	NEP 30/90	C ARG 69	YEM 15/84	ZIM 12/91	UGA 92/70	ISR 3/63	FRA 2/73
8	IND 1/89	Peru 69	C ₁ Noville	ZIM 22/89	BOT 11/77	ZIM 2/84	MAY 2/90	BEL 2/79
9	HKN 14/90	A Zambia	C Pando	ZAM 2/92	UGA 3/91	P26/90 HV5	TUR 11/2000	ITL A/89
10	TUN 6/89	A ₃₁ COL 69	IND 51/79	KNP 1/88	UGA 5/70	P27/90 DSA39	MYA 2/97	HKN 1/80
11	ETH 3/90	KEN 3/64	SAU 9/74	P30/90 Cher30	CIV 5/90	P20/90 Cher32	IRN 15/2001	HKN 1/82
12	JOR 1/90	NYE 8/89	C ₁ Oberbayern	KNP 6/88	KEN 8/91	ZIM 4/83	LAO 1/96	HKN 1/76

Fig. 1.

A Titration of CHO cell culture supernatant fluid containing soluble recombinant integrin against purified inactivated antigens of each of the seven serotypes of FMD virus plus SVD virus (at 1 ug/ml)



B Reaction of purified inactivated antigens of each of the seven serotypes of FMD virus plus SVD virus (at 1 ug/ml) added directly onto plates previously blocked with blocking buffer 2

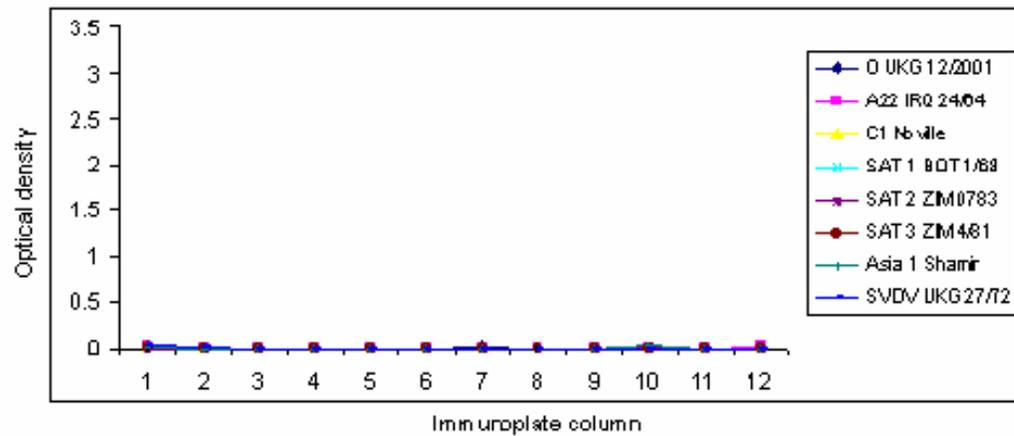


Fig. 2. Homotypic reactions of cell culture grown antigens of each of the seven serotypes of FMD virus plus SVD virus in ELISAs using combinations of reagents for capture/detection of virus as indicated by the captions for sections A to F.

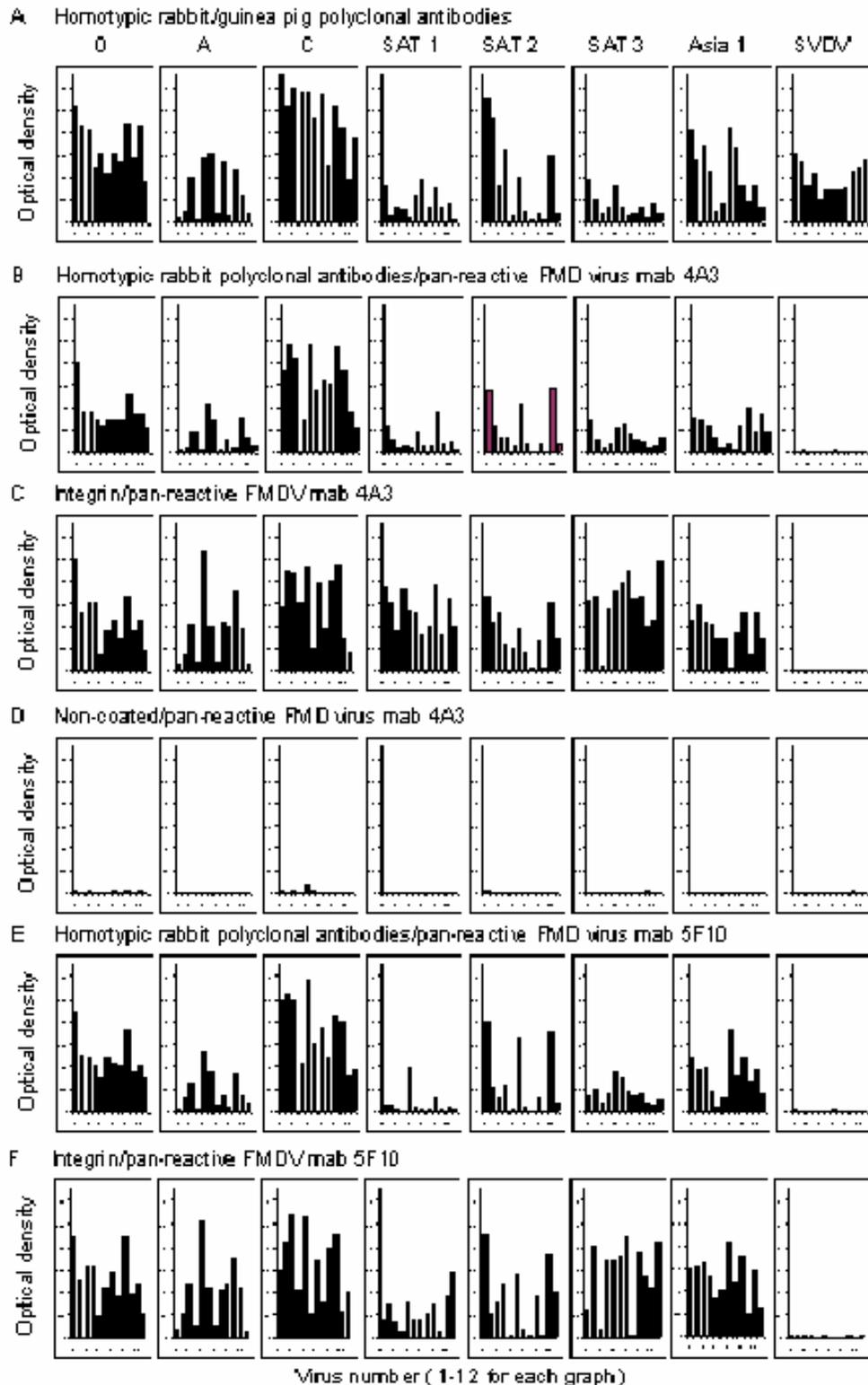
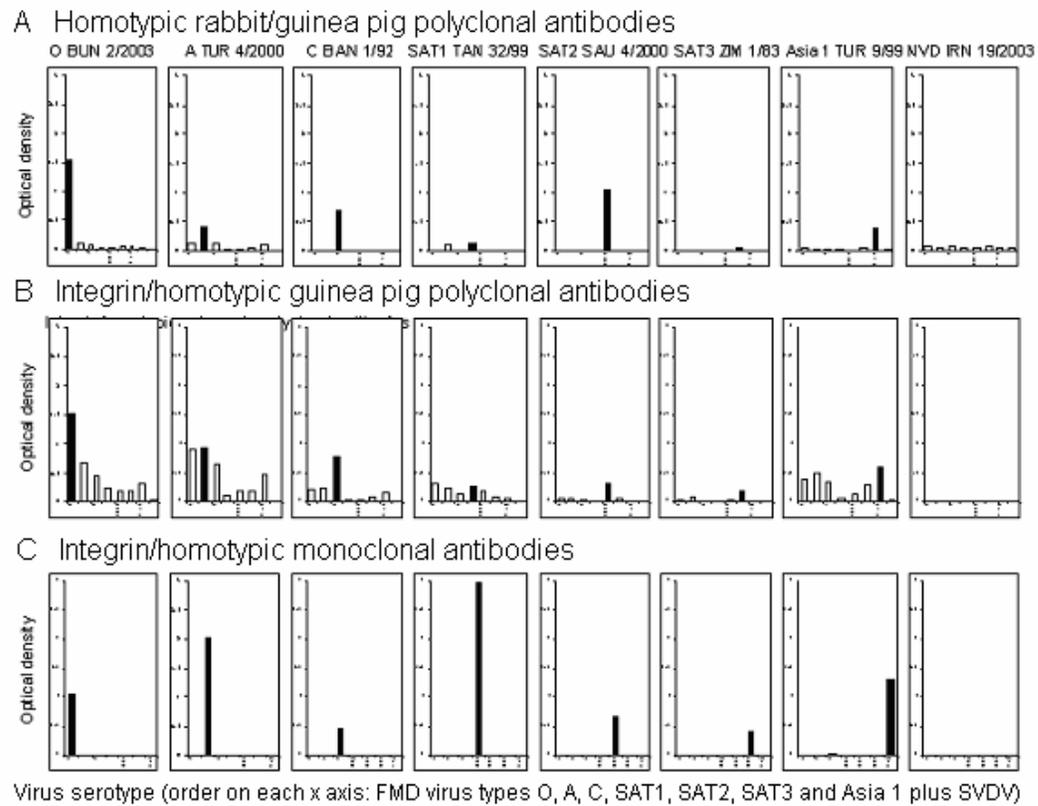


Fig. 3. Homo- and heterotypic reactivity of epithelial suspensions of each of the seven serotypes of FMD virus (serotype and strain as indicated by the graph headings) and a negative sample (NVD, no virus detected) using combinations of reagents for capture/detection of virus (as indicated by the captions for sections A to C). Homotypic reactions as bold plots and heterotypic reactions as empty plots.



Development of Secondary Standards for the Foot-and-Mouth Disease Solid Phase Competition ELISA and Internal Quality Control using Shewhart like Control Charts

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Abstract:

Objectives: To present a possible method for developing and monitoring secondary standards for the FMDV SPC ELISA in light of quality management according to OIE guidelines, international harmonisation and standardisation and in response to recommendations of the FAO-EUFMD Research Group.

Materials and Methods: Serum samples from cattle monovalently vaccinated against FMDV type O₁ Manisa, A₂₂ Iraq, A Iran 1996, C₁ Noville and Asia1 Shamir were calibrated against candidate primary reference sera following OIE guidelines. Data normalised against the OD value of the SP control were introduced and compared in two Shewhart like control chart software programmes (MedLabQC and MultiQC).

Results and discussion: A negative, cut-off, weak and strong positive working or secondary standard was established for all mentioned serotypes. Internal quality control was assured by monitoring the day-to-day performance of the test and decision criteria to accept or reject a run were selected based on the obtained results. The best suitable method for our specific requirements was chosen. Since primary reference sera are too valuable to use in every day tests, national reference laboratories should establish their own secondary standards. Internal quality control is performed in our laboratory on these sera to achieve accreditation and international recognition of results. This method describes an easy way to fulfil these requirements and visualise the day-to-day performance of the test. It could be a useful tool in eliminating trade barriers.

Introduction:

Quality control in veterinary laboratories is rapidly gaining importance over the last few years (De Clercq, 1998; Wiegers, 2003). Validation, standardisation and accreditation are becoming familiar items in contemporary diagnostics. If a laboratory aspires to an external quality control scheme, such as ISO/IEC 17025, it is not only imperative to monitor the test's validity in such a way that trends are detectable, but regular use of certified reference materials and/or secondary standards is obligatory as well (ISO/IEC International Standard 17025, 1999).

In the instance of foot-and-mouth disease (FMD), both the Office International des Epizooties (OIE) (Anonymous, 1998; OIE, 2004) and the Research Group of the Standing Technical Committee of European Commission for the control of foot-and-mouth disease of the Food and Agriculture Organization of the United Nations (FAO-EUFMD RG) have recommended the use of reference materials and recording methods to detect potential problems and trends (FAO-EUFMD RG, 2002; FAO-EUFMD RG, 2003).

At a Meeting of Consultants by the Joint FAO/International Atomic Energy Agency (FAO/IAEA) Division of the IAEA in Vienna 1992, the consensus opinion was that at least a strong positive, a weak positive and a negative standard serum should be established for antibody detection (Wright, 1998).

However, for serological assays (virus neutralisation test (VNT) and ELISA) OIE-approved international primary reference standard sera are too valuable to use in every day tests. Therefore, national and regional reference laboratories are encouraged to develop their own secondary standards in direct calibration against the primary reference sera (Wright, 1998).

Back in 1931, Walter Shewhart (1931) introduced the control chart, a powerful tool to monitor control data variation in time and to visualise trends and process improvement. This kind of control procedure was introduced in clinical chemistry by Levey and Jennings (1950). The charts are therefore sometimes referred to as "Shewhart charts" and other times as "Levey-Jennings charts". It was not until 1998 that Jacobson (1998), and later Rebeski and colleagues (2001), described this quality control method for serological assays for diagnosis of infectious diseases.

In the past, several decision criteria have been put forward to accept or reject a run. For example, a run should be rejected if one of the control values exceeds the control limit of three standard deviations from the mean. This criterion was later extended to include more rules (uni-rule versus multi-rule and univariate versus multivariate procedures), called the Westgard rules (Westgard *et al.*, 1977). Software programmes (MedLabQC (<http://www.hekmat.net/medicine/software.htm>), MultiQC (<http://www.multiQC.com>), QC-Record, *etc.*) have since been developed to automate the charting method. Other warning and action criteria based on exponentially weighted moving average (EWMA) and exponentially weighted moving variance (EWMV) for example are said to be

more sensitive in detecting small analytical bias than conventional Westgard rules (Neubauer, 1997), but to our knowledge have never been put into practice for FMD serological assays. The aim of this study is to develop secondary standards and to monitor the day-to-day performance of the SPCE (Mackay *et al.*, 2001) in time by charting the normalised data rather than the raw OD values of these standards and by choosing the best suitable rejection and acceptance criteria.

Materials and Methods:

Development of secondary standards

To obtain positive control sera against five FMD virus strains (type O₁ Manisa, type A₂₂ Iraq, type A Iran 1996, type C₁ Noville and type Asia1 Shamir) two calves were vaccinated per strain using homologous monovalent vaccines (Merial, Pirbright, UK). One calf received a single cattle vaccine dose as prescribed by the manufacturer; the other received an additional boost 5 months post initial vaccination. For the monovaccinated calves blood was collected 5 to 6 months post vaccination, whereas for the boosted calves blood was collected 21 to 28 days post booster. Serum was retained, 0.2 µm filtered and stored at -80°C until use. For a negative control serum a commercially available adult bovine serum batch (Gibco/BRL) was chosen.

The sera were titrated in their homologous SPCE. Dilutions resulting in a percentage inhibition (PI) nearly identical to the PI obtained, in direct comparison, for the cut-off (C/O), weak positive (WP) and strong positive (SP) candidate primary reference sera were determined. The negative serum batch was also compared to the candidate primary negative reference serum (NC) (Kitching, Rendle & Newman, 2000; Paton *et al.*, 2002; Paton, Armstrong & Anderson, 2003).

Single original serum dilutions were made in negative adult bovine serum and calibrated against the candidate primary reference sera for an additional three times. If results were satisfactory, single use aliquots were prepared for each of the four secondary standards (C/O, WP, SP and NC) and again calibrated against the candidate primary reference sera. These secondary C/O, WP, SP and NC sera were subsequently used in daily laboratory diagnostics. On each plate four replicates of a standard competing antibody (Co), the C/O and WP secondary standards (Wright *et al.*, 1993) were incorporated. However, due to smaller variability only two replicates of the SP and NC secondary standards were put.

Charting method

The median of the raw OD values measured at 490 nm for the Co, the secondary C/O, WP and NC serum were normalised against the obtained median OD value for the secondary SP serum (Wright *et al.*, 1993). The normalised data for these control values of each plate were entered in two different charting software programmes, *i.e.* MedLabQC and MultiQC, to monitor the SPCEs performance in time.

MedLabQC is a multi-rule, bivariate Shewhart like control chart using conventional Westgard rules as decision criteria for accepting or rejecting runs. Following warning and action signals are applied within one and between two control values: 1:2s (within), 1:3s (within), 2:2s (within and between), R:4s (within and between), 4:1s (within and between) and 10:m (within and between). For abbreviation refer to Table 1. According to Westgard, Barry and Hunt (1981) a plate should be rejected when a violation of the 1:3s rule occurs. The 1:2s rule is a warning rule for additional control data inspection. Following subsequent inspection, if one or more additional Westgard rules are violated the assay should be considered out-of-control and the plate should be rejected. If not, the plate can be accepted.

MultiQC, contrary to MedLabQC, generates charts based on EWMA (a measure for inaccuracy) and EWMV to judge whether or not the run is in statistical control. Unlike Shewhart charts, this kind of chart not only takes the immediate control value into consideration, but uses the previous values as well, making it statistically more advanced. The current control value average (\bar{x}_t) is multiplied by a weighting factor (w) and added to the $(1 - w)$ weighted sum of all former measurements (z_{t-1}). Thus, at each time ($t = 1, 2, \dots, n$) the test statistic $z_t = wx_t + (1 - w)z_{t-1}$ resulting in a smaller contribution of past data measurements. This weighting factor allows for an increased sensibility in shift detection (Neubauer, 1997).

Results:

Development of secondary standards

During the FAO Collaborative Study Phases XVII Part 1 and 2 for FMD Serology Standardisation candidate primary reference sera for the SPCE type O, type A Iran 1996 and type Asia1 Shamir (part 1: a C/O serum; part 2: a SP, a WP1, a WP2, a WP3, a WP4 and a NC serum) were distributed to 9 laboratories. The sera were tested using our in-house SPCE and the PI compared to the PI obtained by the FAO World Reference Laboratory (WRL) (Pirbright, UK) (Paton *et al.*, 2002; Paton, Armstrong & Anderson, 2003). Secondary standards were derived and tested in parallel. For the SPCE type A₂₂ Iraq, and type C₁ Noville the secondary standards were calibrated against

candidate primary reference standards originating from FAO Collaborative Study Phase XVI (Kitching, Rendle & Newman, 2000). As an example, table 2 summarises the values for the SPCE for type Asia1 Shamir.

The secondary SP, WP and C/O standard for SPCE type Asia1 Shamir are single dilutions of the boosted calf in negative adult bovine serum. The secondary SP standard is a 1 in 2 dilution corresponding to the WRL SP part 2 but adjusted to fall in the linear portion of the dose/response profile (Wright *et al.*, 1997), the WP a 1 in 5 calibrated against the PI of the WRL WP4 part 2 and finally the secondary C/O standard is a 1 in 14 dilution calibrated against the WRL C/O part 1. The secondary NC standard is practically identical to the WRL NC candidate. Similar dilutions were determined and secondary standards were calibrated for the SPCE type O₁ Manisa, type A Iran 1996, type, A₂₂ Iraq and type C₁ Noville.

Charting method

The median raw OD values, measured at 490 nm, of the SPCE secondary standards (WP, C/O, NC) and the Co are normalised against the median OD value of the SP included on the same plate as recommended by Jacobson (1998) to correct for inherent daily variability between runs (due to ambient temperatures, test parameters, *etc.*). The normalised data (see Table 5) were introduced in two different control chart software programmes, *i.e.* MedLabQC and MultiQC.

Figure 1 and figure 2 present the control charts on the data obtained for the SPCE type A Iran 1996 generated by MultiQC and MedLabQC software programmes respectively (data from other FMD types not shown, but yield similar results). In order to estimate the mean (*m*) and standard deviation (*s*) needed to interpret the charts and to calculate the Shewhart control limits, a reference data pool of 20 successive WP, C/O, NC and Co values was selected (Jan 7, 2004 → March 10, 2004) (Westgard, Barry & Hunt, 1981). Table 3 outlines the mean, the standard deviation and the Shewhart/MedLabQC control limits for all four monitored controls.

Setting up a MultiQC chart based on EWMA and EWMV is less straightforward. In order to obtain a meaningful comparison between both software programmes and chart types (a Shewhart chart for the MedLabQC programme and an EWMA chart for the MultiQC programme) the parameters selected to implement the EWMA chart have to be based on the MedLabQC control limits (Neubauer, 1997). The basis of comparison is the selection of a correct average run length (ARL) that defines the risk of false rejections. The ARL, weighting factor (*w*) and the factor (*q*) for the control limits were determined using the Crowder (1989) four-step procedure (for univariate quality control). Table 4 sums up these values for the Co and the three secondary standards. The EWMA is represented on the chart as a continuous red line.

The EWMV monitors the imprecision of the analytical process. Briefly, it is displayed on the chart as green bars that turn reddish when the imprecision is significantly increased in comparison to the imprecision of the reference pool. Although valuable, it will not be discussed for practical reasons.

When comparing the two software chart programmes for the SPCE type A Iran 1996, both indicate that the run performed at March 17, 2004 must be rejected. A random error has occurred for the WP value (exceeds the upper limit of both the MedLabQC and the MultiQC chart).

No other rejecting should be made based on the MultiQC chart. Nevertheless, the EWMA line visualised both upward and downward trends (upward trend: May 6 → May 25; downward trends: March 12 → April 4 and May 26 → July 1), signalling possible shifts in assay accuracy.

However, the Westgard rules were violated on several other occasions. Table 6 lists all these violations and the decisions made according to Westgard, Barry and Hunt (1981) for these MedLabQC charts.

Discussion:

Assay characterisation parameters, like repeatability, analytical and diagnostic sensitivity and specificity, are essential for assay validation. However, they are no longer sufficient to achieve and/or maintain accreditation. Assay validation is an incremental and continuous process (Jacobson, 1998) and its performance should be monitored in time (ISO/IEC International Standard 17025, 1999).

More specifically, for serological assays charting methods should be established based on the reference standards (Wright *et al.*, 1997; Wright, 1998). The international consensus is to include at least a strong and weak positive control to the test as well as a negative control (Wright, 1998).

In the instance of the solid phase competition ELISA for antibody detection to foot-and-mouth disease virus, (candidate) primary reference sera (SP, WP, C/O and NC) are available for SPCE serotype O, A, C and Asia. These should be regarded as extremely valuable and may only be used for specific purposes (harmonisation, calibration and standardisation) (ISO/IEC International Standard 17025, 1999). It is, therefore, recommended that national and regional reference

laboratories establish their own secondary and/or working standards to use in daily diagnostics (FAO-EUFMD RG, 2003).

We describe a possible method for developing secondary standards (SP, WP, C/O and NC) by vaccinating calves and calibrating their sera against the candidate primary reference sera supplied by the FAO WRL. The tested dilutions gave nearly identical results as the PI obtained for the candidate primary reference sera (table 2) and can therefore be included on each plate as assay performance monitoring tool.

The quality control data obtained with the standard competing antibody and WP, C/O and NC (median OD values normalised against the median OD value of the SP) were successfully introduced in two easy-to-implement control chart software programmes, MedLabQC and MultiQC. At first glance, MedLabQC generates more warning signals than MultiQC (table 6). However, when inspecting the data more closely for the EWMA chart, all additional warning signals of the MedLabQC programme are detectable as upward or downward trends of the EWMA (red line). For example, the warning signals on March 12 and from April 1 to 7 are part of the downward trend from March 12 to April 4; the warning signals from June 14 to July 1 equal the downward EWMA trend from May 26 to July 1. Thus, performing trend analysis on the MultiQC generated chart makes the chart at least equally sensitive to shifts as the MedLabQC generated one and has the added advantage that these trends are detected earlier (e.g. June 14 → July 1 for MedLabQC versus May 26 → July 1 for MultiQC).

Furthermore, the MedLabQC decision rules reject 3 additional plates in comparison to the MultiQC decision criteria, *i.e.* June 28, July 5 and July 9. Taking a closer look at the control values generated on June 28 and July 9 a proportional downward, respectively upward, shift of all 4 control values is observed, meaning that the WP, C/O, NC sera and the standard competing antibody all react in a similar fashion. Thus, that routine sera data interpretation is not hindered. The tested sera would be proportional lower, respectively higher, than on previous occasions, but would be scored correctly (positive/negative). Rejecting the run and repeating it on a later date would be a waste of precious laboratory time. The same is true for July 5.

Selecting one software programme and the statistics behind it over another comes down to choosing between two types of error (type I error or false rejection and type II error or false acceptance). Although the decision criteria for the MedLabQC programme are appealing (no statistics needed, in fact no automated software programme needed, easy to interpret, *etc.*), it is our opinion that the MultiQC software programme for this specific assay provides the best balance between error detection and false rejection. It is not only at least as sensitive as the conventional Shewhart charts (presented data), but also more flexible since different weighting factor can be selected to increase trend detection (Neubauer, 1997). Moreover, correlations between control values (*i.e.* multivariate quality control) can be calculated and integrated in the decision criteria system (for an overview see Hotelling, 1947). Furthermore, EWMV provides a reliable tool to measure imprecision (not discussed). However, we acknowledge that other control chart types may be in use and could be of equal value (Blacksell *et al.*, 1994).

This kind of assay performance monitoring is not only a useful tool for accreditation means and day-to-day internal quality control, it could also be essential in achieving international mutual recognition of test results and thereby eliminating trade barriers.

Conclusions:

- Control charts are helpful tools in visualising potential problems with the assay
- Each laboratory should choose the charting method that best meets its requirements
- Internal quality control is essential in achieving mutual recognition of results, in eliminating trade barriers and in maintaining accreditation

Recommendations:

- All national and regional reference laboratories for FMD should establish their own secondary or working standards
- All national and regional reference laboratories would benefit greatly from using control charts to monitor the assay's validity in time
- National and regional reference laboratories are stimulated to present their internal quality control data for coordination by the FAO WRL

References:

Anonymous. 1998. Guidelines of the Office International des Epizooties for laboratory quality evaluation, for international reference standards for antibody assays and for laboratory proficiency testing. *Rev. sci. tech. Off. Int. Epiz.*, 17 (2): 600-609.

- Blacksell, S.D., Gleeson, L.J., Lunt, R.A. & Chamnanpood, C.** Use of combined Shewhart-CUSUM control charts in internal quality control of enzyme-linked immunosorbent assays for the typing of foot and mouth disease virus antigen. *Rev. sci. tech. Off. Epiz.*, 13 (3): 687-699.
- Crowder, S.V.** 1989. Design of exponentially weighted moving average schemes. *J. Qual. Technol.*, 21: 1881-1887.
- De Clercq, K.** 1998. Implementation of quality assurance in national foot and mouth disease laboratories, based on the guidelines of the Office International des Epizooties. *Rev. sci. tech. Off. Int. Epiz.*, 17 (3): 786-795.
- European Commission for the Control of Foot-and-Mouth Disease.** 2002. Diagnostics – Antibody detection. *Item 6 of the Session of the Research Group of the Standing Technical Committee*, Cesme, Izmir, Turkey, 17-20 September, p14-17.
- European Commission for the Control of Foot-and-Mouth Disease.** 2003. Diagnostics – Reference sera. *Item 5 of the Session of the Research Group of the Standing Technical Committee*, Gerzensee, Berne, Switzerland, 16-19 September, p12-13.
- Hottelling, H.** 1947. Multivariate quality control. In: Eisenhart, C., Hastay, M. & Wallis, W.A., eds. *Techniques of statistical analysis*, NewYork: McGraw-Hill, p111-184.
- ISO/IEC International Standard 17025.** 1999. General requirements for the competence of testing and calibration laboratories. *International Organisation for Standardisation (ISO)*, ISO Central Secretariat, 1 rue de Varembé, Case Postale 56, CH – 1211, Geneva Switzerland, First edition, chapter 5.9, p19.
- Jacobson, R.H.** 1998. Validation of serological assays for diagnosis of infectious diseases. *Rev. sci. tech. Off. Int. Epiz.*, 17 (2): 469-486.
- Kitching, P., Rendle, T. & Newman, B.** 2000. FAO collaborative study phase XVI: Establishing reference standards for FMD serology. *European Commission for the Control of Foot-and-Mouth Disease. Report of the Session of the Research Group of the Standing Technical Committee*, Borovets, Bulgaria, 5-8 September, p224-230.
- Levey, S. & Jennings, E.R.** 1950. The use of control charts in the clinical laboratories. *Am. J. Clin. Pathol.*, 20: 1059-1066.
- Mackay, D.K.J., Naci Bulut, A., Rendle, T., Davidson, F. & Ferris, N.** 2001. A solid-phase competition ELISA for measuring antibody to foot-and-mouth disease virus. *J. Virol. Meth.*, 97: 33-48.
- MedLabQC.** 2004 (available at www.hekmat.net/medicine/software.htm).
- MultiQC.** 2004 (available at www.multiQC.com).
- Neubauer, A.S.** 1997. The EWMA control chart : properties and comparison with other quality-control procedures by computer simulation. *Clin. Chem.*, 43: 594-601.
- OIE.** 2004. Office International des Epizooties. The World Health Organisation. Manual of diagnostic tests and vaccines for terrestrial animals. Paris, France, Fifth edition, chapter I.1.2.
- Paiba, G.A., Anderson, J., Paton D.J., Soldan, A.W., Alexandersen, S., Corteyn, M., Wilsden, G., Hamblin, P., Mackay, D.K. & Donaldson, A.I.** 2004. Validation of a foot-and-mouth disease antibody screening solid-phase competition ELISA (SPCE). *J. Virol. Methods.*, 115 (2): 145-158.
- Paton, D.J., Armstrong, R.M., Turner, L.S., Hamblin, P.A., Corteyn, M., Gibson, D. & Anderson, J.** 2002. FAO collaborative study phase XVII: Standardisation of FMD antibody detection. *European Commission for the Control of Foot-and-Mouth Disease. Report of the Session of the Research Group of the Standing Technical Committee*, Cesme, Izmir, Turkey, 17-20 September, p226-234.
- Paton, D., Armstrong, R. & Anderson, J.** 2003. An update on progress with the FAO collaborative studies for FMD serology standardisation, phases XVII and XVIII. *European Commission for the Control of Foot-and-Mouth Disease. Report of the Session of the Research Group of the Standing Technical Committee*, Gerzensee, Berne, Switzerland, 16-19 September, p102-115.
- Rebeski, D.E., Winger, E.M., Ouma, J.O., Kong Pages, S., Büscher, P., Sanogo, Y., Dwinger, R.H. & Crowther, J.R.** 2001. Charting methods to monitor the operational performance of ELISA method for the detection of antibodies against trypanosomes. *Vet. Parasit.*, 96: 11-50.
- Shewhart, W.A.** 1931. Economic control of quality of manufactured product. *Van Nostrand, New York (republished by ASQC Quality Press, Milwaukee, WI, 1980)*.
- Westgard, J.O., Groth T., Aronsson T. & de Verdier C.H.** 1977. Combined Shewhart-Cusum control charts for improved quality control in clinical chemistry. *Clin. Chem.*, 23 (10): 1881-1887.
- Westgard, J.O., Barry, P.L. & Hunt, M.R.** 1981. A Multi-rule Shewhart chart for quality control in clinical chemistry. *Clin. Chem.*, 27 (3): 493-501.

- Wieggers, A.L.** 2003. Valid methods: the quality assurance of test method development, validation, approval, and transfer for veterinary testing laboratories. *J. Vet. Diagn. Invest.*, 15: 303-310.
- Wright, P.F., Nilsson, E., Van Rooij, E.M.A., Lelenta, M. & Jeggo, M.H.** 1993. Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis. *Rev. sci. tech. Off. Int. Epiz.*, 12 (2): 435-450.
- Wright, P.F., Tounkara, K., Lelenta, M. & Jeggo, M.H.** 1997. International reference standards: antibody standards for the indirect enzyme-linked immunosorbent assay. *Rev. sci. tech. Off. Int. Epiz.*, 16 (3): 824-832.
- Wright, P.F.** 1998. International standards for test methods and reference sera for diagnostic tests for antibody detection. *Rev. sci. tech. Off. Int. Epiz.*, 17 (2): 527-533.

Table 1. The Westgard control rules (Westgard, Barry & Hunt, 1981)

Abbreviation	Explanation	Example
1:2s	One control value exceeds the control limit set as the mean (m) \pm 2 standard deviations (s). It is a warning rule for additional inspection of control data.	The normalised median secondary WP standard (in short WP value) $< m - 2s$
1:3s	One control value exceeds the control limit set as the $m \pm 3s$. This is the usual rejection criterion on a Shewhart control chart.	NC value $> m + 3s$
2:2s	Two consecutive control values exceed the same limit ($m + 2s$ or $m - 2s$). Consecutive being two different control values on the same plate or the same control value on two different plates.	<u>1 plate:</u> WP value $> m + 2s$ AND Co value $> m + 2s$ <u>2 plates:</u> plate 1: C/O value $< m - 2s$ AND plate 2: C/O value $< m - 2s$
R:4s	Within a plate, the range (R) between the two control values exceeds 4s. Between two successive plates, the difference (R) for the same control value exceeds 4s, one exceeding the $m + 2s$, the other exceeding the $m - 2s$.	<u>1 plate:</u> WP value $< m - 2s$ AND C/O value $> m + 2s$ <u>2 plates:</u> plate 1: NC value $< m - 2s$ AND plate 2: NC value $> m + 2s$
4:1s	Four consecutive control values exceed the same limit ($m + 1s$ or $m - 1s$). This can occur between two different control values on two successive plates, as well as within one control value by inspection of four successive plates.	<u>2 plates:</u> plate 1&2: WP value $> m + 1s$ AND plate 1&2: C/O value $> m + 1s$ <u>4 plates:</u> plate 1,2,3&4: Co value $< m - 1s$
10:m	Ten consecutive controls values fall on the same side of the m. These values occur on five successive plates for two different controls or on ten successive plates for the same control value.	<u>5 plates:</u> plate 1 \rightarrow 5: C/O value $> m$ AND plate 1 \rightarrow 5: WP value $> m$ <u>10 plates:</u> plate 1 \rightarrow 10: NC value $< m$

Table 2. Development of secondary standards for SPCE type Asia1 Shamir (data presented as PI)

Standards	WRL SPCE	In-house SPCE
WRL SP candidate part 2	98	90
WRL WP1 candidate part 2	92	89
WRL WP2 candidate part 2	90	89
WRL WP3 candidate part 2	69	73
WRL WP4 candidate part 2	63	69
WRL C/O candidate serum part 1	42	35
WRL NC candidate part 2		6
In-house SP sec. stand.		75
In-house WP sec. stand.		58
In-house C/O sec. stand.		39
In-house NC sec. stand.		7

Note: At present the WRL is using a slightly modified version of the SPCE (Paiba et al., 2004) with a working cut-off of 60% inhibition.

Table 3. The WP, C/O, NC and Co mean, standard deviation and MedLabQC control limits for the SPCE type A Iran 1996

	m	s	m ± 1s	m ± 2s	m ± 3s
C/O	3.214	0.338	2.876/3.552	2.537/3.891	2.199/4.229
WP	1.957	0.132	1.825/2.089	1.693/2.221	1.561/2.353
NC	4.805	0.582	4.223/5.387	3.651/5.969	3.059/6.551
Co	5.061	0.574	4.486/5.635	3.912/6.209	3.338/6.783

Table 4. The WP, C/O, NC and Co ARL, w and q limits for the SPCE type A Iran 1996

	ARL	w	lower limit	upper limit
C/O	370	0.25	2.019	4.409
WP	370	0.25	1.490	2.424
NC	370	0.25	2.749	6.861
Co	370	0.25	3.033	7.088

Table 5. Data entered into the MedLabQC and MultiQC charts for the SPCE type A Iran 1996

Date	Time	C/O	WP	NC	Co
07/01/2004	10:53:40 AM	2.97	1.94	4.35	4.51
08/01/2004	10:58:38 AM	3.25	2.04	4.81	5.05
14/01/2004	11:07:10 AM	3.55	2.11	5.08	5.25
23/01/2004	11:14:52 AM	3.55	1.96	4.67	5.24
04/02/2004	11:17:18 AM	3.02	1.93	4.66	4.64
05/02/2004	11:19:17 AM	2.65	1.81	3.7	3.94
10/02/2004	3:12:14 PM	2.85	1.83	4.13	4.27
12/02/2004	2:05:47 PM	2.76	1.75	4.38	4.76
16/02/2004	3:34:29 PM	3.12	1.93	4.59	4.87
19/02/2004	2:32:42 PM	2.84	1.88	4.46	4.68
03/03/2004	2:39:52 PM	3.62	2.19	5.49	5.62
03/03/2004	2:42:02 PM	3.49	2.15	5.2	5.55
04/03/2004	1:57:49 PM	2.89	1.76	4.05	4.71
04/03/2004	1:46:19 PM	3.75	2.17	5.6	5.97
08/03/2004	3:33:20 PM	3.78	2.11	5.5	5.76
08/03/2004	3:34:38 PM	3.29	1.96	5.25	5.39
09/03/2004	2:41:49 PM	3.24	1.87	5.36	5.52
09/03/2004	2:42:39 PM	3.46	1.95	5.84	6.05
10/03/2004	4:27:36 PM	3.09	1.9	4.55	4.84
10/03/2004	4:28:44 PM	3.11	1.9	4.43	4.59
12/03/2004	2:04:36 PM	3.39	1.9	5.67	5.89
12/03/2004	2:06:21 PM	3.5	2.14	5.65	5.93
16/03/2004	12:34:53 PM	3.14	1.91	4.72	4.79
16/03/2004	12:35:28 PM	3.67	1.96	5.33	5.5
16/03/2004	1:43:24 PM	3.19	2.04	4.29	4.61
17/03/2004	9:26:03 AM	3.8	2.43	5	5.11
17/03/2004	9:28:35 AM	3.47	2.06	5.62	5.8
19/03/2004	2:16:56 PM	3.25	2.03	4.22	4.52
22/03/2004	4:55:27 PM	2.85	1.95	3.87	3.99
25/03/2004	9:02:48 AM	3.23	1.96	4.5	4.95
29/03/2004	12:51:12 PM	2.82	1.9	3.79	3.97
01/04/2004	2:35:21 PM	3	1.88	4.02	4.36
01/04/2004	4:19:27 PM	3.22	1.9	4.3	4.6
05/04/2004	4:39:31 PM	3.52	1.88	4.34	4.84
05/04/2004	4:47:06 PM	2.94	1.88	4.19	4.24
06/04/2004	2:13:09 PM	3.26	2.07	4.34	4.52
07/04/2004	2:04:47 PM	3.32	2.04	4.52	4.54
08/04/2004	2:05:36 PM	3.52	1.95	5.06	5.22
16/04/2004	1:19:53 PM	3.37	2.03	4.88	5.09
20/04/2004	4:28:52 PM	3.41	1.96	4.78	5.08
22/04/2004	5:15:37 PM	3.28	1.99	4.4	4.56
22/04/2004	5:23:42 PM	3.45	2.06	4.76	5.01
27/04/2004	2:22:09 PM	2.61	1.8	3.93	4.16
28/04/2004	1:15:15 PM	2.97	1.86	4	4.07
28/04/2004	1:22:57 PM	3.12	1.94	4.07	4.2
03/05/2004	3:06:02 PM	3.3	2.03	4.63	4.66
06/05/2004	3:18:52 PM	2.92	1.81	4.03	4.23
06/05/2004	3:19:09 PM	3.43	2.01	4.7	4.96
10/05/2004	3:00:41 PM	3.52	2.02	5.13	5.3
18/05/2004	2:57:19 PM	3.62	2.06	5.91	6.02
25/05/2004	1:17:51 PM	3.68	2	5.72	6.11
26/05/2004	3:21:09 PM	3.38	1.83	4.36	5.31
27/05/2004	12:22:32 PM	3.37	1.99	5.15	5.16
07/06/2004	12:53:05 PM	2.96	1.93	4.24	4.36
07/06/2004	2:40:03 PM	2.92	1.84	4.68	4.9
08/06/2004	1:47:45 PM	2.88	1.8	4.04	4.42
09/06/2004	2:32:37 PM	3.1	1.99	4.32	4.44
14/06/2004	4:10:31 PM	2.96	1.88	4.28	4.58
14/06/2004	4:11:28 PM	3.03	1.91	3.96	4.66
14/06/2004	4:11:48 PM	3.29	1.94	4.69	5.06
15/06/2004	2:01:02 PM	2.68	1.79	3.8	4.17
15/06/2004	2:03:50 PM	3.13	1.9	4.1	4.3
15/06/2004	2:04:24 PM	3.02	1.89	4.23	4.63
28/06/2004	3:12:30 PM	2.53	1.68	3.7	3.79
29/06/2004	2:05:34 PM	3.12	1.85	4.62	4.9
01/07/2004	3:34:42 PM	2.76	1.75	3.99	4.01
01/07/2004	3:35:22 PM	2.71	1.79	3.79	3.86
01/07/2004	3:36:25 PM	3.22	1.91	4.41	4.76
01/07/2004	3:37:36 PM	3.14	1.9	4.34	4.85
01/07/2004	3:38:54 PM	2.84	1.85	4.16	4.4
02/07/2004	4:14:25 PM	2.75	1.78	4	4.13
02/07/2004	4:14:42 PM	2.92	1.81	4.2	4.48
02/07/2004	4:14:59 PM	2.94	1.88	4.2	4.47
02/07/2004	4:15:31 PM	2.87	1.85	4.32	4.55
05/07/2004	4:56:57 AM	3.04	1.91	4.19	4.35
05/07/2004	4:57:46 AM	2.67	1.79	4.24	4.48
05/07/2004	4:58:36 AM	3.19	1.99	4.19	4.1
05/07/2004	4:58:57 AM	2.58	1.63	4.3	4.44
06/07/2004	2:01:05 PM	3.21	1.85	4.88	5.03
08/07/2004	2:36:14 PM	3.27	2.03	5	4.96
08/07/2004	2:37:37 PM	3.56	2.07	5.27	5.28
08/07/2004	2:55:31 PM	3.03	1.79	4.26	4.47
09/07/2004	1:41:37 PM	3.92	2.23	6.1	6.39
20/08/2004	1:35:39 PM	3.44	2.05	4.79	4.95
26/08/2004	3:21:28 PM	3.14	2	4.58	4.7
31/08/2004	1:57:12 PM	3.33	2.02	4.77	4.88

Table 6. Westgard violations for the SPCE type A Iran 1996 using the MedLabQC programme

Date	Time	violations C/O	violations WP	violations C/O & WP	violations NC	violations Co	violations NC & Co	Decision
12/03/2004	2:06:21 PM						4:1s	accept plate
01/04/2004	2:35:21 PM						4:1s & 10:m	accept plate
01/04/2004	4:19:27 PM						10:m	accept plate
05/04/2004	4:39:31 PM						10:m	accept plate
05/04/2004	4:47:06 PM						10:m	accept plate
06/04/2004	2:13:09 PM						10:m	accept plate
07/04/2004	2:04:47 PM				10:m	10:m	10:m	accept plate
27/04/2004	2:22:09 PM	1:2s						accept plate
28/04/2004	1:15:15 PM						4:1s	accept plate
28/04/2004	1:22:57 PM						4:1s & 10:m	accept plate
03/05/2004	3:06:02 PM						10:m	accept plate
06/05/2004	3:18:52 PM						10:m	accept plate
06/05/2004	3:19:09 PM						10:m	accept plate
25/05/2004	1:17:51 PM						4:1s	accept plate
14/06/2004	4:10:31 PM						10:m	accept plate
14/06/2004	4:11:28 PM						10:m	accept plate
14/06/2004	4:11:48 PM						10:m	accept plate
15/06/2004	2:01:02 PM						10:m	accept plate
15/06/2004	2:03:50 PM						4:1s & 10:m	accept plate
15/06/2004	2:04:24 PM				10:m	10:m	10:m	accept plate
28/06/2004	3:12:30 PM	1:2s			4:1s & 10:m	1:2s & 10:m	10:m	reject plate
29/06/2004	2:05:34 PM			10:m	10:m	10:m	10:m	accept plate
01/07/2004	3:34:42 PM			10:m	10:m	10:m	10:m	accept plate
01/07/2004	3:35:22 PM		10:m	10:m	10:m	10:m	4:1s & 10:m	accept plate
01/07/2004	3:36:25 PM		10:m		10:m	10:m	10:m	accept plate
01/07/2004	3:37:36 PM		10:m		10:m	10:m	10:m	accept plate
01/07/2004	3:38:54 PM		10:m		10:m	10:m	10:m	accept plate
02/07/2004	4:14:25 PM		10:m		10:m	10:m	4:1s & 10:m	accept plate
02/07/2004	4:14:42 PM		10:m		10:m	10:m	10:m	accept plate
02/07/2004	4:14:59 PM		10:m	10:m	4:1s & 10:m	10:m	10:m	accept plate
02/07/2004	4:15:31 PM		10:m	10:m	10:m	10:m	10:m	accept plate
05/07/2004	4:56:57 AM		10:m	10:m	10:m	10:m	10:m	accept plate
05/07/2004	4:57:46 AM		10:m	10:m	10:m	10:m	10:m	accept plate
05/07/2004	4:58:36 AM				10:m	10:m	10:m	accept plate
05/07/2004	4:58:57 AM	1:2s & 10:m			10:m	10:m	10:m	reject plate
06/07/2004	2:01:05 PM	10:m				10:m		accept plate
08/07/2004	2:36:14 PM					10:m		accept plate
09/07/2004	1:41:37 PM	1:2s			1:2s	1:2s	2:2s	reject plate

Figure 1. The MultiQC chart for monitoring the SPCE type A Iran 1996

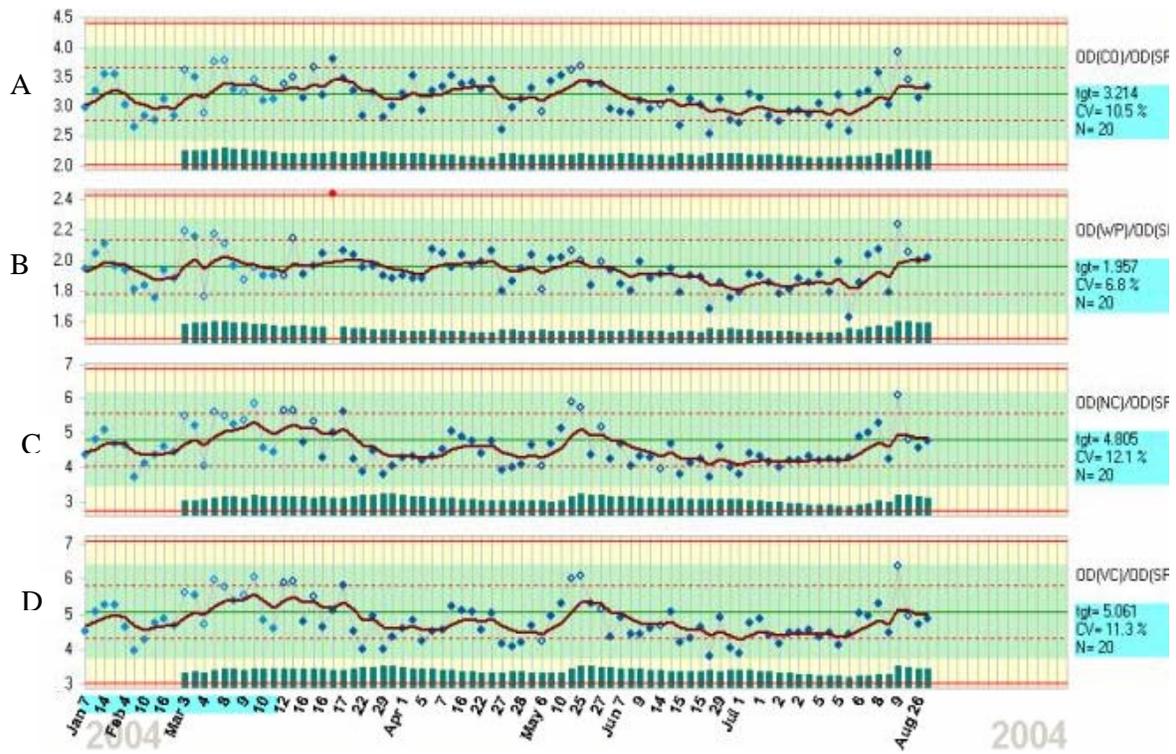
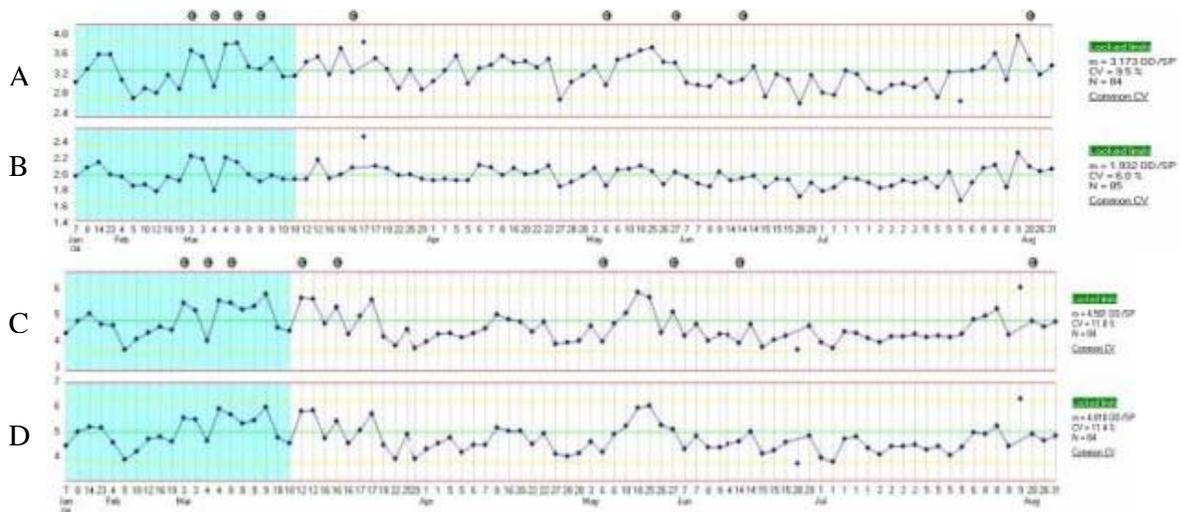


Figure 2. The MedLabQC chart for monitoring the SPCE type A Iran 1996



The A chart represent the data obtained for the C/O secondary standard. Chart B is based on the data for the WP secondary standard. Chart C displays the NC secondary standard data and chart D the Co values.

Prediction of protection by FMD vaccines on the basis of LPBE results

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Abstract

Liquid-phase-blocking ELISA (LPBE) titres of cattle vaccinated against serotypes A or Asia were correlated with vaccine potency values (PD50) determined by cattle challenge tests according to the European Pharmacopoeia. The LPBE was found to be well suited for the prediction of protection by vaccine batches and will allow to replace the challenge test for many routine batch tests.

Introduction

The assessment of potency of foot-and-mouth disease (FMD) vaccines according to the European Pharmacopoeia (EP) requires that vaccine batches are tested in groups of cattle inoculated with different doses of the vaccines so that the quality of a batch can be expressed in terms of fifty percent protective doses (PD50). This test method can be replaced by other methods provided that a correlation to the PD50 method is established. Animal welfare concerns, the limited capacity of high security stables and the risk that virus excreted by infected animals may escape to the environment prompted us to look for an alternative potency test. Previous studies with neutralization assays (Hecke et al., 1960; Stellmann et al., 1968, Pay and Parker, 1977, Pay and Hingley, 1986) had already shown a correlation between SNT titre and protection. However, neutralization tests are usually less reproducible than ELISAs. In the 1980th, R. Ahl established a method for the assessment of vaccine potency in cattle by measuring the antibody response in vaccinated cattle in a plaque reduction test based on a cell suspension plaque assay with BHK21 CT cells (Ahl et al., 1990). While the plaque reduction test solved the problem of reproducibility, it was very laborious and difficult to transfer to another lab. Several groups looked for an alternative potency test based on the liquid-phase blocking sandwich ELISA (LPBE) for the detection of antibodies against foot-and-mouth disease virus, which had been published by Hamblin et al in 1986. In 1989, Hamblin and coworkers published that LPBE antibody levels in cattle sera following vaccination were highly related to protection. They considered the ELISA to be more reliable than the VN and recommended its use for the evaluation of immunological responses following infection as well as following vaccination. Furthermore, the LPBE does not require infectious virus, but works also with inactivated virus (Ferris et al., 1990) allows to reduce disease security risks. Van Maanen and Terpstra, 1989, compared the LPBE and VNT for the assessment of vaccine potency and concluded that, because the SNT and the LPBE are highly correlated and the LBE is more reproducible, the LPBE should predict protection more reliably than the SNT. Amadori et al., (1990) stated that the plaque reduction test and LPBE gave precise estimates within certain limits of potency and recommended a combination of these tests. The LPBE was also established at the BFAV, now Friedrich-Loeffler-Institut (FLI). Preliminary results on the correlation of LPBE titres of cattle vaccinated against serotypes A or Asia with vaccine potency values (PD50) determined by cattle challenge tests will be reported below. In South-America, the LPBE, in a variation employing monoclonal antibodies, was extensively and successfully used for in-vitro testing of FMD vaccines. In 1993, Periolo et al. published a paper on the large-scale use of the LPBE for the evaluation of protective immunity against aphthovirus in cattle vaccinated with oil-adjuvanted vaccines in Argentina. It was found that, with few exceptions, animals with titres of at least 2.1 log₁₀ were protected against challenge with serotypes C85, A87, O1 Caseros and A79. Further data were published by Robiolo et al. (1995). The lowest expected protection (LEP) at a 95% confidence of 245 FMD commercial vaccines was calculated from the LPBE titres of cattle sera obtained from 3920 animals at 60 days postvaccination (d.p.v.) and challenged with live virus at 90 d.p.v. It was found that LEP evaluation was highly specific (able to predict the failure in 100% of the cases) although its sensitivity (ability to predict the approval) was only about 65%. It was possible, nevertheless, to improve the evaluation by using an alternative coefficient (Ro), exclusively dependent on the number of animals exhibiting the highest and lowest LPBE titres in a particular vaccine trial. This coefficient was capable of predicting the approval of 90% of the vaccines, yet maintaining acceptable levels of safety. Based on these results and further studies (Smitsaart et al., 1998) challenge was finally discontinued in Argentina. Unfortunately, data from Argentina can not be pooled with data from Europe, mainly because of the different challenge regimes. In Argentina, lots of 16 animals were vaccinated and challenged at 90 days d.p.v. with one of four virus strains. If 13 out of the 16 animals were protected, the vaccine batch passed. If at least 11 animals were protected, the manufacturers could apply for a retrial. Serum samples from vaccinated and control cattle were collected 60 and 90 d.p.v. and the level of FMDV specific antibodies were determined. In Europe, however, 3 groups of five cattle are vaccinated with receive different volumes of vaccine. 21 d.p.v., the animals are challenged and eight days later the test is read and the PD50 content is calculated, usually by the Kärber method. A vaccine has passed if it contains at least 3 PD50, but the customer may ask for higher values, e.g. 6 PD50 for emergency vaccines. The LPBE was also used to examine the degree of

relation between viruses and predict heterologous protection (Kitching, 1989). Lunt et al. (1994) published an improved method, measuring the LPBE titres at equal OD values for the test virus and the reference virus, which attempted to increase the accuracy of the test.

Materials and Methods

Cattle were vaccinated with aluminium hydroxide vaccines and oil vaccines, type A22 (n=7) and A24 (n=3) as well as Asia 1 Shamir (n=10) of a European manufacturer. The vaccines included commercial as well as some experimental vaccine batches. Vaccine potency was determined according to the European Pharmacopoeia. Three groups of five animals received 1 dose, ¼ dose and 1/16 dose respectively. Sera were taken 21 d.p.v. and titres were determined in the LPBE following the protocol of the World Reference Laboratory for FMD with slight modifications. OD-readings were analysed by an Excel program, using interpolation to obtain precise titres. The group mean LPBE titre for each group of animals that had received a particular (full or reduced) dose of vaccine and the PD50 values determined for this batch of vaccine were used to calculate the regression between titre and protection. For groups that had received a reduced dose, the PD50 value was divided by 4 and 16 respectively.

Results

Data on individual animals showed a large "grey zone", where protection could not be predicted. For type A this "grey zone" ended at a titre of about 2.4, for type Asia at a titre of about 2,8 log₁₀ units. Pooled data for 10 batches of A vaccines and 10 batches of Asia vaccines revealed a correlation between log₁₀ LPBE titres and protection (log₁₀ PD50) of R²=0,8365 and R²=0,8988, respectively.

Discussion

McCullough et al., 1992, found that neither the SNT nor ELISAs were able to predict the outcome of an infection of vaccinated cattle with certainty. They stated that these assays do not measure immunological protection but only antibody responses and described a "three zone" phenomenon: In the "white zone" antibody titres are high and donor animals likely to be protected; in the "black zone" antibody titres are low and donor animals are likely to be susceptible to infection and in the "grey zone" antibody titres are intermediary and no interpretation should be made with respect to protection. Looking at the results for individual animals, the findings of McCullough et al. could be confirmed, but using group mean titres resulted in correlation for types A and Asia in the range of 0.8 to 0.9. By this method, it could be confirmed that LPBE titres correlate well with protection in cattle, at least for serotypes A and Asia. Further statistical analysis, including probit analysis will be performed. It would be a great advantage to include sera from other manufactures in the study to investigate whether correlations established for one type of vaccines are also valid for vaccines from other sources. For serotype O, we are not yet satisfied with the correlation found for 10 batches tested so far are. The reasons for this are being investigated in cooperation with the manufacturer. The phenomenon certainly is not due to technical problems with the LPBE, which is highly reproducible. WRL reagents gave similar results when used to test some of the sera. Currently, we are retesting all the sera by VNT in order to compare the results. We are also trying to establish a monolayer plaque reduction test that is less laborious than the plaque test established by Ahl (1990). We intend to use these methods also for heterologous tests in the frame of the FMD_ImproCon Project financed by the European Commission. This work will also contribute to the objectives of the "Position Paper on Requirements for Vaccines against Foot-and-Mouth Disease" issued by EMEA, in particular the reduction of challenge experiments.

References:

Ahl, R., Haas, B., Lorenz, R. J. & Wittmann, G. 1990. Alternative potency test of FMD vaccines and results of comparative antibody assays in different cell systems and ELISA. Report of the Meeting of the Research Group of the Standing Technical Committee of the European Commission for the Control of FMD, Lindholm, Denmark; FAO Rome, pp 51-60.

Amadori, M., Archetti, I.L., Tollis, M., Buonavoglia, C & Panina, G.F. 1991. Potency assessment of foot and mouth disease vaccines in cattle by means of antibody assays. *Biologicals*. 19: 191

EMEA. 2004. *Position Paper on Requirements for Vaccines against Foot-and-Mouth Disease*, Committee for Veterinary Medicinal Products, EMEA/CVMP/775/02-Post-Consultation

Ferris NP, Kitching RP, Oxtoby JM, Philpot RM & Rendle R. 1990. Use of inactivated foot-and-mouth disease virus antigen in liquid-phase blocking ELISA. *J Virol Methods*. ; 29(1):33-41.

Hamblin C, Barnett IT & Hedger RS. 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. I. Development and method of ELISA. *J Immunol Methods*; 93(1):115-21.

Hamblin C, Barnett IT & Crowther JR. 1986. A new enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. II. Application. *J Immunol Methods*; 93(1):123-9.

Hamblin C, Kitching RP, Donaldson AI, Crowther JR & Barnett IT. 1987. Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus. III. Evaluation of antibodies after infection and vaccination. *Epidemiol Infect.* 1987; 99(3):733-44.

Hecke, F., Uhlmann, W. & Lorenz, R. 1960. Bericht über die Ergebnisse des Großversuchs 1957-1958 zur Wirksamkeitsprüfung der Maul-und-Klauenseuche - Konzentratvakzine nach Pyl und der Originalvakzine nach Waldmann und Kobe. IV. Beziehung zwischen Serumneutralisation und Immunität. *Mh.Tierheilkunde* 12: 1-11.

Kitching RP, Knowles NJ, Samuel AR & Donaldson AI. 1989. Development of foot-and-mouth disease virus strain characterisation-a review. *Trop Anim Health Prod*;21(3):153-66.

Lunt RA, Linchongsabongkoch W & Gleeson LJ. 1994. A modified liquid phase (LP) blocking ELISA used to assess type O foot-and-mouth disease virus antigenic variation in Thailand. *Vet Microbiol.* ;42(1):79-90.

McCullough KC, Bruckner L, Schaffner R, Fraefel W, Muller HK & Kihm U. 1992. Relationship between the anti-FMD virus antibody reaction as measured by different assays, and protection in vivo against challenge infection. *Vet Microbiol.*;30(2-3):99-112.

Periolo OH, Seki C, Grigera PR, Robiolo B, Fernandez G, Maradei E, D'Aloia R, & La Torre JL. 1993. Large-scale use of liquid-phase blocking sandwich ELISA for the evaluation of protective immunity against aphthovirus in cattle vaccinated with oil-adjuvanted vaccines in Argentina. *Vaccine*; 11(7):754-60.

Smitsaart EN, Zanelli M, Rivera I, Fondevila N, Compained D, Maradei E, Bianchi T, O'Donnell V & Schudel AA. 1998. Assessment using ELISA of the herd immunity levels induced in cattle by foot-and-mouth disease oil vaccines. *Prev Vet Med.*;33 (1-4):283-96.

Van Maanen C. & Terpstra C. 1989. Comparison of a liquid-phase blocking sandwich ELISA and a serum neutralization test to evaluate immunity in potency tests of foot-and-mouth disease vaccines. *J Immunol Methods.*, 13;124(1):111-9.

Pay, T.W.F. & Hingley, P.J. 1986. The use of serum neutralising antibody assay for the determination of the potency of foot-and-mouth disease vaccines in cattle. *Dev.Biol.Stand.* 64: 153-161.

Pay, T.W.F. & Parker, N.J. 1977. Some statistical and experimental design problems in the assessment of FMDV vaccine potency. *Develop. Biol. Stand.* 35: 369

Robiolo, B., Grigera, P.R., Periolo, O.H., Seki, C., Bianchi, T., Maradei, E. & La Torre, J.L. 1995. Assessment of foot and mouth disease vaccine potency by liquid phase blocking ELISA: a proposal for an alternative to the challenge procedure in Argentina. *Vaccine*, Vol. 13, No.14, pp1346 - 1352.

Stellmann, C., Bornarel, P., Lang, R. & Terre, J. 1968. Controle quantitatif du vaccin antiaphtheux. Analyse statistique de la relation liant les titres d'anticorps neutralisant au pourcentage de protection bovine. *Rec.Med.Vet. (Alfort)* 144: 325-351

Addition of saponin to double oil emulsion FMD vaccines enhances specific antibody responses in cattle and pigs

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Introduction:

Single oil emulsion (SOE) vaccines have been extensively used in cattle for effective control and eradication programs against foot-and-mouth disease (FMD) in South America (Sutmoller *et al.*, 2003).

Double oil emulsion (DOE) vaccines have proved to induce early protective response in different target species (Doel *et al.*, 1994), particularly in pigs, therefore systematic vaccination with DOE has been limited to this species (Sutmoller *et al.*, 2003). The safety and efficacy of SOE and DOE vaccines have been demonstrated in different studies (Barnett *et al.*, 1996, PIADC-PANAFTOSA, 1975), but little information is available on trials comparing their performance in both cattle and pigs.

We have previously shown the enhancing effects of saponin on the immune response of cattle vaccinated with single oil emulsion (SOE) vaccines (Smitsaart *et al.*, 2000). Saponins extracted from *Quillaia saponaria* are complex chemical adjuvants and have the capacity to stimulate adaptative immunity, by inducing strong Th1 and Th2 responses and cytotoxic T-lymphocyte (CTL) activity (Marciani, 2003).

In this work, we studied immune responses induced by double oil emulsion (DOE) vaccines containing saponin, in both pigs and cattle. Antibody responses following a single dose of SOE and DOE vaccines were compared in both species under controlled conditions. In addition, the safety of these formulations was assessed.

Materials and Methods:

Vaccines: Vaccines were formulated at laboratory scale with various concentrations of inactivated, polyethylene glycol-concentrated, FMDV antigen derived from the O1 Campos strain. SOE vaccines were prepared by adding 40% v/v of aqueous phase to 60% v/v of oily phase (90% Marcol 52, Exxon, France/10% Montanide 888, Seppic, France). Mixtures were left overnight, at 4°C on a magnetic stirrer (300 rpm) followed by homogenisation using an Ultraturrax T 25 (IKA, Works, Inc., USA) at 25°C for 2.5 min at 24000 rpm. DOE vaccines were prepared by adding 50% w/w of aqueous phase to 50% w/w of oily phase (Montanide ISA 206, Seppic, France) and emulsification at 25°C for 12 min at 2000 rpm with a Eurostar power control-visc emulsifier (IKA Works, Inc USA). Saponin-vaccines contained 3 mg per dose of saponin (Quest, Ireland) which had been added to the aqueous phase (Table 1). Formulated vaccines were tested for sterility, conductivity, viscosity, 140S content (after emulsion disruption), safety (in the mouse and guinea pig) and stability at different temperatures (DOE: 25°C and 4°C, SOE: 4°C, 37°C and 56°C); the drop test was also applied. In addition, SOE vaccines had to comply with the centrifuge test and glycerine test (Casas Olascoaga *et al.*, 1990, Dossier Montanide ISA 206, Seppic, France).

Animals and experimental design: Fifty cattle and 50 pigs which were seronegative to FMDV were inoculated intramuscularly with 2 ml/dose of each formulation (Table 1).

Serology: at different intervals after vaccination, specific antibody responses were determined by liquid-phase blocking sandwich ELISA (Robiolo *et al.*, 1995) and by the microserum neutralization test (Rweyemamu *et al.*, 1977) using baby hamster kidney (BHK21 clone 13) cell monolayers (Table 1).

Local reaction detection: the inoculation site was examined at each time that animals were bled. The local tissue reaction was further assessed when cattle were slaughtered in a commercial abattoir at 70 days post vaccination (dpv); at post mortem examination, inflamed tissue at the site of inoculation was dissected away from the surrounding tissue and weighed.

Statistical analysis: one way ANOVA and two tailed unpaired Student's *t*-test were used to calculate differences in ELISA antibody titers between groups of animals. For each group, the mean weight of inflamed tissue at the inoculation site was compared with the reference value (30 g) and statistical significance determined by one tailed Student's *t*-test. This reference value was obtained from a database constructed by the Pathology Department of the National Sanitary Authorities (SENASA) and based on official vaccine potency tests which were conducted on a total of 5000 cattle for 294 commercial vaccine batches approved during 1990-1996.

Results:

Saponin enhances immune responses of DOE vaccines in cattle and pigs

DOE-vaccinated groups, both cattle and pigs, reached peak antibody levels at 23-24 dpv, regardless of whether or not saponin was included in the vaccine. Similar antibody profiles were apparent by virus neutralization and ELISA tests in both cattle and pigs (Trial 1-2, Fig 1).

DOE vaccines to which saponin has been added (DOE-saponin) induced higher antibody titres in cattle at 23 dpv ($P < 0.05$) and in pigs at 50 dpv ($P < 0.05$) when compared with DOE vaccines lacking saponin (Fig 1).

In cattle, the enhancing effects of saponin were only apparent in the group which had received vaccine with a low antigen payload (5 μg of 140S per dose). In the group which received DOE vaccine without saponin, the antibody titre decreased between 23 and 60 dpv ($P < 0.05$). Moreover, a greater proportion of the cattle receiving DOE-saponin vaccines had protective antibody levels when compared with those receiving vaccine without saponin (Fig 2-B).

On the contrary, in pigs, the enhancing effects of saponin were observed with both low and high antigen payloads (5 and 20 μg of 140S/dose). As in cattle, the proportion of pigs which had high antibody titres ($\log_{10} > 2.5$) was greater in the group of animals vaccinated with DOE-saponin vaccine than in those receiving vaccine without saponin (Fig 2-A).

When comparing the variation in the immune responses of cattle and pigs, less variation in antibody titres was found in pigs than in cattle. However, greater variation in antibody titres was observed in the group of pigs which received DOE lacking saponin than in the group of pigs which received DOE-saponin vaccine (Fig 1-A and B).

SOE-saponin vaccine induced higher antibody responses in pigs than DOE-saponin vaccine

SOE-saponin vaccine induced higher levels of antibody in pigs at 10 dpv ($P < 0.05$) and at 60 dpv ($P < 0.01$) than similar DOE-saponin vaccine (Fig 3-B). In cattle, differences in antibody titres between the groups receiving these formulations were not statistically significant at any bleeding time (Fig 3-A).

As observed in trial 1-2, DOE-saponin-vaccinated groups (both pigs and cattle) reached peak antibody titers at 20-30 dpv whereas SOE-saponin-vaccinated groups did not reach peak values until 60 dpv. In this regard, a significant increase in antibody titres was detected in SOE-saponin-vaccinated group at 60 dpv when compared to 30 dpv ($P < 0.05$) (Fig 3-B).

DOE-saponin and SOE-saponin formulations proved to be safe in both pigs and cattle

None of the vaccines produced systemic or visible local reactions. The local tissue reaction at the site of inoculation in vaccinated cattle was quantified and in each group the mean weight of inflamed tissue at this site was less than 20 g, not significantly different from the reference value (Table 2). No correlation was found between the extent of the local tissue reaction and antibody titers after vaccination (data not shown). These vaccine formulations produced a local reaction characterized as either a granuloma (localized nodule) or diffuse granulomatous inflammation. No specific pattern of local reaction (localized or diffuse) was associated with any particular vaccine formulation, with the presence or absence of saponin or with different types of emulsion. However, it should be noted that only one group of cattle vaccinated with a formulation lacking saponin was included in post-mortem studies, reducing the statistical power of this analysis. In this study, vaccinated cattle displayed a diffuse local reaction more often than a localized nodular reaction.

Discussion:

Saponins have been widely used as veterinary adjuvants and are generally included in aluminium hydroxide-adjuvanted vaccines. Saponins induce strong Th1 and Th2 responses and moderate CTL responses to some proteins, probably as a result of forming mixed protein-saponin micelles (Cox and Coulter, 1997). Partially-purified saponins are simple to formulate and generally safe (Cox and Coulter, 1997).

In South America, single-oil emulsion vaccines have been developed and applied for systematic vaccination of cattle population since 1990 (Sutmoller *et al.*, 2003). Water-in-oil, single oil emulsion vaccines based on mineral oil (Marcol 52) and Montanide 888 as the emulsifier resulted in fluid emulsions which were easy to prepare, safe and of adequate stability (Casas Olascoaga, 1990). Double oil emulsion vaccines based on Montanide ISA 206 have low viscosity and have performed well in pigs.

For effective vaccination, it is desirable that the immune response conferred by vaccines is strong and homogeneous, inducing high levels of protective antibodies and a long duration of immunity in most vaccinated individuals. In this study (trial 1) the ability of saponin-adjuvanted vaccines to achieve uniformly high antibody titres in pigs and a longer duration of immunity than vaccines which lack saponin, has been demonstrated. We found that animals receiving DOE-saponin vaccine with a low antigenic payload reached maximum antibody levels at 30 dpv and that this antibody level was

maintained at least until 60 dpv in both species. Conversely, when the equivalent vaccine without saponin was administered to cattle, antibody levels decreased after 30 dpv, as previously reported for DOE –Montanide ISA 206 vaccines (Barnett *et al.*, 1996). In another report, Hunter (1996) showed that cattle vaccinated with either DOE or SOE emulsions reached peak antibody levels at 30 dpv and maintained high antibody titres for 6 months, although he did not specify which type of oil was used in the vaccine formulations.

The adjuvant capacity of saponin in DOE and SOE formulations was evaluated in cattle and pigs after single vaccination with O1 Campos antigen. The results in pigs showed that SOE-saponin vaccine elicited significantly higher antibody levels than DOE-saponin vaccine. Similarly, Barnett *et al.* (1996) have reported single oil-in-water emulsion vaccine to be more effective than DOE at stimulating a response against A24 Cruzeiro virus.

In our study, antibody levels to O1 Campos virus as determined by ELISA were lower in cattle than in pigs, whereas neutralizing antibodies were similar in both species or higher in cattle than in pigs. Although these results do not agree with an earlier report (Barnett *et al.* 1996), the difference might be explained by differences in the virus strain, antigen payload and serological assay which were used in both studies.

Additionally, we observed greater variation in the antibody response among individual cattle than in individual pigs. This may simply be a species difference or may be due to a combination of factors: vaccine formulation, FMDV strain and animal species. This finding would suggest that more than five individual cattle would be required in each experimental group to that statistical tests have adequate power to explore the significance of differences between groups.

One possible problem associated with the use of saponin is its possible adverse effect on vaccinates. Our results demonstrated the safety of vaccines containing saponin in cattle and pigs and a negligible local tissue reaction at the site of vaccine inoculation. This was confirmed following post mortem studies in cattle, in which the extent of tissue inflammation at this site was quantified. From previous studies recorded at SENASA, it was established that where the inflamed tissue associated with local reaction to vaccine exceeded 150 grams weight, this had a detrimental effect on muscle function, diminished the quality of the carcass and was more likely to lead to rejection of the beef at the slaughterhouse. In our study, none of the formulations assayed developed localised nodules which exceeded 37 g weight (data not shown).

In Argentina, SOE- saponin- adjuvanted vaccine has been used for many years. In the last four years more than 400 million doses of vaccine have been administered to cattle and no adverse effects have been reported associated with the use of saponin.

Conclusions:

- Addition of saponin to double oil emulsion vaccines based on Montanide ISA 206 significantly enhanced the immune response in vaccinated pigs and cattle.
- Saponin-SOE vaccine induced higher antibody responses in pigs than saponin-DOE vaccine
- No adverse side effects were observed after administration of single or double oil emulsion vaccines containing saponin.
- Oil-saponin-vaccines can be used for rapid and effective immunization of susceptible animal population against the disease

Recommendations:

- Research should continue to investigate the protective capacity of these vaccine formulations.
- Further studies are encouraged to characterize specific immune response associated with the adjuvant effect of saponin components.

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References:

Barnett, P., Pullen, L., Williams, L. & Doel, T.R. 1996. International bank for FMD vaccine: assessment of Montanide ISA 25 and ISA 206, two commercially available oil adjuvants. *Vaccine* 14(13): 1187-1198.

Casas Olascoaga, R., Augé de Mello, P., Abaracón, D., Gomes, I., Alonso Fernández, A., Mesquita, J.A., Darsie, G.C., Pinkoski, D.I., Deak, J.G., Gubel, J.G., Barbosa, J.R. 1990. Production and control of oil-adjuvanted foot-and-mouth disease vaccines. *Bol. Centr. Panam. Fiebre Aftosa* 56: 3-51.

Cox, J.C. & Coulter, A.R. 1997. Adjuvants-a classification and review of their modes of action. *Vaccine* 15 (3): 248-256.

- Doel, T.R., Williams, L. and Barnett, P.** 1994. Emergency vaccination against FMD: rate of development of immunity for the carrier state. *Vaccine* 12(7): 592-600.
- Marciani, D.J.** 2003. Vaccine adjuvants: role and mechanisms of action in vaccine immunogenicity. *Drug Discovery Today*. 8(20): 934-943.
- Hunter, P.** 1996. The performance of Southern African Territories serotypes of foot-and-mouth disease antigen in oil adjuvanted vaccines. *Rev. sci. tech. Off. int. Epiz.* 15 (3): 913-922
- PIADC-PANAFTOSA. Plum Island Animal Disease Center, Pan American Foot-and-Mouth Disease Center,** 1975. Foot-and-mouth disease vaccines. I. Comparison of vaccines prepared from virus inactivated with formalin and adsorbed an aluminium hydroxide gel with saponin and virus inactivated with acethylethylenimine and emulsified with incomplete Freund's adjuvant. *Bol. Centr. Panam. Fiebre Aftosa*, 19-20, 9-16
- Robiolo, B., Grigera, P., Periolo, O., Seki, C., Bianchi, T., Maradei, E. & La Torre, J.** 1995. Assessment of foot-and-mouth disease vaccine potency by liquid-phase blocking ELISA: a proposal for an alternative to the challenge procedure in Argentina. *Vaccine*. 14: 1346-1352.
- Rweyemamu, M.M., Pay, T.W.F. & Parker, M.J.** 1977. Serological differentiation of foot-and-mouth disease virus strains in relation to selection of suitable vaccine viruses. *Dev. Biol. Stand.* 35: 205-214.
- Smitsaart, E., Mattion, N.; Filippi, J.; Robiolo, B.; Periolo, O.; La Torre, J. & Bellinzoni, R.** 2000. *Enhancement of the immune response induced by the inclusion of saponin in oil adjuvanted vaccines against foot-and-mouth disease.* Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of FMD. Borovets, Bulgaria. App 32. 255-262.
- Sutmoller, P., Barteling, S.S., Casas Olascoaga, R., Sumption, K.J.** 2003. Control and eradication of foot-and-mouth disease. *Virus Res* 91: 101-144.

Table 1: Vaccine composition and experimental design

Trial (species)	Vaccine	Number animals/group	Saponin	140S content (µg/dose)	Adjuvant	Bleedings (dpv*)	Post mortem studies
1 (Pig)	DOE/20/sap	5	Yes	20	ISA 206	0,24,50	ND
	DOE/5/sap	5	Yes	5			
	DOE/20	5	No	20			
	DOE/5	5	No	5	Marcol 52-Montanide 888		
	SOE/20/sap**	5	Yes	20			
	SOE/5/sap	5	Yes	5			
2 (Cattle)	DOE/20/sap	5	Yes	20	ISA 206	0,8,23,60	70 dpv
	DOE/5/sap	5	Yes	5			ND
	DOE/20	5	No	20			70 dpv
	DOE/5	5	No	5	Marcol 52-Montanide 888		ND
	SOE/20/sap	5	Yes	20			70 dpv
	SOE/5/sap	5	Yes	5			ND
3 (Pig)	DOE/10/sap	10	Yes	10	ISA 206	0,10,20,60	ND
	SOE/10/sap	10	Yes	10	Marcol 52-Montanide 888		
4 (Cattle)	DOE/10/sap	10	Yes	10	ISA 206	0,15,30,60	70 dpv
	SOE/10/sap	10	Yes	10	Marcol 52-Montanide 888		

* dpv: days post vaccination. ND: not done. **82% of protection in cattle by protection to podal generalization test (PGP) at 30 dpv.

Table 2: Local reaction and post-mortem results of cattle vaccinated with DOE and SOE formulations containing or lacking saponin.

Vaccine*	Trial	Deep palpation 15 dpv	Post mortem results-70 dpv			
		Local reaction > 8 cm/total	Media weight (g)	SD	Type of reaction**	
					Localized	Diffuse
DOE/20/sap	2	0/5	12.86	3.76	80%	20%
DOE/5/sap	2	0/5	ND	ND	ND	ND
DOE/20	2	0/5	12.88	5.06	60%	40%
DOE/5	2	0/5	ND	ND	ND	ND
SOE/20/sap** *	2	0/5	10.22	2.92	40%	60%
SOE/5/sap	2	0/5	ND	ND	ND	ND
DOE/10/sap	4	0/10	13.14	3.9	30%	70%
SOE/10/sap	4	0/10	15.66	8.49	20%	80%

* see composition in Table 1 (DOE/SOE denotes type of emulsion, number indicates µg of 140S/dose, sap denotes addition of saponin). dpv: days post-vaccination. ND: not done. SD: standard deviation.

** Percentage of cattle with localized or diffuse local reaction. ***82% of protection in cattle by protection to podal generalization test (PGP) at 30 dpv.

Figure 1: Mean group antibody responses of pigs of trial 1 (Panels A and B) and cattle of trial 2 (Panels C and D) after vaccination with DOE formulations of O1 Campos strain. Panels A and C: ELISA antibody titres. Panels B and D: neutralizing antibody titres. References x axis: see table 1. * : significant difference compared to its equivalent in antigen content without saponin ($P < 0.05$). Δ : significant difference compared to 62 dpv ($P < 0.05$). T-bars represent the standard deviation.

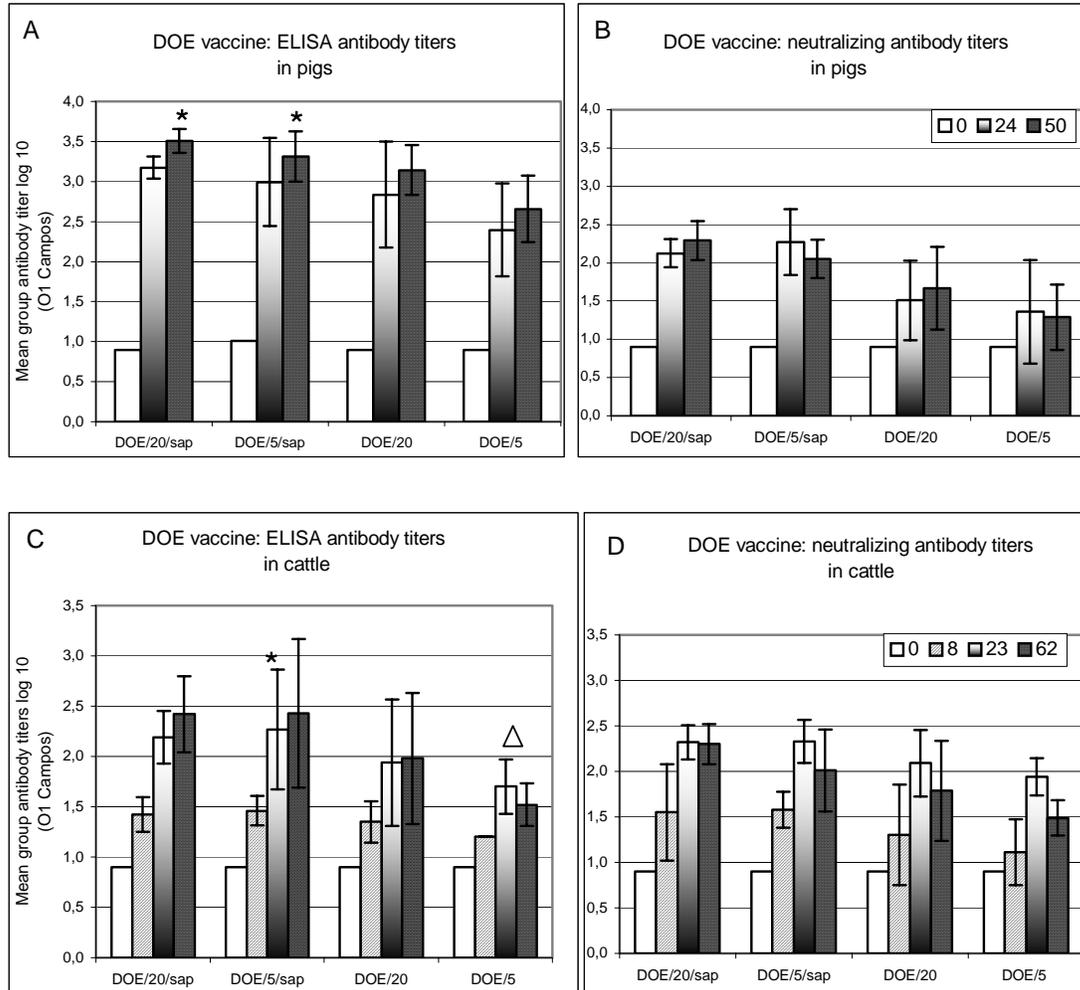


Figure 2: Frequency of pigs of trial 1 with ELISA titres above $\log_{10} 2.5$ (Panel A), and: frequency of cattle of trial 2 with ELISA levels compatible with protection ($>\log_{10} 1.85$) (Panel B) after vaccination with DOE or SOE vaccines containing or lacking saponin.

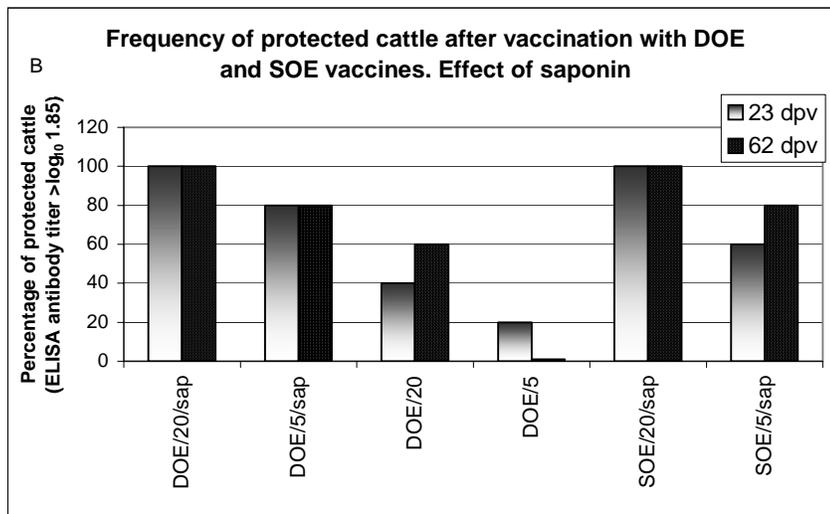
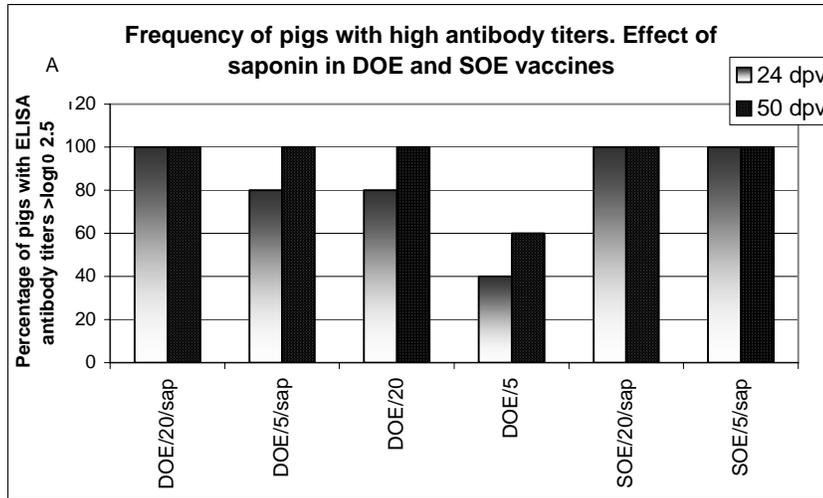
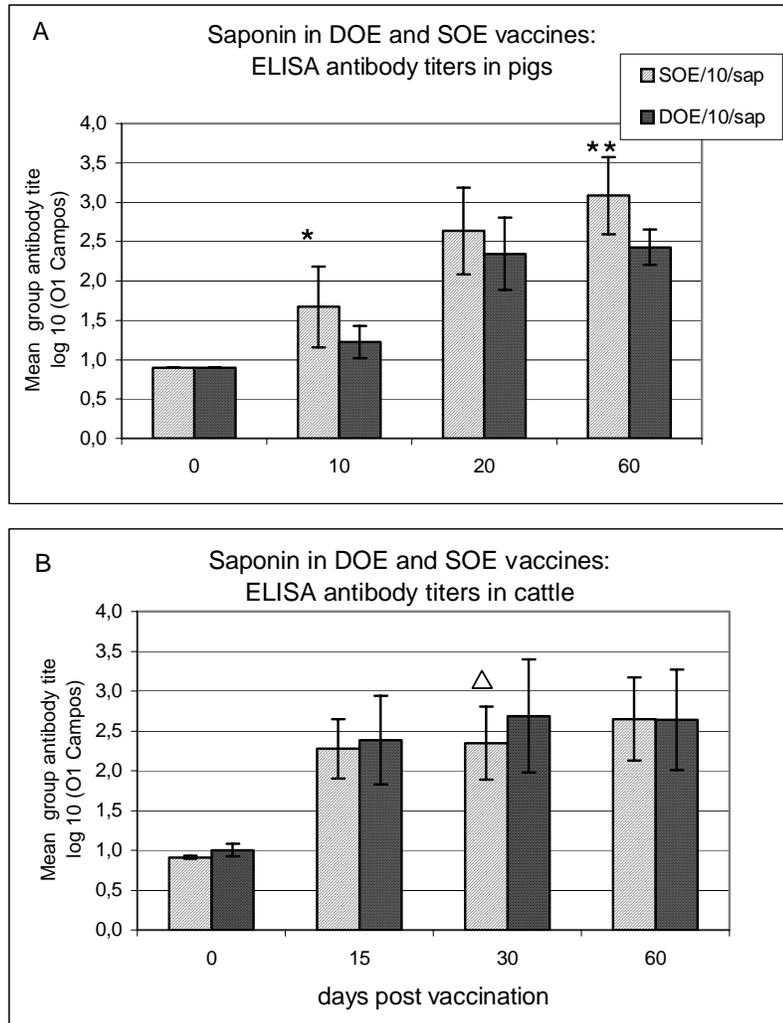


Figure 3: Mean group ELISA antibody responses of pigs of trial 3 (Panel A) and cattle of trial 4 (Panel B) vaccinated with DOE and SOE vaccines containing saponin. * significant difference vs DOE vaccine ($P < 0.05$). ** very significant difference vs DOE vaccine ($P < 0.05$). Δ : significant difference to 60 dpv ($P < 0.05$). T-bars represent the standard deviation.



Harmonising regulatory requirements for FMD vaccines within the European Union

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The recently adopted Commission Directive governing control of Foot-and-Mouth Disease (FMD) within the EU (Directive 2003/85/EC) places emphasis on vaccination as a method of control that should be considered in the first, rather than the last instance. There is therefore a greater likelihood that FMD vaccines will be used to control future incursions of the disease into the EU. In parallel, and as shown during the 2001 outbreak of FMD in the UK, the competent authorities responsible for consumer protection are paying ever increasing attention to the safety of products of animal origin. This increases pressure to ensure that vaccines used to control outbreaks of FMD are authorised to the same standards as vaccines used to control any other animal disease. This article outlines the authorisation procedures that are available for FMD vaccines within the EU, explains the measures currently in progress to make authorisation a more realistic option and describes the opportunity that currently exists to amend existing legislation to make authorisation more likely to happen in practice.

A marketing authorisation (MA) must be obtained before any veterinary medicinal product (VMP) may be placed on the market within the EU. The technical requirements that products must meet are specified in the annexes to Directive 2001/82/EC, as amended by Directive 2004/28/EC, and are described in more detail in general and specific guidelines and in the European Pharmacopoeia (Ph. Eur.). These requirements are the same whatever the route by which an MA is obtained. A national MA may be obtained by submission to the national competent authority, and subsequent approval, of a dossier demonstrating compliance with the requirements of the Directive. This MA may then be recognised by one or more other Member States of the EU through the Mutual Recognition procedure, allowing the product to be placed on the market in those member states. Recently, Council Regulation 726/2004 has been introduced which allows vaccines for diseases subject to Community control measures to be authorised through the Centralised Procedure. In this procedure, a dossier is submitted to the European Medicines Evaluation Agency and is evaluated by the Committee for Veterinary Medicinal Products (CVMP). If deemed compliant with the requirements, an authorisation is then issued by the European Commission which is valid in all Member States of the European Union.

The only exception to the requirement for an MA is an emergency provision that applies in the event of a serious disease epidemic. In such cases, Member States may permit the release of an unauthorised medicine, provided that the Commission is informed of the detailed conditions of use and provided that no authorised product is available for the disease concerned. Up until recently, this provision would have been used for the release of FMD vaccines from the strategic antigen reserves held by the EU in the EU FMD Antigen Bank. In most cases where FMD vaccines have been either formulated or actually used on a national basis, this has relied on national authorisations that had not been subject to the mutual recognition procedure. There is currently only one FMD vaccine that has been assessed as fully compliant with the requirements of Directive 2001/82, as amended, and whose authorisation has been mutually recognised.

It is important to recognise that obtaining a marketing authorisation is only the first step to release onto the market of an authorised product. Having obtained an MA, all batches of product released onto the market must pass a batch release procedure either by the manufacturer alone or through an official batch control procedure. Official batch control of immunological VMPs is not compulsory and is not harmonised throughout the EU. However, most member states apply some form of official batch control to FMD vaccines as vaccination against FMD is required under EU legislation to be under official control. The objective of official batch control is to demonstrate that the batch to be released is of the same composition and quality as the batches on which the authorisation dossier was based.

Several factors make authorisation, and subsequent release, of FMD vaccines an unattractive option for manufacturers. First, the existence of provisions allowing the release of unauthorised vaccines in the event of an emergency acts as a disincentive to manufacturers to go to the expense and inconvenience of obtaining an MA and to authorities in requiring them to have one. However, whilst an outbreak of FMD is certainly an emergency, the preparation and storage of antigens in advance of need is not. There is no reason why such antigens, and vaccines formulated from them, should not be subject to normal

regulatory requirements. Furthermore, as mentioned above, competent authorities for consumer protection are now likely to prevent products from animals which have been vaccinated with unauthorised products from entering the human food chain. Second, FMD vaccines are a 'special case' in regulatory terms. MAs for vaccines usually cover the release of a vaccine that contains a set number and amount of antigens in a defined formulation of excipients. In the case of FMD, an authorisation must permit the release of vaccines containing any of up to 10 or 20 different antigens (strains) alone or in combination. It is not feasible to predict, let alone test, all of these antigenic combinations, presenting particular problems in regulatory terms. In addition, in the event of incursion of a new strain of FMD into the EU, it may be necessary rapidly to adapt a field strain to become a new vaccine strain and to incorporate this into a new vaccine. Under current legislation, a new vaccine would require a new authorisation with all the consequent bureaucracy, expense and delay that this involves. Finally, once authorised, official batch control remains problematic. The definitive test for potency of FMD vaccines is currently the Ph. Eur. challenge test in cattle. The Ph. Eur. monograph on FMD vaccines for ruminants contains a specific allowance that permits release in emergency of batches formulated in exactly the same manner, and containing the same antigens, as a trial batch which has previously been shown to pass the challenge test. In practice however, manufacturers rarely wish to release exactly the same antigens in exactly the same formulation. The costs of performing challenge tests on every batch of antigen produced are prohibitive and the animal welfare implications are unacceptable. Serological alternatives to challenge therefore need to be adopted for batch release. However, the logistical and ethical difficulties of establishing statistically valid correlations between antigen load, serological titre and protection for every antigen, and every possible combination of antigens, to the standards usually required for regulatory purposes makes this an impossible solution to achieve.

In order to address the technical and scientific challenges to authorisation of FMD vaccines, the CVMP established an *ad hoc* group to prepare a Position Paper on requirements for FMD vaccines (EMA/CVMP/775/02). The group comprised representatives of the CVMP Immunologicals Working Party, the European Department for the Quality of Medicines (EDQM), the Office International des Epizooties (OIE), the Research Group of the EUFMD Commission, the EMA and the European Commission. FMD vaccine manufacturers were invited as experts and observers. At the same time, though as separate exercises, the Research Group of the EUFMD Commission was consulting with the Ph. Eur. on changes to the FMD monograph and the OIE was producing the 5th Edition of the OIE Manual. The involvement of the EDQM and the OIE in the *ad hoc* group ensured consistency of the CVMP requirements with those of the European Pharmacopoeia and the OIE Manual respectively.

Through the involvement of all parties, the position paper puts forward practical means whereby manufacturers can demonstrate that their products comply with the requirements of the annex to Directive 2001/82, as amended. The paper covers issues such as quality requirements for demonstrating freedom from contamination with extraneous agents; removal of non-structural proteins from concentrated antigen stock so that the vaccine will be suitable for detection of infected animals in a vaccinated population as part of a 'vaccinate to live' policy; how new strains might be added to an authorisation in emergency situations; how potency may be demonstrated by means other than challenge in animals, and a range of other issues of particular relevance to FMD vaccines.

The position paper describes technical solutions through which FMD vaccines can be shown to meet EU standards of quality, safety and efficacy. However, Directive 2001/82, as amended, and the associated variation regulation 1084/2003, does not currently make specific allowance for the multiplicity and interchangeability of antigens that are necessary for an FMD vaccine authorisation, nor for the rapid addition of new antigens in the event of an emergency. In the field of human disease, special provisions exist to allow human influenza vaccine strains to be updated on a regular basis in response to recommendations from WHO Reference Laboratories. A similar, but even more flexible, legislative basis is required for FMD vaccines. The annexes to Directive 2001/82, as amended, are currently being reviewed following the recent review and amendment of EU pharmaceutical legislation. This represents an ideal opportunity to create a sound legal base for the authorisation of FMD vaccines in the EU from which the technical guidance in the position paper can be operated.

In conclusion, the European Commission is urged to amend the annexes to Directive 2001/82, as amended, and Commission Regulation 1084/2003 to make specific provision for the exceptional requirements of FMD vaccines. In this way the authorisation of FMD vaccines will be promoted within the EU which will be of benefit to both animal health and consumer protection.

*The Position Paper may be downloaded in full from the website:
<http://www.emea.eu.int/pdfs/vet/press/pp/077502en.pdf>*

Conclusions

- that authorisation of FMD vaccines is desirable in the interests of animal health and consumer protection
- that sufficient general guidance on the requirements for authorisation already exists in the European Pharmacopoeia, the OIE Manual and in EU legislation and guidelines
- that the recently adopted Position Paper EMEA/CVMP/775/02 on 'Requirements for Vaccines against Foot-and-Mouth Disease' provides additional, specific guidance on the requirements for authorisation of FMD vaccines within the EU
- that this position paper may serve as a useful model for regulatory agencies in other regions

Recommendations

- Member Countries of the EU FMD Commission should use authorised FMD vaccines wherever possible
- Manufacturers should obtain marketing authorisations for their FMD vaccines in any country or region where they might be used
- The European Commission should amend the annexes to Directive 2001/82, as amended, and Commission Regulation 1084/2003 to make specific provision for the exceptional requirements of FMD vaccines

Foot-and-Mouth Disease Vaccine Strain Selection and Development

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The antigenic diversity of foot and mouth disease FMD viruses is well known and frequently prompts questions on the selection and potential efficacy of inactivated vaccines. A common benchmark to assess the probable efficacy of a given vaccine strain in relation to a field isolate is the range of definitions published by the World Reference Laboratory for FMD (Ferris and Donaldson, 1992) and based on the r_1 value derived from the neutralisation of the field virus by sera raised against the vaccine strain. The definitions are as follows:

$r_1 = 0$ to 0.19 . These values represent a highly significant serological variation from the reference vaccine strain. Where possible, it would be advisable to use a vaccine strain with a closer relationship to the field virus. However, in an emergency, a potent vaccine of the type used as a reference in the test may provide adequate protection, especially if administered on more than one occasion.

$r_1 = 0.2$ to 0.39 . These values represent an area of concern. They show significant differences from the reference strain, but protection may be satisfactory if a sufficiently potent vaccine is employed.

$r_1 = 0.4$ to 1.00 . These values are not significantly different from the reference vaccine strain as measured by the particular test system used.

Thus a low r_1 value gives cause for concern and will often stimulate the development of a new vaccine strain. Against this background, a critical question is the extent to which significant antigenic variant viruses emerge and the cover or otherwise which could be expected from current vaccine strains.

There are two reasons to develop a new vaccine strain. Firstly, there is the recognition by experts, including the vaccine producers and the reference laboratories, that a significantly different virus has appeared in a region and may/will warrant the development of a new vaccine strain. This recognition is invariably based on a serological assay such as the virus neutralisation test but may be supported by sequence analysis of the VP1 protein. One such example was the emergence of a new A strain in Iran in 1996. Meril responded by developing a new vaccine strain, referred to as A Iran 96, which is now widely used in the Middle East as well as being the choice of some antigen banks. The second reason is concerned with those countries where regular vaccination programmes are employed and the epidemiological situations are relatively stable. Such situations support the obvious concept of preparing a vaccine strain from a local field isolate so that the field viruses and vaccine strains are matched as closely as possible. In this paper, the focus will be on the emerging strains and the threat they represent to world animal health.

Contrary to some 'opinions' on the fringes of foot and mouth disease vaccine research and development, antigenic variation does not represent an insurmountable problem in terms of the ability of the manufacturers to keep pace with the commonly observed level of variation in the field. Additionally, evasion of vaccine-induced protective immunity by selection of escape mutants does not appear to be a common phenomenon. There is both a large amount of anecdotal as well as experimental evidence to support these statements including the highly successful vaccination programmes employed in southern South America and Western Europe, prior to these regions deciding to stop vaccination to facilitate, *inter alia*, exports of animals and animal products. Nevertheless, these general statements require expansion with particular respect to the properties of the different serotypes of the virus.

The O serotype is the most widespread and certainly shows moderate levels of strain variation in the field with, occasionally, more extreme variants. Nevertheless, there are two main lineages of vaccine strains represented by the old (in excess of 30 years since their development) O strains of Europe and South America (OBFS 1860, O Lausanne and O Campos) and the equally old strains of the Middle East and Asia (represented by strains such as O Manisa and O-3039). In the case of the latter group of viruses, there are other valuable strains of O serotype in use. However, established virus strains such as O Manisa provide good cover for many first occurrence situations. Indeed, we found an exceptionally good r_1 match between the UK 2001 outbreak strains and our Manisa vaccine strain within two days of the first report of the disease.

The A serotype is quite different, exhibiting a broad range of antigenic variants including the A24 strains typified by the Cruzeiro 1955 isolate which is widely used in South America, the A22 viruses, typically A22 Iraq 24/64 which is quite widely used in the Near and Far East, and the more recent A

variants emerging in the Middle East (A Iran 94, 96, 99 and 2001) and Asia (A Malaysia 97). With the A serotype, it is certainly advisable to use vaccine strains appropriate for the local field isolates. However, a more careful examination of the A serotype distribution worldwide does appear to indicate some stability of the situation in South East Asia and South America whereas the Middle East appears at first sight to represent a 'hot-spot' where, until recently, significant variants have emerged reasonably frequently. It is tempting to speculate that this apparent frequency may be due to increased sampling within the region as a result of the very positive actions of veterinary authorities, notably those of Iran. With the A serotype, it very much remains a position of continued vigilance.

The antigenic diversity of both the C and Asia1 serotypes is considerably less. In the case of the C serotype, it was common practice to use only one of several C strains during the period that this serotype was more prevalent and, nowadays, reports of the virus are very infrequent. In the case of the recent isolation of a C virus in Northern Brasil, there seems no basis to assume that the conventional C South America vaccine strains will not adequately cover the situation. While more prevalent, Asia1 field strains are very well covered by a single strain such as Asia1, Shamir. This is consistent with the report of Samuel and Knowles (2001) which pointed to the constraints that appear to be operating with the Asia1 serotype where all viruses described since 1954, the date that the serotype was first described, are members of a single toptotype.

In the case of the SAT viruses, the sequence data alone points to significant variation within each of the three serotypes (Vosloo, 1992) which would give very great cause for concern if any field strains substantially escaped from Africa and established in the Middle East or further afield. In recent years, only SAT2, the most prevalent of the three serotypes, has threatened to do this with excursions into Saudi Arabia and North Africa. To date, it seems that an appropriate vaccine, SAT2 Eritrea, along with other control measures, have kept these field viruses in check. Nevertheless, there is a critical need to continue to monitor the SAT serotypes and determine more precisely the efficacy of existing SAT vaccine strains in relation to their ability to protect against significantly different field isolates.

In this article, it has been stated that antigenic variation does not represent an insurmountable problem in relation to protection by vaccination. An important prerequisite in this respect is the need to monitor comprehensively the antigenic characteristics and worldwide distribution of field viruses so that vaccines can be selected or developed within an acceptable time frame. Despite this approach, new antigenic variants occasionally emerge with little warning and there is no option but to use an existing vaccine strain while a more appropriate vaccine strain is developed. While this is a relatively infrequent event, evidence exists which demonstrates that protection can be achieved using a high potency vaccine where the level of homology between the vaccine and field/challenge viruses is low. One of the most recent anecdotal examples was the situation during the 1996 outbreak in the Balkans when A22 Iraq vaccine was used, apparently successfully, against a field isolate which was substantially different from the vaccine. Experimental reports have primarily focussed on the O and A serotypes using one or two way challenge studies in cattle. Barteling *et al* (1997) demonstrated that O Manisa vaccine made from European Bank antigens gave 6.7 PD₅₀ against challenge with a significantly different O virus, O Greece 1994. It must be added that the O Manisa vaccine gave approximately 20 PD₅₀ when tested by homologous challenge. Protection between distantly related O strains has also been demonstrated with O Lausanne vaccine followed by O Lausanne or O Manisa challenge (96% for homologous protection against 64% for the heterologous protection. Lombard *et al*, 1979). Given the recognised antigenic variability of the A serotype, it is perhaps more surprising to observe cross-protection with A virus vaccines. Using A22 Iraq vaccine and A Saudi 23/86 challenge virus, Schermbrucker demonstrated greater than 85% protection with two consecutive doses of vaccine, 85% representing the level of protection equivalent to 3 PD₅₀ (cited by Doel, 2003). Heterologous protection has also been demonstrated with other A serotype viruses. In the case of A Iran 96 vaccinated cattle challenged with A Iran 99, Bruckner and Griot (2002) showed good levels of protection after a double dose, single dose or 1/3 dose of vaccine. Similar results were reported by Favre *et al* (1981) who demonstrated 99.5% homologous and 40% heterologous protection using A5 Allier vaccination and A5 Allier or A24 Argentina challenge. A similar result was seen when A24 vaccinated cattle were challenged with A24 Argentina (94% protection) or A5 Allier (79% protection). Clearly, it would be valuable to extend these early studies to some of the more recent field isolates and vaccine strains with particular reference to the SAT viruses and the use of high potency vaccines typically found with antigen banks.

Finally, it is important to recognise that vaccine strain development, when it is required, may not be a simple matter. Table 1 lists some of the issues to be considered in developing a new vaccine strain. One particular issue is the availability of field isolates and it is, regrettably, a frequent problem that only a few viable samples are obtained from a new outbreak of the disease. In this sense, perhaps the greatest vulnerability in terms of ability to respond rapidly and effectively to an emerging disease situation in a region (and, therefore, its potential spread beyond the borders of the region) is our

ignorance of the viruses circulating in the field. The frequent and timely submission of field samples to the reference laboratories and the corresponding availability to the vaccine producers is, without doubt, one of the most important weapons in the control of the disease by vaccination.

References

Barteling, S.J., Swam, H., Anemaet, D.A.J., Tuyn, C. & Vreeswijk, J. 1997. Foot-and-mouth vaccines from the European Vaccine Banks seem to be sufficiently stable for emergency vaccination. *In* Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease. Poiana-Brasov, Romania, 23-27 September. Food and Agriculture Organisation (FAO), Rome, pp. 68-70.

Bruckner, L & Griot, C. 2002. FMD Vaccines. Potency testing in the target species. *In* Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease. Izmir, Turkey, 17-20 September. Food and Agriculture Organisation (FAO), Rome, p. 327.

Doel, T.R. 2003. FMD Vaccines. *Virus Research*, 91: 81-99.

Favre, H., Lombard, M., Juve, J. & Lopez, G. 1981. Etudes serologique et immunologique des souches vaccinales A5 Allier France 1960 et A24 Argentine 1968. *In* Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease. Tubingen, Germany, 29 September to 1 October. Food and Agriculture Organisation (FAO), Rome, pp. 58-61.

Ferris, N.P. & Donaldson, A.I. 1992. The World Reference Laboratory for Foot and Mouth Disease: a review of thirty-three years of activity (1958-1991). *Rev. sci. tech. Off. int. Epiz.* 11: 657-684.

Lombard, M., Brun, A. & Duret, C. 1979. Serological and immunological studies of the Near-East foot-and-mouth disease virus strains of O1 Manisa 1969 and O1 OLTU 1977. *In* Report of the Twenty-Third Session of the European Commission for the Control of Foot-and-Mouth Disease. Rome, 27-30 March. Food and Agriculture Organisation (FAO), Rome, pp. 89-98.

Samuel, A.R. & Knowles, N.J. 2001. Foot and mouth disease type O viruses exhibit genetically and geographically distinct evolutionary lineages (topotypes). *J. Gen. Virology*, 82: 609-621.

Vosloo, W., Knowles, N.J. & Thomson, G.R. 1992. Genetic relationships between southern African SAT-2 isolates of foot-and-mouth-disease virus. *Epidemiol Infect*, 109: 547-558.

Table 1. Some of the Issues and Problems with Development of New FMD Vaccine Strains

1. Lengthy time scale, logistics and high cost with particular respect to full regulatory testing and licensing.
2. Failure to adapt to suspension culture. Some isolates only grow in monolayers.
3. Failure to grow to commercially viable yields.
4. Screening hampered by insufficient quality, quantity, numbers of field isolates.
5. Rare properties such as 146S sensitivity to inactivant (eg. Sat 2 Kenya 227/66) and propensity to aggregate which compromise process recoveries.
6. Slow growth at scale-up thus reducing the annual production capacity.
7. Disqualification of an isolate because of a high risk of rejection by regulatory authorities. (e.g. use of a bovine isolate from a country recognised as 'high risk' in terms of BSE).

Novel immunological approaches for emergency FMD vaccines inducing mucosal immunity

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Introduction

Vaccination has the potential to reduce large-scaling culling to control future FMD outbreaks in Europe. Due to the rapidity of FMDV replication and spread, the development of vaccine formulations inducing early protection is critical for an emergency scenario. Our strategy is to stimulate innate antiviral immune defence by targeting natural interferon producing cells (NIPC). One possibility are CpG oligonucleotides (ODN), shown to induce non-specific protection in murine models. This requires the identification and characterization of CpG motifs targeting NIPC representing the main source of interferon- α (IFN- α) production in the immune system. Induction of mucosal immunity is another critical element to improve current vaccines. Neutralization of FMDV at mucosal surfaces by antibodies could prevent virus entry and establishment of the carrier status. Consequently, a second goal of our investigations is to induce mucosal immunity following parenteral vaccination. To this end, the immunological barrier separating the systemic from mucosal immunological compartments has to be overcome.

Materials and Methods

Porcine plasmacytoid DC were enriched from peripheral blood and stimulated with various CpG ODN. INF- α in culture supernatants was detected by ELISA. Pigs were vaccinated with FMDV vaccine adjuvanted with CpG ODN, and the antiviral response monitored using INF- α and Mx protein detection. Modulation of chemokine receptor expression and DC migration was analysed by flow cytometry and chemotaxis assays.

Results and discussion

CpG type A capable of inducing strong INF- α responses by stimulating plasmacytoid DC were identified and characterized. Their in vivo effect on the innate immune system is under current investigation to determine optimum doses and formulations, and to test in challenge experiments. Current concepts forming a basis for mucosal immunity induction will be presented, based on modulation of DC and lymphocyte migration. In vitro culture systems enabling a systematic and controlled approach to the question are currently and will be discussed.

Cytokine and Toll-like receptor mRNAs in the nasal-associated lymphoid tissues in cattle during foot-and-mouth disease virus infection

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Abstract

Introduction: A number of studies have shown the importance of tissues in the pharyngeal area in foot-and-mouth disease virus (FMDV) infection during acute disease and persistence. **Materials and Methods:** To explore characteristics of the local mucosal immune response in bovine nasal-associated lymphoid tissues (NALT) to FMDV infection during acute disease and persistence, a quantitative real-time RT-PCR assay was used to measure mRNA expression of bovine cytokines IFN- α , β and γ , IL-2, IL-1 α , TNF- α , and Toll-like receptors (TLR)-3 and -4. The NALT from dorsal soft palate were collected from cattle at 7 dpi and from carriers and non-carriers at 64 dpi. Experimental infection was with FMDV type O UKG34/2001 isolate. **Results:** In comparison with uninfected animals, expression of IFN- α mRNA was significantly increased in NATLs during acute disease. An increased expression of IFN- γ and IL-1 α mRNA was also observed but was much lower than IFN- α . There was a slight increase in mRNA expression of TNF- α and IL-2. During persistence, the level of IFN- α mRNA expression in carrier cattle was lower than non-carrier cattle. In contrast, the level of TNF- α mRNA expression in carrier cattle was much higher than in non-carrier cattle. For TLRs, an increased expression of TLR-4 but not TLR-3 in NALT during the acute stage of infection was observed when compared with uninfected animals. No differences in the expression of TLR-3 and -4 mRNA were detected in NATLs between carrier and non-carrier cattle. **Conclusion:** IFN- α mRNA was significantly up-regulated in bovine NALT in response to FMDV infection during the acute stage of disease. TNF- α mRNA was significantly up-regulated in bovine NALT during persistence.

Introduction

Foot and mouth disease virus (FMDV) is a member of the *Picornaviridae* which causes a highly contagious disease of cloven-hoofed animals and is one of the most economically important diseases of livestock worldwide (Alexandersen *et al.*, 2003b). Importantly, persistent infection (so-called carrier stage) can occur following clinical or sub-clinical disease in either naive or vaccinated ruminants (reviewed in Alexandersen *et al.*, 2002). A carrier is defined as an animal from which live virus can be recovered for longer than 28 days after exposure (Salt, 1993, Alexandersen *et al.*, 2002). Persistent infection appears to occur only in a proportion of virus-exposed ruminants in which virus becomes undetectable eventually (Alexandersen *et al.*, 2002). In contrast, pigs infected with FMDV do not become persistently infected. The significance of persistently infected animals (carriers) lies in their potential to act as a source of new outbreaks (Bengis *et al.*, 1986, Dawe *et al.*, 1994) and make control efforts even more costly. For example, during the 2001 UK outbreak, a key issue was whether or not to vaccinate. There were concerns that some animals would become subclinical carriers in which disease would be masked by vaccination and that this would adversely affect trade prospects.

The mechanism(s) by which FMDV persistence is established and maintained remains unclear. The acquisition of FMDV infection occurs most often by the inhalation of infectious aerosol and uptake of virus at oral/respiratory mucosal epithelial surfaces. A number of studies have shown the importance of the pharyngeal tissues in FMDV infection during acute disease and persistence (reviewed in Alexandersen *et al.*, 2002, 2003b, Zhang and Alexandersen, 2004). The epithelial cells of the bovine pharyngeal region (soft palate and pharynx) have been identified as the site for FMDV persistence in ruminants (Zhang and Kitching, 2001). Recently, we have shown that the presence of viral RNA in tissues of the bovine dorsal soft palate correlated with the presence of infectious virus in oesophago-pharyngeal (OP) fluid samples (Zhang and Alexandersen, 2004) and also that the half-life of viral clearance in probang fluid samples is prolonged early on in animals that subsequently becomes carriers (Zhang *et al.*, 2004). However the factors that control FMDV infectivity in the pharyngeal mucosa have not been described, although evidence suggests that the cell-mediated immunity may be involved in the clearance of persistent virus (Ilott *et al.*, 1997, Childerstone *et al.*, 1999). Improving our understanding of this will provide fundamental knowledge to help develop an effective

strategy for FMD control. This paper investigates characteristics of the local mucosal immune response in bovine nasal-associated lymphoid tissues (NALT) in the pharyngeal region to FMDV infection during acute and persistent stages in order to determine associations of FMDV-induced changes with viral persistence/clearance.

Materials and Methods

Ten standard Compton steers (Holsteins) 6-10 months of age were used. This included two uninfected animals used as controls. Animals for the infection studies were placed as pairs of two or four together in individual rooms in a biosecure animal building. Half of the animals in each room were selected at random and inoculated by subepidermo-lingual injection of approximately 0.5 ml of FMDV O UKG 34/2001 as described elsewhere (Alexandersen *et al.*, 2003a, Zhang *et al.*, 2004) while the other half of the animals were kept as direct contacts throughout the experiment. After inoculation, animals were monitored for clinical signs of disease and the rectal temperatures were recorded daily until 10 days after inoculation. Samples of OP-fluid and serum were collected before the start of the experiment (negative controls) and at intervals after infection. OP-fluid samples were collected using a probang cup (Sutmoller and Gaggero, 1965) and diluted in an equal volume of Eagle's-HEPES medium (pH 7.2) containing 5% foetal calf serum (FCS) and stored at -80°C until required. Each OP-fluid sample was assayed on primary bovine thyroid (BTY) cells to determine the presence of infectious virus as described (Snowdon, 1966). RNA was extracted from OP fluid samples and serum with MagNA Pure LC NA Extraction Kit II (Roche, UK) as described previously (Zhang and Alexandersen, 2004). RNA was eluted in 50 μl of elution buffer and stored at -80°C until used. The level of viral RNA in samples was quantified by a quantitative RT-PCR assay as described previously (Zhang and Alexandersen, 2004).

NALT were collected at post-mortem from dorsal soft palate of cattle randomly selected and killed at the indicated time after infection and immediately put into RNAlater (Ambion, UK), then stored at -20°C until required. mRNA was extracted from tissues samples with MagNA Pure LC mRNA Extraction Kit II (Tissues) (Roche, UK) as described previously (Zhang and Alexandersen, 2004). mRNA was eluted in 50 μl of elution buffer and stored at -80°C until used.

mRNA expression for the cytokine IFN- α , β and γ , IL-1 α , and TNF- α were quantified using a GeneAmp 5700 Sequence Detection System (Applied Biosystems, UK). RNA was reverse transcribed to cDNA with the TaqMan reverse transcriptase kit (Applied Biosystems, UK), followed by PCR amplification with the TaqMan Master Mix kit (Applied Biosystems, UK) in the presence of specific forward and reverse primers and fluorescently labelled probes specific for each gene of interest, using 1 μL of cDNA in a 25 μL final volume for 50 cycles. Similar analyses were also performed on mRNA of the housekeeping gene GAPDH on each sample. For each gene of interest, we confirmed that efficiency of amplification was comparable to that of GAPDH (data not shown), permitting calculations of the amounts of gene induction as described below.

mRNA RNA expression for TLR 3 and 4 and IL-2 were quantified using the Master SYBR Green kit (Eurogenics, UK), using 1 μL of cDNA in a 25 μL final volume. Briefly, PCR was carried out in a GeneAmp 5700 Sequence Detection System (Applied Biosystems) for 40 cycles. Amplification specificity was checked using melting curve following the manufacturer's instructions. Similar analyses were also performed on mRNA of the housekeeping gene GAPDH on each sample. The efficiency of amplification was comparable to that of GAPDH (data not shown), and extents of gene induction were calculated as described below.

Quantitative RT-PCR data were analysed with the comparative C_T method ($\Delta\Delta C_T$). Briefly, first the difference (ΔC_T) between the C_T values of the target and the normaliser: $\Delta C_T = C_T$ (target) - C_T (normaliser) are calculated for each sample to be quantified. One of these samples is chosen as the reference for each comparison to be made. The comparative $\Delta\Delta C_T$ calculation involves finding the difference between the sample ΔC_T and the reference ΔC_T . The last step in quantification is to transform these values to absolute values. The formula for this is: Comparative Expression Level = $2^{-\Delta\Delta C_T}$. Data of cytokine expression were statistically evaluated for significance using the t-test (using MINITAB release 12.21 software). A P value < 0.05 was considered statistically significant.

Results

All animals inoculated with FMDV type O UKG 34/2001 developed signs of clinical disease within 1-2 days post inoculation (dpi) and in contact cattle around 3-5 days. Viral RNA and infectious virus in OP-fluid samples was positive by a quantitative RT-PCR assay and virus isolation assay on BTY cells from around 1 dpi (data not shown). Cattle were confirmed as carriers of FMDV by recovery of

infectious FMDV from OP-fluid samples collected at 28 dpi (Table 1). OP-fluid samples collected from two carriers at the time when the animals were killed was positive by virus isolation assay on BTY cells and the viral RNA loads was $5.2 \pm 0.7 \log_{10}$ copies/ml determined by a quantitative RT-PCR assay.

Table 1. Clinical profiles and recovery of FMDV in the OP-fluid samples of experimentally infected animals

Animal ID	dpi ^a	Infection route ^b	Status ^c	IV ^d
UD34	64	Inoculated	Noncarrier	P
UD35	7	Inoculated	Acute	P
UD36	7	Contact	Acute	N
UD37	64	Contact	Noncarrier	N
UD40	64	Inoculated	Carrier	P
UD41	64	Inoculated	Carrier	P
UD42	64	Contact	Noncarrier	N
UD43	64	Contact	Noncarrier	N

^adays post inoculation. For direct contact animals, it is defined as days after contact.

^binfection were by subepidermo-lingual injection or by contact.

^ca carrier is defined as an animal from which live virus can be recovered for at least 28 days after exposure

^dOP-fluid samples collected at the day the animals were killed were assayed on BTY cells to determine the presence of infectious virus (IV).

^PIV-positive; ^N: IV-negative. ND: no samples taken at the time the animal was killed.

The presence of viral RNA in tissues of the bovine dorsal soft palate has been demonstrated (Zhang and Alexandersen, 2004). But the factors that control FMDV infectivity in the pharyngeal mucosa are unclear. Therefore, profile of the mucosal cytokines IFN- α , β and γ , IL-2, IL-1 α , TNF- α and TLR-3 and -4 at mRNA levels in NALT tissues collected from dorsal soft palate of FMDV-infected cattle were analysed by a quantitative RT-PCR assay. As shown in Figure 1, at 7 dpi (n=2) expression of IFN- α mRNA was significantly increased (100000 times increase) in NALT. The increased expression of IFN- γ (6 ± 6 times increase), IFN- β (6 ± 2 times increase) and IL-1 α mRNA (4 ± 1 times increase) was also observed but at a much lower extent than IFN- α . There was a slight increase in mRNA expression of TNF- α and IL-2 but at a lower extent than IL-1 α . During persistence, the level of TNF- α mRNA expression in carrier cattle (1100 ± 65 times increase) was significantly higher than in non-carrier cattle (1 ± 0.7 times increase; $P = 0.027$). The level of IFN- β mRNA expression (26 ± 3 times increase) in carrier cattle was also higher than non-carrier cattle (18 ± 3 . times increase) but there were no significant differences ($P = 0.26$). In contrast, the level of IL-1 α mRNA expression (4 ± 3 times increase) in carrier cattle was lower than non-carrier cattle (6 ± 1 times increase). The level of IFN- α mRNA expression in carrier cattle was also lower than non-carrier cattle but there was no significant differences ($P = 0.92$).

The major innate recognition system for microbial invaders in vertebrates is now thought to be the Toll-like receptor (TLR) family (Beutler *et al.*, 2003). Therefore, TLR-3 and -4 response in NALT during infection was also studied. As shown in Figure 3, an increased expression of TLR-4 (5 ± 3 times increase) but not TLR-3 in NALT during the acute stage of infection was observed when compared with uninfected animals. During persistence no increased production of TLR-3 was observed in NALT from both carrier and non-carrier cattle. For TLR-4 mRNA an increased expression of TLR-4 from both carriers and non-carriers was observed when compared with uninfected animals but there was no significant difference between them ($P = 0.7$).

Discussion

The experiments described here present a quantitative analysis of the levels of cytokine and TLR mRNA in NALT tissues of cattle experimentally infected with FMDV O UKG 34/2001. Data from a single experiment provide evidence that a difference exists between carrier and non-carriers. In exploring the mucosal cytokine profile at mRNA levels in FMDV-infected cattle, we found the most obvious differences in the expressions of TNF- α , exhibiting a significant increase in carrier cattle. This may be due to the presence of viral RNA in pharyngeal region of carriers (Zhang and Alexandersen, 2004), which may represent a continued stimulation of the cells producing the cytokine. It has been

shown that TNF are involved in the polyclonal expansion of B cells in human retrovirus infection (Higuchi *et al.*, 1997), as well as in chronic lymphocytic leukaemia (CLL) (Waage and Espevik, 1994). Therefore significant increase in expression of TNF in carriers may explain in part why high level of IgA was detected in OP fluid and saliva samples in carriers (Archetti *et al.*, 1995). It is obviously important to work out how this cytokine influences FMD virus replication in the pharyngeal region and to find out where the TNF- α is coming from and whether it has any direct role in local protection.

A significant elevation of IFN- α mRNA was seen in NALT. An increased expression of IFN- γ was also observed but at a much lower extent than IFN- α . It has been hypothesised that the initiation of FMDV persistence is correlated with the amount of interferon produced in the cells. FMDV strains modified by passage in alternate hosts or repeated passage in cell cultures have reduced virulence in cattle and, in contrast to more virulent wild-virus, will induce the production of IFN (Sellers, 1963) with a correlation between lack of virulence in cattle and increased IFN production (Sellers and Mowat, 1968) The ability of FMDV to form plaques in cell culture is apparently correlated with suppression of IFN- α and β (Chinsangaram *et al.*, 1999). Recent evidence has shown IFN- γ , to be a potent inhibitor of FMD virus in persistently

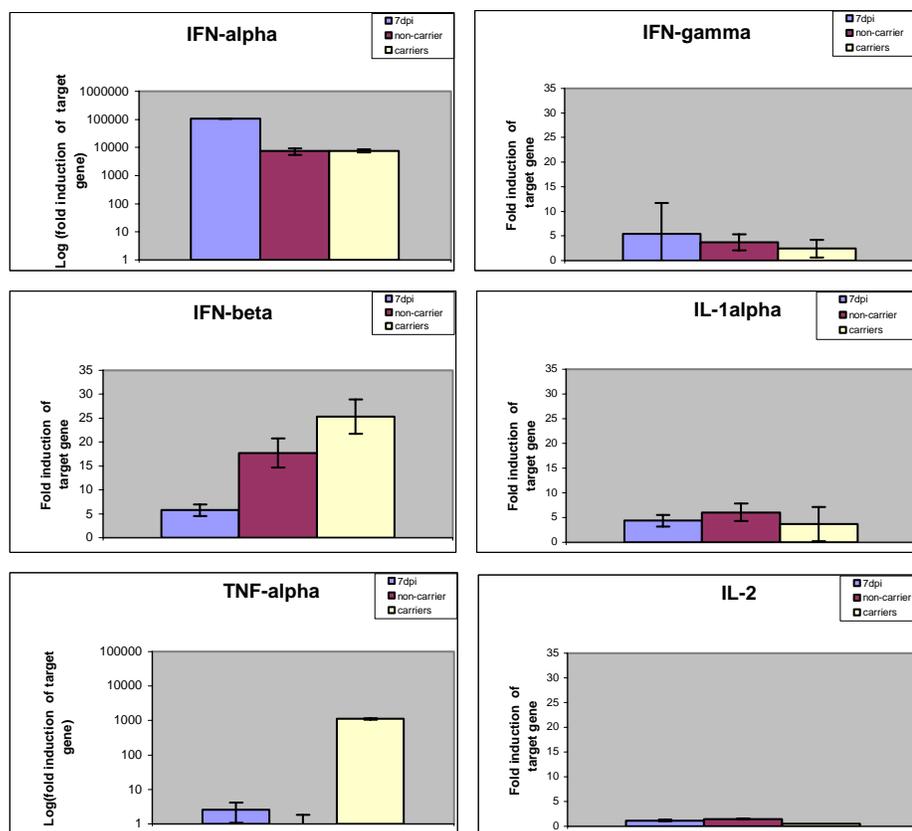


Figure 1. Cytokine response in NALT during infection. NALT were collected at 7 and 64 dpi from infected animals with FMDV UKG34/2001 and then cytokine mRNAs were studied by a real-time RT-PCR. Data represent mean \pm SD from at least two animals at each time point. Bars represent standard error of the mean.

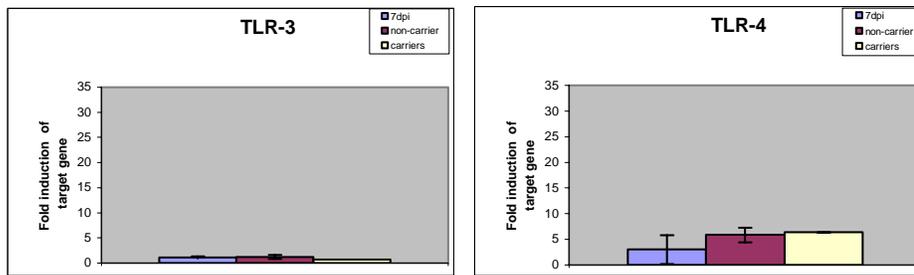


Figure 2. TLR mRNA expression in NATL during infection. NATL were collected at 7 and 64 dpi from infected animals with FMDV UKG34/2001 and then TLR mRNAs were studied by a real-time RT-PCR. Data represent mean \pm SD from at least two animals at each time point. Bars represent standard error of the mean

infected bovine epithelial cells *in vitro* (Zhang *et al.*, 2002). Other studies have shown that IFN- α and β inhibit FMDV replication (Chinsangaram *et al.*, 2001). These evidences suggest that one mechanism controlling infection *in vivo* might be mediated through the inhibitory effect induced by IFNs. IFN- γ is produced primarily by natural killer cells and sub-populations of T-cells during the innate and adaptive phases of a response against viral infection. Agents that promote T-cell activation should induce IFN- γ synthesis, and it is known that double-stranded RNA, gram-positive bacterial components and endotoxins stimulate IFN- γ , often following the release of IL-12. Virus persists in pharyngeal tissues in carrier cattle (Zhang and Alexandersen, 2004). Double-stranded RNA would be a likely by-product of FMD replication, which could theoretically act as a stimulus for IFN- γ . The significance of this finding needs further study and it will be important to find out where the IFN is coming from and whether it has any direct role in local protection. Further studies are also required to conclusively investigate the shift of cytokine expression pattern during acute to persistent stages and in animals that clear the infection compared to carrier animals.

Recent evidences suggest that TLRs and their signalling pathways involve type I IFN induction in response to virus-specific molecular patterns (Doyle *et al.*, 2003). In this study, an increased expression of TLR-4 was observed in NALT during FMDV infection, which may indicate a role in inducing expression of type I IFN mRNA in response to FMDV infection. In contrast, TLR-3 mRNA expression was not affected by FMDV infection, which may indicate that TLR3 may be not required for the generation of effective antiviral responses to FMDV infection, although it has been assumed that TLR-3 is likely to play an important role in host defence against virus infections (Doyle *et al.*, 2003, Harte *et al.*, 2003). The concept is also supported by a recent study showing that the absence of TLR3 does not alter either viral pathogenesis or impair host's generation of adaptive antiviral responses to these viruses (Edelmann *et al.*, 2004). However, it does not rule out that the physiological amount of viral dsRNA naturally made during viral infection were sufficient to stimulate through TLR-3 a biological meaningful response to affect the outcome of infection. Further studies are needed to find out if TLRs has important role in local protection.

Conclusions

IFN- α mRNA was significantly up-regulated in bovine NALT in response to FMDV infection during the acute stage of disease. TNF- α mRNA was significantly up-regulated in bovine NALT during persistence.

Recommendations

Mucosal cellular immune responses may have an important role in controlling FMDV persistence. More work is required at mRNA and proteins levels so that virus and host effects during infection can be elucidated at mucosal surface.

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Reference

- Alexandersen, S., Quan, M., Murphy, C., Knight, J. & Zhang, Z.** 2003a. Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *J Comp Pathol*, 129(4): 268-82.
- Alexandersen, S., Zhang, Z. & Donaldson, A.I.** 2002. Aspects of the persistence of foot-and-mouth disease virus in animals--the carrier problem. *Microbes Infect*, 4(10): 1099-110.
- Alexandersen, S., Zhang, Z., Donaldson, A.I. & Garland, A.J.** 2003b. The pathogenesis and diagnosis of foot-and-mouth disease. *J Comp Pathol*, 129(1): 1-36.
- Archetti, I.L., Amadori, M., Donn, A., Salt, J. & Lodetti, E.** 1995. Detection of foot-and-mouth disease virus-infected cattle by assessment of antibody response in oropharyngeal fluids. *J Clin Microbiol*, 33(1): 79-84.
- Bengis, R.G., Thomson, G.R., Hedger, R.S., De Vos, V. & Pini, A.** 1986. Foot-and-mouth disease and the African buffalo (*Syncerus caffer*). 1. Carriers as a source of infection for cattle. *Onderstepoort J Vet Res*, 53(2): 69-73.
- Beutler, B., Hoebe, K., Du, X. & Ulevitch, R.J.** 2003. How we detect microbes and respond to them: the Toll-like receptors and their transducers. *J Leukoc Biol*, 74(4): 479-85.
- Childerstone, A.J., Cedillo-Baron, L., Foster-Cuevas, M. & Parkhouse, R.M.** 1999. Demonstration of bovine CD8+ T-cell responses to foot-and-mouth disease virus. *J Gen Virol*, 80 (Pt 3): 663-9.
- Chinsangaram, J., Koster, M. & Grubman, M.J.** 2001. Inhibition of L-deleted foot-and-mouth disease virus replication by alpha/beta interferon involves double-stranded RNA-dependent protein kinase. *J Virol*, 75(12): 5498-503.
- Chinsangaram, J., Piccone, M.E. & Grubman, M.J.** 1999. Ability of foot-and-mouth disease virus to form plaques in cell culture is associated with suppression of alpha/beta interferon. *J Virol*, 73(12): 9891-8.
- Dawe, P.S., Flanagan, F.O., Madekurozwa, R.L., Sorensen, K.J., Anderson, E.C., Foggin, C.M., Ferris, N.P. & Knowles, N.J.** 1994. Natural transmission of foot-and-mouth disease virus from African buffalo (*Syncerus caffer*) to cattle in a wildlife area of Zimbabwe. *Vet Rec*, 134(10): 230-2.
- Doyle, S.E., O'Connell, R., Vaidya, S.A., Chow, E.K., Yee, K. & Cheng, G.** 2003. Toll-like receptor 3 mediates a more potent antiviral response than Toll-like receptor 4. *J Immunol*, 170(7): 3565-71.
- Edelmann, K.H., Richardson-Burns, S., Alexopoulou, L., Tyler, K.L., Flavell, R.A. & Oldstone, M.B.** 2004. Does Toll-like receptor 3 play a biological role in virus infections? *Virology*, 322(2): 231-8.
- Harte, M.T., Haga, I.R., Maloney, G., Gray, P., Reading, P.C., Bartlett, N.W., Smith, G.L., Bowie, A. & O'Neill, L.A.** 2003. The poxvirus protein A52R targets Toll-like receptor signaling complexes to suppress host defense. *J Exp Med*, 197(3): 343-51.
- Higuchi, M., Nagasawa, K., Horiuchi, T., Oike, M., Ito, Y., Yasukawa, M. & Niho, Y.** 1997. Membrane tumor necrosis factor-alpha (TNF-alpha) expressed on HTLV-I-infected T cells mediates a costimulatory signal for B cell activation--characterization of membrane TNF-alpha. *Clin Immunol Immunopathol*, 82(2): 133-40.
- Ilott, M.C., Salt, J.S., Gaskell, R.M. & Kitching, R.P.** 1997. Dexamethasone inhibits virus production and the secretory IgA response in oesophageal-pharyngeal fluid in cattle persistently infected with foot-and-mouth disease virus. *Epidemiol Infect*, 118(2): 181-7.
- Salt, J.S.** 1993. The carrier state in foot and mouth disease--an immunological review. *Br Vet J*, 149(3): 207-23.
- Sellers, R.F.** 1963. Multiplication, interferon production and sensitivity of virulent and attenuated strains of the virus of foot-and-mouth disease. *Nature*, 198: 1228-9.
- Sellers, R.F. & Mowat, G.N.** 1968. Interference between modified and virulent strains of foot-and-mouth disease virus. *Arch Gesamte Virusforsch*, 23(1): 20-6.
- Snowdon, W.A.** 1966. Growth of foot-and mouth disease virus in monolayer cultures of calf thyroid cells. *Nature*, 210(40): 1079-80.
- Sutmoller, P. & Gaggero, A.** 1965. Foot-and mouth diseases carriers. *Vet Rec*, 77(33): 968-9.
- Waage, A. & Espevik, T.** 1994. TNF receptors in chronic lymphocytic leukemia. *Leuk Lymphoma*, 13(1-2): 41-6.
- Zhang, Z. & Alexandersen, S.** 2004. Quantitative analysis of foot-and-mouth disease virus RNA loads in bovine tissues: implications for the site of viral persistence. *J Gen Virol*, 85(Pt 9): 2567-75.
- Zhang, Z., Murphy, C., Quan, M., Knight, J. & Alexandersen, S.** 2004. Extent of reduction of foot-and-mouth disease virus RNA load in oesophageal-pharyngeal fluid after peak levels may be a critical determinant of virus persistence in infected cattle. *J Gen Virol*, 85(Pt 2): 415-21.
- Zhang, Z., Hutching, G., Kitching, P. & Alexandersen, S.** 2002. The effects of gamma interferon on replication of foot-and-mouth disease virus in persistently infected bovine cells. *Arch Virol*, 147(11): 2157-67.
- Zhang, Z. & Kitching, R.P.** 2001. The localization of persistent foot and mouth disease virus in the epithelial cells of the soft palate and pharynx. *J Comp Pathol*, 124(2-3): 89-94.

Full Protection in Pigs against FMDV Challenge following Single Dose of Synthetic Emergency FMD Vaccine

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Abstract:

Introduction: The UBITH[®]-VP1 synthetic peptide-based vaccine for FMDV O, formulated as a water-in-oil emulsion, was evaluated in an emergency application single dose efficacy trial by the National Institute of Animal Health in Taiwan. Two groups having six 8-12 week old pigs each were given single injections of either 2.0 ml or 1.0 ml of the UBITH[®]-VP1 vaccine, by intramuscular injection. The animals were then challenged 28 days later according to the pig vaccine potency protocol described in the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2004), by injecting 10^{5.0} TCID₅₀ of FMDV O/TAW/97 into the heel bulb. Vaccine performance from an earlier 2 dose protocol was also characterized. **Results:** Following virus inoculation, none of the vaccinated pigs showed clinical signs over the 14-day observation period. In contrast, the 2 unvaccinated control animals developed signs of generalized FMD. No adverse effects were recorded among the pigs following vaccination throughout the pre-challenge period. Protection was not correlated to neutralizing antibodies. The 2 dose protocol indicated cellular immunity mediated by T lymphocytes secreting IFN-gamma as a correlate of immunity. **Discussion:** A safe and convenient-to-manufacture high potency emergency FMD vaccine demonstrating full protection against FMDV_{O1Taiwan} in pigs has been developed. Given that the target antigen component for the vaccine was the G-H neutralizing epitope of VP1, the pigs were immunized by a marker vaccine. Vaccinates are therefore readily distinguished serologically from virus-infected counterparts.

Introduction

Chemically inactivated virus vaccines are available for immunization against foot-and-mouth disease (FMD) in affected countries. Although this type of conventional vaccine is generally accepted to be effective, its preparation involves large-scale propagation of virulent virus in BHK cells. This is risky and certainly inconvenient to manufacture in the context that the viruses must be contained and complete inactivation of the virions must be ensured. Indeed, vaccine preparations containing viruses that have not been inactivated in the process have been reported to be associated with FMD outbreaks (Beck and Strohmaier 1987; King et al 1981). Given these considerations, UBI and UBI-Asia have, for the past six years, focused on the development of synthetic marker vaccines for FMD, to provide advantages of ease of manufacture, safety and serological distinction from infection by nonstructural protein ELISA tests. The project has involved the creation of a conformational G-H loop peptide from the viral VPI protein, which is known to contain a major virus neutralizing antibody-inducing epitope (Pfaff et al 1982;

Acharya et al 1989; Bittle et al 1982; Brown et al 1999). An immunogenic vaccine peptide was then designed by linking the VP1 fragment having a consensus sequence G-H loop from serotype O to a promiscuous UBITH[®] T-helper epitope (Fig. 1). Swine immunized twice with a vaccine having this synthetic peptide as immunogen were protected from challenge by FMDV O₁ Taiwan virus (Wang et al 2002; 2004). In the present communication we report on the efficacy of our peptide-based vaccine administered once in an emergency vaccine immunization/challenge protocol. Swine given one injection of the vaccine preparation, UBITH[®]FMD (04-3S-1) with the UBITH[®]-VP1 construct (Fig. 1), were fully protected from challenge by FMDV O₁ Taiwan. Preliminary results suggest that efficacy of this emergency vaccine preparation is attributed to cellular immunity mediated by interferon gamma (IFN- γ)-secreting T lymphocytes.

Materials and Methods

Peptide synthesis

Peptide for vaccine formulation was produced by synthesis on a solid-phase support using an Applied Biosystems Peptide Synthesizer Model 430A, and Fmoc protection for the α -NH₂ terminus and side chain protecting groups of trifunctional amino acids. The UBITH[®]-VP1 peptide having the combinatorial library T helper epitope (UBITH[®]1_{comb}, Fig. 1) was prepared by providing a mixture of the desired amino acids at the specified positions. Completed peptides were cleaved from the solid support and side chain protecting groups removed by 90% trifluoroacetic acid. The combinatorial immunogen for the vaccine was characterized by size exclusion chromatography to a specification that requires 90% of the integrated area to exceed a mass threshold limit value, and by Edman degradation for N-terminal amino acid analysis. Liquid phase cyclization of the G-H loop serotype O consensus peptide was accomplished by pH adjustment and monitored by colorimetric assay using Ellman's reagent until disulphide bond formation was at least 90% complete.

Vaccines, vaccination and virus challenge

Vaccine was prepared by dissolving the synthetic FMDV VP1 immunogen shown in Fig. 1 in water at 50 μ g/ml and formulating with Seppic Montanide ISA 50v into water-in-oil emulsions (1:1 by volume). 12 week-old LYD (Landrace x Yorkshire x Duroc) pigs that were weaned at 3 week of age were maintained in the animal facility at the Animal Technology Institute Taiwan, Chunan, Miaoli, Taiwan. All animals were checked to ensure they were FMDV-free and free of prior vaccination by testing their sera for FMDV-specific neutralizing antibodies. Each pig was injected intramuscularly at the back of the ear once, or, in an earlier study, twice at 4 weeks apart with either 1.0 ml or 2.0 ml of the UBITH[®] FMD (04-3S-1) vaccine preparation, or with 2.0 ml of the Merial FMDV O Taiwan inactivated virus vaccine. The swine were observed for localized reactogenicity and for other adverse reactions to the vaccines. Animals that were given the one dose of UBITH[®] FMD (04-3S-1) and two unvaccinated control animals were bled for serological assays on day 0 and 4 weeks later, prior to being challenged by inoculation with 1×10^5 TCID₅₀ of FMDV O₁ Taiwan into the heel bulbs of their forelegs inside the P3 facility at the National Institute for Animal Health, Tamsui, Taiwan, in accordance with a recommended challenge protocol (OIE 2004) except that the recommended challenge dose was exceeded 10-fold. Experimental animals were

monitored for clinical signs of FMD over a 14-day observation period. These included daily recording of body temperature, and observations of whether the pigs had become lame, and had acquired vesicular lesions on the coronary bands of their legs and on their snouts. In the earlier study group where pigs were given two injections of the vaccine four weeks apart, sera were collected weekly after each vaccination, and NA titers were determined. Concurrently, PBMCs were stimulated with the complete vaccine immunogen (Fig. 1) or the VP1 fragment alone to evaluate cellular responses to these recall antigens by measuring IFN- γ .

Virus

Virus (FMDV O/TAW/97) that was not passaged in the BHK cell line was prepared by collecting the vesicular fluid from FMDV-infected pigs. After centrifugation to remove contaminating cells and tissue, virus-containing supernatant was titrated to determine TCID₅₀, and stored in liquid N₂ until use for challenge studies and virus neutralizing antibody (NA) assays.

VP1 ELISA

The commercial VP1 test kit developed by UBI was used to quantitate serum antibodies generated against the consensus serotype O VP1 vaccine component in experimental animals. The assay was performed according to the protocol that was provided with the kit. Briefly, 100 μ l of diluted serum samples were added to individual assay microtitre wells and allowed to react at 37°C for 1 hour. The sample wells were then washed 6 times with UBI® Wash Buffer. A standardized preparation of HRP-conjugated ImmunoPure® Protein A/G (Pierce Chemical Co., Rockford IL, USA) was added to each well and incubated for 30 minutes at 37°C. The wells were again washed 6 times and 100 μ l of the substrate solution, 3,3',5,5'-tetramethylbenzidine (TMB), was added. After 15 min incubation, reactions were stopped by the addition of 1.0 M H₂SO₄ and absorbance at 450 nm was measured in an ELISA reader (EL x 405 model).

Serum IFN- γ quantitation

A sandwich ELISA was used to quantitate IFN- γ in the serum samples collected from pigs before vaccination and on day 28 before they were challenged with FMDV O_{1 Taiwan}. The assay was carried out by first coating individual wells of the Maxisorb ELISA plates (Nunc) with the capture antibody (purchased from PBL Biomedical Laboratories, USA) at 0.5 μ g per well. Sites not occupied by the immobilized antibodies were blocked by adding 200 μ l of ELISA blocking buffer (UBI) into each assay well. After washing the plates 4 times each time with 200 μ l of PBS containing 0.025% Tween 20 (PBS-T20), serum samples individually diluted in ELISA dilution buffer (UBI) at 1 in 10, 25 and 75 were added to the assay wells. The plates were incubated for 1 hr at 37°C to allow binding of the cytokine to the solid phase anti-IFN- γ capture antibody. The plates were then washed 4 times with PBS-T20, and the detection anti-IFN- γ antibody was added to the assay wells at 0.25 μ g per well. Following incubation for 1 hr at 37°C, the plates were washed, and 100 μ l of protein A conjugate (UBI) was added to each test well. Excess conjugate was washed off (6 times with PBS-T20) after the 1 hr incubation period was completed, and 100 μ l of the recommended substrate solution (UBI) was then added for reaction

detection. Colour development was stopped 30 min later by adding 50 µl of 1N H₂SO₄, and colour intensity was recorded by measuring absorbance at 450 nm.

Results were calculated by comparing the measured A₄₀₅ values obtained from an individual serum against a calibration curve that was constructed by assaying dilutions of a standard recombinant IFN-γ (PBL Biomedical Laboratories, USA) under the same test conditions. The IFN-γ response in the serum of each animal at 28 days post vaccination was represented by subtracting the value measured for the animal before immunization. Final results were expressed as pg/ml of serum.

Assay of IFN-γ produced by PBMC

PBMC (peripheral blood mononuclear cells) were harvested from heparinized blood of experimental animals by diluting the heparinized blood 1:1 with serum free RPMI 1640 culture medium (Gibco). 10.0 ml of the diluted blood was then layered onto 9.0 ml of Ficoll-Paque™ Plus density gradient (Amersham Biosciences) in a 50.0 ml sterile centrifuge tube, and lymphoid cells were separated from red blood cells by centrifugation at 2000 rpm for 35 minutes. The cell layer at the interface of serum and Ficoll-Paque™ Plus was drawn off. Cells were washed twice with serum free RPMI 1640 by centrifugation at 1000 rpm for 10 min, and resuspended in culture medium [RPMI 1640 supplemented with 10.0% fetal bovine serum (Gibco), recommended concentrations of L-glutamine, and penicillin/streptomycin mix (Gibco)] at 2.5 x 10⁶ cells/ ml. PBMC were then cultured at this concentration in individual wells of a 24 well culture plate (Nunc) in the presence of 10.0 µg of the vaccine immunogen or the VP1 fragment of the vaccine construct. Negative control cultures containing PBMC alone without stimulating antigen were also set up. All cultures were kept at 37°C for 3 days in a 5.0% CO₂ incubator. Supernatants were collected 3 days after culture initiation, and IFN-γ contents were measured using the quantitative assay described above.

Neutralizing Antibody (NA) Assay

The quantitative assay for antibodies that neutralize FMDV O_{1 Taiwan} was done against BHK-21 cells in flat-bottomed microtitre plates. The test was an equal volume test in 200 µl. 50 µl of diluted serum collected from the individual experimental animal before vaccination or at day 28 post-immunization was mixed with a 50 µl aliquot containing 200 TCID₅₀ of FMDV O_{1 Taiwan} and incubated for one hour at 37°C. 100 µl of culture medium [MEM (Gibco) supplemented with 10.0% fetal bovine serum] containing 2.5 x 10⁵ BHK-21 cells was then added to each assay. Cultures were examined microscopically after 48 hours for cytopathic effect (CE). Titres were expressed as the reciprocal of the final dilution of serum giving 50% inhibition of the virus-induced CE.

Results

Humoral responses

Swine in trial groups 1 and 2 given single immunizations with UBITH[®] FMD (04-3S-1) at 2.0 and 1.0 ml doses, respectively, were found to have mounted moderate anti-peptide antibody responses as compared to the two unvaccinated control animals, based on the reactivities of their sera to the VP1

antigen ELISA (Table 1). The virus neutralizing antibody (NA) assays showed an uneven distribution of biological activities in the serum samples of the experimental pigs (Table 2). The results of the two antibody assays indicate only a loose correlation between antigen-binding (ELISA) titre and NA. For example, the highest ELISA titre of 3.082 (in Log₁₀ scale) was measured in the serum samples of pig 152 that also had the highest NA titre of 16, the lowest ELISA titre was measured in swine 57 found to be devoid of NA, and pigs 170 and 172 also were correlated in their ELISA and NA titers. However, sera from pigs 151, 155, 158, and 166 in trial group 1, and sera from pigs 153 and 159 in trial group 2 all had comparable ELISA titres but an NA titre of 11 was found only in 153; while the 151, 159 and 166 sera had lower detectable virus neutralizing activities, and NA was not detected in the sera from 155 and 158.

The inconsistent NA titres that were measured in the two trial groups from the one-dose vaccination protocol were consistent with the earlier observation that our vaccine does not induce high NA in response to the first immunization of a two-dose protocol given to pigs. However, the two dose protocol was quite capable of eliciting significant NA titers ranging from >16 to > 300 in response to the second injection (Figure 2).

Virus challenge

None of the animals in the single-vaccinated groups showed significant body temperature elevation of 40°C and above following virus challenge. In contrast, unvaccinated control pig 1063 had temperatures of 40.5 °C and 40.0 °C on post-challenge days 4 and 5 respectively. This animal developed clinical signs of FMD from day 4 post- challenge. Vesicles were found on the coronary bands of its forelegs, and vesicular lesions were then found on its snout on day 5. Unimmunized pig 1064 had a higher than normal temperature of 41.5 °C on day 3, and its fever lasted until day 6. Concomitant with initial body temperature elevation, all 4 legs of 1064 had vesicles on day 3, and by day 4 vesicles were also found on its snout. Both control pigs were lame by day 5. In contrast, FMD-associated clinical signs were not found in any of the single dose vaccinated swine (Table 2). There was no clear pattern of correlation between the pre-challenge humoral responses and protective immunity in this one dose protocol.

Cellular immune responses

Figure 3 shows that the sera of 11 out of the 12 immunized pigs of trial groups 1 and 2 contained higher levels of IFN-γ on day 28 post vaccination (before virus challenge) as compared to the day 0 levels. However, serum IFN-γ levels did vary substantially among these vaccinated pigs. The highest serum cytokine levels of over 1000 and 600 pg/ml were detected in pigs 160 and 158. respectively. Moderate IFN-γ levels of between 100 – 260 pg/ml were found in animals 151, 152, 154 and 159; while low serum IFN-γ levels of 25 – 80 pg/ml were measured in the remaining pigs of the two trial groups (with the exception of animal 172 which had no detectable IFN-γ).

These data are consistent with our observation from the previous 2 dose protocol that the PBMC of pigs vaccinated with UBITH[®] FMD (04-3S-1) could be re-stimulated in vitro to secrete IFN-γ by the vaccine immunogen or its VP1 component (Figure 4).

Discussion

An efficacious synthetic peptide-based FMD vaccine was developed by UBI and UBI-Asia for emergency application in swine. The efficacy trial conducted with our vaccine preparation, UBITH[®] FMD (04-3S-1) showed that a single injection of either 2.0 ml or 1.0 ml was protective in swine against challenge by FMDV O₁ Taiwan without producing adverse reactions. An interesting feature of this VP1 peptide-based vaccine appeared to be that while it did induce consistent anti-peptide antibodies directed against the G-H loop, as evaluated by the ELISA titres (Table 1), there was only a loose correlation between the anti-peptide antibody responses and virus neutralizing activities, with no consistent NA responses. Despite the inconsistent levels of serum NA, the swine were found to have acquired full protective immunity against challenge with FMDV O₁ Taiwan with a single dose of either 1.0 ml or 2.0 ml of the UBITH[®] FMD (04-3S-1) vaccine preparation.

To further seek immune correlates of protection, we have investigated the T cell effector immune mechanisms induced by UBITH[®] FMD (04-3S-1). Pigs at day 28 post-immunization were found to have a tendency toward elevated serum IFN- γ responses. Two alternative explanations can account for this observation. First, it is possible that the kinetics of induction of IFN- γ secreting lymphocytes in these outbred pigs vary since the different MHC gene products they express would regulate the T and B cell responses that are generated. Thus, the high, moderate, low, as well as the lack of IFN- γ (in pig 172) that is detected would simply reflect the accumulation versus degradation of the cytokine at the time the blood of the animals was collected. Second, the frequencies of IFN- γ secreting lymphocytes induced by vaccination could differ in these pigs since this phenomenon is again a MHC-controlled event. In this case, higher and lower frequencies of IFN- γ secreting cells induced would account for their capacities to release more or less of the cytokine respectively. Having considered these interpretations separately, it is entirely possible that these events could take place concomitantly to account for the variable elevations of serum IFN- γ that were monitored. With respect to the second possibility, we have observed from the previous two-injection study that the immunized pigs did contain circulatory T cells that secreted IFN- γ upon in vitro re-stimulation with the vaccine immunogen, or the VP1 component alone of that vaccine construct (Figure 4). This observation from a two dose vaccine protocol supports an interpretation that the increased serum IFN- γ levels measured in pigs of the two trial groups from the one dose protocol can be attributed to vaccine-specific T cells that secrete this cytokine. In view of this finding and our other finding that our vaccine needs two injections to elicit protective levels of NA, but that animals given one injection of the vaccine are fully protected, it would appear that effective Th1 responses, as represented by the IFN- γ are responsible for the initial protection against FMDV infection (Figure 5). However, it is believed that both effective humoral and cellular immunity are required to confer long-term protection from FMDV.

We were limited to the investigation of NA and IFN- γ as correlates or surrogate correlates of immunity against FMDV in this report because of the availability of reagents at the present time. Apart from the possible effect of IFN- γ , it cannot be concluded whether other effector and/or regulatory immune mechanisms may also participate in the protection against FMDV O₁ Taiwan conferred by the UBITH[®] FMD

(04-3S-1) vaccine. In any case, the role of IFN- γ in promoting antigen-processing in the induction of MHC class I-restricted T cells, which are important for protection against virus infections, has been well documented (Van Hall et al 2000; Appay et al 2002). A more recent study has demonstrated that FMDV infection in cattle resulted in the induction of MHC class I-restricted CD8 effector cells (Childerstone et al 1999). In this regard, it remains to be confirmed whether our vaccine indeed elicits IFN- γ secreting CD8 cells that play a major role in protection against FMDV.

Effector responses in pigs induced by the peptide vaccine formulation are being investigated in further potency trials. The PD₅₀ for the peptide-based vaccine will be determined in those trials as part of the pending approval process for the vaccine by the Council of Agriculture of Taiwan. A UBITH®-VP1 vaccine formulation has already been approved for use in pigs by the Ministry of Agriculture of China.

Conclusions

A potent synthetic peptide marker vaccine for FMD has been developed for use in pigs.

The vaccine elicits IFN- γ responses after one or two doses and NA after two doses.

This vaccine is being commercialized for use in major markets.

Recommendations

Further studies by the investigators are needed to characterize the correlates of immunity and to determine the PD₅₀.

The investigators need to initiate discussions with the Biological Standards Commission regarding specifications for a synthetic peptide-based FMD vaccine.

References

- Acharya, R., Fry, E., Stuart, D., Fox, G., Rowlands, D.J. & Brown, F. 1989 The three-dimension structural of foot-and mouth disease virus at 2.9A resolution. *Nature* 337: 709-716.
- Appay, V., P. R. Dunbar, M. Callan, P. Klenerman, G. M. Gillespie, L. Papagno, G. S. Ogg, A. King, F. Lechner, C. A. Spina, S. Little, D. V. Havlir, D. D. Richman, N. Gruener, G. Pape, A. Waters, P. Easterbrook, M. Salio, V. Cerundolo, A. J. McMichael, and S. L. Rowland-Jones. 2002. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. *Nat. Med.* 8:379-385.
- Beck, E. & K. Strohmaier. 1987. Subtyping of European foot-and-mouth disease virus strains by nucleotide sequence determination. *J. Virol.* 61:1621-1629.
- Bittle, J.L., Houghten, R.A., Alexander H, Shinnick, T.M., Sutcliffe, J.G., Lerner, R.A., Rowlands, D.J., Brown, F. 1982. Protection against foot-and mouth disease by immunization with a chemically synthesized peptide predicted from the viral nucleotide sequence. *Nature* 298: 30-33.
- Brown, F., Benkirane, N., Limal, D., Halimi, H., Newman, J.F., Van Regenmortel, M.H., Briand, J.P. & Muller, S. 1999. Delineation of a neutralizing subregion within the immunodominant epitope (GH loop) of foot-and-mouth disease virus VP1 which does not contain the RGD motif. *Vaccine* 18: 50-56.
- Childerstone AJ, Cedillo-Baron L., Foster-Cuevas M and Parkhouse RME. 1999. Demonstration of bovine CD8⁺ T-cell responses to foot-and-mouth disease virus. *J. Gen Virol* 80: 663-669.

King, A.M.Q., Underwood, B.O., McCahon, D., Newman, J.W.I. & Brown, F. 1981 Biochemical identification of viruses causing the 1981 outbreaks of foot-and-mouth disease in the UK. *Nature* 293:479-480.

Office International des Epizooties. 2004. Chapter 2.1.1 Foot-and-mouth disease. In *Manual of diagnostic tests and vaccines for terrestrial animals*, 5th edition, Paris (available at www.oie.int/eng/normes/mmanul/A_summry.htm, updated 23.07.2004).

Pfaff, E., Mussgay, M., Bohm, H.Q., Schulz, G.E. & Schaller, H. 1982 Antibodies against a preselected peptide recognize and neutralize foot and mouth disease virus. *EMBO J* 1(7):869-874.

Van Hall, T., Sijts, A., Camps, M., Offringa, R., Melief, C., Kloetzel, P. M. & Ossendorp, F. 2000. Differential influence on cytotoxic T lymphocyte epitope presentation by controlled expression of either proteasome immunosubunits or PA28. *J. Exp. Med.* 192:483-494.

Wang, C.Y., Chang, T.Y., Walfield, A.M., Ye, J., Shen, M., Chen, S.P., Li, M.C., Lin, Y.L., Jong, M.H., Yang, P.C., Chyr, N., Kramer, E. & Brown, F. 2002. Effective synthetic peptide vaccine for foot-and-mouth disease in swine. *Vaccine* 20: 2603-2610.

Wang, C.Y. 2004. Artificial T helper cell epitopes as immune stimulators for synthetic peptide immunogens. US patent no. 6,713,301 B1, United Biomedical Inc.

Table 1. Anti-peptide humoral responses generated in pigs immunized with UBITH[®] FMD vaccine

Trial group	Vaccine/Dose	Pig number	VP1 ELISA antibody titre (Log ₁₀)	
			Before Immunization	Before virus challenge ^a
1	UBITH [®] FMDV (04-3S-1) (2.0 ml)	151	1.502	2.846
		152	1.500	3.082
		155	1.494	2.724
		158	1.499	2.839
		166	1.500	2.807
		168	1.501	2.624
2	UBITH [®] FMDV (04-3S-1) (1.0 ml)	153	1.504	2.822
		154	1.504	2.094
		159	1.500	2.748
		160	1.501	2.468
		170	1.501	2.575
		172	1.503	2.572
Control	None	1063		1.505
		1064		1.501

^a Day 28 post-immunization.

Table 2. Serum FMDV neutralizing antibody titres and protection from virus challenge by one-dose vaccine

Pig number	NA titre ^a		FMD signs ^c following virus challenge
	Before Immunization	Before virus challenge ^b	
151	≤ 3	6	None
152	≤ 3	16	None
155	≤ 3	≤ 3	None
158	≤ 3	≤ 3	None
166	≤ 3	4	None
168	≤ 3	8	None
153	≤ 3	11	None
154	≤ 3	≤ 3	None
159	≤ 3	4	None
160	≤ 3	4	None
170	≤ 3	8	None
172	≤ 3	11	None
1063	≤ 3	≤ 3	From 4 days post challenge
1064	≤ 3	≤ 3	From 3 days post challenge

^aNA titres were determined as described in Materials and methods. A titre of ≤ 3 was considered to be insignificant.

^bTests were done at 4 weeks post-vaccination, before inoculation with challenge virus

^cClinical signs of FMD observed as described in Materials and methods

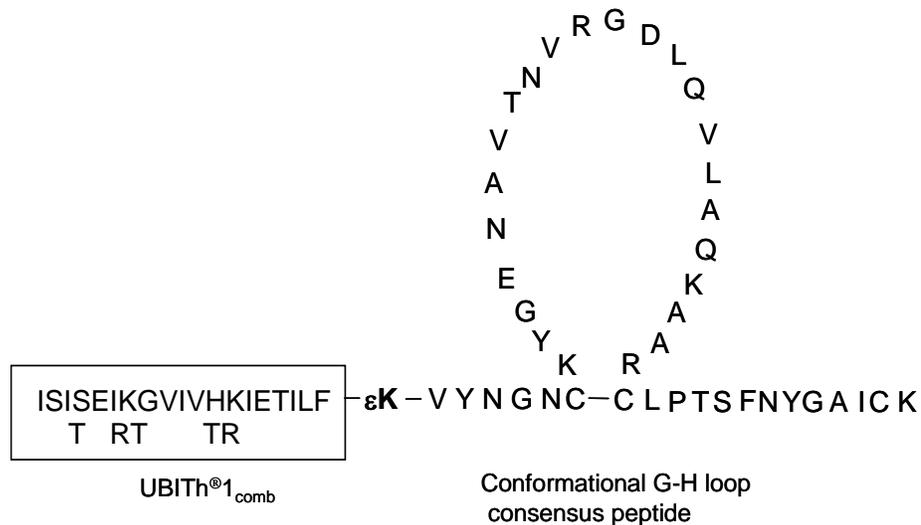


Figure 1. Schematic representation of the UBITH[®]1_{comb}-VP1 FMD vaccine construct

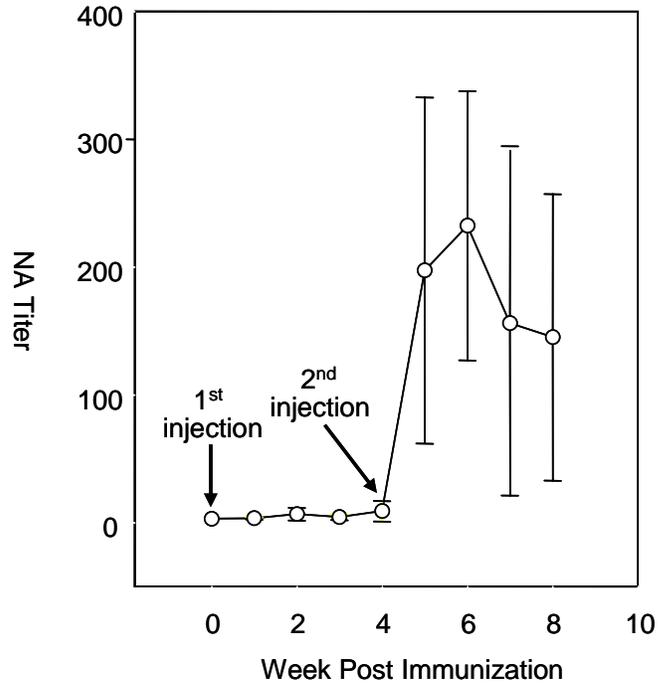


Figure 2. Serum NA titres measured in pigs given 2 injections of UBITH® FMD (04-3S-1). 8 FMDV-free pigs, 8-12 weeks of age, were immunized twice with doses 4 weeks apart. Sera were collected weekly and assayed for NA activities after each immunization.

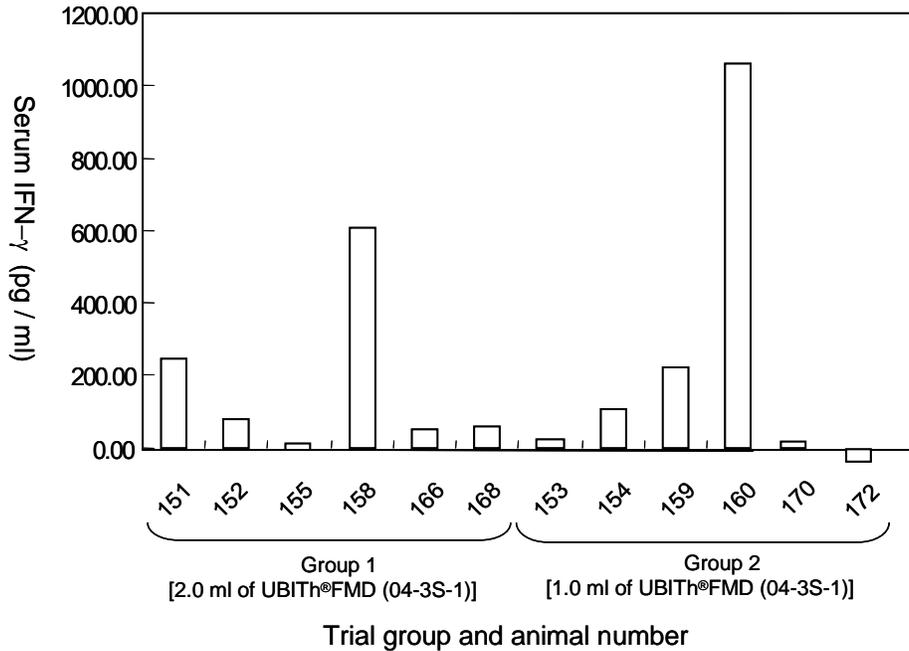


Figure 3. Serum IFN-γ levels of vaccinated pigs. Animals were bled on day 28 before challenge. Final values were corrected for by subtraction of day 0 baseline values

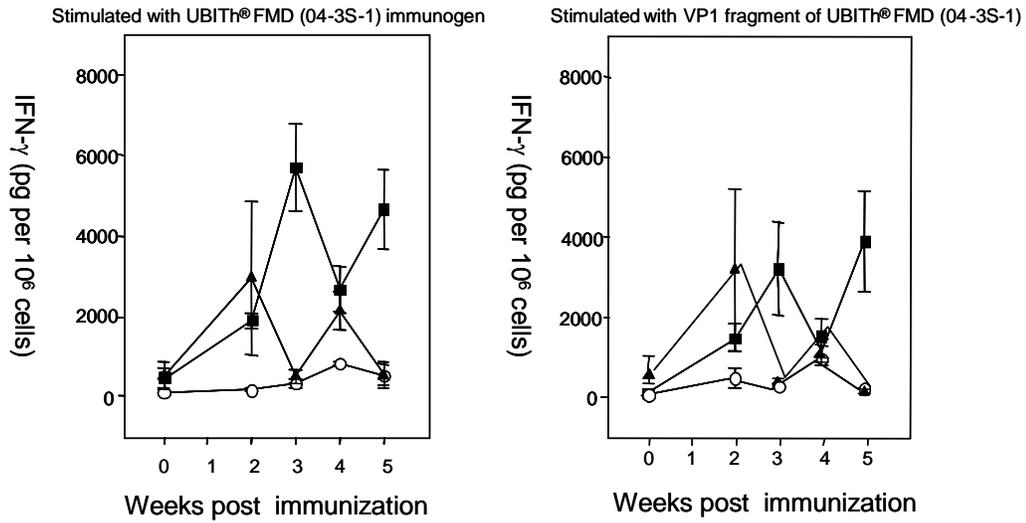


Figure 4 Cellular immune responses induced by UBITH[®] FMD (04-3S-1) (■) or Merial FMD vaccine (▲) in pigs given 2 immunizations. 8 or 5 FMDV-free pigs were immunized twice with UBITH[®] FMD (04-3S-1) or Merial FMD_{O1 Taiwan} vaccine at 4 weeks apart. 5 unvaccinated pigs were used as negative control (O). Animals were bled weekly, and their serum NA titers and the capability of their PBMCs to respond to recall vaccine antigens with IFN- γ response were determined as described in Materials and methods.

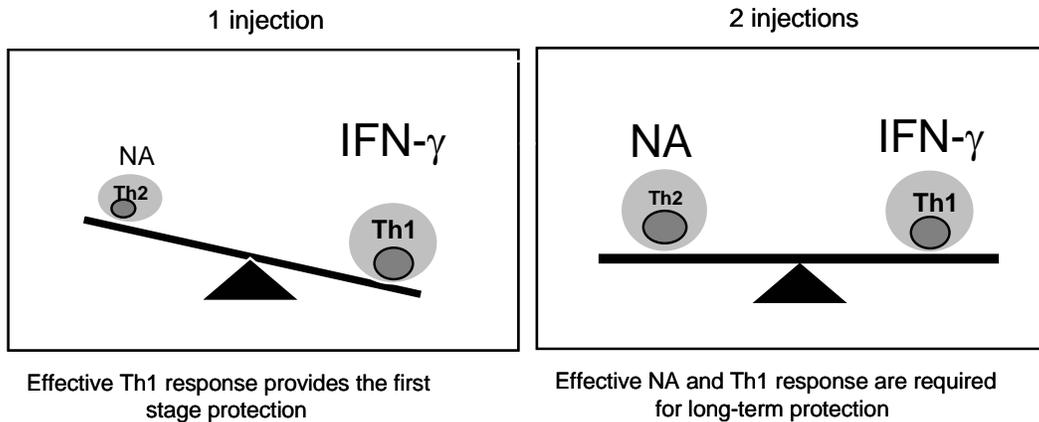


Figure 5. Schematic representation for protection against FMDV based on the study with UBI's UBITH[®]FMDV (04-3S-1) vaccine.

Early development of Adenovirus-vectored FMD vaccine and antiviral

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Few effective intervention tools are available today to control FMD outbreaks. These include culling and slaughter of all animals in infected and neighboring contact premises and strict quarantines of the affected areas. These measures lead to massive accumulation of thousands of animal carcasses for disposal with the consequent disruption of commerce, tourism and transportation. Vaccination has been proposed as a tool to decrease susceptibility of animal populations in ring-vaccination schemes. Current vaccines have been designed and tested for protection at 3-4 weeks post-vaccination, creating a window of susceptibility in the vaccinated population. Previous experiments at PIADC have shown that recombinant Adenovirus-vectored FMD empty capsid (VLP) can protect swine and bovine against direct FMDV challenge by 7 days post vaccination. In addition Adenovirus-vectored porcine interferon can protect swine against FMD challenge in as little as 24 h and for as long as 5 days post injection. The combination of vaccine and antiviral would close the window of susceptibility and could serve as emergency intervention tools to rapidly control FMD outbreaks. We are now taking these two products through early development utilizing a cell line approved for vaccine production and producing experimental vaccine lots under "Good Laboratory Practice" conditions in partnership with a private industry partner. With these products we will be evaluating novel approaches combining vaccination and antiviral therapy as intervention tools to rapidly control and minimize the impact of FMD outbreaks in the United States.

Significantly enhanced immune responses induced by a FMD DNA vaccine in swine using a protein antigen boost

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Abstract:

Introduction: It has previously been shown that a FMD DNA vaccine containing the “empty capsid” cassette-structural protein precursor P1 and the non-structural proteins 2A, 3C and 3D (pCDNA3.1/P1-2A3C3D, P1) combined with an adjuvant plasmid expressing porcine granulocyte macrophage colony stimulating factor (poGM-CSF) induced neutralising antibodies to FMDV and conferred partial protection against live virus challenge in swine. Current studies are aimed at enhancing the immune responses from this DNA vaccine further in swine by incorporating a prime/boost vaccination strategy. Increasing the priming dose of FMD DNA vaccine (600 µg) and GM-CSF (400 µg) and combining with a protein boost induced an average anti-FMDV antibody titre which was up to 30 times higher than that following conventional vaccination.

Materials and Methods: Groups of pigs were immunised with P1 and poGM-CSF plasmids via the intramuscular/ intradermal route (i.m./i.d) once, twice or three times which was followed 3 weeks later by a protein boost of inactivated FMDV antigen and FMDV 3D protein via the i.m. or i.d. route.

Results: FMDV specific immune responses were significantly increased following the protein antigen boost of the P1 DNA vaccinated pigs.

Conclusion: FMDV P1 DNA vaccination followed by an inactivated FMDV antigen/3D boost could be a more efficient vaccination strategy in this model.

Introduction:

Vaccination with a DNA plasmid by various routes has been shown to elicit protective immune responses to the encoded antigen in a variety of animal models (Ulmer *et al.* 1993; Somasundaram *et al.* 1999; Lodmell *et al.* 1998). This novel approach is particularly attractive for several reasons: the antigen is endogenously synthesised and processed and therefore more closely mimics natural infection. This results in the antigen being presented via both MHC class I and class II pathways generating both humoral and cellular immune responses. The use of plasmid DNA as a vaccine can also trigger innate immunity in the host as an effect of the unmethylated CpG motifs in the bacterial plasmid backbone (Yankaucka *et al.*, 1993; Wolff *et al.*, 1992; Klinman *et al.*, 2004). Additionally, DNA vaccines are non-infectious, easy to prepare, inexpensive, and are stable at room temperature reducing cold chain requirements (Babiuk *et al.*, 2000; Gurunathan *et al.*, 2000; Cichutek 2000). DNA vaccines have the potential to provide a more effective and cheaper vaccine for economically important domestic animals such as cattle and pigs and are particularly advantageous over the conventional FMD vaccine because they do not require high-security containment facilities for manufacture, and are easy to manipulate for incorporation of marker genes or covering against various serotypes and field isolates in an outbreak.

The structural proteins of FMDV VP0, VP3 and VP1 were produced when the P1-2A precursor was cleaved by the viral protease 3C. One of each of these proteins can form into protomers and five protomers assemble into a pentamer. An icosahedral capsid particle is then assembled with twelve pentamers. When this capsid particle lacks the RNA genome, they are called “empty capsids” (Yafal and Palma, 1979; Rombaut *et al.*, 1991; Abrams *et al.*, 1995). It was found that empty capsid particles are capable of inducing antibody responses at a similar level to that induced by the whole virus (Rowlands *et al.*, 1975; Grubman *et al.*, 1985; Francis *et al.*, 1985). Taking this observation together with the finding that the non-structural protein 3D stimulates a strong humoral and cellular immune response in the host (Foster *et al.*, 1998), a P1 FMDV DNA vaccine was constructed containing an “empty capsid” gene cassette-P1-2A, 3C and 3D. Partial protection against homologous O1 Lausanne virus challenge was induced in pigs after three immunisations of this P1 plasmid. The antibody responses induced by this FMD DNA vaccine was improved by co-administration of a plasmid encoding porcine granulocyte macrophage colony stimulating factor (GM-CSF) (Cedillo-Barron *et al.*, 2001). Furthermore, it has been found that increasing the amount of P1 plasmids and poGM-CSF DNA plasmids from 300 µg and 200 µg each to 600 µg and 400 µg respectively improved the immune response to FMDV in vaccinated pigs in a recent study performed in our group (unpublished data). This study was aimed at optimising this vaccination protocol to enhance the antibody and cellular responses induced by FMDV DNA immunisation of pigs by employing the prime/boost strategy, and simplifying the DNA vaccination protocol by reducing the injection intervals without decreasing the specific immune responses in vaccinated animals.

Materials and Methods:

Animal experiment

12 large white cross-bred Landrace pigs, weighing 20-25 Kg, were housed as four groups of three. Each animal received 600µg of P1 plasmids and 400µg of adjuvant plasmid poGMCSF dissolved in sterile saline via two 1 ml shots in each hind leg muscle followed by administration of the remaining 0.5 ml, which was equally split intradermally (i.d.) into the dorsal surface of either ear. The vaccination regime for different groups were as outlined below. Briefly, Pigs of groups 2, 1 and 3 were vaccinated with DNA plasmids once, twice or three times at 3 weeks interval, respectively, and this was followed by a protein antigen boost with 7.5 µg of O1 Lausanne inactivated antigen and 20 µg of FMDV 3D protein via the i.d. route. The group 4 pigs received two DNA immunisations 3 weeks apart followed by a protein antigen boost, as other groups but via the i.m. route. Serum samples were taken regularly on a weekly basis after each vaccination until the experiment was terminated at 7 days (group 3) or 21 days (groups 1, 2 and 4) post protein antigen boost. Samples prior to vaccinations were also collected for background level assessment.

1. pcDNA3.1/P1-2A3C3D (600µgms) + GMCSF (400 µgms) 2 times + i.d. protein boost
2. pcDNA3.1/P1-2A3C3D (600µgms) + GMCSF (400 µgms) once + i.d. protein boost
3. pcDNA3.1/P1-2A3C3D (600µgms) + GMCSF (400 µgms) 3 times + i.d. protein boost
4. pcDNA3.1/P1-2A3C3D (600µgms) + GMCSF (400 µgms) 2 times + i.m. protein boost

Serum samples from single conventional O1 Lausanne vaccinated pigs (each pig receiving 6.5 µg of antigen per dose) using vaccine supplied from the International Vaccine Bank, Pirbright Laboratory, IAH, UK were also used for testing the FMDV specific antibody and neutralising antibody responses, and the results were compared to those obtained in the DNA vaccinated pigs.

Detection of FMDV specific antibody responses

The antibody responses to FMDV in serum were analysed by an indirect sandwich ELISA using inactivated O1Kaufbeuren (O1K) virus. The antibody in serum samples from P1 DNA vaccinated pigs was detected using rabbit anti-porcine antibodies HRP conjugate (DAKO). Antibody titres were expressed as the reciprocal of the highest serum dilution with an OD value at least two times that of the serum samples at 0 day. Neutralising antibodies to FMDV in serum samples were detected by microneutralisation assay using porcine kidney RSB cells and FMDV O₁ Kaufbeuren virus (Golding *et al.*, 1976). The neutralising antibody titres were calculated as the log₁₀ of the reciprocal antibody dilution required for 50% neutralisation of 100TCID₅₀ virus.

Delayed type hypersensitivity (DTH) test

To investigate the existence of any T cell mediated immunity following P1 DNA vaccination, all four groups pigs were administered with O1 Lausanne inactivated antigen and/or recombinant protein FMDV 3D, 21 days following the last DNA vaccination. Each of P1 vaccinated pigs received either 7.5 µg/0.1ml unpurified antigen or 20 µg/0.1ml 3D protein (diluted in endotoxin free PBS) via the intradermal route on one side of the abdomen. PBS alone was used as a control. The pigs were monitored daily and the skin, measured as the induration (diameter of raised skin or inflammation) at the site of intradermal injection, 2 days later. A measurement greater than a 2mm increase in skin thickness/swelling was considered as a positive response.

Results

FMDV antibody responses and neutralising antibody detection

Antibodies to FMDV were demonstrated after the second DNA vaccination and increased significantly at 7 days post protein antigen boost which were then maintained until the end of the experiment in all animals representing groups 1, 3 and 4 (Figure 1a, c and d). The highest anti-FMDV antibody titre (animal VB57 in group 1) was 204800 which is about 64 times higher than that observed in single conventional vaccinated pigs (Figure 3a). Antibody responses to FMDV were induced following protein antigen boost in all group 2 animals lasted for 5 weeks (Figure 1b). The highest antibody titre from the group 2 pigs was the same as that observed in group 3 pigs before the protein antigen boost (Figure 1c) or the single conventional vaccinated pigs (Figure 3a), but was about 64 times lower than that observed in group 1 pigs. There was no significant difference in the antibody responses to FMDV following the protein antigen boost, either i.d. or i.m. among groups 1, 3 and 4 (Table 1), although the antibody responses induced in three times P1 DNA vaccinated pigs (group 3) were greater than those in pigs vaccinated twice with the P1 DNA construct (groups 1 and 4) prior to the protein antigen boost

Neutralising antibody responses were demonstrated as early as 21 days post primary DNA vaccination in one animal from each of groups 2 and 4. All animals in groups 1, 3 and 4 produced neutralizing antibodies after the second DNA vaccination, and their ability to neutralize FMDV O1 Kaufbeuren virus was significantly enhanced after the protein antigen boost (Figure 2a, c and d). The highest

neutralizing antibody titre was recorded in group 3 and was about one \log_{10} higher than that observed in any of the single conventional vaccinated pigs (Figure 3b). The neutralising antibodies induced in group 2 pigs (single vaccination) were also elevated after the protein antigen boost, however the highest titre was more than one \log_{10} lower than that obtained in groups 1, 3 and 4 pigs. There was no significant difference in the neutralizing antibody responses among groups 1, 3 and 4 after either i.d. or i.m. protein antigen boost. (Table 1), although group 1 gave the best neutralizing antibody responses prior to the protein antigen boost.

Delayed type hypersensitivity (DTH) test

When a protein antigen boost was administered via the i.d. route, as a DTH test, the skin reaction was recorded. The results are summarised in Table 2. Two animals from each of groups 1 and 2 showed clear DTH reactions to FMDV antigen. One animal from each of groups 1, 2 and 3 gave a weak response to FMDV antigen. Two animals from group 2 and one animal from group 3 showed DTH responses to FMDV 3D protein. In general, animals from group 2 displayed stronger DTH responses than those from group 3.

Discussion

A number of FMD DNA vaccines have been developed and partial protection against virus challenge was induced in DNA vaccinated animals (Ward *et al.*, 1997; Huang *et al.*, 1999; Benvenisti *et al.*, 2001; Wong *et al.*, 2000; 2002). However, the induced neutralising antibodies, which are a vital for vaccine efficacy, was slower and at a lower level than that from a conventional vaccine (Ward *et al.*, 1997; Huang *et al.*, 1999). The enhancement of immune responses using various DNA based prime boost strategies has also been documented (Hanke *et al.*, 1998; Gonzalo *et al.*, 2002; Robinson, 2003; Moore and Hill, 2004) and demonstrated to be effective for FMDV in mice when using a plasmid encoding VP1 followed by a VP1 peptide (Shieh *et al.*, 2001). To improve the efficacy of a FMD DNA vaccine previously constructed (Cedillo-Barron *et al.*, 2001), we have examined several vaccination parameters including incorporation of protein antigen boost strategies to induce stronger protective immunity in pigs.

The results in this study show that antibody responses to FMDV were generated after the secondary P1 vaccination and increased if pigs received the tertiary vaccination. However, both FMDV specific antibody responses and neutralizing antibody responses were significantly enhanced following the i.d. or i.m. antigen boost in all vaccinated animals (Figure 1 and 2). There was no difference in either total antibody or neutralizing antibody responses after protein boost following two or three DNA vaccinations. The route of protein antigen administration i.d. or i.m. as a boost showed no significant difference in either specific antibody responses or neutralizing antibody responses. The average total FMDV antibody titre after the i.m. antigen boost in the twice P1 DNA vaccinated pigs was about 14 times higher and the neutralizing antibody titre was about one \log_{10} higher than those observed in the single conventional vaccinated pigs (Table 1). This result is important in considering the practical application of this scheme in the field as a single i.m. boost is far more practical to undertake. Results from animals given a single P1 DNA vaccination followed by a protein antigen boost suggest that this relatively simple vaccination regime can induce similar levels of both FMDV specific and neutralising antibody in animals to those induced by a single dose of conventional vaccine. Although much further work is required to examine longevity of such a response, protection from challenge with live virus and the degree of sterile immunity conferred, these results support the theory that a prime boost regime combining a DNA prime and protein boost could potentially be an effective new approach to FMDV vaccination.

Results from the DTH test suggest that the more times an animal has been vaccinated with DNA plasmid the lower the DTH response observed. Animals vaccinated only once with plasmid showed a marked response to the antigen, however those vaccinated 3 times showed little or no reaction at all. This suggests that repeated DNA vaccination can cause desensitization to the antigen in this system. These data also suggest that repeated DNA vaccination to achieve high antibody titres may have an adverse effect on the cellular response supporting the theory that the prime/boost regime of 1 or 2 vaccinations with DNA followed by a protein boost is a much more effective regime for optimizing both humoral and cellular responses.

Overall, the best and simplest vaccination procedure observed was 2 immunizations at 3 week intervals with 600 μ g P1-2A3C3D plasmid combined with 400 μ g of the GM-CSF plasmid 'adjuvant' via i.m. / i.d. delivery each time followed 3 weeks later by an i.m. boost of 20 μ g recombinant 3D and 7.5 μ g of O1 Lausanne vaccine antigen. An effective protective immune response encompassing both the cellular and humoral arms of the immune system combined with a practical immunization regime is vital in the development of any vaccine which could be used in the field. We have confirmed the efficacy of a prime boost regime for FMD DNA vaccination in at least one natural target host, pigs.

Such encouraging results suggest that a prime boost strategy is worthy of further investigation and a good candidate approach in the development and application of new generation FMD vaccines.

Conclusions:

- FMDV DNA (P1) vaccination followed by an inactivated FMDV antigen and protein 3D boost may be a more effective vaccination strategy in swine.
- Specific immune responses to FMDV were significantly improved in pigs receiving two P1 DNA vaccinations and protein antigen boost than a single DNA vaccination followed by protein antigen boost.

Recommendations:

- Further assessment on DNA vaccination strategies and regimes that incorporate prime/boost regimes that explore the potential for further improvement and/or refinement.

Acknowledgements:

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References:

Abrams, C. C., King, A. M. Q. & Belsham, G. J. 1995. Assembly of foot-and-mouth disease virus empty capsids synthesized by a vaccinia virus expression system. *J. Gen. Virol.*, 76: 3089-3098.

Babiuk, L. A., van Drunen Littel-van den Hurk S., Loehr B. I. & Uwiera R. 2000. Veterinary applications of DNA vaccines. *Dev. Biol. (Basel)*, 104: 73-81.

Benvenisti, L., Rogel, A., Kuznetzova, L., Bujanove,r S., Becker, Y. & Stram, Y. 2001. Gene gun-mediate DNA vaccination against foot-and-mouth disease virus. *Vaccine*, 19: 3885-3895.

Cedillo-Barrón, L., Foster-Cuevas, M., Belsham, G. J., Lefevre F. & Parkhouse R. M. E. 2001. Induction of a protective response in swine vaccinated with DNA encoding foot-and-mouth disease virus empty capsid proteins and the 3D RNA polymerase. *J. Gen. Virol.*, 82: 1713-1724.

Cichutek, K. 2000. DNA vaccines: development, standardization and regulation. *Intervirology*, 43: 331-338.

Collen, T., Baron, J., Childerstone, A., Corteyn, A., Doel, T. R., Flint, M., Garcia-Valcarcel, M., Parkhouse, R. M. E. & Ryan, M. D. 1998. Heterotypic recognition of recombinant FMDV proteins by bovine T-cells: the polymerase (P3D_{pol}) as an immunodominant T-cell immunogen. *Virus Res.*, 56: 125-133.

Foster, M., Cook, A., Cedillo, L. & parkhouse, R. M. E. 1998. Serological and cellular immune responses to non-structural proteins in animals infected with FMDV. *Vet. Quart.*, 20(Suppl.2): S28-S30.

Francis, M. J., Fry, C. M., Rowlands, D. J., Brown, F., Bittle, J. L., Houghten, R. A., & Lerner, R. A. 1985. Immunological priming with synthetic peptides of foot-and-mouth disease virus. *J. Gen. Virol.*, 66: 2347-2354.

Golding, S. M., Hedger, R. S. & Talbot P. 1976. Radial immno-diffusion and serum neutralisation techniques for the assay of antibodies to swine vesicular disease. *Res. Vet. Sci.*, 20: 142-147.

Gonzalo, R. M., del Real, G., Rodriguez, J. R., Rodriguez, D., Heljasvaara, R., Lucas, P., Larraga, V. & Esteban, M. 2002. A heterologous prime-boost regime using DNA and recombinant vaccinia virus expressing the Leishmania infantum P36/LACK antigen protects BALB/c mice from cutaneous leishmaniasis. *Vaccine*, 20: 1226-1231.

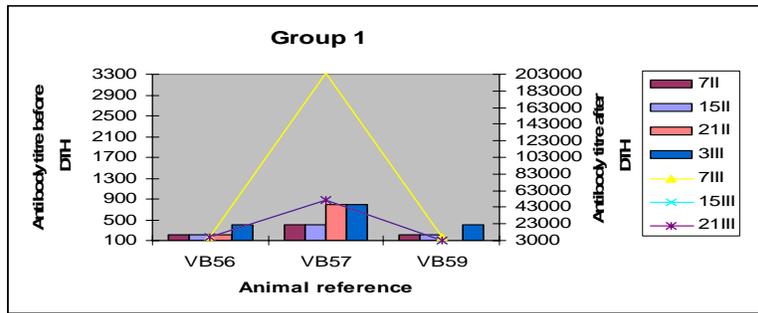
Grubman, M. J., Morgan, D. O., Kendal, J. & Baxt, B. 1985. Capsid intermediates assembled in a foot-and-mouth disease virus genome RNA-programmed cell-free translation system and infected cells. *J. Virol.* 56: 120-126.

Gurunathan, S., Klinman, D. M. & Seder, R. A. 2000. DNA vaccines: immunology, application, and optimization. *Annu. Rev. Immunol.*, 18: 927-974.

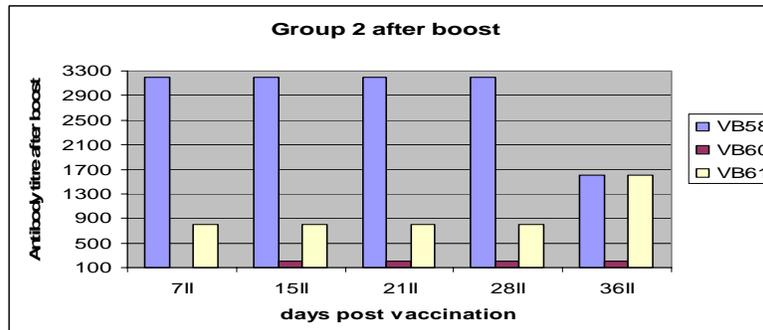
Hanke, T., Blanchard, T. J., Schneider, J., Hannan, C. M., Becker, M., Gilbert, S. C., Hill, A. V., Smith, G. L. & McMichael, A. 1998. Enhancement of MHC class I-restricted peptide-specific T cell induction by a DNA prime/MVA boost vaccination regime. *Vaccine*, 16: 439-435.

- Huang, H., Yang, Z., Xu, Q., Sheng, Z., Xie, Y., Yan, W., You, Y., Sun, L. & Zheng, Z.** 1999. Recombinant fusion protein and DNA vaccines against foot and mouth disease virus infection in guinea pig and swine. *Viral Immunol*, 12: 1-8.
- Klinman, D.M., Currie, D., Gursel, I. & Verthelyi, D.** 2004. Use of CpG oligodeoxynucleotides as immune adjuvants. *Immunol. Reviews*, 199: 201-216.
- Lodmell, D. L., Ray, N. B. & Ewalt, L. C.** 1998. Gene gun particle-mediated vaccination with plasmid DNA confers protective immunity against rabies virus infection. *Vaccine*, 16: 115-118.
- Moore, A.C. & Hill, A.V.** 2004. Progress in DNA-based heterologous prime-boost immunization strategies for malaria. *Immunol Rev.*, 199: 126-143.
- Robinson, H.L.** 2003. Prime boost vaccines power up in people. *Nat. Med*, 9: 642-643.
- Rombaut, B., Foriers, A. & Boeye, A.** 1991. *In vitro* assembly of poliovirus 14S subunits: identification of the assembly promoting activity of infected cell extracts. *Virology*, 180(2): 781-787.
- Rowlands, D. J., Sangar, D. V. & Brown, F.** 1975. A comparative chemical and serological study of the full and empty particles of foot-and-mouth disease virus. *J. Gen. Virol.*, 26: 227-238.
- Shieh, J. J., Liang, C. M., Chen, C. Y., Lee, F., Jong, M. H., Lai, S. S. & Liang, S. M.** 2001. Enhancement of the immunity to foot-and-mouth disease virus by DNA priming and protein boosting immunization. *Vaccine*, 19: 4002-4010.
- Somasundaram, C., Takamatsu, H., Andreoni, C., Audonnet, J. C., Fisher, L., Lefèvre, F. & Charley, B.** 1999. Enhanced protective response and immuno-adjuvant effects of porcine GM-CSF on DNA vaccination of pigs against Aujeszky's disease virus. *Vet. Immunol. Immunopathol.*, 70: 277-287.
- Ulmer, J. B., Donnelly, J. J., Parker, S. E., Rhodes, G. H., Felgner, P. L., Dwarki, V. J., Gromkowsk, S. H., Deck, R. R., De Witt, D. M., Friedman, A., Howe, L. A., Leaner, K. R., Martinez, D., Perry, H. C., Shiver, J. W., Montgomery, D. C. & Liu, M. A.** 1993. Heterologous protection against influenza by injection of DNA encoding a viral protein. *Science*, 259:1745-1749.
- Ward, G., Rieder, E. & Mason, P. W.** 1997. Plasmid DNA encoding replicating foot-and-mouth disease virus genomes induces antiviral immune responses in swine. *J. Virol.*, 71: 7442-7447.
- Wolff, J. A., Ludtke, J. J., Acsadi, G., Williams, P. & Jani, A.** 1992. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. *Hum. Mol. Genetics*, 1: 363-369.
- Wong, H. T., Cheng, S. C., Chan, E. W., Sheng, Z. T., Yan, W. Y., Zheng, Z. X. & Xie Y.** 2000. Plasmids encoding foot-and-mouth disease virus VP1 epitopes elicited immune responses in mice and swine and protected swine against viral infection. *Virology*, 278: 27-35.
- Wong, H. T., Cheng, S. C., Sin, F. W., Chan, E. W., Sheng, Z. T. & Xie, Y.** 2002. A DNA vaccine against foot-and-mouth disease elicits an immune response in swine which is enhanced by co-administration with interleukin-2. *Vaccine*, 20:26412647.
- Yafal, A. G. & Palma, E. L.** 1979. Morphogenesis of foot-and-mouth disease virus. I. Role of procapsids as virion precursors. *J. Virol.*, 30: 643-649.
- Yankaucka, M. A., Morrow, J. E., Parker, S. E., Abai, A., Rhodes, G.H., Dwarki, V.J. & Gromkowski, S. H.** 1993. Long-term anti-nucleoprotein cellular and humoral immunity is induced by intramuscular injection of plasmid DNA containing the NP gene. *DNA Cell Biol.*, 12: 771-776

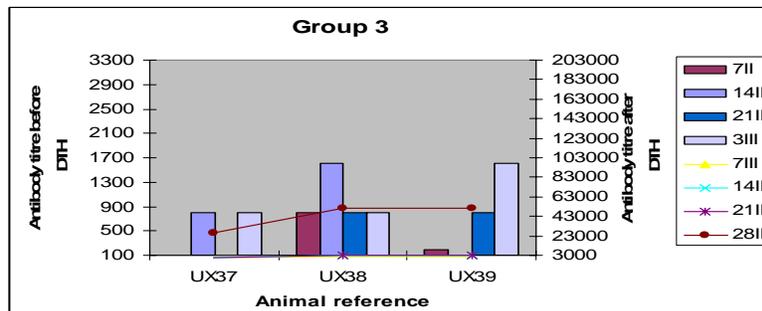
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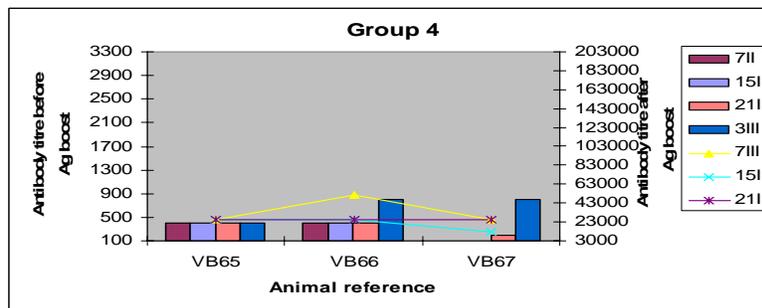
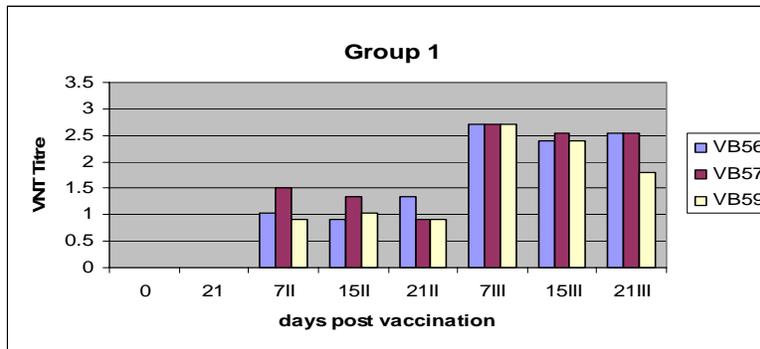
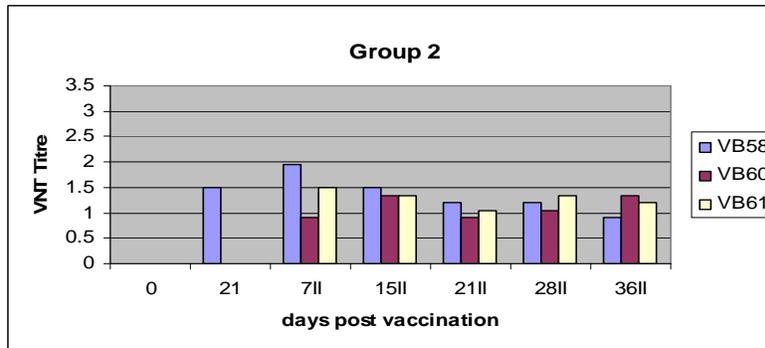


Figure 1. FMDV specific antibody titres in P1 vaccinated pigs. a, b, c and d represent results from groups 1, 2, 3 and 4, respectively. Columns represent antibody titres before the tertiary P1 vaccination in figure 1c or DTH/ antigen boost in figure 1a and d using the left hand Y axis scale, while lines represent antibody titres obtained after tertiary P1 vaccination or DTH/ antigen boost accordingly using the right hand Y axis scale. II: days post the secondary vaccination in groups 1, 3 and 4, but DTH in group 2; III: days post the tertiary vaccination in group 3, or the DTH/antigen boost in groups 1 and 4.

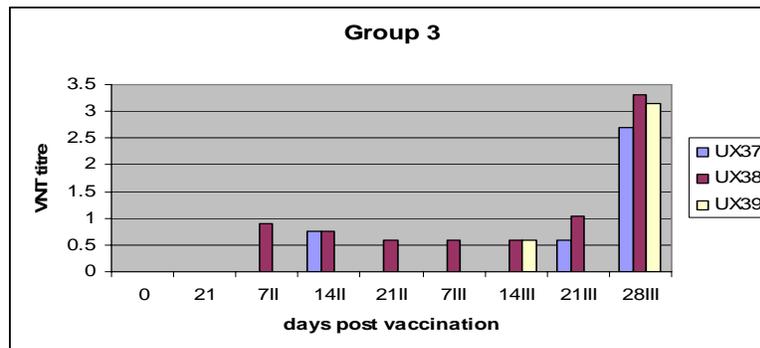
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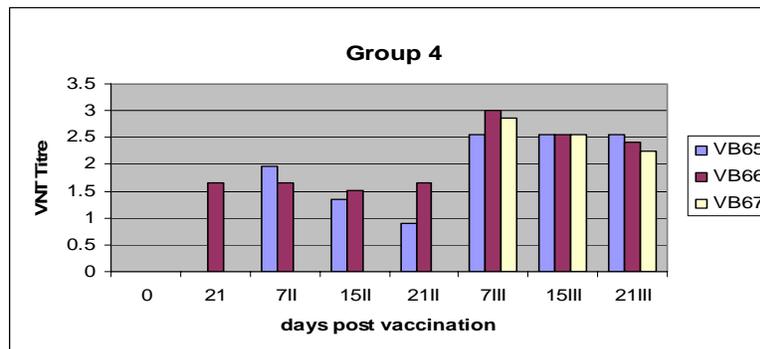
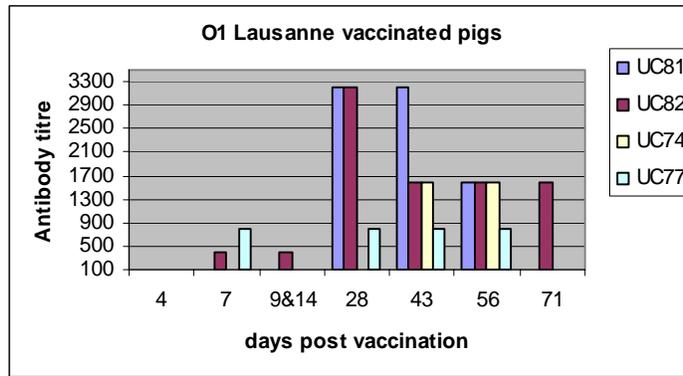


Figure 2. Neutralising antibody titres in P1 vaccinated pigs. a, b, c and d represent results from groups 1, 2, 3 and 4, respectively. II: days post the secondary vaccination in groups 1, 3 and 4, but DTH in group 2; III: days post the tertiary vaccination in group 3, or the DTH/antigen boost in groups 1 and 4.

a)



b)

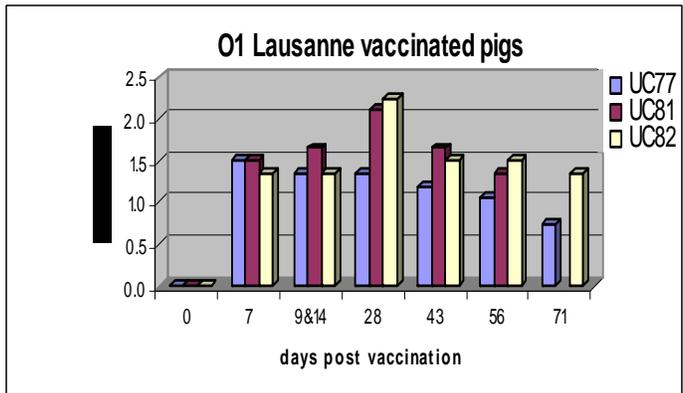


Figure 3. Immune responses induced in O1 Lausanne single vaccinated pigs. a: FMDV specific antibody titres. b: neutralising O1 Kaufbeuren antibody titres.

Table 1. Comparison of average antibody titres among four groups of P1 vaccinated pigs

Groups of P1-2A3C3D vaccinated	P1 vaccination (times)	FMDV protein boost	FMDV antibody titres		Neutralising antibody titres	
			0 days post protein boost	7 days post protein boost	0 days post protein boost	7 days post protein boost
1	Twice	i.d.	367	72533	1.05	2.71
2	Once	i.d.	-	1367	0.5	1.45
3	Three	i.d.	2400	42667	0.55	3.05
4	Twice	i.m.	333	34133	0.85	2.8
O1 lausanne vaccinated	once		2400		1.9	

Table 2. DTH responses in P1 vaccinated pigs

Groups	Animals	FMDV 3D	FMDV antigen	PBS
1	VB56	2	30	0
	VB57	0	40	0
	VB59	2	5	0
2	VB58	13	20	0
	VB60	0	5	0
	VB61	13	20	0
3	UX37	10	5	0
	UX38	0	0	0
	UX39	5	0	0

Immunogenicity and protection conferred by DNA vaccines based on FMDV minigenes in a mouse model

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Abstract

We have studied the potential of DNA vaccines based on viral minigenes corresponding to three major B- and T-cell FMDV epitopes coexpressed with different target signals aiming to optimize their antigenic presentation and thus their immunogenicity. A collection of pCMV plasmids expressing the BTT epitopes [(133-156)VP1-(11-40)3A-(20-34)VP4 from isolate Cs8c1] fused to different target signals (ubiquitin, LIMP-II, a signal peptide (SP) and CTLA-4), was produced. As a first approach, we have studied the immune response induced and the protection conferred by different vaccine candidates in a mouse model. NIH Swiss mice (non-syngeneic) received 3 IM doses of plasmid and neutralizing antibodies in serum after the third dose were analysed by a plaque reduction assay. Vaccinated mice were challenged with the homologous FMDV and viremia at 48 hours post-infection was determined.

From all mice immunized with minigene-bearing plasmids, only one of the animals immunized with the BTT tandem epitopes fused to the signal peptide developed specific neutralizing antibodies. At day 2 post FMDV challenge, while control mice immunized with pCMV showed high titers of virus in their blood the only animal that developed neutralizing antibodies after DNA vaccination was protected against FMDV infection. Furthermore, 7 more animals did not show viremia at 48 h post infection, even in the absence of detectable antibodies prior to challenge. The best vaccine candidate resulted to be the plasmid expressing the 3 viral epitopes alone. While protection was always lower to 25% for the rest of the plasmids, 80% of the mice immunized with pCMV-BTT were protected.

We have demonstrated the protective capacity of a DNA vaccine based on FMDV minigenes in a mouse model. Work must be done to elucidate the mechanisms involved in protection and to determine the protective capacity of our vaccines in natural FMDV hosts.

Introduction

Despite their immunogenicity, peptide vaccines based on a major B cell epitope (B) at the G-H loop of VP1 FMDV capsid protein, have shown to confer partial protection to FMDV (Taboga et al., 1997). Previous work in our laboratory has identified two major T cell epitopes, located in the VP4 structural protein (TVP4) and in the non-structural polypeptide 3A (T3A) (Sobrino et al., 2001). An ideal vaccine should provide a complete immune response: both humoral and cellular responses. In an attempt to improve the immunogenicity of these epitopes after DNA vaccination *in vivo*, we decided to use successful strategies previously described in our lab and in others (Boyle et al., 1997, 1998; Rodríguez & Whitton, 2000). Thus, we fused our antigens to ubiquitin to enhance CTL responses or to the LIMP-II target signal to improve the CD4-T cell responses. At the other hand, in an attempt to optimize B cell responses, we targeted our epitopes to the cell membrane or to the professional antigen presenting cells (APCs) by adding a signal peptide (sp) or by fusing them to CTLA4.

Handling of a large number of vaccine candidates (multiple plasmid constructs) exponentially increase the number of animals to be used, a requirement hard to be afforded with FMDV natural hosts. Thus, as a first approach, a mouse model has been developed and used to assess the immunogenicity of minigene-based DNA vaccines. Despite mice are not natural hosts for FMDV, this species has been shown useful to study the immune response against FMDV (Collen et al., 1989; Fernández et al., 1986).

Methods

Plasmid Generation. A plasmid had been previously constructed carrying in tandem the epitopes B-TVP4, including a ClaI restriction site between both epitopes (B-epitope corresponds to VP1 137-156 and T corresponds to VP4 20-34 residues of the type C FMDV isolate Cs8-c1) (Domenech et al., unpublished results). The sequence corresponding to epitope T3A (residues 11-40 of 3A) was amplified by PCR using a plasmid carrying the whole 3A protein as template, and primers including a ClaI restriction site. This restriction site was used to clone the fragment amplified into the ClaI restriction site between epitopes B and T, to obtain the BTT construct. These amplicons included proper restriction sites at their ends to facilitate their cloning alone or fused to different target signals in the pCMV plasmid (Clontech) under the control of an eukaryotic promoter. Thus, the ORFs were cloned in the following plasmids: pCMV (to express the epitopes alone), pCMV-LIMP II and pCMV-Ubiquitin in which FMDV epitopes were expressed as fusions with LIMP II (Class II targeting) or ubiquitin (Class I targeting), respectively. Furthermore, FMDV epitopes were cloned in plasmids

pCMV-SP (signal peptide) and pCMV-CTLA4 to drive the antigens to the membrane and to APCs, respectively. The sequence of the diverse constructs was confirmed by automatic sequencing and the plasmids were produced free of endotoxins in a large scale (QIAGEN kits) and used to inoculate mice. *In Vitro Expression.* Monolayers of BHK-21 cells were transfected by using the lipofectamine-Plus reagent (GIBCO-BRL). At 48 h after transfection, cells were immunoperoxidase-stained using a MAB against the FMDV B epitope.

Immunization And Infection. All experiments were done using the NIH Swiss strain of mice. For DNA immunization, 3 doses of 100 µg each of plasmid were administered intramuscularly. Two control groups were included: animals vaccinated with 2 doses of 100 µg of synthetic peptide A24, corresponding to positions (138-156) VP1 of FMDV Cs8c1; and animals vaccinated with 2 doses of 10⁶ pfu, BEI-inactivated, of FMDV C-S8c1 (Sáiz et al., 1992). These antigens were IP inoculated as 1:1 emulsion in complete (1st injection) or incomplete (second) Freund's adjuvant. For challenge, 10³ pfu of FMDV Cs8c1 were inoculated in the footpad.

Virus titration. Viremia was followed by infecting monolayers of IBRS-2 cells in M96 plates with serial dilutions of serum and monitoring of the cytopathic effect at 72 hpi. Titre was defined as the reciprocal of the serum dilution (log10) causing cytopathic effect (cpe) in 50% of the wells. Titers lower than 1.3 were considered as negative, since this was the value corresponding to the first dilution tested (1/20).

Immune responses. FMDV specific antibodies were detected both by a trapping ELISA against unpurified CS8c1 virus captured using a polyclonal antiserum and by a plaque-reduction neutralization assay (Mateu et al, 1987). Neutralization titres are expressed as the reciprocal of the serum dilution (log10) that caused 50% of plaque reduction.

For the ICCS assay, spleen cells from mice infected 5 days in advance with FMDV, were stimulated directly *ex vivo* O/N with different viral stimuli. Three hours before incubating the cells with specific, labeled antibodies, Brefeldin A (10 µg/ml) was added to the cultures to avoid cytokine secretion. Cells were surface stained with an anti-CD4 antibody, permeabilized and labeled with an anti-IFN gamma antibody. Double positive cells (CD4+ and IFN +) were plotted by using a Flux Cytometer.

Results

Construction of Plasmids Expressing Viral Epitopes

We first generated different versions of plasmid pCMV expressing the B cell epitope alone (B) and in tandem with TVP4 (BT) or with TVP4 and T3A (BTT) (Table 1). Before testing the plasmids *in vivo* we checked their expression after transfection in BHK-21 cells by immunostaining with a specific monoclonal antibody against the B epitope. Only cells transfected with pCMV-BTT, expressing the three epitopes, showed a specific signal (Table 1 and Fig 1a). Conversely, no expression was detected upon transfection with plasmids pCMV-B and pCMV-BT. Thus, BTT was selected to characterize the potential of minigene-based DNA vaccine to elicit protective immune responses to FMDV.

In order to explore the effect of directing the expression of the BTT tandem minigene to different cell compartments, new plasmids were constructed in which BTT was fused to different target signals. The expression of the B cell epitope was detectable in all cases (figure 1, panel e). Relative to what observed with plasmid pCMV-BTT, a higher number of cells were positive when a signal peptide was fused to the BTT (pCMV-sp-BTT). This increase was not detected when BTT was co-expressed with Ubiquitin, LII or CTLA4 (Fig 1, panels b,c,d).

A Mouse Model For Fmdv Immunization And Challenge

An initial experiment was performed to characterize the viremia and the humoral immune response induced upon infection of different strains of mice with FMDV C-S8-c1. In outbred NIH-Swiss mice infected with 10³ pfu, the virus was detected in blood as soon as at 24 hpi, reaching a peak at 48 hpi and being the virus completely cleared at 72 hpi (figure 2a). 10-20% of the infected animals died between days 3 and 10 p.i. Viral clearance in blood at 3 dpi correlated with the detection of neutralizing antibodies in sera. The titers of neutralizing antibodies reached a peak as early as at 8 dpi, which remained for months (figure 2b).

To characterize the level of protection developed by convalescent mice, animals were challenged with the same dose of FMDV C-S8c1 and both viremia and antibody response were followed at different times post-infection. None of the pre-infected animals showed virus in blood at any time after the re-infection, while control naïve mice showed a typical viremia, as described previously. These results clearly demonstrate that preimmunization of mice with a sub lethal dose of virus confers total protection against a subsequent challenge with the homologous virus. Mice immunized with a single dose of a BEI-inactivated FMDV (C-S8c1) vaccine also induced neutralization titres equivalent to those reached in infected animals (see table 2), and a 100% protection when animals were challenged after two doses of vaccine (see figure 3).

DNA Vaccination

Groups of NIH Swiss mice received 3 im injections of each of the minigene-bearing plasmids, as indicated in Table 2. As controls, 8 mice were injected with the empty plasmid (pCMV) and 4 animals

were infected with 10^3 pfu of FMDV C-S8c1. An additional group of mice was immunized twice with a synthetic peptide corresponding to the B epitope included in the minigene constructs. All mice were bled 10 days after the last inoculation and the sera were used to detect FMDV specific antibodies by ELISA and by a neutralization assay. As expected, pCMV injected mice did not develop specific antibodies, while 100% of the surviving virus-infected mice developed high titres of neutralizing antibodies (>2.4) (table 2). However, from the 30 mice immunized with minigene-bearing plasmids, only one, belonging to the group of mice immunized with the BTT fused to the signal peptide (pCMV-sp-BTT), developed specific neutralizing antibody titers (1.6). One of the five animals immunized with peptide A24 also developed neutralizing antibodies (titer 2.0).

Protection Of Immunized Animals Against Viral Challenge

To evaluate the protective capacity of the minigene-bearing plasmids, all mice were challenged with 10^3 pfu of the homologous FMDV C-S8 at least two months after the last antigen dose. Animals were bled at 48 h post-infection. As expected, the animal that developed neutralizing antibodies after inoculation with pCMV-sp-BTT was fully protected against FMDV infection, as estimated by the lack of viremia at day 2. Interestingly, 7 out of the 30 animals immunized with minigene-bearing plasmids did not show viremia at 48 h post infection, in spite of not having developed detectable neutralizing antibodies prior to challenge (figure 3). The higher protection, in 4 of the 5 animals analysed, was conferred by plasmid pCMV-BTT that expressed the BTT minigene alone. Unexpectedly, protection was lower to 25% for the rest of the plasmids designed to drive the antigens to different antigen presenting pathways, while 50% of the mice immunized with peptide A24 were protected. As expected, control mice immunized with pCMV (empty plasmid) exhibited high titers of virus in their blood at day 2 post FMDV challenge (figure 3). The protection observed by the pCMV-BTT vaccine seemed to correlate with the induction of specific CD4-T cell responses against FMDV. At the time of sacrifice it was possible to detect CD4 T cells that specifically secrete IFN γ in response to *ex vivo* stimulation with the specific FMDV peptides in at least two of the pCMV-BTT protected mice (figure 4).

Discussion

Our previous results demonstrated the capacity of sequences corresponding to T cell epitopes TVP4 or T3A to provide *in vitro* T help leading to the production of neutralizing antibodies, when presented as fusion peptides with the major B cell site from capsid protein VP1 (Blanco et al., 2000; 2001). These results opened the possibility of designing subunit vaccines including B and T cell epitopes relevant for the induction of protection against FMDV. As the efficient synthesis of long peptides is still an unsolved problem, the possibility of expressing these minigene constructs in DNA expression vectors is an interesting possibility to test its immunogenicity in animal models. Here, we have used this approach in combination with a mouse model system for FMDV immunization and challenge that allows the analysis of a considerable number of variables, using statistically significant numbers of animals.

The lack of expression in cells transfected with pCMV-BT and pCMV-B might be due, among other possibilities, to the instability of these short peptides (39 amino acids the longer) in the cytoplasm of transfected cells. The detection of expression when the T3A epitope was included (pCMV-BTT) could be related to an improvement in the stability of the tandem peptide expressed (69 amino acid).

When driving the BTT polypeptide to different cell compartments, expression of the B cell epitope was detectable in all cases (figure 1). When compared to pCMV-BTT, the only construct resulting in a higher number of positive cells was pCMV-sp-BTT in which the presence of a signal peptide seems to improve the stability of the BTT polypeptide (Fig 1, panel e). The lower number of positive cells obtained after transfection with the rest of the plasmids might be explained by the rapid degradation (pCMV-BTT-LII, pCMV-Ubq-BTT; panels b and c respectively) or by the fast secretion of the BTT epitopes to the milieu (pCMV-CTLA4BTT, panel d).

The contribution of the humoral response to the *in vivo* protection against FMDV has been clearly established along the years. In particular, a strong correlation is found in convalescent and conventionally vaccinated animals between neutralizing activity in sera and protection against FMDV challenge (reviewed in Sobrino et al., 2001). The analysis of the inhibition of viremia upon FMDV challenge in the mouse model developed has provided interesting information on the protective immune responses elicited by the pCMV derivatives expressing the different versions of BTT epitopes studied. As expected, the only animal that developed neutralizing antibodies after immunization with pCMV-sp-BTT was solidly protected against FMDV infection. However other animals were also protected in the absence of detectable neutralizing antibodies prior to challenge (table 2 and figure 3). Thus, 4 of the 5 mice that received pCMV-BTT were able to clear the virus upon challenge. Driving BTT to the MHC I and MHC II presenting pathways does not improve either the specific antibody production or the protection conferred. However, fusing the epitopes to a strong signal peptide improved both their *in vitro* expression and the induction of protective neutralizing antibodies. Interestingly, a correlation is observed between protection to challenge and the induction of FMDV specific CD4-T cell responses in mice immunized with pCMV-BTT. We are currently working in the

characterization of the T cell responses elicited by these plasmids as well as assessing the efficacy of these DNA immunization strategies in the pig, an important natural host for FMDV.

Acknowledgements

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References

Blanco E, McCullough K, Summerfield A, Fiorini J, Andreu D, Chiva C, Borrás E, Barnett P & Sobrino F. (2000). Interspecies major histocompatibility complex-restricted Th cell epitope on foot-and-mouth disease virus capsid protein VP4. *J Virol.* 74(10), 4902-7.

Blanco E, Garcia-Briones M, Sanz-Parra A, Gomes P, De Oliveira E, Valero ML, Andreu D, Ley V & Sobrino F. (2001). Identification of T-cell epitopes in nonstructural proteins of foot-and-mouth disease virus. *J Virol.* 75(7), 3164-74.

Boyle JS, Koniaras C & Lew AM. (1997). Influence of cellular location of expressed antigen on the efficacy of DNA vaccination: cytotoxic T lymphocyte and antibody responses are suboptimal when antigen is cytoplasmic after intramuscular DNA immunization. *Int. Immunol.* 12, 1897-1906 .

Boyle JS, Brady JL & Lew AM (1998). Enhanced responses to a DNA vaccine encoding a fusion antigen that is directed to sites of immune induction. *Nature* 392, 408-411.

Collen T, Pullen L & Doel TR. (1989). T. Cell dependent induction of antibody against foot-and-mouth disease virus in a mouse model. *J. Gen. Virol.* 70, 395-403.

Fernandez FM, Borca MV, Sadir AM, Fondevila N, Mayo J & Schudel AA. (1986) Foot-and-mouth disease virus (FMDV) experimental infection: susceptibility and immune response of adult mice. *Vet. Microb.* 12, 15-24 .

Mateu MG, Rocha E, Vicente O, Vayreda F, Navalpotro C, Andreu D, Pedrosa E, Giralt E, Enjuanes L & Domingo E. (1987) Reactivity with monoclonal antibodies of viruses from an episode of foot-and-mouth disease. *Virus Res.* 8, 261-274 .

Rodriguez F & Whitton JL. (2000). Enhancing DNA Immunization. *Virology* 268, 233-238 .

Sobrino F, Saiz M, Jimenez-Clavero MA, Nunez JI, Rosas MF, Baranowski E & Ley V. (2001). Foot-and-mouth disease virus: a long known virus, but a current threat. *Vet Res.* 32(1), 1-30. Review.

Sáiz JC, Rodríguez A, González M., Alonso F & Sobrino F. (1992). Heterotypic lymphoproliferative response in pigs vaccinated with foot-and-mouth disease virus. Involvement of isolated capsid proteins. *J. Gen. Virol.* 73, 2601-2607.

Taboga O, Tami C, Carrillo E, Nunez JI, Rodriguez A, Saiz JC, Blanco E, Valero ML, Roig X, Camarero JA, Andreu D, Mateu MG, Giralt E, Domingo E, Sobrino F & Palma EL. (1997). A large-scale evaluation of peptide vaccines against foot-and-mouth disease: lack of solid protection in cattle and isolation of escape mutants. *J. Virol.* 71 (4), 2606-2614 .

Table 1. Plasmids expressing FMDV minigenes used in this study

PLASMID	VIRAL EPITOPES (FMDV C-S8c1)	EXPRESSION
pCMV	none	Negative
pCMV-B	(133-156)VP1	Negative
pCMV-BT	(133-156)VP1-(20-34)VP4	Negative
pCMV-BTT	(133-156)VP1 - (11-40) 3A - (20-34)VP4	Positive (fig.1)

Table 2. Neutralizing antibodies after inoculation of mice

Inoculum	SN positive ^a / total mice	SN titre
pCMV	0 / 8	---
pCMV-BTT	0 / 5	---
pCMV-BTT-L II	0 / 5	---
pCMV-Ubq-BTT	0 / 5	---
pCMV-CTLA4-BTT	0 / 5	---
pCMV-sp-BTT	1 / 4	1.6
Plasmid mix	0 / 6	---
Peptide A24	1 / 5	2.0
BEI-inactivated virus	5 / 5	> 2.3
C-S8c1 virus	4 / 4	> 2.4

^a values < 1.0 were considered as negative.

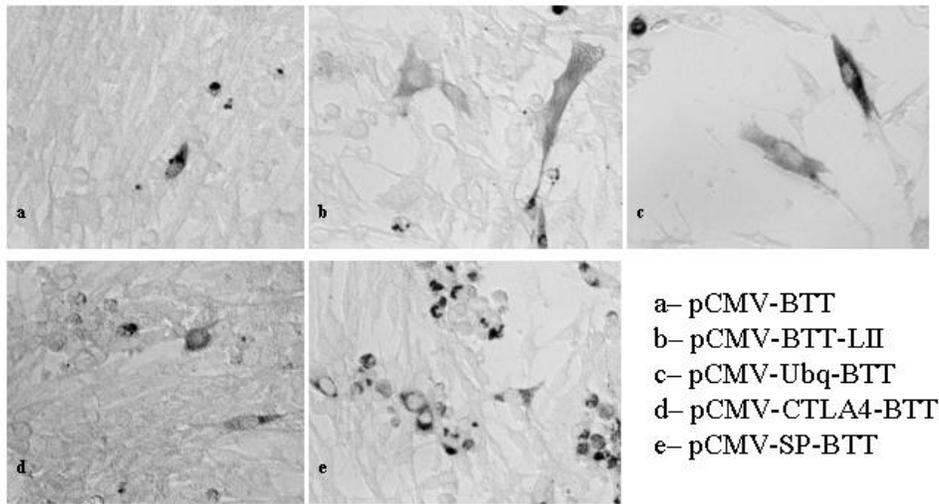


Figure 1. Expression of viral epitopes in BHK-cells transfected with plasmids coding for the BTT epitopes fused to different target signals

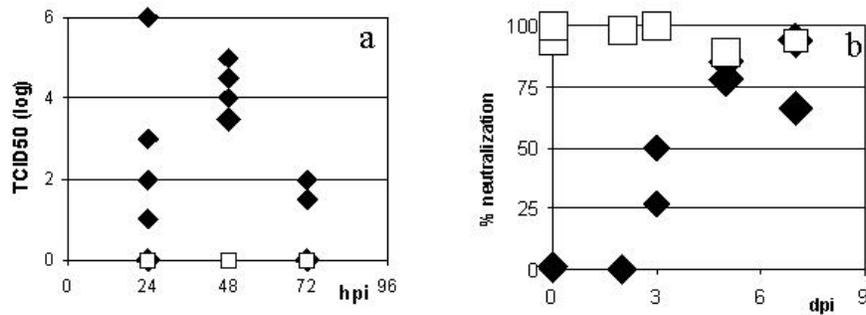


Figure 2. Viremia (a) and development of neutralizing antibodies (b) in Swiss mice infected with FMDV. Black, naïve animal; white, preimmunized animals.

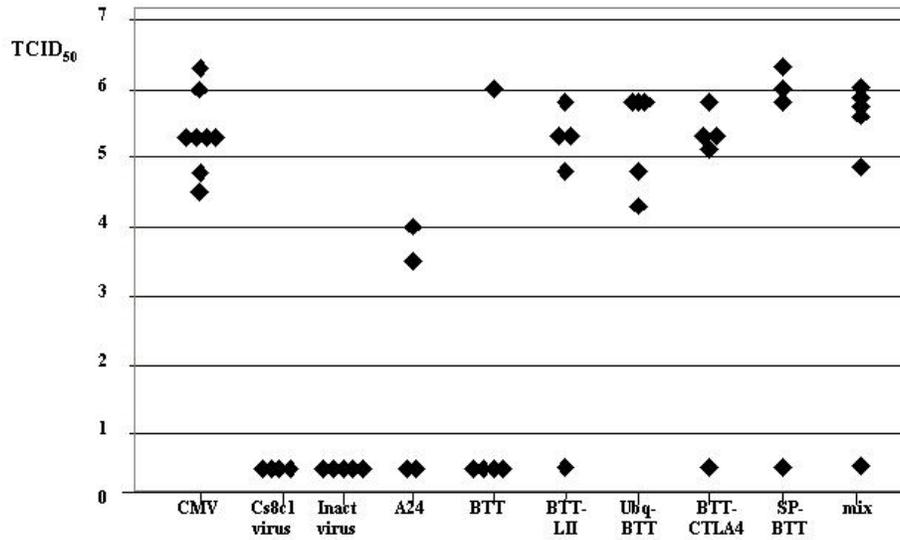


Figure 3. Virus in blood at 48 hpi.

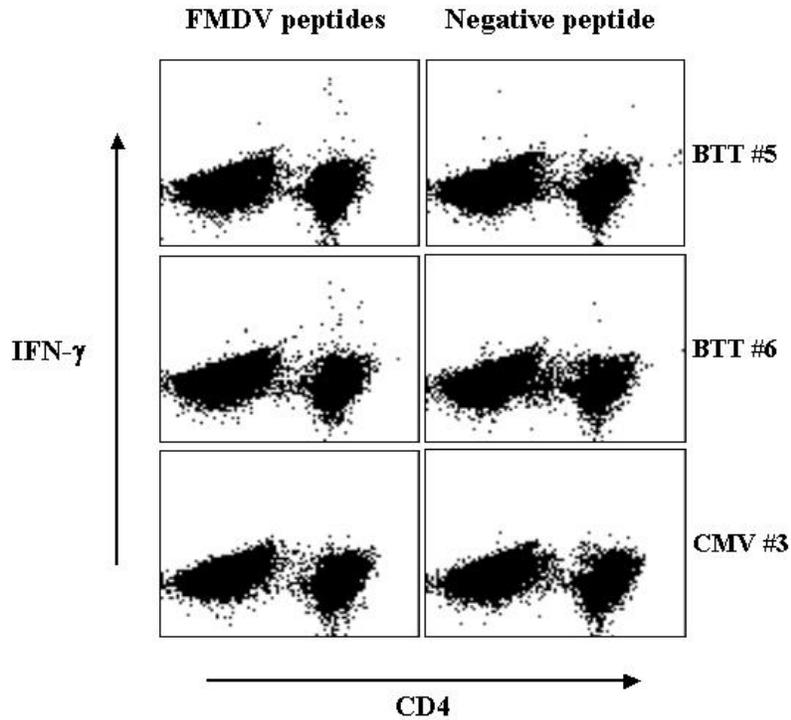


Figure 4. Cell response (ICCS) at 5 days post FMDV challenge .

OIE standards and recommended procedures for Foot and Mouth Disease (FMD)*

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Preventing the spread of disease through international trade is one of the primary objectives of the World Organisation for Animal Health (OIE). This is accomplished by establishing international standards and guidelines that facilitate trade while minimising the risk of introducing diseases such as Foot and Mouth Disease (FMD). The OIE Standards are contained in the *Terrestrial Animal Health Code* (the *Terrestrial Code*) and the *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (the *Terrestrial Manual*).

Since 1995 the standards developed by the OIE were formalised as international standards by the Agreement on the Application of Sanitary and Phytosanitary Measures (SPS) of the World Trade Organization (WTO).

For this purpose the OIE has developed international standards to minimise the risk of the spread of FMD through international trade. These standards include: procedures for surveillance and prompt reporting of FMD outbreaks; requirements that should be met for a country or zone to be defined as free from FMD with vaccination or without vaccination; recommendations for the safe importation of animals, products, semen, and embryos into an FMD free country or zone; guidelines for surveillance for FMD, and the general provisions that countries should meet to reduce the risk of spread of FMD through trade. The *Terrestrial Manual* describes in detail the various tests and vaccines for FMD diagnosis and control. It provides a list of prescribed tests; these are tests that are required by the OIE *Terrestrial Code* for the international movement of animals and animal products and are considered optimal for determining the health status of animals.

During the OIE 71st and 72nd General Session, the International Committee approved a new criteria for OIE listed diseases (international spread, significant spread within native populations, zoonotic potential, emerging disease), notification and epidemiological information, a resolution for the adoption of a new procedure for Validation and Certification of Diagnostic assays (Re. XXIX/71st Gen. Ses.), introduction of the concept of freedom from FMD virus circulation, new standards for FMD vaccines (potency, purity) and FMD diagnostic laboratories, and important advances in the validation of the NSP index test for bovines. Finally the OIE participated in the June meeting of the United Nations Sub-Committee of Experts on the transport of Dangerous Goods (UNSCETDG) in Geneva and presented a document with the amendments to be incorporated in the 2005-2006 ICAO Technical Instructions.

Discussion Paper on the risks posed by FMD carriers occurring amongst vaccinated cattle

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Abstract

The presence of FMD in many parts of the world implicates that veterinary authorities and stakeholders in Europe always will have to accept the risk of incursions of FMD.

Although vaccination by itself does not produce carriers, vaccinated ruminants when exposed to FMD virus some can become carriers (*vaccinated carriers*). When discussing vaccination policies as a means of controlling FMD outbreaks, the perceived "carrier risk" appears to be the main argument against such policy.

Historical evidence shows that healthy bulls occasionally transmitted FMD to susceptible livestock. However, so far such evidence does not exist for vaccinated carriers. Also, under a variety of experimental conditions, transmission of FMD from recovered as well as vaccinated carriers has not been demonstrated. Still, (according to the norms of the OIE Terrestrial Code) if vaccination is used to eradicate FMD it takes longer to obtain the FMD free status than after stamping-out.

Is that policy justified? What is the level of risk of the presence of vaccinated carriers and how does it compare with other FMD risks that the European society and international trade organizations have been willing to take?

The purpose of this discussion paper is:

- to explore if a "benchmark" for acceptable risk can be defined;
- to explore the types and accepted levels of FMD risks Europe has been exposed to in the past;
- to compare, those risks to the levels of risk posed by recovered and vaccinated virus carriers.

We conclude that current policies for controlling outbreaks and the consequences for export trade as yet are not based on risk assessment but on a "zero risk" approach. Reconsideration of that approach is needed to give veterinary authorities and livestock industry the best and safest options for FMD control while enabling safe international trade.

1 Introduction to risks and risk perception

As long as we live we have to deal with dangers or hazards. The *probability* that a danger becomes reality we call a risk. This risk may be dependent on natural or geographic conditions (earthquakes, storms, floods etc.), but also on human action (i.e. eating, crossing a street, driving a car, flying etc.). Humans often accept risks for economical (financial) reasons, or for having certain experiences that are considered as pleasant or exciting or both (e.g. parachute jumping). What one person may experience as an acceptable risk is unacceptable to others.

A risky situation may be more acceptable when it produces clear, demonstrable benefits for certain (powerful) groups or to society as a whole (e.g. transport in general, transport of dangerous chemicals or explosive materials). If people feel that they have control over a situation it is perceived as less risky (e.g. driving a car). When they are not in control, the situation is often perceived as a higher risk (e.g. flying an airplane as a passenger).

The question is *"Is it possible to examine potential risky situations in a qualitative or quantitative fashion and thus give people and their representatives facts on which to base essential (political) decisions intended to minimize particular risks?"*

2 Risks of introduction of FMD

FMD-free countries run a constant risk of introduction of the disease simply because FMD occurs in many parts of the world. Many human activities that increase that risk are accepted or are considered as inevitable, as for instance travel and trade. The last is particularly risky when it comes to trade of animals that are susceptible to FMD and products from such animals.

The events in 2001 clearly showed in South America and in Europe that even importation from countries with an OIE status "free of FMD without vaccination" is not without risks. In other words, trading of animals or animal products means acceptance of the risks of FMD introduction.

We have experienced escapes from diagnostic and research laboratories and vaccine production facilities that were perceived to be safe. In the eighties, vaccines considered to be well inactivated have caused

outbreaks. The recent occurrence of type C in Brazil – after almost 10 years of absence – is another indication that such risks still might exist.

Europe has – under certain conditions - imported millions of tons of meat from areas in the world where the disease occurred. Travel is increasing and with it illegal imports of animals and animal products from all over the world. Even the “circle culling” that has been applied to control FMD outbreaks can be considered a risky undertaking.

With respect to FMD there are a number of ways to look at those risks (Starr et. al. 1976):

- Evaluation of a risk immediately after the occurrence of an adverse event. Results of that evaluation can be used to make policy decisions for eventual similar events in the future (e.g. risks of swill feeding, risks of rest stops for cattle like in Mayenne and risks of markets where FMD has been transmitted).
- Statistical evaluation of risk, based on available data such as series of epidemiological FMD data. In the case of rare events, statistical analysis is often not very useful.
- Analysis of risk scenarios to evaluate the probabilities that something in a procedure may go wrong and the consequences of such a failure. That method uses objective and subjective data, as well as historical information. It makes allowance for the amount of uncertainty of the available data and information. The method is suitable for the analysis of risks related to the import of animal and animal products (Vose, 1997)
- Perceived risk, usually based on intuitive interpretation of anecdotal/historical and/or scientific information. The unknown size of the risk and fear of politicians of being held responsible if things go wrong often lead to excessive safety measures to minimize or prevent that risk (e.g. risks of BSE being transmitted to humans).

3. Risks of carriers

The risk that carriers may cause FMD outbreaks is generally perceived as an unacceptable risk (Salt, 1993). Since vaccinated cattle when exposed to diseased livestock may become healthy carriers (in this paper they will be referred to as *vaccinated carriers*), the importation of vaccinated animals into countries that are free of FMD is prohibited (Article 2.1.1.10 of Appendix 3.8.6 of the OIE Code).

Also, the *perceived* risk of vaccinated carriers is reflected in same Appendix, Art. 2.1.1.7. For a country to recover the free status, the waiting period after an outbreak in accordance with this is:

- sub b) **3 months** after the slaughter of all vaccinated animals where a stamping-out policy and serological surveillance are applied ;
- sub c) **6 months** after the last case or the last vaccination where a stamping-out policy, emergency vaccination **NOT** followed by the slaughtering of all vaccinated animals, and serological surveillance are applied, provided that a serological survey based on the detection of antibodies to nonstructural proteins (NSP) of FMD virus demonstrates the absence of infection in the remaining vaccinated population.

Clearly, the risk that cattle from sub-clinical infected herds outside the culling zones may become carriers is not considered. The risk of vaccinated carriers and the consequences for export trade have been used as arguments against the use of vaccine to control outbreaks or to justify the wholesale killing of healthy vaccinated livestock (like in The Netherlands). Although the bad experiences in Europe with the circle culling practiced during the 2001 outbreaks has favored the use of vaccination to control and eradicate future outbreaks in the EU, export will still be hampered by longer periods which results the loss of markets. No compensation payments are made for these types of damages and losses.

Since we cannot exclude that some vaccinated cattle might come in contact with diseased livestock and thus may become carriers, it is legitimate to ask:

- “How large would be the chance that they become carrier?”
- “How large would be the risk that such vaccinated carriers would come in contact with susceptible animals?”
- “How large would be the risk that, in that case, they will transmit FMD?”

A further question would be: “Would the risk posed by vaccinated carriers be larger than the risk of (sub-clinical infected) carriers remaining after large scale stamping-out operation?” Finally, “How do these risks compare with other risks of FMD introduction that are accepted by the international community?”

4. Accepted risks with respect to FMD introduction

Europe has accepted the risk of FMD virus incursions under various circumstances. However, the veterinary authorities of the European countries have become more and more risk adverse because the

EU was practically FMD free for more than a decade. The discontinuation of systematic vaccination of the cattle in 1992 resulted in fully susceptible populations in Europe. This created a risky situation because several outbreaks occurred on the borders of the EU that were dealt with by zonal or ring vaccination and stamping-out (Sutmoller et al., 2003). Even after the catastrophic outbreaks in the U.K. and The Netherlands in 2001 authorities apparently accept these consequences of the present eradication policies because of trade benefits.

To complicate matters even more, different sectors of the livestock industry have different priorities. For instance, under present regulations, certain livestock sectors may prefer large-scale stamping-out measures, only because export of their products will open up earlier. In addition, supermarket chains do not want products from European animals vaccinated against FMD, because they expect consumer resistance, even though such products have never shown to be a human or animal health risk (Sutmoller et. al 2003, Sutmoller & Casas 2003). It is interesting to note that consumers readily accept meat from vaccinated cattle from South American countries and have done so for decades.

Therefore, in view of these social and economic different interests, any acceptable risk must be defined in terms of: "for whom and under which circumstances".

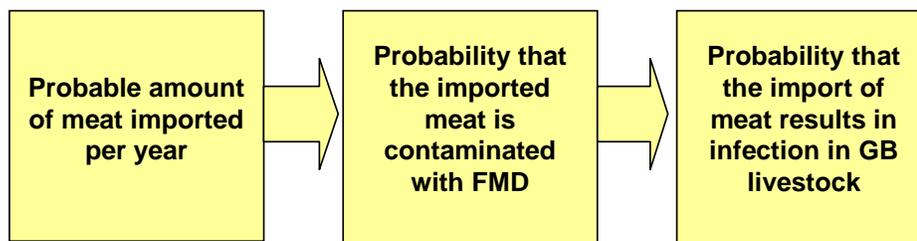
Can we develop a "benchmark" for acceptable risk (of carriers) that would accommodate these different positions? If the level of carrier risk would be equal or less than that benchmark, would that influence the position of the veterinary community with regard to the length of the waiting periods to obtain the FMD free status after the last case of FMD had occurred?

A way to develop such a benchmark might be to look at other FMD risks that Europe has been exposed to and has accepted, such as, the risk of illegal importation of animal products and the risk of legal large scale importation of meat from South America.

5. Risk of importation of illegal animal products.

At the 35th General Session of this Commission, Hartnett et al., presented a paper on a risk assessment for the illegally import of meat and meat products contaminated with FMD virus into Great Britain and the subsequent exposure of GB livestock.

The model consists of three modules each describing a distinct stage in the process that could lead to an outbreak of FMD in GB. The model framework is shown in the figure below:



Together, these modules would represent the various transfer pathways of the virus from the country of origin to the infection of livestock in GB.

In the first module the illegal flow of meat and meat products from different regions was estimated. The estimated gross annual illegal flow into GB would amount to 700 tons of meat and meat products. Ship and airline waste were excluded from the scope of the study.

For the second module, the probability that illegally imported meat or meat products would contain FMD virus was derived from:

- the annually incidence of FMD in each of the countries of origin;
- the length of time the animals have infectious FMD virus in the tissues (viremic animals);
- the virus load of contaminated products;
- the persistence of FMD virus in each contaminated product as a consequence of processing and storage time.

The information on FMD incidence in countries of origin was mainly obtained from OIE and the WRL, but with a factor of 40% for the global average level of under-reporting. The number of viremic animals was calculated from the incidence level.

The estimated amount of contaminated meat and meat products illegally imported per year, according to the results of the second module, had a mean value of 95 kg, with 90% certainty of a volume between 30 and 244 kg per year. However, these values are likely very uncertain and possibly underestimated, in view of the over-simplification of that module.

In addition, the third module was very complex and estimated the probability that through several pathways, contaminated, illegally imported meat and meat products would infect livestock in GB.

The final conclusions, combining the results of the 3 modules were:

- With 90 % certainty there would be a probability of 1 FMD outbreak in GB within a range of 41-1100 years, with a mean of 1 outbreak in 130 years as a result of the illegal import of meat.
- Approximately 95% of the estimated risk would be associated with illegal meat arriving in personal baggage in quantities that are destined for commercial use.

Could the results of above risk assessment be used to approximate the involuntary risk for the whole of Europe as a result of illegally imported meat and meat products?

The following items might be considered:

1. *Flaws in the design of the model.*
2. *European borders are more open than those of GB and have larger links with endemic areas.*
3. *Europe has a much larger number of foreign national residents than GB and a massive tourist interchange of people carrying animal products.*
4. *Absence of database(s) required to estimate inflow of illegal meat into Europe.*
5. *Ship and airline waste.*

Considering the above factors we can conclude that Europe probably is running a risk that is at least a ten times higher than was calculated for GB for illegally imported meat. In that case Europe would run a risk of one outbreak within 4 to 100 years with a most likely probability of one outbreak within 13 years.

6. Risk that Europe has been willing to take with the importation of meat from South America

In addition to the risk caused by illegally imported meat and other animal products Europe has accepted the risk of legally imported meat from South America and some other countries (e.g. Botswana) with sporadic FMD or with a fair chance of FMD introduction.

The importation of South American meat can be divided in 3 different phases of risk:

- Prior to the years 1967-68 the risk that meat from infected animals was exported must have been considerable, because of the many outbreaks in South America and the lack of efficient controls.
- Following the epidemic of 1967-68 in Great Britain, risk reduction measures were introduced that resulted in the development of a high level of technology and inspection in slaughterhouses and packing plants in South America. In spite of the enormous volumes of these imports and many FMD outbreaks that still occurred in the countries of origin, there were no further introductions of FMD.
- During the third phase several of the South American countries obtained the status "FMD free with or without vaccination". The OIE Code established long waiting periods and severe requirements to obtain such status. Presently, in addition, the countries have to demonstrate the absence of infection and circulation of the virus in the livestock population. The perceived risk of carriers, justifiable or not, played a predominant role in the rationale for the development of these rules.

Several papers have described the risk of the importation of meat from South America in qualitative or quantitative terms (Astudillo 1997, CCE/CISFAPS 1997, Suttmoller 2001a, Yu et.al 1997). Basically all use the scenario pathway as shown in Figure 1. The probability of a "Yes" or "No" answer for each of the events in the scenario must be evaluated to obtain the final level of risk of exporting FMD contaminated meat.

To obtain an answer to the first and second question in Figure 1 the epidemiological situation of the exporting country must be evaluated, as well as the number of herds required for the volume of meat to be exported. The third question in Figure 1 evaluates the degree of compliance and probabilities of failure of the EU export requirements. This set of risk mitigation measures are based on strict rules and controls of the source of the cattle and conditions of slaughter plant. They were developed following the extensive FMD outbreaks in the U.K. during the period 1967-1968. In addition, EEC Directives (EEC 1986) required

the maturing of carcasses and removal of bones, lymph nodes and large blood vessels from the meat, in order to destroy or remove FMD virus and reduce the risk of importation of quantities of FMD.

Risk assessments done so have supported the contention that under present EU requirements - inspection at the farm of origin, systematic vaccination of the cattle, slaughterhouse and meat inspection, maturing and deboning of carcasses - import of meat, even from regions that are not FMD free, are an acceptable risk. Carriers are not an issue in this respect (Sutmoller 2001b)

For the past decades the EU has been importing annually some 250,000 tons of meat from the Southern Cone of South America (Argentina, Brazil, Paraguay and Uruguay; data obtained from FAOSTAT, 2004) and has accepted the risk related to that importation.

Astudillo et al. (1997) estimated that if an infected farm would send cattle to the slaughterhouse the probability that a contaminated carcass would pass all inspections when following the EU rules would be just over 1:1000, (90% confidence limit). However, the virus load of the contaminated meat would decrease with a factor of 100 by maturation and deboning.

Sutmoller (2001b) estimated the risk of importing 10,000 ton of meat obtained from a FMD free zone with 500,000 cattle herds, that suffered a limited incursion of FMD. The calculated with probability was $10^{-6.6}$ (1: 4,000,000, 90% confidence limit) that 10,000 tons of meat would contain meat from at least meat from one infected herd. The estimated reduction of the virus load of the contaminate meat due to maturation and deboning was 95%. With an annual importation of 250,000 ton by the EU the probability would be >1: 100,000 that all inspections would fail.

If the zone would have and 500 infected herds per year the risk would be 100 times higher, but with that number of outbreaks meat export operation would already have been put on hold.

Thus the levels of risk for meat imported according to EU rules are extremely small and justifiably acceptable.

7. Carriers as a source of FMD outbreaks

The risk of carriers in the context of this discussion paper is the likelihood of carriers transmitting FMD to susceptible livestock causing new FMD outbreaks. To study this problem we may have to quantify how many carriers remain after the various methods of dealing with an outbreak. A compilation of the results of surveys done after recent outbreaks in Europe and South America might provide such an answer. Next we can ask *"What is the evidence that carriers transmitted FMD to susceptible livestock?"*

7.1 Historical evidence

Fogedby (1963) made an extensive review of historical evidence of carriers transmitting FMD to susceptible animals. We completed the data from his review with other sources (Machado 1969, Salt 1993, Sutmoller et al., 1967, Sutmoller et al. 2003, Thomson 1996) which resulted in Tables 1 and 2

Table 1 shows that for the past 150 years there have only been 9 documented cases in which transmission occurred, 7 of which involved healthy bulls. Thomson (1996) reported on two events in Zimbabwe during 1989 and 1991. The cattle were moved around in Zimbabwe before they reached the herd where the outbreak occurred and there was suspicion that might have transmitted FMD from earlier outbreaks. Although the RNA sequences of the viruses involved were quite similar, there are a number of question marks with respect to the evidence that these animals did indeed cause the outbreaks. The carrier status was not verified for the cattle that supposedly caused the outbreak and only one carrier was found in the herds from which they originated.. The gender of these animals was not reported, but there might have been bulls in the cattle that were moved. In our view the evidence that carriers were involved in these two events is rather disputable.

Table 2 shows field evidence that transmission of FMD by carriers has not been shown in numerous experiments, carried out under a large variety of circumstances, to study FMD transmission from carriers to susceptible contact cattle or pigs. These negative results were obtained even though the total number of overall contact days is very large.

7.2 Controlled transmissions experiments

Controlled experiments to study FMD transmission from carriers to susceptible contact cattle or pigs have all given negative results although the total number of overall contact/days in these experiments was very large as well. Dr Tenzin at Utrecht University is reviewing all the literature and compiling the

information on this subject. A statistical analysis is carried out, but results are not available yet (pers. com. Tenzin & Stegeman, 2004)

7.3 Sexual transmission

It is striking that bulls are involved in practically all the historical cases with circumstantial evidence of carriers causing outbreaks in susceptible herds (Table 1). *"Is there other evidence that in the past carrier bulls could have been responsible for transmission of FMD?"*. Pustiglioni (1973) reported that 7 of 22 bulls that had not suffered from FMD for at least 6 months had FMDV in their semen. There are no reports either to support or contradict these findings. More recently, field evidence was reported of sexual transmission from carrier buffalo bulls to susceptible animals (Thomson, 1996). Bastos et al. (1999) suggested that sexual transmission of the disease from carrier buffalo bulls to buffalo cows or domestic cows could occur, because FMD virus SAT3 was isolated from both semen and from sheath washes from a naturally infected African buffalo. They considered this a persistent infection since the virus genotype was not recently circulating in the buffalo herd. They speculated that virus in the sheath-wash of the buffalo bull presumably originated from the mucosal epithelial tissues of the prepuce. The finding could explain the mechanism of the maintenance of FMD of the SAT virus types in small isolated buffalo populations. Further investigation of FMD transmission by sexual contact with recovered bulls is urgently required. To our knowledge, in the numerous "regular" contact experiments sexual contact was never considered or attempted. However, if sexual transmission of FMD by recovered bulls would occur, it could explain why the contact experiments only produced negative results. Most importantly, it also could explain most of the historical evidence on which the perceived carrier risk is based. It might be the missing piece of the puzzle that has kept us occupied for the past 50 years!

Discussion

In the late fifties and early sixties, in the absence of systematic vaccination in endemic areas, the percentages of carriers in the cattle populations were very high (Van Bekkum, 1959, Suttmoller & Gaggero, 1965). However, surveys by staff of the Panamerican FMD Center in later years showed a very much reduced percentage of carriers in endemic areas when vaccine was commonly used to control FMD outbreak situations. In addition to vaccination, other zoo-sanitary measures such as - immediate quarantine and slaughter of outbreak farms and a movement stand still of animals - will further reduce the number of carriers in a vaccinated population.

We have pointed out in a previous paper (Barteling and Suttmoller, 2002) that over the past decades the control of more than 15 outbreaks by vaccination was always successful and that there have been no cases of recurrent disease. Also in Uruguay recurrent disease did not occur even though the extensive outbreak in 2001 was controlled by vaccination of cattle only, sharing the pastures with young cattle, pigs and millions of unvaccinated sheep.

Thus, although all available evidence indicates that the probability of transmission of FMD by vaccinated carriers to susceptible livestock is extremely low - in fact there are no well-documented cases - still this risk is perceived as serious and justifies a longer ban on export trade than when outbreaks are not controlled by stamping-out. It is this perception that in its consequences has led to the killing of millions of cattle and sheep (in the U.K.) and to the killing of hundreds of thousands of healthy vaccinated livestock (in The Netherlands).

However, levels of risk - not perceived risks - must be considered when developing regulations and guidelines for the international movement and trade in animals and animal products. It must also be the most important consideration when deciding on the various options to deal with an outbreak.

Export restrictions of animals and animal products place a heavy penalty on the use of FMD vaccine. As a consequence countries with an important export of livestock or livestock products have - for the earliest regaining of their FMD free status - omitted the use of vaccine after an incursion of FMD. If vaccination had been used, healthy vaccinated cattle were slaughtered in order to avoid long export bans. It is curious that vaccinated pigs also must be destroyed even though pigs have not been shown to become carriers.

However, in all those cases where outbreaks of FMD were controlled by vaccination without slaughter of the vaccinated animals no recurrent FMD occurred caused by vaccinated carriers (Barteling and Suttmoller 2002). Also, products from these animals went into the normal consumption circuit without causing new outbreaks. Suttmoller & Casas, 2003 concluded that with adequate protocols products from vaccinated animals represent an acceptable, "close to zero" animal health risk.

By further analysis of the outbreak in Uruguay (Sutmoller et al., 2003) we can try to get some insight in the size of the risk represented by carriers.

In short, during 2001 Uruguay suffered an extensive outbreak of FMD with over 2057 infected farms among total of 47,057 livestock farms of which 28.795 had cattle and sheep. Only in the first cases stamping-out was applied (total of about 7000 animals were killed) with an immediate movement control of all livestock. At the height of the epidemic there were 40-60 new cases per day with some 20.000 animals. Vaccination was started within a week after the start of the outbreak, first as ring vaccination of the cattle population, but soon vaccination was extended to whole cattle population. Sheep were not included in these vaccinations, even though cattle and sheep used the same pastures. After the first vaccination round the movement restrictions were relaxed, followed by a revaccination of cattle. The epidemic lasted for a total of 8 months.

	Cattle	Sheep	Pigs
Livestock population Uruguay	11,080.980	10,801.376	293,874
Population at risk*	1,518,965	947,879	7,598
Clinically diseased	76,579	236	112
Attack rate	5%	0.02%	
Average farm size of population at risk	738	460	

*population at risk: animals on infected farms and obvious contacts.
(Sources MGAP 2002a & 2002b, Cámara de Representantes 2001)

According several authors (a.o.: Salt 1993, Sutmoller & Gaggero, Sutmoller et al. 1967) it is most likely that about 50- 90% of the susceptible cattle that became clinically diseased on the 2057 infected farms developed the carrier state. In addition there must have been contacts with the clinically affected animals, both cattle and sheep that also resulted in carriers. This means that we can safely assume that during the epidemic some 50.000 carriers have been generated. These carriers used common pastures with unvaccinated sheep (often the sheep/cattle ratio is 1:1, but often higher) and in several districts roaming pig are numerous. Also, in any vaccinated population there are calves with decreasing maternal antibody levels becoming susceptible for the disease. *Still there was no recurrent disease in the year following the last case.* From that we can conclude that one carrier animal represents a risk of transmitting disease in Uruguay was less than 1: 50.000. Of course this risk decreases with time. Systematic vaccination of the cattle population as done in Uruguay also will accelerate the decrease of the number of carriers.

From all the other outbreaks occurring globally during the past decades that were controlled by vaccination - all with no incidents of recurrent disease - we may conclude that likely this risk is even less

In a relatively limited outbreak situation - like the one in The Netherlands – that is rapidly controlled the number of vaccinated carriers will be small when by stamping out of diseased herds is being applied together with immediate stand-still of all livestock movements and ring or zonal vaccination. Elbers et al. (2003) made a sero-survey of wildlife in the epidemic area. For that survey samples were tested from suspect wild deer and farmed deer thought to be at risk or possibly exposed to FMD virus. All samples were negative, but there still the existence of a small number of carriers in the cattle population can not be excluded.

If in the Netherlands the vaccinated animals (and - for arguments' sake – 100 carriers) had been left alive - then the likelihood of carriers causing a recurrence of FMD would be 1:500 (one outbreak as a consequence of 500 epidemics of the size of the 2001 outbreak). However, in view of the findings of Elbers et al. (2003) the total risk may be much smaller – how much smaller we don't know.

This risk could have been further reduced by screening for potential carriers by an a-NSP test and removing potential carriers from the herds. The remaining carrier risk would have been magnitudes smaller and of the order of other accepted FMD risks. Therefore, the use of vaccine should not be hampered by additional trade restrictions, a conclusion that was already made by other arguments (Barteling and Sutmoller, 2002). Also, if rules for export trade had been based on risk evaluation, in the Netherlands the destruction of some 250,000 healthy vaccinated cattle would not have been necessary

In addition, whatever timeframe for the waiting periods to regain the FMD-free status would be agreed upon, it is crucial that after any outbreak, the veterinary service should - to the satisfaction of the international trading community - show the absence of FMD virus circulation, before normal export can be resumed. However, trade in animal products can be resumed as soon as the survey is satisfactorily completed and the absence of FMD virus circulation has been proven.

Conclusions and Recommendations

The subjects that have been considered in this discussion paper can be summarized as follows:

- Science-based risk assessments should be made to compare the different risks associated with different eradication methods used in animal and animal products.
- For illegally imported meat Europe may run a risk of one outbreak within 4 to 100 years with a most likely probability of one outbreak occurring in 13 years.
- For the importation of 250,000 ton of meat from South America by the EU, produced in accordance with OIE rules and guidelines, the probability is extremely low (less than 1:100,000) that this volume contains meat from at least one infected herd. Carriers do not contribute to that risk.
- One carrier animal represents a risk of transmitting FMD to susceptible contact animals that is likely much less than 1:50,000 (there are no well documented cases of transmission).
- Considering that the risk posed by vaccinated carriers is extremely low and considering the levels of risks that Europe of illegally and illegally imported meat has accepted, the policy of restrictions on export trade when part of the livestock population is vaccinated for controlling outbreaks should be reconsidered.
- If serological surveys after an outbreak are carried out rapidly and efficiently, it shows the international community, that veterinary and laboratory services are efficient and well-organized. Because this is one of the most important risk reduction factors within the risk equation, this element should be fundamental for recovery or obtaining the FMD-free status. Export restrictions should be lifted as soon as the tests have been carried out with negative results.

If the vaccination option is used at an early stage, it will cause the least disruption of social and economic life. If outbreaks are kept limited, e.g. by vaccination, with limited consequences for export trade, it will stimulate veterinary services and stakeholders to notify the disease and to take proper action in the earliest stage possible. This will be another element to safeguard the international community from spread of this disease.

Also, we strongly recommend to investigate whether carrier bulls are able to transmit disease by the sexual route.

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References

- Astudillo V., Sutmoller P., Saraiva V. & López A.** 1997. Risks of introducing foot and mouth disease through the importation of beef from South America. *In* Contamination of animal products: prevention and risks for animal health (P. Sutmoller, ed.). *Rev. sci. tech. Off. int. Epiz.*, **16** (1), 33-44.
- Astudillo, V., Cané, B., Geymonat, D., Kroetz, I. A., Trapani, C., Sutmoller, P., Zotte, A. & Gimeno, E.** 1995. Risk assessment and risk regionalization based on the foot and mouth disease surveillance system in South America. Presented at the OIE Conference on Risk Assessment, Animal Health and Trade, Paris, France, May 23-25.
- Barteling, S.J. & Sutmoller, P.** 2002. Culling versus vaccination: challenging a dogma in veterinary science. Rep. Session Res. Gr. Standing Techn. Comm.Eur. Commission for the Control of FMD, Cesme, Izmir, Turkey, FAO Rome, 113 -121.

Bastos A.D.S., Bertschinger H.Y.J., Cordel C., van Vuuren de W.J., Keet D., Bengis R.G., Grobler D.G. & Thomson G.R. 1999. Possibility of sexual transmission of foot-and-mouth disease from African buffalo to cattle. *Vet. Rec.* 145, 3, 77-79

Bekkum J.G. van, Frenkel H.S., Frederiks H.H.J. & Frenkel S. 1959. Observations on the carrier state of cattle exposed to foot-and-mouth disease virus. *Tijdschrift voor Diergeneesk.*, 84, 1159-1164.

Cámara de Representantes 2001 Comisión de Ganadería, Agricultura y Pesca.- Foro "Lucha contra la Fiebre Aftosa", Palacio Legislativo, 8 de noviembre de 2001, , pp. 65

Casas Olascoaga R., Gomes I., Rosenberg F.J., Auge de Mello, P., Astudillo, Vicente. & Magallanes, N. 1999. Fiebre Aftosa, Book i-xv 1-458 Editora Atheneu, Sao Paulo

CCE/ CISFAPS. 1997. *Qualitative Risk Assessment of Foot and Mouth Disease Virus Introduction Through Importation of Deboned Beef from Argentina.* Center for Computational Epidemiology & Center for the Integrated Study of Food Animal and Plant Systems, Tuskegee University. Final Report Submitted to the USDA/APHIS, June-July 1997, pp 56

Elbers A.R.W., Dekker A., Dekkers L.J.M. 2003 Serosurveillance of wild deer and wild boar after the epidemic of foot-and-mouth disease in the Netherlands in 2001. *Vet. Rec.* November 29, 2003

European Communities 1986. Investigation on the possible effect of electrical stimulation on pH and survival of foot-and-mouth disease virus in meat and offals from experimentally infected animals. Com. of European Communities, Brussels, 44 pp.

Fogedby E: 1963. Review of epizootiology and control of foot-and-mouth disease in Europe 1937-1961. *Eur Comm Control of FMD*, FAO, Rome, 128pp

Hartnett E., Seaman M., Adkin A., Cooper J., Watson E., Cox T. & Wooldridge M Risk Assessment for the Import of Meat and Meat Products Contaminated with Foot and Mouth Disease Virus into Great Britain and the Subsequent Exposure of GB Livestock. 35th General Session of EU FMD Commission, (also available as PDF documents at www.defra.gov.uk/animalh/illegal)

Machado, M., 1969. Aftosa – A historical survey of foot-and-mouth disease and inter-american relations. State University of New York Press, Thurlow Terrace, Albany, NY 12201, 1969. Lib. of Congress cat. Card # 69-11317

MGAP 2002a. Uruguay, "Foot and Mouth Disease Report" 22nd March 2002 , Direccion General Servicios Ganaderos, MGAP, Uruguay Pp 1-10 Submitted to European Union and OIE.

MGAP 2002b. Ministerio de Ganadería, Agricultura y Pesca.- "Uruguay Proposal to the Office International des Epizooties (OIE) International Animal Health Code, Chapter 2.1.1. Foot and Mouth Disease", 31 October, 2002, pp. 20.

MORLEY, R.S. 1993. A model for the assessment of the animal disease risk associated with the importation of animals and animal products. *Rev. sci. tech. Off. int. Epiz.*, 12(4), 1055-1092

Office International des Epizooties (OIE) 2003. Terrestrial Animal Health Code, 11th Ed. OIE, Paris.

Pullar 1965

Pustiglione Netto L. 1973. Carriers in foot-and-mouth disease. *Biologica* (Sao Paulo), **40**, 14-16.

Salt J. S. 1993. The carrier state in foot and mouth disease: an immunological review. *Br. vet. J.*, 149, 207-223.

Starr, C, Rudman R. & Whipple, C. 1976. Philosophical Basis for risks analysis. *Annual Review of Energy*, 1, 652-662

Sutmoller P. 2001a. Assessment of the risk of foot-and mouth disease from the importation of fresh/frozen deboned meat originating from the Brazilian states Rio Grande do Sul and Santa Catarina, 28pp

Sutmoller, P. 2001b. Importation of beef from countries infected with foot and mouth disease: a review of risk mitigation measures *Rev. sc. Tech. Off. Int. Epiz.*, **20** (3), 715-722,

Sutmoller P. & Casas Olascoaga R. 2002. Unapparent foot and mouth disease infection (sub-clinical infections and carriers): implications for control. *Rev. sci. tech. Off. int. Epiz.*, 2002, 21 (**3**), 519-529

Sutmoller P. & Casas Olascoaga R. 2003. The risk posed by the importation of animals vaccinated against foot and mouth disease and products derived from vaccinated animals: a review. – *Rev. Sci. Off. Int. Epiz.*, 2003, **22** (3), 823-835

Sutmoller P., Barteling S.S., Casas Olascoaga R. & Sumption K.J. 2003. Review: control and eradication of foot-and-mouth disease. *Virus Res.*, **91**, 101-144.

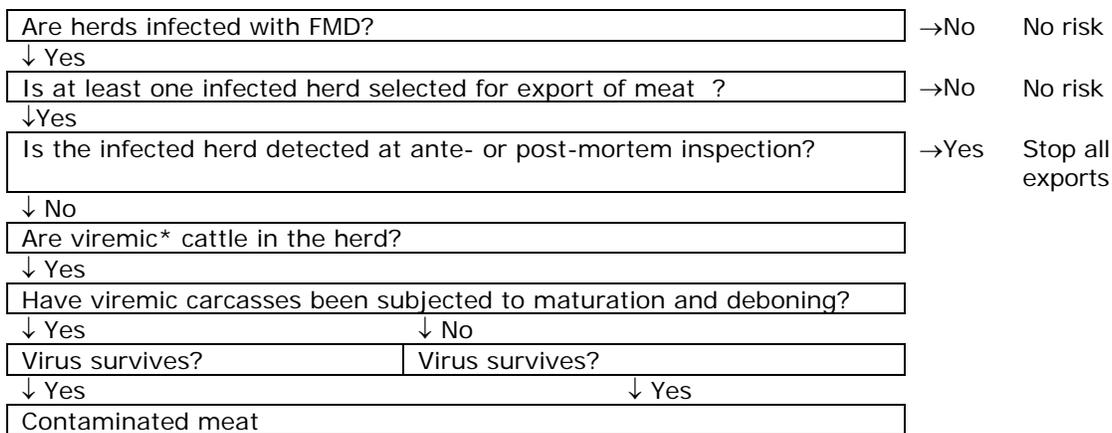
Sutmoller P., Cottral G.E., & McVicar J.W. 1967. A review of the carrier state in foot-and-mouth disease. U.S. Livestock Sanitary Assoc. Proc. 71, 386-395

Sutmoller P. & Gaggero A. 1965. Foot-and-Mouth disease carriers. *Vet. Rec.*, 77, 968-969

Thomson G.R. 1996. The role of carrier animals in the transmission of foot and mouth disease. *In* 64th General Session of the International Committee, Office International des Epizooties (OIE), 20-24 May, Paris. OIE, Paris, 34.

Vose D.J. 1997. Risk analysis in relation to the importation and exportation of animal products. *In* Contamination of animal products: prevention and risks for animal health (P. Sutmoller, ed.). *Rev. sci. tech. Off. int. Epiz.*, **16** (1), 17-29.

Yu P., Habtemariam T., Wilson S., Oryang D., Nganwa D., Obasa M. & Robnett V. 1997. A risk-assessment model for foot and mouth disease (FMD) virus introduction through deboned beef importation. *Prev. vet. Med.*, **30** (1), 49-59.



* Carcasses of cattle with virus circulating in the bloodstream (viremia) are the main concern because those animals will have virus in the muscles, lymphnodes, bone marrow, organs etc. In addition, during exsanguination, slaughter and processing of these animals most likely gross environmental contamination of the slaughter plant will take place.

Figure 1. Scenario Pathway for the assessment of risk of FMD contaminated meat being exported. The probability of a “Yes” or “No” answer for each event must be evaluated to obtain the final level of risk.

Year	Country	Animals involved	Time after being recovered/sub-clinical infected	Time after introduction in susceptible herd
1871/ ' 72	Australia (1)	Bulls from U.K.	unknown	unknown
1897	Sweden (2)	Bull from The Netherlands	unknown	2 weeks
1898	Sweden (2)	Bull from The Netherlands	unknown	5 months
1924	U.K. (2)	Bull and heifer	7 months	1 month
1926	U.K. (2)	Bull and cow	15 months	8 months
1926	U.K. (2)	Bull and heifer	15 months	8 months
1946	Mexico (3)	327 Brazilian Zebu bulls	unknown	2 month
1989	Zimbabwe (4)	Cattle* Zimbabwe	2 years	unknown
1991	Zimbabwe (4)	cattle* Zimbabwe	2 years	Unknown

* Gender of animals not reported.

Table 1. Outbreaks with circumstantial evidence that they are caused by carriers as reviewed by Pullar, 1965 (1), Fogedby, 1963 (2), Machado 1969 (3), Thomson 1996 (4).

Year	Country	Animals involved	Time after being recovered/sub-clinical infected	Size of susceptible contact herd
1914	U.S.A. (2)	740 cattle	3 months cattle 3½ months pigs	50 (young) cattle 50 pigs
1924-'27	Sweden (2)	1500 cattle	1 m - 2½ year	> 10.000 cattle
1926	Argentina (2)	600 cattle	20 days	50 cattle
1926 and following	Argentina (2)	100 – 800 cattle	> 15 days	> 1000 cattle
1928	Switzerland	20 cattle	2-12 months	35 cattle 4 pigs
1945	Mexico (3)	120 Brazilian Zebu bulls	unknown	Mexican national herd
1951 - 1957	South Africa (2)	Approx. 250.000 (aphtization) cattle	4-5 months	Free movement into susceptible herds

Table 2. Experience from the field with potential carriers where no transmission of disease occurred as reviewed by Fogedby 1963 (2), Machado 1969 (3)

The relationship between age and the likelihood of persistence in FMDV infected cattle

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Abstract:

Introduction: After the acute phase of infection a proportion of cattle infected with foot-and-mouth disease virus (FMDV) may become persistently infected and asymptotically carry the virus in their pharyngeal regions for months or years. The host factors that enable this state are not known, but several experiments done previously in our laboratory with cattle of different ages in experiments designed for other purposes have indicated that the percentage of carriers in cattle may be lower in younger than in older animals. The present paper provides evidence of age at the time of infection as a factor in the likelihood of persistence of FMDV. **Materials and Methods:** Nineteen cattle that were between 78 and 176 days old at the time of virus exposure and 13 cattle that were between 221 and 355 days old at the time of virus exposure old were infected with the type O UKG/34/2001 either by intradermo-lingual injection or by direct contact. Clinical observations were performed and the presence of virus in pharyngeal regions (probang samples) assessed by virus isolation on BTY cells and/or fluorogenic PCR (Taqman) assays. **Results:** All animals were infected with FMDV and had virus in their probang samples for at least 7 days after virus exposure. Virus was cleared from the pharynx of cattle at different rates. About 46% (5/11) of animals that were older than 220 days when exposed had virus in their pharynx 28 days after exposure to virus compared to about 7% (1/15) of cattle younger than 180 days. **Discussion:** When exposed to FMDV, cattle younger than about 6 months appear to resist persistent infection, but those older than 7 months are more likely to become carriers. These observations provide opportunities to study the mechanisms for the establishment of persistent infections comparing FMDV infection in these groups of cattle.

Introduction:

Foot-and-mouth disease (FMD) is a severe vesicular disease of cloven-hoofed animals and pigs and has a reputation for rapid and extensive transboundary spread and severe economic consequences for the countries affected (Coetzer *et al.*, 1994). The virus that causes FMD belongs to the *Aphthovirus* genus of the *Picornaviridae* family that are non-enveloped, icosahedral viruses with positive sense RNA genomes. Although the genus consists of 7 serotypes of FMDV O, A, C, Southern African Territories (SAT) 1, SAT 2, SAT 3 and Asia 1, they produce the same disease. Infection with any strain may lead to the production of a proportion of persistently infected ruminants that are symptomless carriers of disease (Sutmoller and Gaggero, 1965; Alexandersen *et al.*, 2002a). In natural infections initial FMDV replication most likely occurs in the epithelia of pharyngeal regions (Burrows *et al.*, 1981; Zhang and Kitching, 2001). A viraemia and generalized infection follow that lead to viral replication and typical lesion formation in the squamous epithelia of the foot, mouth and tongue. This is followed by an antibody mediated clearance of virus from the body. However, in some animals complete elimination does not occur from all organs at the same rate and persistently infected animals that have no detectable virus in any other tissues have virus in their oesophageal-pharyngeal (OP) fluids later than 28 days after infection. The host factors that enable this state are not known. Rates of persistence in different groups of non-vaccinated cattle experimentally infected with Type O FMDV have varied between 17 to 77% (Salt *et al.*, 1996; Zhang *et al.*, 2004). Rates of persistence may also vary with species and the maximum duration of the carrier state ranges from 2 months in water buffalo to 5 years in African buffalo, and 3.5 years in cattle (Alexandersen *et al.*, 2002a). Several experiments done previously in our laboratory with cattle of different ages in experiments designed for other purposes indicated that the percentage of carriers in cattle may be lower in younger than in older animals, and recent studies have indicated that the rate of clearance of FMDV from the pharyngeal region may be related to the likelihood of persistence (Zhang *et al.*, 2004). This paper provides targeted evidence of age at the time of infection as a factor in the likelihood of persistence of FMDV.

Materials and Methods:

In two separate experiments a total of 32 animals of which 19 were younger than 180 days old, called the young group, and 13 that were older than 220 days old called the old group, at the time of viral exposure were used to study persistence of FMDV type O strain UKG 34/2001. In two separate but similar experiments 4 groups of 4 cattle were housed in each room of the microbiologically secure isolation unit. Two cattle in each room were intradermo-lingually inoculated with 0.5 ml of inoculum containing $10^{5.9}$ TCID₅₀ of virus as described previously (Alexandersen *et al.*, 2002b). The other cattle

were allowed free and direct contact with the inoculated cattle. The clinical progress of animals was monitored and scored daily as described by Quan *et al.*, (2004) and Alexandersen *et al.*, (2003); as were rectal temperature measurements, the presence of viraemia, and the presence of infectious virus in OP fluid (probang) samples. Some cattle were removed from the groups at 7, 35 and 59 days after exposure for the collection of tissues for other studies and cattle removed 7 days post infection (dpi) are not included in the calculations.

Blood was collected periodically in 15 ml vacutainer tubes and serum was separated for testing. OP fluid samples were collected with probang cups into equal volumes of 1x MEM with glutamine and 5% foetal calf serum, pH 7.2. Both serum and OP fluid samples were stored at -70°C before use. The infectious viral loads in OP fluid samples were quantified by virus isolation in primary bovine thyroid (BTY) cells (Snowdon, 1966). The magnitude of viraemia was quantified by fluorogenic real-time RT-PCR (Taqman) assays essentially as described previously (Alexandersen *et al.*, 2002c; Zhang and Alexandersen, 2003), as they were in OP fluids samples.

Results:

All experimental cattle became infected with FMDV and developed some clinical signs of disease. Inoculated animals developed disease 1-2 days after inoculation while animals in contact with them developed signs about 3-4 days later. The mean clinical scores of older inoculated cattle peaked at 2 days after inoculation (score=3) and, in the same age group, the animals that were in direct contact peaked 6 days after exposure (score=3) to inoculated cattle. In contrast mean peak clinical scores were seen on day 5 for both the inoculated and the direct contact cattle in the younger cattle although the inoculated scored higher at 4 than the direct contacts which scored 2. Mean rectal temperatures in young cattle that were inoculated peaked 2 days after infection at 40.2°C while those in contact peaked at day 4 at 39.2°C . In older inoculated cattle the mean peak temperature was 40.9°C and occurred on day 2 while the older cattle in direct contact peaked on day 3 at 39.4°C .

Most cattle had virus in their OP fluid samples for at least 5 days after virus exposure. After the initial deposition of virus by inoculation, peak viral amounts in probang samples were detected 2-4 dpi for inoculated and 3-6 dpi for contact animals. Persistent infections in carrier animals were confirmed by the demonstration of infectious virus in the OP fluid samples taken 28 dpi or later. About 46% (5/11) of animals that were older than 220 days when exposed had virus in their pharynx for more than 28 days after exposure to virus compared to about 7% (1/15) of cattle younger than 180 days.

In one of the experiments, mean peak viral loads in OP fluid samples from inoculated cattle regardless of the age group was 2 days post inoculation, but mean peak viral load of young contact infected animals of which none were carrier was 5 days post inoculation compared to 3 days post inoculation for similar older cattle. In these groups virus was cleared from the pharynx at different rates where young cattle had a mean clearance rate of 0.047 with a half-life of virus presence of about 15 hours compared to 0.016 and 43 hours, respectively, for older cattle.

Discussion:

When exposed to FMDV, cattle younger than about 6 months appeared to resist persistent infection, but those older than 7 months were more likely to become carriers. Clinical and experimental factors were examined in order to provide some explanations for these differences, and overall, the onset of disease was more rapid and more severe in the older cattle which were more evident in the cattle infected by direct contact. Younger cattle eliminated the virus more efficiently than older cattle, but this could also represent either the inability of the virus to influence this environment towards persistent infection or a host factor that inhibits persistence. These observations and other work presented at this meeting by our research group suggest that host factors and viral factors may be important in the establishment of persistence, and as more data becomes available in the course of other experimental work, the definition between old and young cattle and the likelihood of persistence will become clearer. This phenomenon will provide opportunities to study the mechanisms for the establishment of persistent infections comparing FMDV infection in these groups of cattle.

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References:

Alexandersen, S., Quan, M., Murphy, C., Knight, J. & Zhang, Z. 2003. Studies of quantitative parameters of virus excretion and transmission in pigs and cattle experimentally infected with foot-and-mouth disease virus. *J. Comp. Pathol.* 129: 268-282

- Alexandersen, S., Zhang, Z. & Donaldson, A.** 2002a. Aspects of the persistence of foot-and-mouth disease virus in animals—the carrier problem. *Microb. Infect.*, 4: 1099–1110.
- Alexandersen, S., Zhang, Z., Donaldson, A. I. & Garland, A. J. M.** 2002b. The pathogenesis and diagnosis of foot-and-mouth disease. *J. Comp. Pathol.*, 129: 1-36.
- Alexandersen, S., Zhang, Z., Reid, S., Hutchings, G. & Donaldson, A. I.** 2002c. Quantities of infectious virus and viral RNA recovered from sheep and cattle experimentally infected with foot-and-mouth disease virus O UK 2001. *J. Gen. Virol.*, 83: 1915-1923.
- Burrows, R., Mann, J. A., Garland, A. J., Greig, A. & Goodridge, D.** 1981. The pathogenesis of natural and simulated natural foot-and-mouth disease infection in cattle. *J. Comp. Pathol.*, 91: 599-609.
- Coetzer, J. A. W., Thomsen, G. R., Tustin, R. C. & Kriek, N. P. J.** 1994. Foot-and-mouth disease. In: *Infectious Diseases of Livestock with Special Reference to Southern Africa*, J. A. W., Coetzer, G. R., Thomsen, R. C., Tustin and N. P. J., Kriek (Eds) Oxford University Press, Cape Town, pp. 825–852.
- Quan, M., Murphy, C. M., Zhang, Z. & Alexandersen, S.** 2004. Determinants of early foot-and-mouth disease virus dynamics in pigs. *J. Comp. Pathol.*, (In Press).
- Salt, J. S., Mulcahy, G. & Kitching, R. P.** 1996. Isotype-specific antibody responses to foot-and-mouth virus in sera and secretions of carrier and non-carrier cattle. *Epidem. Infect.* 117: 349-360.
- Snowdon, W. A.** 1966. Growth of foot-and mouth disease virus in monolayer cultures of calf thyroid cells, *Nature*, 210: 1079-1080.
- Sutmoller, P. & Gaggero, A.** 1965. Foot-and-mouth diseases carriers. *Vet. Record*, 77: 968–969.
- Zhang, Z. & Alexandersen, S.** 2003. Detection of carrier cattle and sheep persistently infected with foot-and-mouth disease virus by real-time RT PCR assay. *J. Virol. Methods*, 111:95-100.
- Zhang, Z. & Kitching, R. P.** 2001. The localization of persistent foot and mouth disease virus in the epithelial cells of the soft palate and pharynx. *J. Comp. Pathol.*, 124: 89-94.
- Zhang, Z., Murphy, C, Quan, M., Knight, J. & Alexandersen, S.** 2004. Extent of reduction of foot-and-mouth disease virus RNA load in oesophageal–pharyngeal fluid after peak levels may be a critical determinant of virus persistence in infected cattle. *J. Gen. Virol.*, 85: 415–421.

**Evaluation and use of foot-and-mouth disease virus non-structural protein ELISA tests in
Pigs in Hong Kong**

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Introduction

Foot-and-mouth disease (FMD) is endemic in Hong Kong. Being one of the participants of the IAEA/FAO research coordination meetings, we have been evaluating several foot-and-mouth disease virus (FMDV) non-structural protein (NSP) ELISA tests using pig samples collected in Hong Kong. This paper provides our evaluation results and discusses the potential application of these tests in understanding the disease in Hong Kong.

Materials and Methods

The diagnostic sensitivity and specificity of 4 commercially available and research FMDV NSP ELISA test kits were calculated using about 400 pig serum samples collected in Hong Kong. Additionally, about 2000 clinically normal pig serum samples were also tested.

Results and Discussion

There was no significant difference in diagnostic specificity between the 4 test kits in pigs, but there were significant differences in diagnostic sensitivity between these kits which appeared to be dependent on the duration after FMDV post-exposure. There was some evidence of sub-clinical infection in vaccinated pigs. The application of different test kits should be guided by the "Fit for purpose" principle. Additional field samples are planned to be collected and tested for refining the diagnostic sensitivity of these tests in pigs in Hong Kong and appropriate samples will be stored for contributing to the NSP standard serum bank to be established by the IAEA/FAO.

**Serial release testing for FMD ELISA kits:
necessity of official control**

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Abstract

Introduction: To avoid excessive variation between serials of FMD ELISA kits, a lot of laboratories feel the need for standardising serial release testing. At this moment our laboratory performs a control on each serial of ELISA kits that will be used for FMD screening. This paper shows that serial control is necessary to guarantee the quality of the future screening results.

Materials and Methods: For the Ceditest™ FMDV-type O ELISA kit (1 serial) and the Ceditest™ FMDV-NS ELISA kit (2 serials) the diagnostic specificity, the analytical sensitivity, intra plate and inter plate variation are determined. Also an evaluation of a reference panel and of the cut-off region of the serial is done: theoretical cut-off is compared to kit cut-off and a dilution series of a positive sample is analysed on a curve.

Results: Diagnostic specificities of 100% (FMDV-O) and 98.8% (FMDV-NS) are achieved. Intra and inter plate coefficients of variation are below 10% for negative sera (based on OD) and positive sera (based on PI). The results for the reference panel are satisfactory. The ratio theoretical cut-off OD to kit cut-off OD is always higher than 1. The distribution curve for the dilution series is acceptable in the cut-off region. The results for all 3 serials are satisfactory.

Discussion: As we consider that a priori control (marketing authorisation dossier) does not provide a sufficient guarantee for the quality of the serials, we propose to perform serial release testing on each serial of FMD ELISA kits that will be used in routine testing. Guidelines and international control could be the responsibility of an official organisation like EDQM, OIE, FAO WRL or a regional reference laboratory. In the last case mutual recognition of regional serial release testing has to exist.

Introduction

We can emphasize that large difference in quality between batches of the same ELISA could occur. To guarantee a high grade of reliability of the FMD Antibody ELISAs, it is necessary to test (if possible partly by the producer) each serial (lot - batch) and to describe its capacities with regard of sensitivity and specificity (Forschner & Lehmacher, 1992). As a consequence, some countries established governmental regulations for batch quality control. For example in the USA and Canada, all kits, regardless of proposed use, receive a primary evaluation for sensitivity, specificity and reproducibility by the governmental approving agency prior to licensing.

Also, to ensure consistent performance characteristics, licensing authorities in both the USA and Canada require manufacturers to submit a report of the test results for each batch or lot of kits produced. Batches with satisfactory test results are approved for marketing. This licensing authority, however, may select random samples for confirmatory testing. In other countries, marketing is allowed upon certification that a batch was produced and monitored in accordance with a 'good manufacturing practice' marketing authorisation. As in the USA and Canada, animal health authorities may require additional testing of kits used in disease control programmes sponsored by the government. Generally, the purpose of such testing is to ensure that the performance of the kit is appropriate for the proposed use. Most countries require retention of samples from each batch of kits for future examination should problems arise (Morgan, 1998).

At this moment our laboratory performs an elaborate control on each FMDV ELISA serial that could be used for screening. This paper shows that serial control is necessary to guarantee the quality of our future screening results.

Materials and Methods

Kits and sera:

- Ceditest™ FMDV-type O ELISA kit, serial number 04K009 and Ceditest™ FMDV-NS (non-structural proteins) ELISA kit, serial numbers 03K067 and 04K024, all supplied by Cedi Diagnostics, Lelystad, the Netherlands;
- 88 different negative field sera, originating from natural non-infected animals (negative in virus neutralisation or reference ELISA);
- A positive FMDV type O and a positive FMDV type A serum;
- A strong positive FMDV type O and a strong positive FMDV type A serum;
- An FMDV negative serum, used for titration of the strong positive serum: Foetal calf serum (Gibco);
- A negative FMDV serum pool (swine, sheep and bovine) originating from experimental non infected animals (negative in virus neutralisation or reference ELISA);

- A reference panel of 24 different FMDV positive and negative sera: 9 sera from experimentally infected animals (positive in virus neutralisation or reference ELISA), 9 sera from vaccinated animals (positive in virus neutralisation or reference ELISA for that specific virus type) and 6 sera from naive (negative in virus neutralisation) animals.

These sera and their dilutions are conserved at $-20^{\circ}\text{C}\pm 5^{\circ}\text{C}$. Once thawed, the control sera are conserved at 5°C and used within 5 days.

Kit performance evaluation:

Results are always analysed according to the specifications of the test kit.

Determination of specificity of the serial:

On 1 plate 88 negative field sera are tested.

A minimum specificity of 98% is required.

Determination of analytical sensitivity of the serial:

- On 3 different microplates a dilution series (1/2 to 1/1024) was tested. We used sera with a titre of approximately 64. The variation between the positive detection limits of the 3 microplates may not exceed 1 dilution.

Determination of intra- (repeatability) and inter-plate variation (reproducibility) of the serial:

- For negative sera: each negative serum pool (swine, sheep, bovine) was tested 10 times on 3 different microplates.

CV's are based on OD values and are determined per plate and for each species separately and overall. Intra-plate CV's and standard deviations have to be ≤ 0.10 .

The absolute difference between the overall CV and the CV per microplate has to be ≤ 0.05 . So the inter-plate CV may not exceed the intra-plate CV with more than 5%.

- For positive sera: 6 dilutions of the strong positive sample were tested 4 times on 3 different microplates.

Intra-plate CV's are based on PI values and are determined per microplate for each serum dilution separately. Per microplate average CV's and standard deviations of these CV's were calculated. Average CV's and overall average CV have to be ≤ 0.10 . Also, standard deviations and overall standard deviation have to be < 0.05 .

Inter-plate CV's are based on PI values and are determined per position for each dilution separately (for each dilution series 4 CV's are calculated). The average CV for each dilution and the overall average CV have to be ≤ 0.10 ; the standard deviations of CV's obtained from each position separately and the overall standard deviation have to be < 0.10 .

- The inter-plate variation of the reference panel was evaluated by testing the panel on 3 different microplates.

The positive samples have to be positive in the ELISA, negative samples have to be negative in the ELISA. The inter-plate CV based on OD values is calculated for each sample separately. The average CV has to be ≤ 0.10 and the standard deviation on these CV's has to be < 0.10 .

Evaluation of the cut-off region of the serial:

- The theoretical cut-off limit may not exceed the kit cut-off. Because the OD values of the negative samples follow a Gaussian distribution, 99.865% of all the true negative sera will have an OD that is above the kit cut-off OD, when the ratio 'theoretical cut-off to kit cut-off' is higher than 1. Theoretical cut-off limit (overall, swine, sheep, bovine) = (average OD of negative sera – (3 x the standard deviation)).
- A dilution series (1/2 – 1/1024) of a positive sample was tested in single on 3 different microplates. Obtained OD values are presented in a graph (OD vs. (1/dilution)). Special attention is given to the steepness of the curve in the cut-off region. The steeper the curve in this region, the better.

Results:

Determination of specificity of the serial:

- FMDV-O: 100%
- FMDV-NS: 98.8% for both serials

Determination of analytical sensitivity of the serial:

All serials detect a higher dilution as being positive than the detection limit (64). The variation between these results does not exceed 1 dilution.

Determination of intra-plate variation (repeatability) of the serial:

- For negative sera: results are presented in table 1. The results are satisfactory for the FMDV-O serial 04K009 and the FMDV-NS serial 03K067. The CV on microplate 1 of FMDV-NS serial 04K024 is too high.
- For positive sera : results are presented in table 2. All results are satisfactory.

Determination of inter-plate variation (reproducibility) of the serial:

- For negative sera : results are presented in table 3. All results are satisfactory.
- For positive sera: results are presented in table 4. All results are satisfactory.

- For each serial, the reference panel was tested on 3 different microplates. All results are satisfactory: positive sera give a positive result, negative sera give a negative result, type O vaccinated sera are positive in the FMDV-O ELISA and negative in the FMDV-NS ELISA. Although, for the FMDV-O serial 04K009, the average CV is too high. This is due to some high positive samples, which give a high CV when based on OD.

Evaluation of the cut-off region of the serial:

- Determination of theoretical cut-off limit: overall results and the results of the bovine serum pool are presented in table 5.

The negative bovine samples ratio for the FMDV-NS serial 04K024 is too low on microplate 1. We also see a slightly lower ratio for this type of sera on the other two microplates. It is advisable to re-test this part of the control. The distribution of the dilution series (1/2 to 1/1024) for the FMDV-O ELISA is presented in figure 1. The curve for the FMDV-O serial is the steepest in the kit cut-off region (see figure 1 and table 5). The steepness of the FMDV-NS serial 04K024 seems a bit less than the 03K067 serial (not shown).

Discussion

In our opinion a priori control, like for example the marketing authorisation dossier, does not provide enough guarantees for the future quality of the serials that will be brought to the market. Therefore we propose to perform serial release testing on each ELISA serial that will be used in routine testing.

In our laboratory an elaborate quality control is performed on each incoming FMD ELISA serial (FMDV type O as well as FMDV-NS).

The serial or batch will only be released when the parameters under evaluation are at least equivalent to the parameters predicted by the producer of the kit. The evaluation of the kit control and the decision to release a serial are the full responsibility of the FMDV reference laboratory.

To ensure the quality of each serial, the specificity, analytical sensitivity, intra- and inter-plate variation, cut-off region and a reference panel are evaluated.

We put the minimum specificity level to 98% (2% false positives allowed). In all serials tested here this minimal criterion is met.

For detection of the analytical sensitivity, the dilution that still gives a positive result in virus neutralisation also has to give a positive result in our ELISA.

Some authors say that the expression of results in OD has to be abandoned in favour of PP (percent positivity), referenced to a positive reference serum (Desquesnes, 1997; Wright, 1993) and owing to inherent differences among assay systems, antibody activities should be expressed in relative rather than in absolute terms. According to them it is recommended that the antibody activity of the strong positive standard should denote 100% positivity. The activities of the weak positive and negative standards should then be expressed as relative percentages (Wright, 1997). For batch control, we prefer to evaluate the inter- and intra-plate variation, by using the raw OD values for the negative samples and the percent inhibition (PI) values for the positive samples. Because strong positive samples always give high CV's when this CV is based on OD and negative samples often give high CV's when this CV is based on PI, we decided to use the fitness for purpose rule: CV's of negative samples are based on OD, CV's of positive samples are based on PI.

Our results show that in some cases CV's are much lower than 10%. Here we could consider re-evaluating and tightening the criteria for this particular part of the ELISA control.

The reference panels for the FMD ELISAs give the expected results. When this part of the control consistently gives wrong results, rejection of the serial should be considered.

One of the most important parts of the control is the evaluation of the cut-off region. A consistent problem in this part of the control certainly means the rejection of the serial. When the ratio of the theoretical cut-off to the kit cut-off is higher than 1, this means that minimum 99.865% of the truly negative samples will be negative in the test. In all three serials tested here this is the case. However, a ratio of 0.946 is detected for the bovine samples in the FMDVNS serial 04K024 on microplate 1.

As the results of the other two microplates also show lower ratios than the serial 03K067, it could be necessary to retest this part of the control to confirm these results. To exclude the possibility of cross-contamination of the positive samples next to the concerning negative samples, this retest has to be performed without the positive samples. Another possibility is to calculate the ratio of the theoretical cut-off to the kit cut-off with the 88 negative samples used for detection of the specificity. As important as the previous part of the control is the distribution of the dilution series. The steepness of the curve should be high enough in the cut-off region. For the FMDV-NS ELISA we see that its steepness is slightly lower for the serial 04K024 than for 03K067.

If one or more of the criteria mentioned above is not met, one retest is allowed to re-evaluate this part of the control. When again a problem should occur, rejection of the serial should be considered.

For this type of control is very elaborate, it could be considered that the kit manufacturer performs part of the control or the whole control by himself and that he gives the results to the licensing authority.

In any way, in our opinion the determination of analytical sensitivity, specificity, the evaluation of the reference panel and of the cut-off region should also be performed by the official control organisation (licensing authority).

In some other countries, like the USA and Canada, such regulations already exist.

In the 'guidelines for licensing of veterinary diagnostic test kits in Canada' (Anonymous, 1997) for example, potency tests are performed by the manufacturer on each serial of a kit to ensure that the components are functioning as expected. The tests are based on measuring the level of reactivity of the kit positive controls, the kit negative controls, other control reagents and an independent reference panel of test samples from animals classified as diseased or non-diseased. To pass, all samples must fall within the specified range. Here also one re-test is allowed. For kits having a quantitative outcome, with antigen or antibody fixed to a solid phase, samples of the control reagents should be tested a minimum of 4 times on at least 5 different solid phase units. For each sample tested, the manufacturer should report the number of replications, the mean, the standard deviation, the coefficient of variation and the 95% confidence limits. For each new serial produced, a minimum of 2 kits, together with sufficient volumes of the panel of reference test samples for the potency test described in the Outline of Production, positive, negative and other control reagents should be submitted to the Biologics Evaluation Laboratory (BEL) for serial release testing. The manufacturer should keep an equivalent number of kits, pending a request from the Veterinary Biologics and Biotechnology Section (VBBS)/BEL to submit additional samples. For a proportion of the serials produced each year, BEL will test to verify the absence of technical problems in using the kit under laboratory conditions (not for importation of kits found satisfactory by USDA). If a kit is to be used in a Canadian Food Inspection Agency federal disease control program the manufacturer may be required to submit additional kits and reference test samples for serial release. The specific material and information required for a 'program' kit will be negotiated with each manufacturer on a case-by-case basis by the Science Advisory and Management Division and the Animal Health Division.

On the other hand, in the 'Veterinary services Memorandum 800.73' (USDA, 2002) the panel for serial release testing should contain the following samples: samples from negative or uninfected animals, samples from strongly positive animals and weakly positive animals, samples generating assay values just above and just below the cut-off value between positive and negative classification, samples from animals with reactivity to closely related (potentially cross-reactive) antigens and/or vaccinated animals, samples from animals reactive for only one, or a subset, of antigens for kits that detect reactivity to more than 1 antigen. The reference panel should include sera from at least 20 individual animals. Smaller panels may be considered for diseases where samples are difficult to obtain or validate. Pooled sera should be avoided, if possible. A single serum sample may be used more than once in the panel to evaluate error among replicate samples, but a single serum sample must not be diluted with normal serum to produce multiple samples with different reactivity's.

In Europe, such regulations do not yet exist. We therefore propose to create, similar to the control on batch release for human biologicals at the European Directorate for the Quality of Medicines (EDQM), an 'Official Control Authority Batch Release for Animal Biologicals'. In this way an Official Medicines Control Laboratory will evaluate each individual manufacturer's batch of biological products before the batch is placed on the market. The Official Medicines Control Laboratory could, in our case, be an official veterinary reference laboratory. As for human biologicals, EDQM can then be responsible for the co-ordination in providing an international veterinary serial licensing certificate. On the other hand, guidelines and international control could also be the responsibility of any other official organisation like the FAO WRL, an OIE regional reference laboratory or, at the limit a national reference laboratory. In all last cases mutual recognition of serial release testing has to exist.

Conclusions:

- A marketing authorisation dossier does not provide enough guarantees for the future quality of the diagnostic serials that will be brought to the market.
- The performance characteristics control of each ELISA serial is essential for mutual recognition of test results.
- Batch control is a part of Internal Quality Control.

Recommendations:

- An international recognised minimum control protocol should be established for batch control of diagnostics.
- An international body or regional reference centres should be recognised for the co-ordination in providing an international veterinary serial licensing certificate.

- In the absence of an international recognised body, all National Reference Laboratories using test kits have to guarantee their results by batch testing.

Acknowledgments:

Joëlle Wachel, technician at the Veterinary Agrochemical Research Centre, Belgium.

References:

- Anonymous**, 1997, Guidelines for Licensing of Veterinary Diagnostic Test Kits in Canada, Veterinary Biologics Guideline 3.19E, Version VBGL0.319E.01, Veterinary Biologics and Biotechnology Section, Canadian Food Inspection Agency, Ottawa, 20 pp.
- Desquesnes M**, 1997, Standardisations internationale et régionale des épreuves immuno-enzymatiques : méthodes, intérêts et limites, Rev. Sci. tech. Off. Int. Epiz., 16 (3), 810-823.
- Forschner E & Lehmacher W**, 1992, ELISA-systems for surveillance programs of animal infectious diseases: optimization by pretesting and safety definition of production serials, Dtsch. Tierärztl. Wschr., 99, 87-91
- Morgan AP**, 1998, Regulatory control of veterinary diagnostic test kits, Rev. Sci. Off. Int. Epiz., 17(2), 562-567
- United States Department of Agriculture (USDA)/Animal and Plant Health Inspection Service (APHIS)/Center For Veterinary Biologics (CVB)**, 2002, Veterinary Services Memorandum no. 800.73, Washington, DC, 13 pp.
- Wright PF, Nilsson E, Van Rooij EM, Lelenta M, Jeggo MH**, 1993, Standardisation and validation of enzyme-linked immunosorbent assay techniques for the detection of antibody in infectious disease diagnosis, Rev. Sci. Tech. Off. Int. Epiz., 12 (2), 435-450
- Wright PF, Tounkara K, Lelenta M, Jeggo MH**, 1997, International reference standards : antibody standards for the indirect enzyme-linked immunosorbent assay, Rev. Sci. Tech. Off. int. Epiz., 16(3), 824-832.

Table 1

		FMDV-O		FMDV-NS	
		C.V.	≤0.10?	C.V.	≤0.10?
Overall results (all species) :	Microplate 1	0.043	OK	0.078-0.103*	OK-NOK*
	Microplate 2	0.049	OK	0.083-0.084*	OK-OK*
	Microplate 3	0.056	OK	0.096-0.084*	OK-OK*
Swine:	Microplate 1	0.016	OK	0.049-0.066*	OK-OK*
	Microplate 2	0.023	OK	0.032-0.054*	OK-OK*
	Microplate 3	0.035	OK	0.030-0.029*	OK-OK*
Sheep:	Microplate 1	0.026	OK	0.056-0.070*	OK-OK*
	Microplate 2	0.041	OK	0.056-0.044*	OK-OK*
	Microplate 3	0.050	OK	0.052-0.036*	OK-OK*
Bovine:	Microplate 1	0.027	OK	0.053-0.112*	OK-NOK*
	Microplate 2	0.041	OK	0.044-0.043*	OK-OK*
	Microplate 3	0.040	OK	0.050-0.041*	OK-OK*

*serial 03K067 and 04K024

Table 2

FMDV-O				FMDV-NS			
Dilution	Intra-plate CV			Dilution	Intra-plate CV		
	Microplate 1	Microplate 2	Microplate 3		Microplate 1	Microplate 2	Microplate 3
1/100	0.006	0.007	0.005	1/2	0.007-0.018*	0.005-0.024*	0.011-0.022*
1/200	0.014	0.018	0.013	1/4	0.009-0.058*	0.007-0.066*	0.008-0.027*
1/400	0.056	0.019	0.038	1/6	0.007-0.051*	0.012-0.014*	0.030-0.061*
1/600	0.023	0.025	0.051	1/8	0.034-0.022*	0.035-0.027*	0.028-0.060*
1/800	0.019	0.047	0.078	1/10	0.031-0.096*	0.041-0.040*	0.038-0.081*
1/1000	0.018	0.065	0.063	1/12	0.078*	0.090*	0.087*
Average CV ≤0.10?	0.023 OK	0.030 OK	0.041 OK		0.018-0.054* OK-OK*	0.02-0.044* OK-OK*	0.025-0.056* OK-OK*
Standard deviation <0.05?	0.017 OK	0.021 OK	0.028 OK		0.014-0.031* OK-OK*	0.017-0.029* OK-OK*	0.013-0.027* OK-OK*
Overall CV ≤0.10?	0.031 OK				0.020-0.051* OK-OK*		
Overall standard deviation <0.05?	0.023 OK				0.014-0.028* OK-OK*		

*serials 03K067 and 04K024

Table 3

	FMDV-O			FMDV-NS		
	CV	overall CV - CV of microplate x	≤0.05?	CV	overall CV - CV of microplate x	≤0.05?
Microplate 1	0.043	0.0146	OK	0.078	0.0144	OK
Microplate 2	0.049	0.0083	OK	0.103*	0.0044*	OK*
Microplate 3	0.056	0.0013	OK	0.083	0.0100	OK
All plates (overall)	0.057			0.084*	0.0227*	OK*
				0.086	0.0036	OK
				0.084*	0.0228*	OK*
				0.093		
				0.107*		

*serial 04K024

Table 4

FMDV-O					FMDV-NS				
Dilution	Inter-plate CV				Dilution	Inter-plate CV			
	position 1	position 2	position 3	position 4		position 1	position 2	position 3	position 4
1/100	0.009	0.005	0.005	0.009	1/2	0.009	0.010	0.003	0.005
1/200	0.006	0.017	0.023	0.013		0.046*	0.053*	0.036*	0.029*
1/400	0.054	0.082	0.029	0.017	1/4	0.020	0.027	0.023	0.023
1/600	0.056	0.061	0.060	0.070		0.032*	0.064*	0.031*	0.046*
1/800	0.095	0.081	0.099	0.069	1/6	0.021	0.028	0.025	0.030
1/1000	0.020	0.057	0.064	0.078		0.090*	0.063*	0.055*	0.017*
					1/8	0.042	0.036	0.038	0.034
						0.048*	0.053*	0.052*	0.007*
					1/10	0.065	0.055	0.050	0.050
						0.110*	0.085*	0.036*	0.047*
					1/12	0.063*	0.073*	0.053*	0.152*
Average CV	0.040	0.050	0.046	0.043		0.031	0.031	0.028	0.028
≤0.10?	OK	OK	OK	OK		0.065*	0.065*	0.044*	0.050*
Standard deviation	0.035	0.032	0.034	0.033		0.022	0.016	0.018	0.016
<0.10?	OK	OK	OK	OK		0.030*	0.012*	0.011*	0.053*
Overall CV	0.051					0.030			
≤0.10?	OK					OK			
Overall standard deviation	0.026					0.017			
<0.10?	OK					OK			

*serial 04K024

Table 5

Overall OD results for the negative swine, sheep and bovine samples

	FMDV-O						FMDV-NS					
	OD _{average}	St.dev.	Theor. Cut-off	Kit cut-off	ratio	>1?	OD _{average}	St.dev.	Theor. Cut-off	Kit cut-off	ratio	>1?
P1	1.733	0.074	1.512	1.059	1.427	OK	1.459	0.114	1.116	0.749	1.490	OK
P2	1.641	0.080	1.400	0.968	1.446	OK	1.354*	0.139*	0.937*	0.865*	1.083*	OK*
P3	1.758	0.098	1.463	1.029	1.422	OK	1.539	0.127	1.158	0.787	1.472	OK
Overall	1.711	0.098	1.417	1.019	1.391	OK	1.566*	0.132*	1.170*	0.974*	1.201*	OK*
							1.590	0.153	1.131	0.888	1.273	OK
							1.467*	0.124*	1.097*	0.920*	1.192*	OK*
							1.529	0.142	1.104	0.808	1.366	OK
							1.463*	0.157*	0.993*	0.920*	1.079*	OK*

OD results for the negative bovine samples

	FMDV-O						FMDV-NS					
	OD _{average}	St.dev.	Theor. Cut-off	Kit cut-off	ratio	>1?	OD _{average}	St.dev.	Theor. Cut-off	Kit cut-off	ratio	>1?
P1	1.646	0.044	1.515	1.059	1.430	OK	1.342	0.071	1.130	0.749	1.508	OK
P2	1.562	0.064	1.371	0.968	1.416	OK	1.233*	0.138*	0.818*	0.865*	0.946*	NOK*
P3	1.670	0.066	1.471	1.029	1.430	OK	1.391	0.061	1.208	0.787	1.535	OK
overall	1.626	0.074	1.405	1.019	1.379	OK	1.414*	0.061*	1.231*	0.974*	1.263*	OK*
							1.401	0.071	1.189	0.888	1.339	OK
							1.313*	0.054*	1.153*	0.920*	1.253*	OK*
							1.378	0.070	1.167	0.808	1.444	OK
							1.320*	0.117*	0.970*	0.920*	1.054*	OK*

*serial 04K024

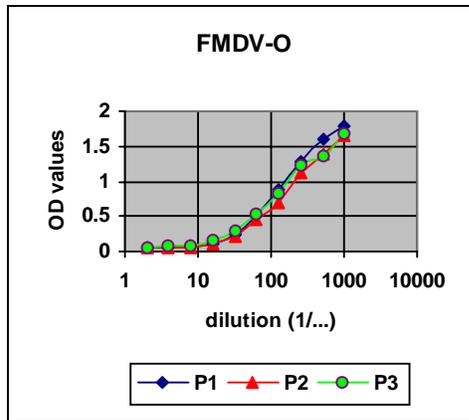


Figure 1. The curve for the FMDV-O serial is the steepest in the kit cut-off region (also see table 5).

The FMD-NS ELISA, the most sensitive test to detect FMDV infected animals in a vaccinated population

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Introduction

Foot-and Mouth disease is one of the most important infectious diseases, and outbreaks with devastating consequences still occur. The disease can be controlled by vaccination, however a critical issue is the occurrence of carrier animals and the risk they pose in transmitting the virus. The aim of this study was to evaluate the most sensitive method to detect carriers.

Material and methods

Seventeen Holstein-Friesian cattle, vaccinated with a full dose, a quarter dose, one-sixteenth dose of FMDV strain A/TUR 14/98, and two not vaccinated were challenged with 10,0000 cattle ID₅₀. four weeks after vaccination. Serum and probang samples were taken prior to vaccination and infection and after infection at regular intervals until 2 years after infection. Samples were tested for the presence of FMDV (by virus isolation), FMDV viral genome (by real-time PCR), FMDV-specific IgA antibodies (by ELISA), antibodies against FMDV non-structural proteins (by Ceditest FMDV-NS ELISA, as described by KJ Sorensen et al., 1998) or neutralising antibodies.

Results

All cattle became carriers. All inoculated cattle developed high titres of neutralising antibodies which remain high during the entire experiment, only 14 out of 17 had an intermittently IgA antibody response in the oropharyngeal fluid. However, all animals developed antibodies against the non-structural proteins and became positive in the Ceditest[®] FMDV-NS ELISA as early as 6 days post infection and remain positive until the end of the sampling period.

Conclusion

Based on our results, the Ceditest[®] FMDV-NS ELISA is the most sensitive method to detect carriers in a vaccinated cattle population.

Reference

Sorensen et al (1998). Arch Virol 143: 1461-1476

3 ABC ELISA for the diagnosis of FMD in Egyptian sheep

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Abstract

Sheep play an important role in the epidemiology and transmission of foot and mouth disease (FMD). Moreover, FMD is suspected to have been transmitted to sheep in which infection is frequently sub-clinical. So, it is of importance to identify animals which have been exposed to the virus and have developed antibodies. Such animals may become carriers and thus be a potential source of new outbreaks. In addition to capsid proteins, certain non-structural proteins (NSP) are also produced during the process of infection by FMD virus (FMDV) and against which immunoglobulins may be formed. The most reliable single NSP indicator is the poly-protein 3 ABC, antibodies to which appear to provide conclusive evidence of previous infection, whether or not the animals have also been vaccinated. Three groups of sheep (4 animals in each group) were used in this study. The first group (Group 1) were infected with FMDV O1/93/Aga-Egypt intradermo-lingually and the second group (Group 2) were in contact with Group 1 from day 1. The third group (Group 3) was vaccinated and revaccinated four weeks later with BEI-inactivated and aluminium hydroxide gel adjuvanted FMD-vaccine. Groups 1 and 2 were observed for clinical manifestations especially for vesicular lesions in the buccal cavity. Serum samples were collected from the three groups along with two sheep which were kept as controls. The serum neutralization test (SNT), a sandwich ELISA and an FMDV-3 ABC ELISA were used for the detection of antibodies to FMD virus. The results showed that the three groups of sheep produced serum-neutralizing (SN) antibodies. In Group 1 SN antibodies appeared as early as 2 days post infection (DPI), although protective levels were not evident until 7 DPI; these protective levels persisted until 40 weeks post infection (WPI). Antibodies were detected in Group 2 from 5 days post exposure (DPE) and protective levels from 8 DPE which persisted until 36 weeks post exposure (WPE). Group 3 had protective antibody levels from 12 days post vaccination (DPV) until 28 weeks post vaccination (WPV). Buccal lesions were observed in Groups 1 and 2; these differed in both severity and distribution but particularly affecting the tongue, lips and gums. Vesicular lesions progressed to become eroded areas and were subsequently healed; all lesions being completely healed by 12 days after either infection or exposure. Sheep in Groups 1 and 2 developed antibodies against FMDV-3 ABC antigen. NSP antibodies were detectable from 10 DPI until 40 WPI in Group 1 and from 15 DPE until 48 WPE in Group 2. Group 3 did not develop NSP antibodies during the course of the study. Therefore demonstrating the usefulness of the CHEKIT-FMD-3 ABC ELISA for the differentiation between infected and vaccinated sheep, which will be very important for FMD control in Egypt.

Introduction

FMD is a contagious disease of cloven hooved animals which has a great potential to cause severe economic loss. FMD is endemic in two-thirds of OIE (Office Internationale des Epizooties) member countries where it is an economic problem and also provides a reservoir of virus which can spread into virus-free areas (Clavijo et al., 2004). Ganter et al. (2001) mentioned that goats and sheep might be carriers, so they play an important role in the epidemiology and transmission of FMD whilst Patil et al. (2002) calculated that sheep and goats constitute the majority of the world's FMD-susceptible livestock. Kitching and Hughes (2002) indicated that sheep and goats are highly susceptible to infection with FMD by the aerosol route. The virus probably most often infects sheep and goats by direct contact. On the other hand, Kitching and Mackay (1994) found that the incubation period in sheep following infection with FMDV is usually between 3-8 days, but can be as short as 24 hours following experimental infection, or as long as 12 days. Davis (2002) reviewed that FMDV is excreted during viraemia for some days, thereafter as serum antibodies develops viraemia decreases and the animal ceases to be infectious as the lesions heal. DeClercq (2002) stated that the FMDV excretion starts 24-48 hours before the onset of clinical signs and declines with the appearance of FMD specific circulating antibody at around 4-5 days post infection. Hughes et al. (2002a) revealed that the optimal dose to infect sheep and for producing in-contact transmission is about 10^4 TCID₅₀. Moreover, Hughes et al. (2002b) mentioned that lesions may fail to develop in approximately 25% of infected sheep; a further 20% may develop only a single observable lesion. In this respect, Arafa (1980) stated that the vesicle appeared on the epithelium of the tongue at 1 DPI and on the gums at 3 to 4 DPI in sheep, while Blood et al. (1985) observed that characteristic the FMD lesions usually develop between 3 and 8 DPE. Amas et al. (2003) found that contact sheep with infected pigs had developed gross lesions consistent with FMD by 5 DPE. In 1964, Dellers

and Hyde detected virus neutralizing antibodies 60 hours post inoculation (which persisted for at least 147 days) in sheep. Peak antibody titres were detectable from 10 DPI. On the other hand, diagnosis of FMD is made by the demonstration of FMD viral antigen or nucleic acid in samples of tissue or fluid. Detection of specific humoral antibody can also be used for diagnosis. The detection of antibody to non-structural proteins (NSP) of FMDV has been used to identify past or present infection (DeDiego et al., 1997; Brocchi et al., 1998; Dekker et al., 1998 and Malirat et al., 1998). Sorensen et al. (1998) detected 3-ABC antibodies from day 10 after experimental infection of sheep. During the course of infection, immune responses to NSP appear later than responses to structural proteins of the virus. Following experimental infection antibodies to the 3-ABC antigen could not be detected earlier than 10 DPI in sheep (Chung et al., 2002). Perhaps the most reliable single NSP indicator is the poly-protein 3 ABC, antibodies to which appear to provide conclusive evidence of previous infection (Makay et al., 1998) whether or not the animals have also been vaccinated. Sorenson et al. (1998) stated that antibodies against 3 ABC have been detected up to 395 days post infection in both cattle and sheep whilst Kitching (2002) reported that the 3-ABC antibodies persist more than 12 months. The severity of infection may influence serum levels of NSP antibodies and also the duration for which this antibody is detectable. The aim of this work is to evaluate the usefulness of the CHEKIT-FMD-3-ABC ELISA as a diagnostic tool to differentiate between FMDV-vaccinated and FMDV-infected Egyptian sheep. Differentiating between vaccinated and infected sheep may help in the future FMD control strategy.

Material and Methods

Animals:

Fourteen male sheep of local breed were used. These were all eight months of age and sero-negative against FMDV.

Virus:

Foot and mouth disease virus (FMDV) type O1/93/Aga-Egypt was used to infect sheep. The virus was titrated in baby mice (2-3 days old); the titre was 10^9 MID₅₀/ml. The virus was injected intradermo-lingually, each sheep receiving 10^4 MID₅₀ of virus. Tissue culture adapted virus (on BHK21 cells) was used in SNT and preparation of sandwich ELISA antigen.

Vaccine:

BEI-inactivated, aluminium hydroxide gel and saponin-adjuvanted FMD vaccine, which was locally prepared, was administered subcutaneously at a dose of 1ml per sheep.

Sample:

Serum samples were collected 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 15 (DPI or DPE or DPV) and at 4, 6, 8, 10, 12, 16, 20, 24, 28, 32, 36, 40, 44, 48 and 52 (WPI or WPE or WPV).

Serum neutralization test (SNT):

The SNT was carried out according to OIE Manual (2000) based on the method described by Ferreira (1976). The titers expressed in log₁₀ were calculated according to Karber (1931).

Sandwich ELISA:

The Sandwich ELISA was carried out according to Voller et al. (1976) and Hamblin et al. (1986) for reagent preparation and test method, respectively.

CHEKIT-FMD-3 ABC ELISA:

The *CHEKIT-FMD-3 ABC ELISA* was provided by Bommeli Diagnostics, Liebefeld-Bern, Switzerland. The test was performed as described by the manufacturer using the following calculation formula:

$$\text{Value \%} = \frac{\text{OD sample} - \text{OD negative}}{\text{OD positive} - \text{OD negative}} \times 100$$

OD: Optical Density

Negative : Negative control (OD = 0.063 = 0%)

Positive : Positive control (OD = 1.215 = 100%)

Results and Discussion

Nowadays, the identification of sheep which are currently- or previously- infected with FMDV is very important to any strategy for the control and eradication of FMD, given that apparently healthy sheep may be the source of a new outbreak. The only way to efficiently identify carrier sheep is by detection of antibodies against non-structural proteins of FMDV, such as 3-ABC. NSP antibodies only develop following infection of animals with FMD virus and not after vaccination with purified, inactivated vaccine.

To evaluate the use of an NSP antibody detection assay in differentiating between vaccination and infection,

an experiment was designed with three groups of sheep (with 4 animals in each group). The first group was infected with FMD O1/93/Aga-Egypt, the second group was exposed by contact with the first group from the day on which they were infected and the third group was vaccinated with an inactivated, adjuvanted vaccine (see the design of the experiment).

Table 1 details the lesions observed in the buccal cavity of experimentally-infected and in-contact sheep. In Group 1 lesions on the tongue epithelium were apparent from 3 DPI whilst lesions appeared on the lips and gums at 4 DPI. All the experimentally-infected sheep developed buccal lesions which had healed completely by 8 DPI but no feet lesions were noticed in this group. These results are in agreement with Arafa (1980) who stated that the vesicles appeared on the epithelium of the tongue at 1 DPI and on the gum at 3 to 4 DPI and with Blood et al. (1985), they mentioned that the characteristic lesions usually developed after 3 to 8 DPE. From Table 1 it is also clear that in-contact sheep (Group 2) developed buccal vesicular lesions from 6 DPE and these lesions were found either on the lip or the gum or both; All lesions had healed by 12 DPE. The results also showed that only three of the four in-contact animals were infected. Kitching and Mackay (1994) found that the incubation period of FMDV in sheep is usually between 3 and 8 days. Also, Hughes et al. (2002b) mentioned that vesicular lesions may fail to develop in approximately 25% of infected sheep. Bouma et al. (2004) indicated that it may be possible that contact with a low amount of virus results in a quick immune response. This may prevent widespread replication of the virus in the infected animal so that there are no visible signs of FMD. In general, the lesions in both groups 1 and 2 appeared as vesicles which progressed to become eroded areas and ended with complete healing.

Serum samples were collected from the animals in the three groups at different times post infection, exposure and vaccination. Table 2 details the antibody titres against FMD. Two techniques were used to determine such titres (SNT and sandwich method ELISA) for vaccinated sheep in Group 3. Antibodies were first detected at 6 DPV, reaching protective levels ($1.2 \log_{10}$) by 12 DPV.

The titres continued to increase, reaching maximum levels 10 weeks after primary vaccination (which was 6 weeks after booster vaccination). Protective levels persisted from 12 DPV until 28 WPV; SN antibody titres ranging from 1.2 to $1.85 \log_{10}$. With ELISA detectable antibody levels were slightly greater, ranging from 1.2 to $2.15 \log_{10}$, a difference of about $0.2 \log_{10}$ in measured antibody levels between the two techniques used. These results are consistent with the statement of Hamblin et al. (1986) who explained that the SNT measures those antibodies which neutralize the infectivity of FMD virion, while ELISA probably measure all classes of antibodies even those produced against incomplete and non-infectious virus.

In case of experimentally infected sheep a trace of neutralizing antibodies was detectable in the animal's sera from 2 DPI ($0.35 \log_{10}$) and protective levels from 7 DPI. Protective levels persisted until 40 WPI reaching maximum titers of 1.80 to $1.90 \log_{10}$ between 4 and 28 WPI. When the sera were examined by ELISA a similar difference of about $0.2 \log_{10}$ was measured between antibody levels detected by ELISA and levels detected by SNT. In the case of contact sheep, SN antibodies were not detected until 5 DPE, protective levels were reached at 8 DPE, and these protective levels persisted until either 36 WPE (based on SNT results) or 40 WPE (when measured by ELISA). Once more, ELISA detected slightly higher levels than SNT. Dellers and Hyde (1964) detected virus neutralizing antibodies in sheep 60 hours after inoculation with FMDV, peak titers occurring by day 10 and detectable antibody persisting for at least 147 days. Davis (2002) indicated that FMDV is shed during viraemia for some days, thereafter as serum antibody develops viraemia decreases. Declercq (2002) showed the appearance of circulating FMD specific antibody at around 4 to 5 days post infection.

Table 3 illustrates the detection of FMDV 3-ABC antibodies in sera collected from the three groups of sheep. Vaccinated sheep failed to produce any 3-ABC antibodies as for control sheep. Detectable 3-ABC antibodies started to appear at 10 DPI (about 1% positivity) in experimentally-infected sheep, increased gradually to a maximum (about 100% positivity) at 10 WPI and started to decrease from 24 WPI, reaching about 56% positivity at 36 WPI. The percentage positivity decreased dramatically from 40 WPI and antibody was no longer detectable by 44 WPI. In-contact sheep also developed antibodies against FMD non-structural proteins (3 ABC) by 15 DPE but detectable antibody disappeared completely after 44 WPE. Sorenson et al. (1998) stated that antibodies against 3 ABC have been detected up to 395 days post-infection in both cattle and sheep. They also showed that FMD-3 ABC antibodies could be detected from day 10 after experimental infection of susceptible sheep. In this study, the detection of the FMD 3-ABC antibodies were detected in both infected and in-contact sheep up until 44 weeks post-infection or post-exposure. The duration for which antibody is detectable may depend on the severity of the infection (Kitching, 2002). Moreover, Chung et al. (2002) found that antibodies to the 3 ABC antigen in sheep could not be detected earlier than 10 days after experimental infection.

References

- Amas, S.F.; Pacheco, J.M.; Mason, P.W.; Schneider, J.L.; Alvarez, R.M.; Clark, L.K. and Ragland, D.** (2003): Procedures for preventing the transmission of FMDV to pigs and sheep by personnel in contact with infected pigs. *Vet. Rec.*, 153: 137-140.
- Arafa, M.H.** (1980): Studies on the carrier state of goats exposed to FMDV. M.V.Sc. Thesis, Cairo Univ.
- Blood, D.C.; Radostitis, O.M. and Henderson, J.A.** (1985): *Veterinary Medicine: A textbook of the disease of cattle, sheep, goats and horses.* 8th ed., ELBS and Bailliere Tindal.
- Brocchi, E.; DeDiego, M.I.; Berlinzani, A.; Gamba, D. and DeSimone, F.** (1998): Diagnostic potential of Mab-based ELISAs for antibodies to non-structural proteins of FMDV to differentiate infection from vaccination. Proceedings of concerted action CT93-0909. *Vet. Q.*, 20: 20-24.
- Bouma, A.; Dekker, A. and Mart, C.M. de Jong** (2004): No foot and mouth disease virus transmission between individually housed calves. *Vet. Microbiol.*, 98: 29-36.
- Chung, W.B.; Sorensen, K.J.; Liao, P.C. and Jong, M.H.** (2002): Differentiating FMDV infected pigs from vaccinated pigs by blocking ELISA using non-structural protein 3 ABC as antigen and its application to an eradication program. *J. Cl. Micro.*, 40: 2843-2848.
- Clavijo, A.; Wright, P. and Kitching, P.** (2004): Developments in diagnostic techniques for differentiating infection from vaccination in FMD. *Vet. J.*, 167: 9-22.
- Davis, G.** (2002): Foot and mouth disease. *Review Res. Vet. Sci.*, 73: 195-199.
- DeClercq, K.** (2002): Overview on FMD diagnostic techniques. In *FMD control strategies, Symposium Proceedings, 2-5 June 2002, Lyon, France*, pp. 345-351.
- DeDiego, M.; Brocchi, E.; Makay, D. and DeSimone, F.** (1997): The non-structural polyprotein 3 ABC of FMDV as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. *Archives of Virology*, 142: 2021-2033.
- Dekker, A. and Gijzen, E.** (1998): The possible use of native FMD non-structural protein 3A in a serological screening test. *Vet. Q.*, 20: Suppl. 2.
- Dellers, R.W. and Hyde, J.I.** (1964): Response of sheep to experimental infection with FMDV. *Am. J. Vet. Res.*, 25: 105.
- Ferreira, M.E.V.** (1976): Prubade microneutralization poraestudies de anticueropos de la fibre aftosa. *Bltn. Cent. Panam. Fiebre Aftosa*, 21 (22): 17-24.
- Ganter, M.; Graunke, W.D.; Steng, G. and Worbes, H.** (2001): FMD in sheep and goats. *Dtsch Tierarztl Wochenschr*, 108 (12): 499-503.
- Hamblin, G.; Barnett, I.T.R. and Crowther, J.R.** (1986): A new ELISA for detection of antibodies against FMDV. II. Application. *J. Imm. Meth.*, 93: 123-129.
- Hughes, G.J.; Kitching, R.P. and Woolhouse, M.E.J.** (2002a): Dose dependent response of sheep inoculated intranasally with a type O-FMDV. *J. Comp. Path.*, 127: 22-29.
- Hughes, G.J.; Mioulet, V.; Kitching, R.P.; Woolhouse, M.E.J.; Andersen, S. and Donaldson, A.I.** (2002b): FMD infection of sheep: implications for diagnosis and control. *Vet. Rec.*, 150 (23): 724-727.
- Karber, G.** (1931): 50% end point calculation. *Archiv fur Experimentelle Pathologie und Pharmakologie*, 162: 480-483.
- Kitching, R.P.** (2002): Identification of FMDV carrier and subclinically infected animals and differentiation from vaccinated animals. *Rev. Sci. Tech. Off. Int. Epiz.*, 21 (3): 531-538.
- Kitching, R.P. and Hughes, G.J.** (2002): Clinical variation of FMD: sheep and goats. *Rev. Sci. Tech. Off. Int. Epiz.*, 21 (3): 505-512.
- Kitching, R.P. and Makay, D.K.** (1994): FMD. *State Vet. J.*, 4: 7-10.
- Mackay, D.K.J.; Forsyth, M.A.; Davies, P.R.; Berlinzani, A.; Belsham, G.J.; Flint, M. and Rayon, M.D.** (1998): Differentiating infection from vaccination in FMD using a panel of recombinant non-structural proteins in ELISA. *Vaccine*, 16: 446-459.
- Malirat, V.; Neitzert, E.; Bergmann, I.E.; Marade, E. and Beck, E.** (1998): Detection of cattle exposed to FMDV by means of an indirect ELISA test using bioengineered non-structural polyprotein 3 ABC. *Vet. Quarterly*, 20 (Suppl. 2): S24-S26.
- OIE** (2000): FMD, Chapter 2.1.1. In *Manual of Standard for Diagnostic Test and Vaccine*, 4th ed., 2000, Paris, pp. 77-92.
- Sorensen, K.J.; Brocchi, E.; Mackay, D. and DeSimone, F.** (1997): FMD, detection of antibodies in cattle sera by blocking ELISA. *Vet. Microbiol.*, 23 (3-4): 253-265.
- Voller, A.; Bidwell, D.E. and Ann Bartlett** (1976): ELISA in medicine. *Theory and Practice. Bul. WHO*, 53: 55-56.

Table 1 Design of the experiments

Experimental Groups	Time of sera collection																									
	Days											Weeks														
	2	3	4	5	6	7	8	9	10	12	15	4	6	8	10	12	16	20	24	28	32	36	40	44	48	52
Group 1 (4 sheep)	Infected intradermo-lingually with the virulent FMD O1/93/Aga-Egypt in a dose of 10^4 MID ₅₀ /ml																									
Group 2 (4 sheep)	In-contact with the experimentally infected group of sheep (Group 1) from the first day of infection																									
Group 3 (4 sheep)	Vaccinated subcutaneously with 1ml of BEI-inactivated, aluminium hydroxide gel-adjuvanted vaccine containing 1-2 µg 140S/dose; revaccinated with the same vaccine dose four weeks after primary vaccination																									
Group 4 (2 sheep)	Negative control sheep (Unvaccinated and uninfected)																									

Table 2: Follow-up of antibodies against FMD in different groups of sheep by SNT and sandwich method ELISA expressed in log₁₀

Time of sera collection		Group (1)		Group (2)		Group (3)	
		SNT	ELISA	SNT	ELISA	SNT	ELISA
Days	2	0.35	0.55	0	0	0	0
	3	0.40	0.65	0	0	0	0
	4	0.65	0.75	0	0	0	0
	5	0.65	0.85	0.25	0.25	0	0
	6	0.85	1.20	0.35	0.55	0.35	0.35
	7	1.20	1.35	0.85	1.35	0.35	0.35
	8	1.35	1.50	1.25	1.35	0.65	0.45
	9	1.36	1.50	1.20	1.40	1.00	1.20
	10	1.35	1.65	1.45	1.55	1.00	1.25
	12	1.40	1.65	1.60	1.75	1.20	1.50
	15	1.65	1.75	1.65	1.85	1.25	1.65
Weeks	4	1.85	2.10	1.65	1.85	1.35	1.65
	6	1.85	2.10	1.70	1.85	1.85	2.15
	8	1.80	2.15	1.75	1.90	1.80	2.10
	10	1.82	1.95	1.90	1.95	1.80	1.95
	12	1.80	1.95	1.85	2.15	1.55	1.95
	16	1.85	1.95	1.75	2.15	1.45	1.75
	20	1.90	1.90	1.75	1.95	1.35	1.45
	24	1.85	1.95	1.65	1.90	1.25	1.25
	28	1.80	1.95	1.35	1.65	1.25	1.25
	32	1.50	1.75	1.20	1.40	0.85	1.25
	36	1.45	1.65	1.25	1.45	0.55	0.85
	40	1.25	1.35	1.00	1.20	0.35	0.55
	44	0.80	1.20	0.95	1.00	0	0
	48	0.35	0.60	0.25	0.30	0	0
52	0.25	0.45	0	0	0	0	

Two negative control sheep were seronegative allover the experiment.

The titers of antibodies are the mean of the animals in each group.

Table 3: Tracing of FMD-3 ABC antibodies in different groups of sheep calculated in percent

Time of sera collection		Group (1)		Group (2)		Group (3)	
		OD	%	OD	%	OD	%
Days	2	0.062	0	0.057	0	0.058	0
	3	0.063	0	0.061	0	0.071	0
	4	0.058	0	0.071	0	0.063	0
	5	0.061	0	0.063	0	0.062	0
	6	0.073	0	0.061	0	0.062	0
	7	0.063	0	0.065	0	0.058	0
	8	0.059	0	0.071	0	0.058	0
	9	0.062	0	0.063	0	0.081	0
	10	0.131	10	0.059	0	0.062	0
	12	0.725	57	0.062	0	0.061	0
	15	0.855	60	0.685	56	0.054	0
Weeks	4	0.950	70	0.721	57	0.062	0
	6	1.130	90	0.760	58	0.063	0
	8	1.211	90	0.853	60	0.062	0
	10	1.321	100	0.850	60	0.073	0
	12	1.411	100	0.933	70	0.071	0
	16	1.400	100	0.980	85	0.065	0
	20	1.393	100	1.123	90	0.061	0
	24	0.882	80	1.125	90	0.061	0
	28	0.860	60	1.332	100	0.063	0
	32	0.879	60	1.323	100	0.061	0
	36	0.721	56	1.410	100	0.073	0
	40	0.136	10	0.933	70	0.063	0
	44	0.065	0	0.625	55	0.058	0
	48	0.071	0	0.125	10	0.063	0
52	0.056	0	0.059	0	0.066	0	

*Two negative control sheep were seronegative allover the experiment.
The OD readings are the mean of the animals in each group.*

New strategies for the differentiation of Foot-and-Mouth disease virus-infected from vaccinated animals: Development of a competitive ELISA and a multiplexed Luminex assay

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Introduction

Available vaccines to foot-and-mouth disease (FMD) stimulate the production of antibodies indistinguishable from those produced by infected animals in response to live virus and because vaccinated animals can be infected and become carriers of FMDV, efforts have been made to develop diagnostic test that can differentiate vaccinated animals from those that are convalescent and from those that have been vaccinated and become carriers following subsequent contact with live virus. Using traditional serological techniques it is not possible to distinguish FMD infected animals from vaccinated animals and control authorities have limited possibilities to monitor virus presence or circulation. Currently the detection of antibodies to non structural proteins (NSPs) is the preferred diagnostic method to distinguish virus infected from vaccinated animals. Considerable effort and attention is now being directed toward the development of new methods and techniques for the rapid and accurate detection of anti-NSP antibodies, harmonization and standardization of current diagnostic techniques, as well as the production of defined reagents.

Two new methodologies were evaluated for the detection of NSPs: A competitive ELISA (cELISA), to detect FMD virus antibodies in cattle, sheep and pigs without any modification in the test procedure and a Luminex-base multiplex immunoassay to measure four NSPs simultaneously from a single sample.

Materials and Methods

Non structural proteins cloning

Viral RNA was isolated using TriPure Isolation Reagent from FMDV infected BHK-21 cells following manufacturer instructions (Boehringer Mannheim, Laval, Quebec). The complete gene encoding for the 3ABC, 3D, 3A and 3B proteins were amplified using reverse transcription-PCR using primers derived from the published sequence of FMDV strain O₁/Campos/Brazil/58 (Pereda et al., 2002). The sense primer was designed such that part of the FMDV NSP protein was in the same reading frame as the PET 6xHis tag and the PinPointXa-1 vector sequence (for 3ABC) encoding a consensus biotinylation site. In vitro amplification by RT-PCR and post-PCR analyses were as previously described (Clavijo et al., 2003). Molecular cloning was accomplished by standard techniques (Sambrook et al., 1989).

NSP recombinant proteins expression and in vivo biotinylation

Expression of 6xHis-3ABC, 3D, 3A and 3B recombinant protein was carried out as described previously (Clavijo et al., 2001). The expression of the biotinylated 3ABC was carried out as the 6xHis-NSP recombinant protein, except that LB media supplemented with D-biotin (2µM) and carbenicillin (50µg/mL) was used. The protein expression was induced by adding IPTG to a final concentration of 100µM. For extraction of the recombinant proteins 6xHis-NSP and biotin-3ABC, the cell pellet was resuspended at room temperature with BugBuster reagent (Novagen, Madison, WI, USA). Following cell lysis, the suspension was incubated on a shaking platform at a slow setting for 20 min at room temperature the inclusion bodies were collected.

Solubilization and refolding of recombinant proteins

Recombinant proteins were solubilized by the addition of IB solubilization buffer (50 mM CAPS, pH 11; 0.3% N-lauroylsarcosine; 1 mM DTT). The solubilized protein was dialyzed (20 mM Tris-HCl pH 8.5; 0.1 mM DTT). The refolded 6xHis-NSP recombinant proteins and the biotinylated 3ABC recombinant protein (B-3ABC) were diluted 1:1 in glycerol and stored at -20°C until used as antigen in the cELISA or multiplex Luminex assay.

Guinea pig anti-3B hyperimmune sera

A 15 amino acid peptide from the 3B NSP region was used as antigen to immunize 6 Hartley guinea pigs of about 600-700g. For inoculation, 200 µl of antigen were emulsified with an equal volume of Freund=s complete adjuvant and inoculated into two sites subcutaneously. Subsequent inoculations on dpi 28 consisted of antigen combined with an equal volume of Freund=s incomplete adjuvant. Samples were taken at 0, 27 and 43 days post infection (dpi) and the animals were exsanguinated via cardiac puncture under general anaesthetic on dpi 44. Prebleed and titre checks were performed using the saphenous vein.

Competitive ELISA test procedure

Competitive ELISA (cELISA) was performed by sensitising polystyrene microtiter plates (Nunc-Immuplate, Roskilde, Denmark) overnight with 100 µl/well of 10 µg/mL of streptavidin (Jackson ImmunoResearch Laboratories Inc, West Grove, Pennsylvania) in coating buffer (0.01 M phosphate buffered saline (PBS)(NaCl

0.138 M; KCl - 0.0027 M; pH 7.4) (Sigma-Aldrich, Oakville, ON) at room temperature (20-25°C). After five washes with washing buffer (0.01M PBS, 0.05 % Tween 20), the biotinylated 3ABC recombinant protein was captured by adding 100 µl/well of 1:3,000 dilution of recombinant antigen in coating buffer. After 1 hr incubation at 37°C with agitation, plates were washed five more times and 50 µl of diluted test sera (1:5) in dilution buffer (PBS-T)(NaCl 0.138 M; KCl - 0.0027 M; pH 7.4, 0.05 % Tween 20) containing 1% BSA was applied in duplicate. An equal volume (50 µl/well) of guinea pig anti 3B hyperimmune sera in PBST 1%BSA buffer at an optimal dilution of 1:15,000 was added to plates without washing. The plates were sealed, gently shaken for 1 min, and incubated at 37°C for 1 hr with agitation. After washing five times, 100 µl of horseradish peroxidase-conjugated donkey anti guinea pig IgG (H+L) (Jackson ImmunoResearch Laboratories Inc, West Grove, Pennsylvania) diluted 1:3,000 in dilution buffer supplemented with 10% normal bovine serum was added and incubated for 1 hr at 37°C with subsequent washing. The chromogen solution *O*-phenylenediamine dihydrochloride (OPD)(Sigma-Aldrich, Oakville, ON) was added and the plate was then shaken continuously in dark at room temperature. Colour development was stopped after 15 min with 50 µl/well of 2M sulphuric acid. Two wells of strong positive, weak positive and negative controls derived from sera of the specific species to be tested (bovine, ovine or swine) were included in each plate. The optical density (OD) was determined at 490 nm on an automated ELISA plate reader (Photometer - Multiskan reader, Labsystems). Results were expressed as percentage of inhibition (PI) and calculated based on the mean optical density (OD) values of a duplicate sample, compared with a standard negative reference serum specific for each species corrected for background signal by subtracting the OD of the high positive reference serum. Test results were derived by the following formula: $PI = [(negative\ reference\ serum\ OD - test\ sample\ OD) / (negative\ reference\ serum\ OD - positive\ reference\ serum\ OD)] \times 100\%$.

Multiplexed Luminex assay

Luminex Laboratory MultiAnalyte Profiling (LabMap3) technology was used to develop an immunoassay that measures FMD-NSPs 3ABC, 3A, 3B and 3D specific antibodies in cattle simultaneously and from a single serum sample. Recombinant His-tagged proteins 3ABC, 3A, 3B and 3D were coupled to Penta-His beads (Qiagen LiquiChip Penta-His Bead Set B) overnight as follows. Each protein was diluted in PBS/BSA (10mM NaH₂PO₄, 150mM NaCl, 0.1% w/v BSA) for a final dilution of 1:50 to 1:100 in the coupling reaction. The reaction was set up in microfuge tubes which were then wrapped in tinfoil to prevent photobleaching of the beads. Reactions were incubated overnight at 4°C in an Eppendorf Thermomixer R at 750rpm. Coupling reactions were washed with PBS/BSA and then microfuged at 10,000xg for two minutes. The wash step was repeated, the supernatant was removed and the beads were resuspended in PBS/BSA. The beads (10µl) were counted and made to a final concentration of 125 beads/µl in PBS/BSA. Prior to starting the test, filter plates (MultiScreen-BV, Millipore) were pre-wet with 100µl PBS-T/BSA/NMS (10mM NaH₂PO₄, 150mM NaCl, 0.02% v/v Tween-20, 0.1% w/v BSA, 3% Normal Mouse Serum) and the buffer was then removed by vacuum. For tests involving a single recombinant protein-bead coupling, the following was added to each well: 35µl PBS-T/BSA/NMS, 15µl coupled beads at 125 beads/µl, 50µl diluted serum samples (final 1:200 dilution in test). For the multiplex test 20µl PBS-T/BSA/NMS, 15µl each bead (60ul volume in total) and 20µl 1:40 dilution of serum samples (final 1:200 dilution in test) was added to each well. Filter plates were sealed, wrapped in tinfoil and incubated at 37°C with vigorous agitation for 1 hour. Plates were washed 3 times with 100ul PBS-T (10mM NaH₂PO₄, 150mM NaCl, 0.02% v/v Tween-20). To each well, 50µl PBS-T/BSA/NMS was added followed by 50µl biotin-SP-conjugated goat anti-bovine IgG (H+L) (Jackson ImmunoResearch Laboratories Inc.) diluted to a final concentration of 1:300 in test. The beads were resuspended by pipetting up and down. Plates were again sealed, wrapped in tinfoil and incubated at 37°C with vigorous agitation for 1 hour followed by washing. 50µl PBS/BSA was added to each well along with 50µl 3µg/ml Streptavidin-R-PE (Qiagen), beads were resuspended by pipetting. After a final 1 hour incubation and washing step, beads were resuspended in 100µl PBS/BSA and transferred to a round bottom plate. The plate was then read in the LiquiChip (Qiagen) using Luminex 100 IS software, version 2.2. All data was recorded in MFI units.

Results

Cloning and expression of NSP recombinant protein

The recombinant NSP proteins were expressed in *E. coli* with a six histidine tag at the amino and carboxy terminus as indicated by the sequencing of the pETclones. The 6xHis-NSP fusion proteins 3A, 3B, 3D and 3ABC have an apparent molecular mass of 25, 42, 68 and 55 kDa respectively on SDS-PAGE and immunoblot using HRP-labelled Ni-NTA (Figure 1 and 2). Usually the purification of the 6xHis-NSP proteins started with about 1 g of *E. coli* cells obtained from 200 mL of culture. For the production of the biotinylated 3ABC recombinant protein a fragment of 1,317 bp was obtained from the pET3ABC clone, ligated into the PinPointXa-1 vector and transformed into JM109 *E. coli* cells. A clone was selected for expression of recombinant fusion protein in JM109 cells. The production of this recombinant protein was assessed by SDS-PAGE which showed a protein with a molecular weight of about 62 kDa that was not present in either cell

control or JM109 containing vector only (Figure 3). When HRP-labelled streptavidin was used as a probe in immunoblotting, a strong band was detected corresponding to the B-3ABC fusion protein.

cELISA with the biotinylated recombinant 3ABC protein

The optimal dilutions of antigen and guinea pig hyperimmune sera were predetermined by checkerboard titration. The negative cut off value was set by adding 3 standard deviations of the mean. Frequency distributions of the mean percentage inhibition generated from 200 each bovine, ovine and porcine negative sera showed the negative sera were normally distributed (Figure 4). For a normal population, less than 1% of the test results will exceed this cut off. For the negative populations tested in this study, the mean percentage of inhibition for bovine, ovine and porcine were 7%, -2.7% and -4.1 %, respectively. This gave a cut off of approximately 40%, however, in order to maximise specificity the cut off was increased to 50 % inhibition. Using this cut off value, a specificity of 100% was obtained for bovine, ovine and porcine sera.

Kinetics of the antibody response to NSP antigens

The kinetics of the antibody response to 3ABC antigen in experimentally infected animals was evaluated. Positive reactions could be found in all infected animals as early as 6-7 days post infection (dpi) (Figure 5). There was no difference in the antibody response of cattle, sheep or pigs to the 3ABC recombinant protein when different serotypes of the virus were used. Anti-3ABC antibodies were detected in 10 of 13 cattle at 7 dpi. All 13 cattle were seropositive at 10 dpi and remained positive until the end of the experiment on day 28-30 (Figure 5). All 12 sheep were positive against 3ABC at dpi between 7 and 10 (Figure 5). Some pigs seroconverted as early as 7 dpi, but all were positive at 10 dpi (Figure 5). Recombinant proteins 3ABC, 3D, 3A and 3B conjugated to fluorescent microspheres provided the basis for a novel immunoassay using the Luminex technology to detect the immune response to these antigens. To determine whether the individual NSP immunoassay could be multiplexed, we examined the kinetics of the immune response of experimentally infected animals with different strains of FMDV. Results indicated that the immune response can be detected between 7-14 days post infection (Figure 6). There was also considerable variation in both the magnitude of the overall response to NSPs in individual animals, which in turn may reflect the difference in the NSP immunogenicity and extent of viral replication.

Discussion

Identifying animals that have been infected with FMDV is important for the control of FMD as recovered cattle and sheep frequently remain carriers of the virus and consequently may become the source of new outbreaks of the disease (Alexandersen et al., 2003). The diagnostic challenge is to distinguish infected animals that have been vaccinated or unvaccinated from those that have been only vaccinated against FMDV since both groups have neutralizing antibodies in their sera. Several ELISAs have been developed to distinguish infected animals from those that have been vaccinated, all based on the detection of antibodies to the NSPs of FMD virus (Bermann et al., 2000; Shen et al., 1999; Sorensen et al., 1998). Tests to detect antibodies to the polyprotein 3ABC or part of this protein have so far been the most successful (Mackay et al., 1998).

The majority of available NSP tests use the principle of indirect or blocking ELISA (Shen et al., 1999; Silberstein et al., 1997). In this study, we report the development of a rapid competitive ELISA that utilises the specific binding of a biotinylated 3ABC recombinant fusion protein in a crude bacterial extract to streptavidin-coated plates as a easy single step purification.

The streptavidin system has been used successfully in developing immunoassays and demonstrated good sensitivity and specificity (Clavijo et al., 1998) because of the reduced nonspecific binding and the ability to bind biotin in an interaction that is almost irreversible (Stayton et al., 1999). Streptavidin-biotin interactions are so strong that elution of biotin-tagged proteins from streptavidin-conjugated resins usually requires denaturing conditions.

For the evaluation of the specificity of the 3ABC cELISA a broad spectrum of negative sera from across Canada was analyzed. The antibody response to 3ABC NSP in cattle, sheep and pigs was detected as early as 7 dpi. Although infection of susceptible cattle with FMDV results in a rapid rise (4-5 dpi) of serum-neutralizing antibody which will protect against infection with homologous and antigenically related viruses, the immune response to NSPs is usually delayed (Mackay et al., 1998). Rodriguez et al., 1994 assessed the immunogenicity of different FMDV proteins in swine. After analyzing the specificity of the anti-FMDV antibodies produced against NSPs in sera from infected or vaccinated pigs, they showed that the NSP 3ABC antibodies were detectable from 2 weeks post infection. There is also considerable variation in both the magnitude of the overall response to NSPs in individual animals, which in turn reflected the difference in the NSP immunogenicity and extent of viral replication. Mackay et al., 1998, using a profiling ELISA showed that the antibody response to 3ABC appeared early after infection and antibody to 3ABC could be detected for longer than antibody to any other NSP. The response to 3A, 3B, 3D and 3ABC could be detected in cattle as early as 7-10 dpi and subsequently decreased gradually (Sorensen et al., 1998). However, antibodies to NSPs 3AB and 3ABC in sheep were detected not until 14 dpi as shown by Sorensen et al, 1998. Although our

results were comparable with those obtained by others, an increased sensitivity was seen in early detection of the antibody response to 3ABC in all three species. However, as seen with other NSP tests its use for detection of infection in non-vaccinated animals is limited compared with tests that used structural proteins as antigens due to its low relative sensitivity to detect positive animals early after infection.

We did not see any variation in the detection level when sera from animals infected with different serotypes of FMDV were analyzed. Foster et. al., 1998, performed a series of longitudinal studies of the humoral and cellular immune responses to NSPs in animals infected with different serotypes of FMDV. They observed a highly variable immune responses to all NSPs in short duration.

Although we expect to use this test for the discrimination between infected and vaccinated animals, the 3ABC cELISA will have the same limitations as other NSP tests. Positive diagnosis may be only possible at the herd level because of the great variability in the initiation, specificity and duration of the immune response to the NSPs. This also indicates that the likelihood of detecting or confirming an infected animal is greatly increased if multiple antigens are used as diagnostic reagents (Clavijo et al., 2004).

In conclusion, the use of the streptavidin-biotinylated antigen in the cELISA provides not only a single step purification of the recombinant antigen, but also a surface with a high binding capacity sufficient to make available an excess of reagent and a negligible nonspecific binding. This allows for the highest possible assay sensitivity and specificity. Currently no NSP test has been fully validated and before any can be used for mass screening, it will need to be characterized against a set of standards which should reflect different epidemiological situations. The complexity of FMD and its wide range of hosts make this standardization difficult to achieve, but the availability of a competitive ELISA that can be used in all species may facilitate this process.

Luminex-based technology promises to be a highly specific and efficient method that permits multiplexed antibody characterization to NSPs from a single serum aliquot. Currently this methodology is not designed for high throughput screening and perhaps its major use may be as an alternative to current confirmatory tests for the differentiation of infection from vaccination after a positive or suspicious ELISA result.

References

Alexandersen, S., Zhang, Z., Donaldson, A.I. & Garland, A.J.M., 2003. The pathogenesis and diagnosis of foot-and-mouth disease. *J. Comp. Pathol.* 129:1-36.

Bergmann, I.E, Malirat, V., Neitzert, E., Beck, E., Panizzutti, N., Sanchez, C. & Falczuk, A. 2000. Improvement of a serodiagnostic strategy for foot-and-mouth disease virus surveillance in cattle under systematic vaccination: a combined system of an indirect ELISA-3ABC with an enzyme-linked immunoelectrotransfer blot assay. *Arch. Virol.* 145: 473-89.

Clavijo, A., Lin, M., Riva, J., Mallory, M. & Zhou, E-M. 2001. Development of a competitive ELISA using a truncated E2 recombinant protein as antigen for detection of antibodies to classical swine fever virus. *Res. Vet. Sci.* 70:1-7.

Clavijo, A, Viera-Pereira, P.J. & Bergmann, I. 2003. Use of the reverse transcription polymerase chain reaction (RT-PCR) for the rapid diagnosis of foot and mouth disease in South America. *Vet. Res. Commun.* 27:63-71.

Clavijo, A., Wright, P. & Kitching, P. 2004. Developments in diagnostic techniques for differentiating infection from vaccination in foot-and-mouth disease. *Vet J.* 167: 9-22.

Clavijo, A., Zhou, E-M., Vydelingum, S. & Heckert, R. 1998. Development and evaluation of a novel antigen capture assay for the detection of classical swine fever virus antigens. *Vet. Micro.* 60:155-168.

Mackay, D.K.J., Forsyth, M.A., Davies, P.R., Berlinzani, A., Belsham, G.J., Flint, M. & Ryan, M.D. 1998. Differentiating infection from vaccination in foot-and-mouth disease using a panel of recombinant, non-structural proteins in ELISA. *Vaccine* 16: 446-459.

Pereda, A.J., Konig, G.A., Chimeno Zoth, S.A., Borca, M., Palma, E.L. & Piccone, M.E. 2002. Full length nucleotide sequence of foot-and-mouth disease virus strain O1/Campos/Bra/58. *Arch. Virol.* 147: 2225-2230.

Sambrook, J., Fritsch, E.F. & Maniatis, T. 1989. *Molecular cloning: A laboratory manual*, 2nd ed. Cold Spring Harbor laboratory, Cold Spring Harbor, New York.

Stayton P.S., Freitag, S., Klumb, L.A., Chilkoti, A., Chu, V., Penzotti, J.E., To, R., Hyre, D., Le Trong, I., Lybrand, T.P. & Stenkamp, R.E. 1999. Streptavidin-biotin binding energetics. *Biomol. engineering* 16: 39-44.

Shen, F., Chen, P.D., Walfield, A.M., Ye, J., House, J., Brown, F. & Wang, C.Y. 1999. Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA. *Vaccine* 17: 3039-49.

Silberstein, E., Kaplan, G., Taboga, O., Duffy, S. & Palma, E. 1997. Foot-and-mouth disease virus-infected but not vaccinated cattle develop antibodies against recombinant 3AB1 nonstructural protein. *Arch. Virol.* 142:795-805.

Sorensen, K.J., Madsen, K.G., Madsen, E.S., Salt, J.S., Nqindi, J. & Mackay, D.K.J. 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB, and 3ABC in ELISA using antigens expressed in baculovirus. *Arch.Virol.* 143:1461-1476.

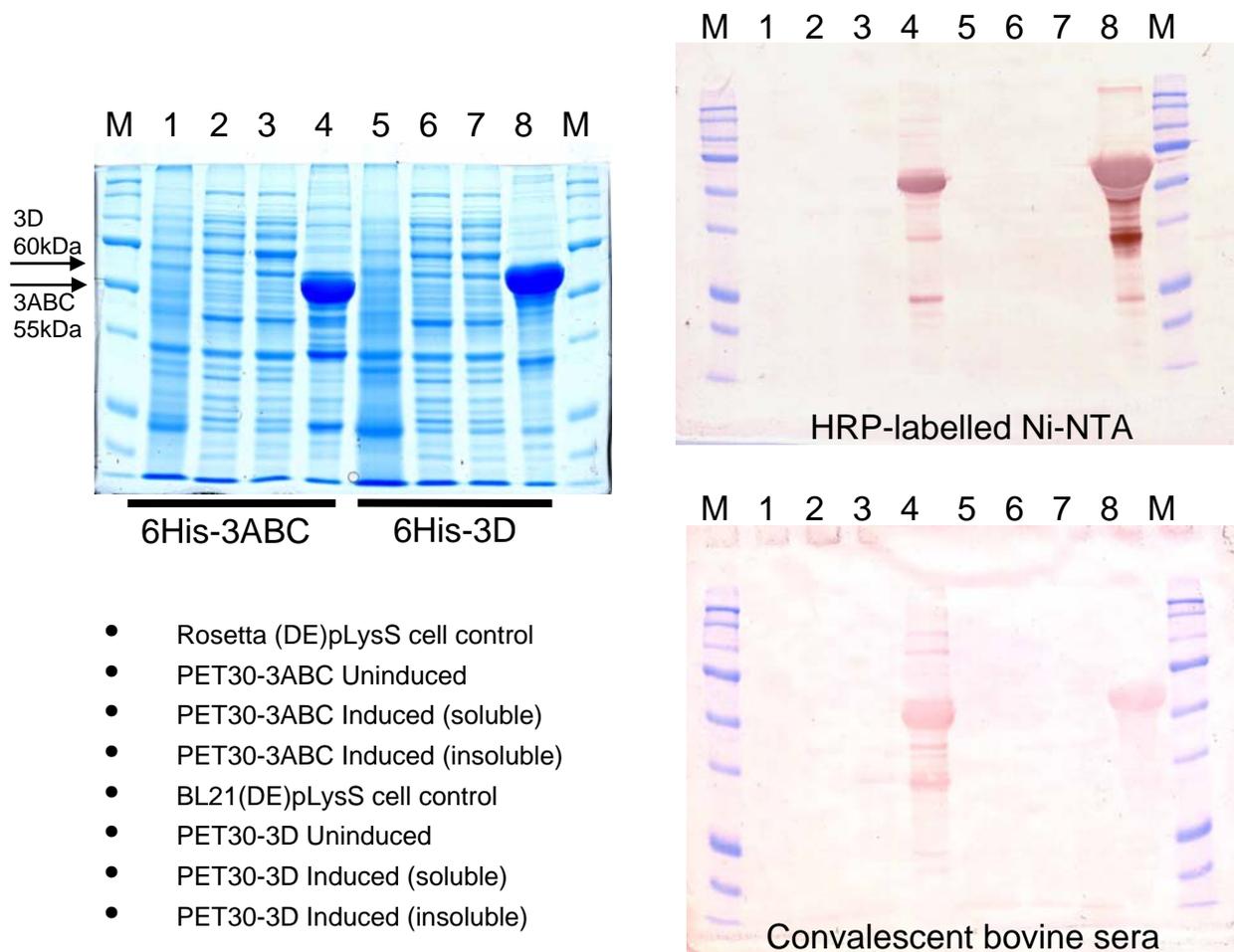


Figure 1. Expression of NSP 3ABC and 3D

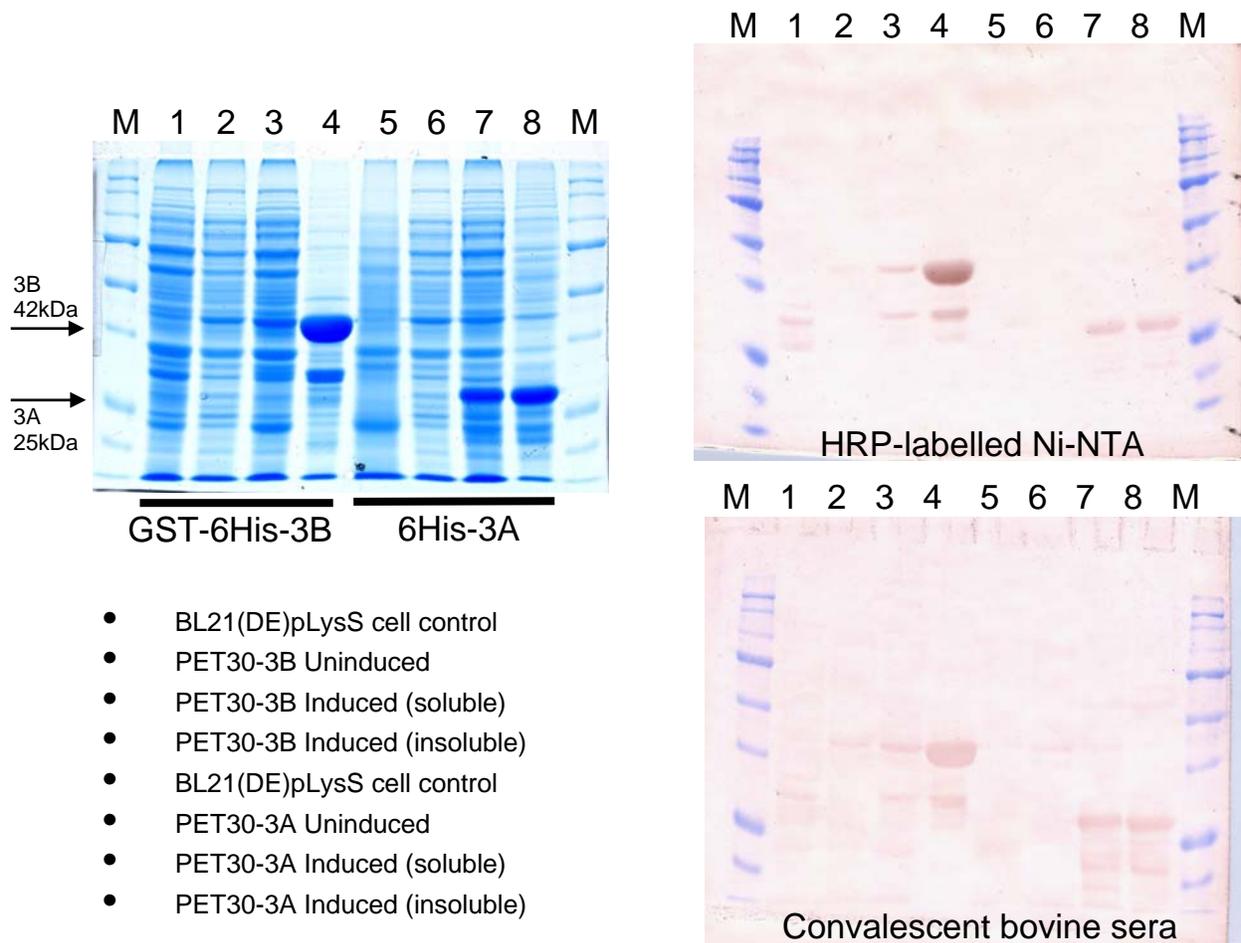


Figure 2. Expression of NSP 3B and 3A

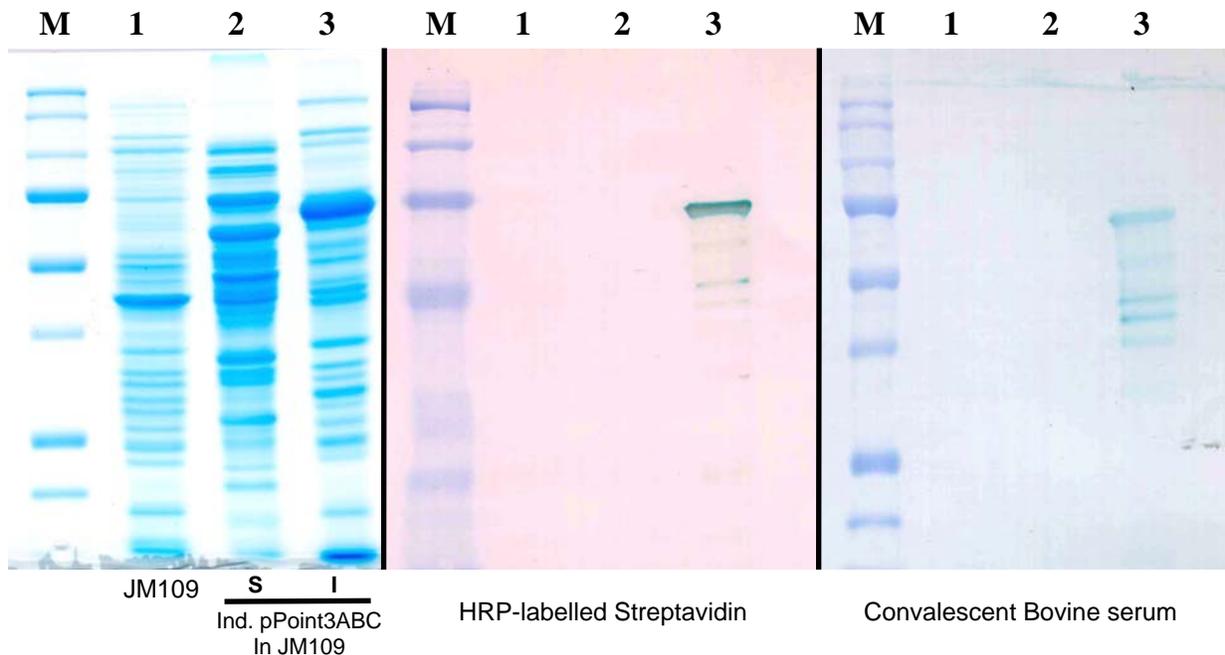


Figure 3. Protein analysis of the pPoint3ABC expression clone. Bacterial cell lysates were resolved using 10% SDS-PAGE and stained with coomassie blue; 1. Soluble extract from JM109 cells; 2. Soluble fraction from induced JM109 cells containing pPoint3ABC expression vector, 3. Insoluble fraction; M. Molecular mass standard in kilodaltons.

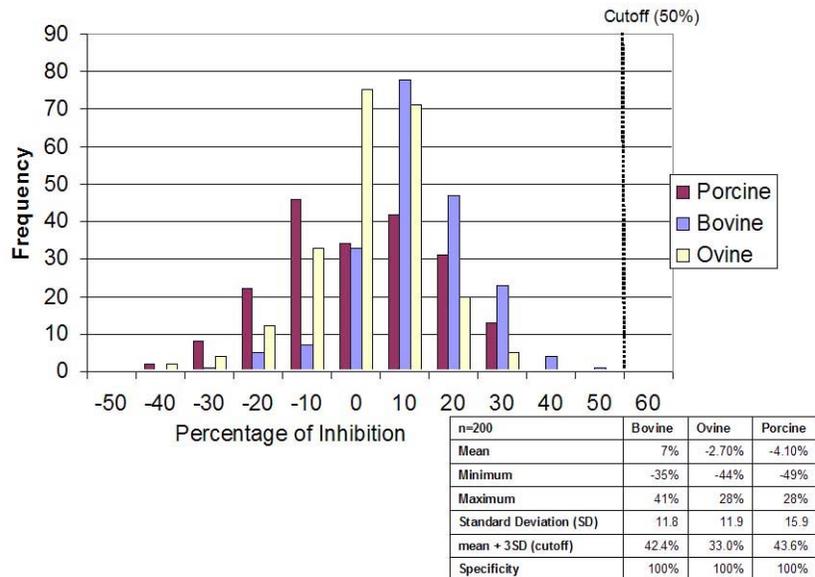
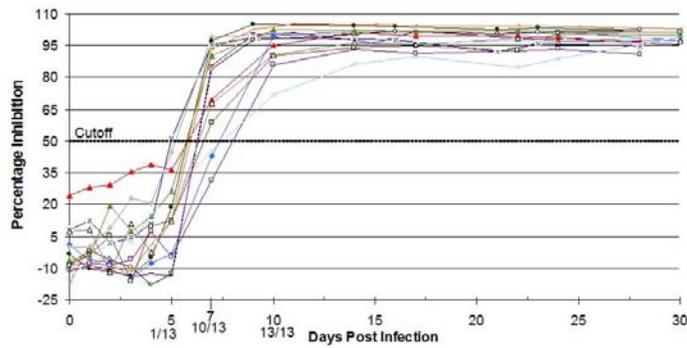
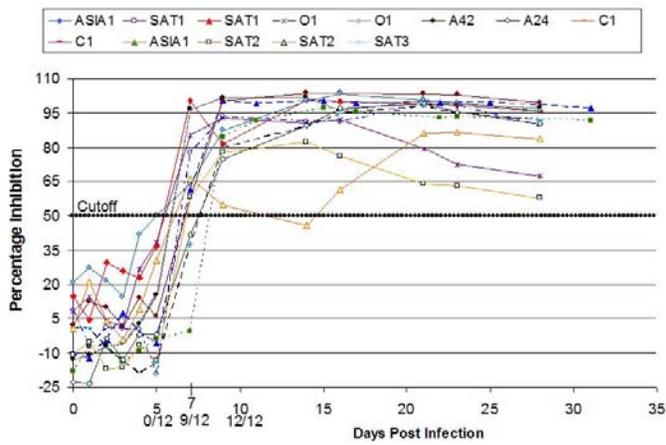


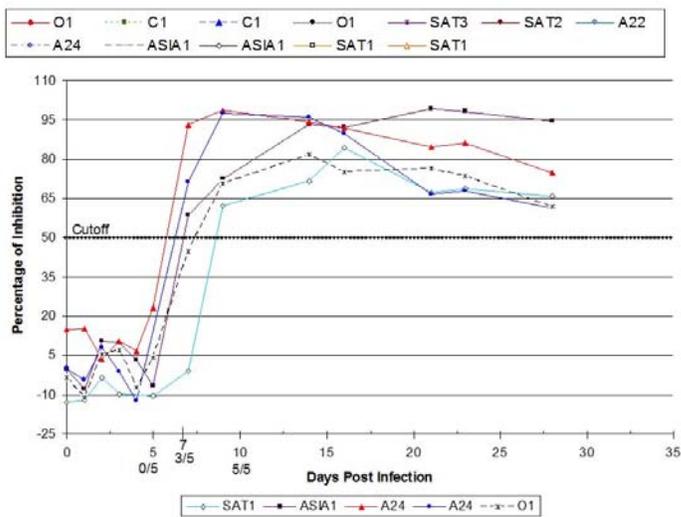
Figure 4. Frequency distribution of negative sera from cattle, sheep and pigs. Sera were tested at a final dilution of 1:10 in the 3ABC competitive ELISA. The vertical line indicates the threshold of the test at 50% inhibition.



A



B



C

Figure 5. Kinetics of the 3ABC antibody response from experimentally infected cattle as detected by the 3ABC cELISA. A. Bovine; B. Sheep; C. Pig.

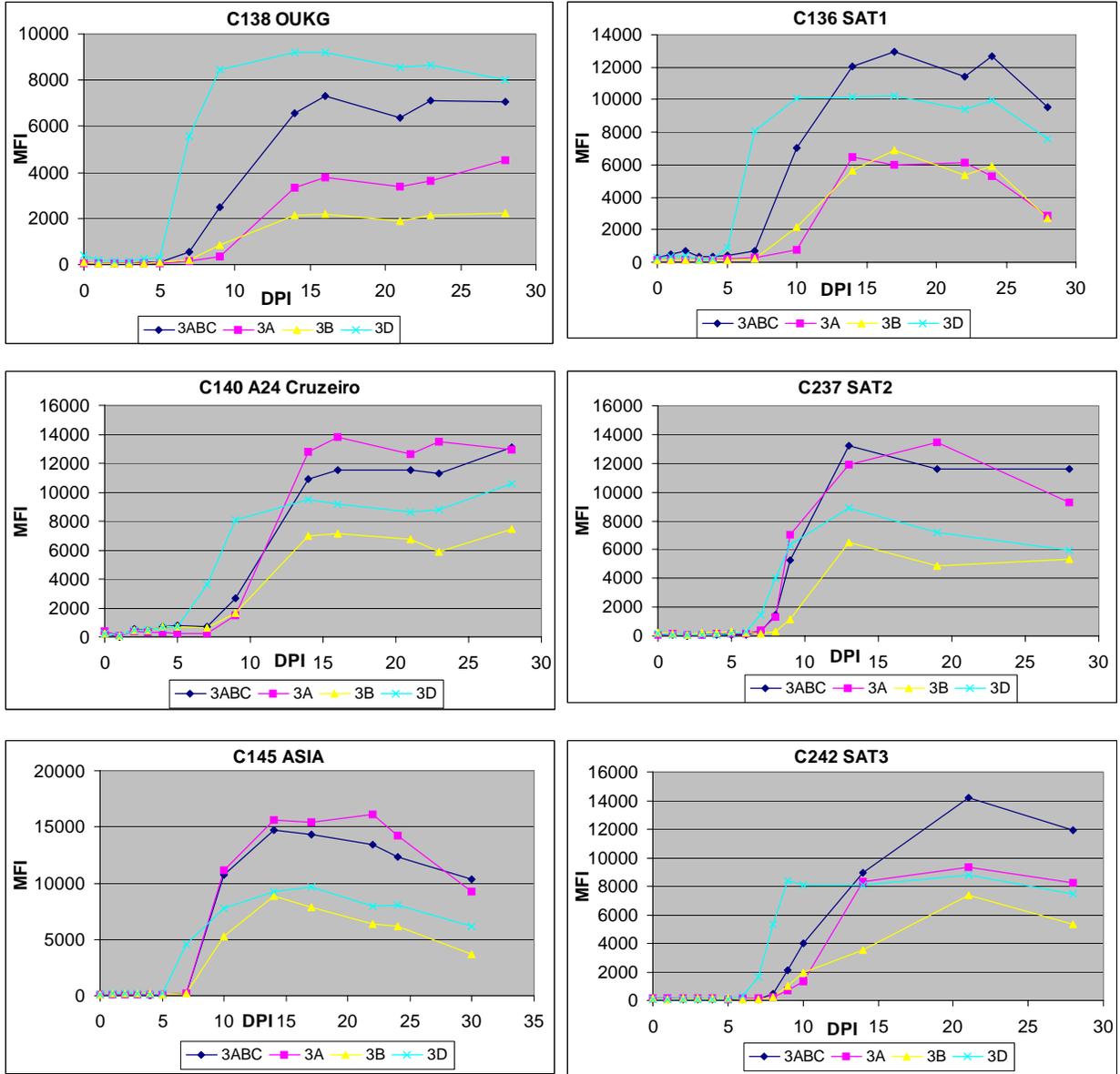


Figure 6. Kinetics of the NSP 3D, 3ABC, 3A and 3B antibody response from experimentally infected cattle as detected by the Luminex-based multiplex immunoassay.

In vitro production of Interferon- γ from whole blood of FMD vaccinated and infected cattle after incubation with inactivated FMDV antigen

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Abstract

Introduction: To aid in the implementation of the "vaccinate-to-live" policy and for declaration of FMD-free status, a number of serological tests are currently available to identify FMDV-infected cattle in a vaccinated population. However, it is difficult to prove complete absence of infection to a high level of certainty and therefore additional methods are needed that could help to confirm sub-clinical infection in vaccinated herds. The main aim of the present study has been to determine whether the detection of cell mediated immune responses to FMDV could be used as a diagnostic tool. **Materials and Methods:** Heparinised blood samples were collected from a vaccine potency test (A Iran 96) and re-stimulated overnight with inactivated FMDV homologous antigen. The supernatant was used to quantify interferon- γ production by a trapping ELISA (Biosource, Europe). **Results:** Interferon- γ production was significantly higher in the blood samples from all vaccinated cattle on day 14 and day 21 post vaccination compared to unvaccinated cattle ($P < 0.05$). The quantity of interferon- γ was significantly higher in full dose vaccine animals than in the other animals on day 21 post vaccination ($p < 0.05$). At all time points post-challenge, the quantity of interferon- γ production was significantly higher in the blood sample from vaccinated animals than from unvaccinated control animals ($P < 0.05$). At 21 days post challenge, vaccinated FMDV carrier animals produced less Interferon- γ than vaccinated non-carrier animals ($p < 0.05$). **Discussion:** Vaccinated and subsequently infected animals appear to have elevated interferon- γ responses and this could be a useful indicator that infection has occurred. Correlations between interferon- γ production and virological protection in challenged animals were compared to those between virus neutralising antibody titre and protection. Future work will aim to establish the cell types responsible for interferon- γ production and the viral antigens that elicit this response.

Introduction

Foot-and-mouth disease (FMD) is a highly contagious disease of domestic and wild cloven-hoofed animals. It is endemic in many countries in Asia, Africa, South America and at the periphery of the European Union. Inactivated whole virus FMD vaccine can protect animals against clinical illness and can reduce the viral spread by reducing replication and excretion of virus in vaccinated and subsequently infected animals. However, the problem associated with FMD vaccination is that it does not necessarily prevent sub-clinical infection which can result in ruminants becoming carriers which may be considered as a potential threat in the spread of the disease. Therefore, serosurveillance is necessary to identify persistent infection in order to regain the FMDV free status. Though a number of serological tests are currently available to identify FMD infected animals within vaccinated herds, it remains difficult to prove complete absence of infection to a high level of certainty. Therefore additional methods are necessary to confirm infection in vaccinated herds. The whole blood interferon- γ (IFN- γ) production assay has been introduced recently for the diagnosis of Tuberculosis in cattle. This assay was originally developed as an adjunct to intra-dermal skin testing (both assays detecting specific cell mediated immune responses) and is based on the production of IFN- γ as a consequence of a memory response to purified protein derivative (PPD) from *M. bovis* [1]. These findings prompted us to investigate the use of a whole blood IFN- γ production assay to identify cattle that have been vaccinated and then infected with FMDV.

Materials and Methods

Animal experiment: A vaccine potency test was carried out in compliance with the current monograph of the European pharmacopoeia 1997: 0063'Foot-and-mouth disease (ruminants) vaccine (inactivated)'. Seventeen female cattle aged 10 months, weighing approximately 200 kg at the outset of the experiment were used for this study. The animals were divided into four groups; the first 3 groups consisted of 5 animals each, whereas the last group contained only two animals (group 4 - unvaccinated controls). The group I animals were vaccinated with a full dose (2ml) whereas the second and third groups received 1/4th (0.5ml) and 1/16th (0.125ml) of the full dose of vaccine (an oil adjuvanted A Iran 96 vaccine), respectively by the intramuscular route. On the 21st day post-vaccination, all the animals were challenged with virulent, cattle-adapted A Iran 96 virus [$10,000 \text{ ID}_{50} / 10^5 \text{ TCID}_{50}$] administered intradermally into two sites on the upper surface of the tongue (0.1ml per site). These animals were then monitored over an 8 day period for any clinical signs of disease and maintained for a further 24 days.

Sample collection: Clotted blood samples for serology, heparinised blood samples for whole blood IFN- γ production assay and oesophago-pharyngeal fluids (probang samples) for virus isolation and for detection of viral RNA were collected at different time points during pre-vaccination, post-vaccination and up to 32 days following challenge.

Virus Isolation: Aliquots of 200 μ l of oesophago-pharyngeal samples were inoculated onto mono-layers of primary calf thyroid (BTY) cells for virus isolation [6]. Cell mono-layers were examined for the appearance of cytopathic effect at 24, 48 and 72 hours post-infection. Serotyping ELISA [6] was performed on the supernatant of the positive tubes to confirm the presence of FMD virus.

Real-time PCR: To detect viral RNA in oesophago-pharyngeal fluids, real-time RT-PCR was carried out as described by Reid and colleagues [7], using a more appropriate probe (kindly provided by S. Alexandersen) which had one base change compared to that used in earlier studies (SAmulti2-P-IR-292-269R).

FMD virus neutralisation and NSP tests: Titres of neutralising FMD antibodies were measured by the micro-neutralisation assay as described in the OIE Manual [6]. Serum samples were also examined for the presence of antibodies against non-structural FMDV poly protein 3ABC using a commercial kit (Cedi-Diagnostics FMDV-NS) based on assay of Sorensen and colleagues [8].

Whole blood IFN- γ production assay: To measure the production of IFN- γ from white blood cells restimulated with FMD vaccine antigen, a whole blood assay was carried out [1] in two steps. The first step consisted of a short-term culture of heparinised whole blood in the presence or absence of FMDV antigen and the second step was a capture ELISA for the measurement of IFN- γ levels in the plasma of the induced blood. The assay was set up within 24 hours of blood collection. For this purpose, concentrated FMDV vaccine antigen (A Iran 96 or O₁ Manisa) without adjuvant was diluted 1:10 in sterilised PBS and centrifuged at 8000 \times g for 10 minutes and then filtered through a 0.2 μ m filter to remove particulate matter. For induction, 50 μ g of the above antigens were added in duplicate to 200 μ l of heparinised blood in tissue culture grade 96 well plates. Negative control antigens, PPDA (M. Avium PPD) at 10 μ g per ml or an equal volume of PBS were added in duplicate to 200 μ l of blood. Phytohemagglutinin (PHA) (10 μ g/ml) and Poke Weed Mitogen (PWM) (10 μ g/ml) were used in duplicate as positive controls. Finally, duplicates of 250 μ l of whole blood were processed without induction to obtain baseline plasma samples. As further specificity controls, blood samples collected from two naïve cattle from another experiment were stimulated with FMDV antigen, PHA, PWM and PBS as above. The plates were incubated at 37°C for 24 hours in a CO₂ incubator. The plates were then centrifuged at 1000 rpm for 10 minutes at 4°C. A 100 μ l volume of supernatant was pipetted out from each well into a new 96 well plate and stored at -20°C until required.

Bovine IFN- γ ELISA kits were obtained from Biosource Europe S.A., Belgium and the ELISA was conducted according to the manufacturer's instructions except 25 μ l of supernatant was diluted with filtered deionised water to a final volume of 100 μ l. IFN- γ standards containing differing, known amounts of interferon were tested in duplicate in the first two columns of each ELISA plate and the quantity of IFN- γ in each unknown sample was estimated from the standard curve obtained from the known standards.

Results:

Clinical signs: Following needle challenge, the control animals suffered from pyrexia on the first and second day following challenge (40.5 to 41°C) and temperatures remained moderately high for three more days (39-39.5°C). Only one animal (UZ54) from the first group of vaccinated cattle showed a slight rise of temperature (39.4-39.5°C) on the first two days following challenge. All of the cattle from the second group except one (UZ62) had an elevated body temperature (40 to 40.8°C) at one day following challenge, whereas all of the animals from the third group suffered from fever (40.1 to 40.9°C) on the first two days following challenge. Severe vesicular lesions were observed in both the control animals on the third and fourth day following challenge, whereas lesions were observed in the back feet of one animal from group 1 (UZ54). All other vaccinated cattle were protected from FMD lesions at the 8th day post- challenge and the vaccine passed the EP test with a calculated potency in excess of 6 PD₅₀.

Virus isolation and real time RT-PCR: Probang samples collected up to 28 days post-challenge from the unvaccinated control animals were found to be positive by RT-PCR and also by virus isolation. Viral RNA and live virus was detected in probang samples as recorded in Table 1. At 28 days after challenge, two animals from group 2 (UZ58 and 60), one animal from group 3 (1/16 dose vaccine) (UZ64) and both control animals were found to be persistently infected by virus isolation. FMDV RNA could also be detected in animals from group 2 (UZ59 and UZ60) so that seven animals in total

comprising five vaccinated and two unvaccinated cattle were positive for virus and/or viral RNA at 28 days after challenge.

Detection of virus neutralising antibody: The virus neutralising antibody responses following vaccination and challenge are shown in Figure 1. All of the cattle were seronegative at the onset of the study. After 21 days post-vaccination, all of the vaccinated animals were seropositive, the titre of all animals except one (UZ64) was greater than 1/64. It appears from the pattern of post challenge neutralising antibody responses (Figure 1) that the plateau titres attained by groups 2 and 3 (1/4 and 1/16 dose vaccine) were influenced by a boosting effect of infection after vaccination. However, infection alone (group 4) induced similar neutralising antibody titres. Interestingly, the neutralising antibody titres of the animals in group 1 (full dose vaccine) were significantly lower than the titres of the other groups on Days 21, 28 and 32 post-challenge ($P < 0.01$). The lower antibody titres of group 1 are probably related to the failure to consistently establish infection after challenge in these animals.

Detection of antibodies against non-structural 3ABC FMD protein: Antibodies against the non-structural 3ABC protein were detected using a commercial 3ABC blocking ELISA (Ceditest FMDV-NS, Cedi-diagnostics, Netherlands). All of the cattle were Ceditest negative pre- and post-vaccination. All of the cattle showed an increase in test reactivity following challenge, although in three animals (UZ55 from group 1 and UZ59 and 62 from group 2) the levels did not at any time surpass the manufacturer's cut-off for seroconversion, which is set at 50%. By the end of the experiment at 32 days post-infection, seven out of the 15 vaccinated and subsequently challenged cattle scored negative in the test. Indeed, two animals UZ59 and UZ62 were virus positive and CEDI-test negative on 28 day post-challenge (Table.3).

Interferon- γ : The ability to detect vaccinated and challenged animals using the production of IFN- γ from re-stimulated whole blood assays was initially determined using the manufacturer's recommended cut-off optical density value of > 0.22 as positive. In the full dose vaccine group (group 1), all of the animals were IFN- γ positive on days 5, 14 and 21 following vaccination. However, by 32 day post-challenge only three of the five animals were IFN- γ positive. In contrast, only one of five animals administered with 1/4 dose vaccine (group 2) was IFN- γ positive on 21 day post-vaccination, also only one of these five animals was IFN- γ positive 32 day post-challenge. However, four of the five animals that received a 1/16 dose of vaccine were IFN- γ positive on 21 day post-vaccination. Similarly, 32 days after subsequent challenge four of the five animals were IFN- γ positive.

Comparisons were made of the mean quantity of IFN- γ produced by the different groups after whole blood re-stimulation with A Iran 96 viral antigen and the results are shown in Figure 2. IFN- γ production was significantly higher in the blood samples from the three groups of vaccinated cattle on day 14 and day 21 post-vaccination, compared to unvaccinated cattle ($p < 0.05$). The quantity of IFN- γ produced was significantly higher in group 1 compared to the other three groups at 21 days post-vaccination ($p < 0.05$). Also, at all time points after challenge, the quantity of IFN- γ produced in the blood samples from vaccinated animals was significantly higher than the quantity produced in the samples from the unvaccinated control animals ($p < 0.05$). The results were re-analysed and the animals grouped as carriers and non-carriers. At 21 days post-challenge, vaccinated FMDV carrier animals produced less IFN- γ in comparison to vaccinated non-carrier animals ($P < 0.05$) (Figure 3).

Discussion:

During the course of a FMDV vaccine potency test in cattle, we analysed samples from the animals to investigate the feasibility of using a whole blood IFN- γ assay as a measure of the cell mediated immune response. This whole blood assay was compared with virus isolation, real-time RT-PCR, neutralising antibody titre and non-structural protein ELISA to identify immune and infected animals.

It was observed that in the cattle receiving the highest payload of vaccine (group 1), local virus replication was considerably reduced and none of the animals became persistently infected. One animal (UZ54) developed a slight fever and hind foot lesions in the early post-infection period, but the only indication of viral replication was the detection of viral RNA eight days post-challenge. The antibody responses of the group 1 animals were also suggestive of a low level of viral replication. The pattern of virus neutralising antibody titres showed little or no boosting effect after challenge. There were also weak post-challenge responses to the non-structural 3ABC protein; animals showed a relatively delayed or incomplete seroconversion, and all scored below the positive threshold by 32 days post-infection (data not shown). Using the IFN- γ assay, three of the five animals were positive at 32 days. The antigen used to stimulate the whole blood assays is predominantly structural proteins; similarly, neutralisation assays detect antibody response to structural proteins. Hence, it is not surprising there is some correlation between the neutralisation assay and the IFN- γ whole blood

assay in these animals that had been vaccinated but in which little or no productive infection could be detected.

In the $\frac{1}{4}$ vaccine dose payload group there was more fever and virus was recovered more frequently from the oropharynx. This persisted to 28 days post challenge in four out of the five animals. Also, there was a clear boosting effect in the pattern of neutralising antibody titre after challenge. Three of these five cattle became seropositive by the CEDI test (3ABC ELISA), although two of the persistently infected cattle did not score positive in the test at 32 days post-infection, emphasising the fact that such tests are not entirely reliable at detecting individual persistently infected animals. The failure to prevent viral replication and persistence in the group did not appear to be due to an inadequate vaccine application, since the titres of virus neutralising antibody at 21 days post-vaccination were very similar to those found in the well-protected, group 1 cattle. Again, this indicates that neutralising antibody titre alone does not correlate with virological protection. Interestingly, IFN- γ production was significantly lower in this group of animals compared to the group of calves receiving the full dose of vaccine (group 1) who were better protected. These findings suggest that a combination of neutralisation titre and whole blood IFN- γ production could provide a better correlation with virological protection.

The third group of cattle that received the lowest payload of vaccine in the form of a 1/16 dose were surprisingly well protected. A lack of correlation between vaccine dose and response in potency test had been recorded earlier [9]. None of the cattle in this group developed foot lesions and virus was only recovered from the oropharynx of a single animal, UZ64, at a single time point, 28 days after infection. However, all of these group 3 cattle showed a marked increase in the levels of neutralising and 3ABC ELISA antibodies after infection, suggesting that there had been considerably more virus replication than was the case for the group 1 cattle. Probably this occurred in the first week of infection and might have been detected by more frequent sampling in this early post-challenge period. However, this was not possible in the present study, as it might have interfered with the evaluation of the vaccine's potency. The mean neutralising antibody titres of the group 3 cattle after vaccination, but before challenge were lower than in the group 2 cattle and this again points to a lack of correlation between neutralising antibody levels and virological protection. However once again, the quantity of IFN- γ produced in the whole blood assay from the animals in group 3 was higher than those of group 2, suggesting a better correlation with virological protection. Rweyemamu and colleagues [10] observed that above a certain vaccine payload, little further benefit is gained with respect to increasing the neutralising antibody response. It may be of interest to revisit these vaccine payload titration experiments and determine whether IFN- γ response in a whole blood assay can be correlated with protection.

In the present study, it was observed that unvaccinated cattle produced very little IFN- γ after infection, but that vaccination primed animals for a strong post-infection response (Figure 1). It would have been useful to have kept some vaccinated animals beyond 21 days post-vaccination, without challenge, to confirm that the dramatic rise after challenge was indeed in response to infection with live virus, although this explanation seems extremely likely. The level of IFN- γ on the day of challenge correlates quite well with protection from persistent infection, with group 1 and 3 cattle showing stronger responses than group 2 and going on to produce fewer FMD carriers. Taking a cut-off of 0.22 OD units, three cattle do not exactly fit this pattern; UZ61 and UZ65 had lower values but did not become carriers, whilst UZ59 had a higher value but remained virus positive. This is not all that surprising, since even amongst naïve animals there is considerable variability in the proportion of carriers engendered by infection. These results suggest that an ability to produce IFN- γ may be a better indicator of post-vaccinal protection against persistent infection than the titre of neutralising antibody. It would be interesting to study more infections in naïve animals to see if any correlation exists between the speed of onset, magnitude and duration of IFN- γ production in these whole blood assays and whether persistent infections develop.

It is still not clear why there was more virus replication in the group 2 cattle in the present study than those in group 3 which received a lower dose of vaccine. The one animal of group 1, in which there was generalisation of disease to involve the feet, is not explained by either IFN- γ responsiveness or levels of neutralising antibody.

IFN- γ responses between five and 14 days after challenge were rather variable even within a particular vaccinated group and some individuals produced very strong restimulation responses. In the two unvaccinated cattle, responses had reverted to baseline levels by 9 days post-infection. Two cattle that went on to become carriers had very high IFN- γ responses at these post-challenge time-points, UZ59 and UZ64, accounting for the lack of a clear difference between carriers and non-carriers (Fig. 2). However, by 21 and 32 days post infection, there was a tendency for non-carriers to have higher levels of IFN- γ than carriers and in group 2, four out of five cattle no longer responded to

FMD restimulation by 32 days post-infection. This contrasted with the situation in groups 1 and 3 where three of five and four of five cattle respectively, still responded at this time-point.

Interestingly, specific IFN- γ production was only transiently detected, up to nine days post-challenge, in the samples from the unvaccinated control animals, suggesting extensive viral replication may be associated with suppression of cellular immune responses [11] as reflected in a reduced capacity to stimulate IFN- γ production in the whole blood assays. The tendency of persistently infected animals to have lower IFN- γ values compared to animals that cleared virus may also be related to the immunosuppressive capacity of the virus.

Conclusions:

- From this initial study, it appears that high levels of IFN- γ production may be found in many vaccinated and subsequently infected cattle, irrespective of the vaccine dose.
- Cell mediated immunity may be playing a role in virological protection.

Recommendations:

- Future work should establish the cell types responsible for interferon- γ production and the viral antigens that elicit this response.

Acknowledgements:

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References:

- 1 **Wood, P.R. & Jones, S.L.** 2001. BOVIGAM (TM): an in vitro cellular diagnostic test for bovine tuberculosis. *Tuberculosis*, 81(1-2), 147-155.
- 2 **Wood, P.R. & Rothel, J.S.** 1994. In-Vitro Immunodiagnostic Assays for Bovine Tuberculosis. *Veterinary Microbiology*, 40(1-2), 125-135.
- 3 **Boehm, U., Klamp, T., Groot, M. & Howard, J.C.** 1997. Cellular responses to interferon-gamma. *Annual Review of Immunology*, 15, 749-795.
- 4 **Barnett, P.V., Keel, P., Reid, S.** et al. 2004. Evidence that high potency foot-and-mouth disease vaccine inhibits local virus replication and prevents the 'carrier' state in sheep. *Vaccine*, 22(9-10), 1221-1232.
- 5 **Vanlierop, M.J.C., Nilsson, P.R., Wagenaar, J.P.A.** et al. 1995. The Influence of Mhc Polymorphism on the Selection of T-Cell Determinants of Fmdv in Cattle. *Immunology*, 84(1), 79-85.
- 6 **Anon.** 2004. Manual of diagnostic tests and vaccines for Terrestrial Animals (mammals, birds and bees) 5th edition, Vol I, OIE. Paris, France.
- 7 **Reid, S.M., Grierson, S.S., Ferris, N.P., Hutchings, G.H. & Alexandersen, S.** 2003. Evaluation of automated RT-PCR to accelerate the laboratory diagnosis of foot-and-mouth disease virus. *Journal of Virological Methods*, 107(2), 129-139.
- 8 **Sorensen, K.J., Madsen, K.G., Madsen, E.S., Salt, J.S., Nqindi, J. & Mackay, D.K.J.** 1998. Differentiation of infection from vaccination in foot-and-mouth disease by the detection of antibodies to the non-structural proteins 3D, 3AB and 3ABC in ELISA using antigens expressed in baculovirus. *Archives of Virology*, 143(8), 1461-1476.
- 9 **Filho, Y.L.V., Astudillo, V., Gomes, I.** et al. 1993. Potency Control of Foot-and-Mouth-Disease Vaccine in Cattle - Comparison of the 50-Percent Protective Dose and the Protection against Generalization. *Vaccine*, 11(14), 1424-1428.
- 10 **Rweyemamu, M.M., Ouldrige, E.J., Head, M. & Ferrari, R.** 1984. The effect of antiserum quality on strain specificity assessment of foot and mouth disease virus by the neutralization reaction. *Journal of Biological Standards*, 12(3), 295-303.
- 11 **Bautista, E.M., Ferman, G.S. & Golde, W.T.** 2003. Induction of lymphopenia and inhibition of T cell function during acute infection of swine with foot and mouth disease virus (FMDV). *Veterinary Immunology and Immunopathology*, 92(1-2), 61-73.

Table 1. Virus isolation and Real time RT-PCR results

Animals	odpc	7dpc	14dpc	21dpc	28dpc
UZ53	-	-	-	-	-
UZ54	-	+	-	-	-
UZ55	-	-	-	-	-
UZ56	-	-	-	-	-
UZ57	-	-	-	-	-
UZ58	-	+	+	+	+
UZ59	-	-	+	-	+
UZ60	-	-	+	+	+
UZ61	-	-	-	-	-
UZ62	-	-	+	-	+
UZ63	-	-	-	-	-
UZ64	-	-	-	-	+
UZ65	-	-	-	-	-
UZ66	-	-	-	-	-
UZ67	-	-	-	-	-
UZ68	-	+	+	+	+
UZ69	-	+	+	+	+

Animals UZ53-57 – Gr1 (full dose), UZ58-62 – Gr2 (1/4 dose), UZ63-67 – Gr3 (1/16 dose) and UZ68-UZ69 – Gr 4 (unvaccinated control). + indicates virus isolation positive, * indicates PCR positive and – indicates virus isolation and PCR negative.

Table 2 Virus neutralising antibody responses in serum of cattle following vaccination and challenge

Animal No	0dpc	7dpc	14dpc	21dpc/0dpc	5dpc	8dpc	14dpc	21dpc	28dpc	32dpc
UZ53	0	11	128	90	178	128	178	128	64	64
UZ54	0	16	90	45	178	708	512	512	178	512
UZ55	0	22	64	178	64	178	256	128	128	90
UZ56	0	16	45	64	256	128	355	256	128	355
UZ57	0	0	22	45	128	90	256	128	512	256
UZ58	0	32	64	90	128	512	1024	1024	256	512
UZ59	0	32	90	128	355	708	708	1413	512	512
UZ60	0	0	45	45	178	1024	1024	1024	708	708
UZ61	0	0	64	128	708	708	1024	1024	1413	1024
UZ62	0	64	64	128	178	1024	708	512	708	1024
UZ63	0	0	16	45	355	1024	1413	1413	1413	1024
UZ64	0	0	16	11	90	1024	355	1024	708	1024
UZ65	0	8	45	64	512	1413	708	1024	1413	1024
UZ66	0	22	45	90	1413	1413	1413	1413	1024	1413
UZ67	0	22	45	178	708	512	1024	708	1413	1024
UZ68	0	0	0	0	32	64	512	1024	355	512
UZ69	0	0	0	0	45	90	128	355	1024	708

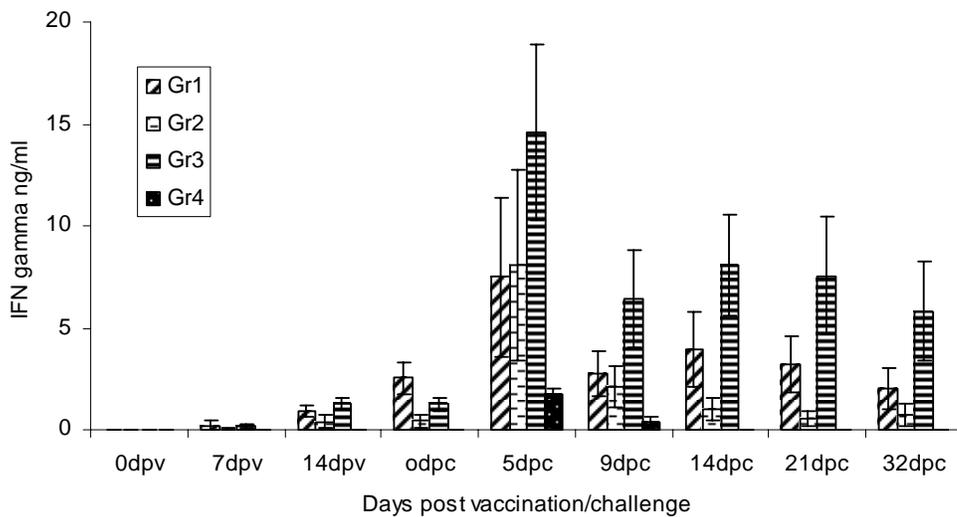
Gr1 (animals UZ53-57) –full dose vaccine, Gr2 (animals UZ58-62) - 1/4 dose, Gr3 (animals UZ63-67) - 1/16 dose, Gr4 (animals UZ68-69) - unvaccinated control

Table 3. Non-structural antibody responses in serum of cattle following vaccination and challenge

Animal no	0dpv	7dpv	14dpv	21dpv/opc	5dpc	9dpc	14dpc	21dpc	28dpc	32dpc
UZ 53	23	25	21	22	19	34	68	61	60	47
UZ 54	-5	-8	-4	-3	3	41	52	51	48	32
UZ 55	6	4	16	16	11	20	41	48	49	46
UZ 56	34	34	36	37	34	57	63	58	56	49
UZ 57	13	16	7	16	13	18	62	34	33	31
UZ 58	0	2	2	-1	0	65	81	85	86	84
UZ 59	-2	-2	-1	-1	0	5	14	42	47	39
UZ 60	12	5	11	8	6	62	58	68	76	75
UZ 61	11	7	5	9	14	70	79	81	82	82
UZ 62	2	-3	2	3	14	20	23	33	44	41
UZ 63	3	-8	1	1	7	43	57	59	59	59
UZ 64	12	14	9	4	6	63	77	84	87	90
UZ 65	4	-1	6	10	16	69	78	77	74	78
UZ 66	4	2	-4	6	4	69	83	85	88	86
UZ 67	2	5	13	8	2	10	46	62	68	65
UZ 68	25	3	1	-	2	73	84	82	81	82
UZ 69	-8	22	20	-	25	76	84	87	91	90

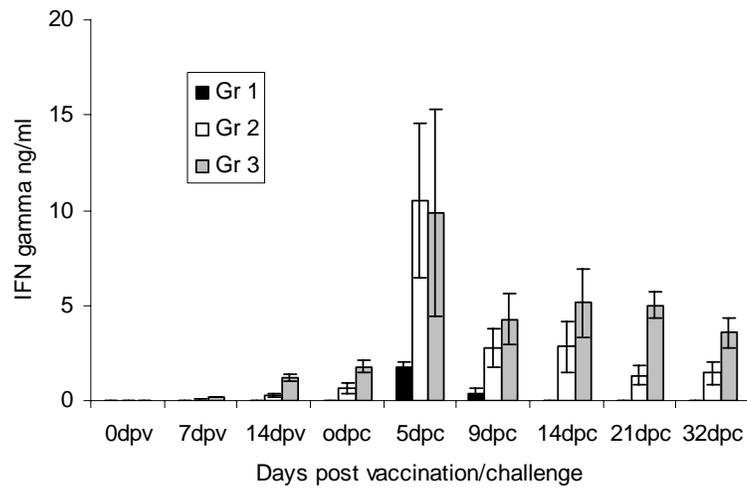
Gr1 (animals UZ53-57) –full dose vaccine, Gr2 (animals UZ58-62) - 1/4 dose, Gr3 (animals UZ63-67) - 1/16 dose, Gr4 (animals UZ68-69) - unvaccinated control

Fig.1 IFN- γ responses in different groups of cattle following vaccination



Gr1 received full dose vaccine, Gr2- 1/ 4 dose, Gr3- 1/16 dose and Gr4- no vaccine.

Fig. 2 Comparison of IFN- γ responses in carrier and non-carrier animals



Gr1- non-vaccinated carrier, Gr2- vaccinated carrier and Gr3- non-carrier.

Secretory IgA as an indicator of oropharyngeal FMDV replication

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Abstract

Introduction: FMDV-specific IgA in oronasal secretions has been reported as a useful indicator of infection in vaccinated cattle. We have developed an ELISA for the detection of IgA to FMDV structural proteins in saliva and evaluated it using sera from cattle following infection or vaccination plus infection. The results have been compared to those of other tests for virus replication and antibody development. **Materials and Methods:** Samples came from naive cattle and from three cattle experiments involving 66 animals and serotype O FMDV. Vaccinated, multiply vaccinated or unvaccinated cattle were challenged by contact with virus-donor cattle. Animals were monitored for clinical signs, virus replication and serological responses and kept for 1-6 months after challenge infection. An ELISA was developed to detect FMDV-specific, salivary IgA using a rabbit capture antibody, whole virus antigen and a commercially available polyclonal anti-IgA conjugate. **Results:** The IgA assay on saliva was specific for infected animals. Vaccination alone, even after two repeat vaccinations rarely resulted in significant levels of IgA being detected. Acute infection lead to the development of elevated salivary IgA and there was a correlation between the continued presence of FMD virus in the oropharynx and elevated levels of salivary IgA. **Discussion:** This new assay has promise for detecting animals that have become persistently infected with FMDV following vaccination.

Introduction

Garland (1974) studied levels of FMDV-specific antibodies in the oral and nasal secretions of FMD vaccinated or infected cattle and found that these were mainly IgA and IgG1 and that they appeared 3-5 days after infection, peaking by 21-28 days. In contrast, killed parenterally administered FMD vaccines produced little secretory antibody that was mainly IgG1, although successive doses of vaccine could elicit IgA. Archetti et al. (1995); Salt et al. (1996) and Amadori et al. (2000) considered that mucosal IgA detection in oesophago-pharyngeal fluids had potential as an indicator of infection. However, Moonen et al., (2004) did not find IgA detection to be a reliable indicator of carrier status in a longitudinal study of FMDV vaccinated and subsequently FMDV inoculated cattle. Here, we report on the development and evaluation of an IgA ELISA with considerable promise for the diagnosis of persistently infected cattle. After initial tests on saliva, nasal and oropharyngeal fluids, saliva was considered the best mucosal fluid to test on account of ease of collection and satisfactory test results.

Materials and methods

Saliva samples were collected from the cheek region of the mouth using cotton tampons pre-dampened by the addition of phosphate buffered saline and held by forceps. In the laboratory, the saliva was extracted from the tampons by squeezing in the barrel of a syringe or by centrifugation. In order to test the specificity of the method, saliva samples were collected from 173 cattle that had not been vaccinated or infected with FMDV, shortly after arrival at our laboratory for a variety of experimental studies. Saliva was also collected at different time-points from three different cattle studies involving vaccination with oil adjuvanted O Manisa and subsequent challenge by contact with donor cattle that had been previously inoculated in the tongue with O UKG 2001. Two of these studies were as described by Cox et al., (this proceedings), except that some animals were retained beyond 28 days for up to 168 days post challenge (dpc, study 1) or up to 105 dpc (study 2). In each case, twenty recipient cattle were vaccinated, whilst 5 remained unvaccinated and all were challenged 21 days after vaccination by 5 days of co-mingling with 5 donor cattle. The vaccinated and unvaccinated recipients and the donors were then split into three separately housed groups. The difference between studies 1 and 2 was that a x10 payload of vaccine was used in study 2. In a third study, six calves were vaccinated on three occasions at 21 day intervals with the x1 payload of vaccine. They were challenged 35 days after the last vaccination by 5 days of co-mingling with 4 donor cattle derived from the unvaccinated control group of study 2, at the point when these calves had been removed after their own 5 days exposure to infection. In each study, samples of blood and oesophago-pharyngeal (OP) fluid were collected periodically for serology and virus detection respectively.

The IgA ELISA was based on a modification of the SPCE (Paiba et al., 2004) using plates coated with rabbit anti-FMDV and subsequently loaded with O Manisa virus capsid antigen. Thereafter, a dilution of saliva was added and IgA was detected with a conjugated polyclonal antibody to bovine IgA. ELISA results were expressed as optical density values. Serology was performed with three commercially available ELISA tests for antibodies to non-structural proteins (NSP) of FMDV according to the manufacturers' instructions. The three tests were the Cedi-Diagnostics FMDV-NS test, the UBI FMDV

NSP ELISA and the Bommeli CHEKIT-FMD-3ABC. Since the Cedi test detected the most infected vaccinates from study 1, it was the only method used to analyse sera from studies 2 and 3.

Results

The frequency distribution of ELISA result values for the 173 saliva samples obtained from FMDV naïve cattle are presented in Fig 1. The median category value was the range from 0.11 to 0.2. At a cut-off of 0.47 (mean plus three standard deviations) the specificity of the test was 97.1%, whereas increasing the cut-off to 0.6 yielded a test specificity value of 99.4%.

None of the vaccinated cattle from any of the challenge tests showed signs of clinical FMD, whereas the control, unvaccinated cattle were all severely affected. Similarly, virus was only detected in the blood of unvaccinated cattle. Results of virus isolation and RT-PCR on OP samples of the cattle from studies 1 and 2 are summarised in Figs 2 and 3 respectively. Despite the absence of clinical signs and viraemia, FMDV was detected in OP fluids from most of the vaccinated cattle as well as in all of the unvaccinated animals. Two groups of animal could be delineated; (1) those from which FMDV could not be detected or detected only transiently, and (2) those that became persistently infected. FMDV was detected in OP samples from nine unvaccinated cattle from study 1 up to and beyond 28 dpc, and from three such cattle in study 2. FMDV was also detected repeatedly in OP samples of two other vaccinated cattle from study 2, up to, but not beyond 28 dpc. FMDV persistence in OP fluids beyond 28 dpc was noted in three unvaccinated cattle from study 2, two regularly (UY95, UY96) and one on an isolated occasion (UY93). In Study 3, no virus was recovered from OP fluids collected at any time after challenge of the multiply vaccinated cattle. However, RT-PCR results are still awaited.

NSP serology revealed seroconversion in all of the unvaccinated contact challenged cattle and in study 1, this result was obtained with all three tests. However, only two sera obtained from the study 1 vaccinates that did not become persistently infected were scored positive by NSP serology (UV20 at 16 dpc and UV8 at 42 dpc – both by the Cedi test). The numbers of persistently infected study 1 cattle that were scored positive by NSP serology is summarised in Fig 4, showing a consistently superior sensitivity with the Cedi test. The two persistently infected cattle that were never scored as NSP seropositive in any test were UV2 and UV14. In the second study, using the Cedi test, 6 animals were scored positive at or beyond 28 dpc on most occasions, namely UY72, UY76, UY79, UY83, UY87 and UY90. From Fig 3, it can be seen that all of these cattle were subclinically infected with FMDV, but only UY76, UY83 and UY90 were found to be persistently infected by virus isolation beyond 28 dpc. Two of the multiply vaccinated cattle (VC14 and VC19) seroconverted in the Cedi test, after challenge. However these two animals revealed higher percentage of inhibition after their third vaccination.

The numbers of persistently infected vaccinated cattle from study 1 scored positive by IgA ELISA, when 0.6 was used as the cut-off OD value, are summarised in Fig 4. An equal or greater proportion was scored positive as when the Cedi NSP serological test was used (up to 8 are positive for IgA and 7 positive for the Cedi test). One persistently infected vaccinated animal was completely undetected by IgA ELISA and this was UV5, which was detected by NSP serology, whilst two animals UV2 and UV14 were not detected at all by NSP serology. In study 2, the six persistently infected cattle (three vaccinates and three non-vaccinates) scored positive by IgA ELISA, although UY90 had a test OD value that fluctuated between 0.3 and 0.7 in the period between 28 and 105 dpc and in the non-vaccinated animal UY93, from which virus was only detected once after 28 dpc, the IgA response was not maintained. Many, but not all of the vaccinated and non-vaccinated cattle identified as transiently infected by virus isolation and RT-PCR were also scored positive in the IgA test; elevated IgA levels were also transient in most cases, but persisted longer in some individuals (UV18, UY72). Representative responses from study 2 are shown in Fig 5.

IgA results for study 3 are depicted in Fig 6. A moderate elevation in IgA was found in some cattle after the second or third vaccination, but only in two cattle, VC15 and VC18, did the OD values exceed 0.5. Two of the cattle showed elevations in FMDV-specific IgA between three and five weeks after challenge and these were also scored positive by the NSP Cedi test.

Discussion

A FMDV-specific IgA ELISA has been developed that gave strong OD signals with saliva from FMDV-infected cattle. Regardless of vaccination status, levels of virus-specific IgA became elevated after infection and persistence of IgA was correlated to viral persistence in OP fluids. Increases in IgA could be detected in vaccinated cattle that had become subclinically infected after exposure to FMD affected donor cattle. The IgA test therefore has considerable potential for the detection of subclinical infection in vaccinated cattle and for identification of persistently infected cattle following the application of a vaccinate-to-live policy. Nevertheless, more work is needed to evaluate the method with larger numbers of samples from the different categories of naïve, vaccinated and vaccinated-and-infected

cattle as well as with samples from other species and after vaccination/infection with other FMDV serotypes. Field testing is also required. These studies are currently underway.

Considerable fluctuation was sometimes noted in the OD values obtained with serial saliva samples obtained from the same animal. This may be related to physiologic changes in the concentration of salivary proteins and requires further study. Test values could benefit from being normalised to the value obtained with a standard control sample.

References

Amadori, M., Haas, B., Moos, A. & Zerbini, I. 2000. IgA response of cattle to FMDV infection in probang and saliva samples. Report of the Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease, Borovets, Bulgaria, 5-8 September 2000. Rome: FAO, Appendix 9: 88-106.

Archetti, I.L., Amadori, M., Donn, A., Salt, J. & Lodetti, E. 1995. Detection of foot-and-mouth disease virus-infected cattle by assessment of antibody response in oropharyngeal fluids. *J. Clin. Microbiol.* 33: 79-84.

Garland, A.J.M. 1974. The inhibitory activity of secretions in cattle against foot and mouth disease virus. PhD thesis, London School of Tropical Hygiene and Medicine, London University and The Animal Virus Research Institute, Pirbright, Surrey May 1974.

Moonen, P., Jacobs, L., Crienen, A. & Dekker, A. 2004. Detection of carriers of foot-and-mouth disease virus among vaccinated cattle. *Vet Microbiol.* 103: 151-60.

Paiba, G. A., Anderson, J., Paton, D. J., Soldan, A. W., Alexandersen, S., Corteyn, M., Wilsden, G., Hamblin, P., Mackay, D. K. J. & Donaldson, A. I. 2004. Validation of a Foot-and-mouth disease antibody screening Solid-phase competition ELISA (SPCE). *J. Virol. Methods* 115: 145-158.

Salt, J.S., Mulcahy, G. & Kitching, R.P. 1996. Isotype-specific antibody responses to foot-and-mouth disease virus in sera and secretions of "carrier" and "non-carrier" cattle. *Epidemiol. Infect.* 117: 349-60.

Acknowledgements

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Fig 1. Frequency distribution for ELISA results with saliva from 173 naïve cattle

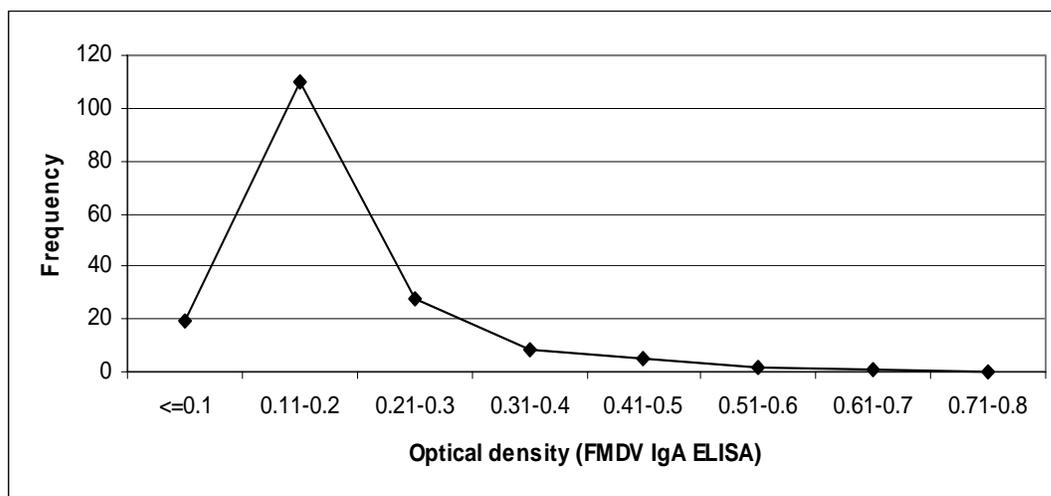


Fig 2. Results of virus isolation and RT-PCR on OP fluids from study 1

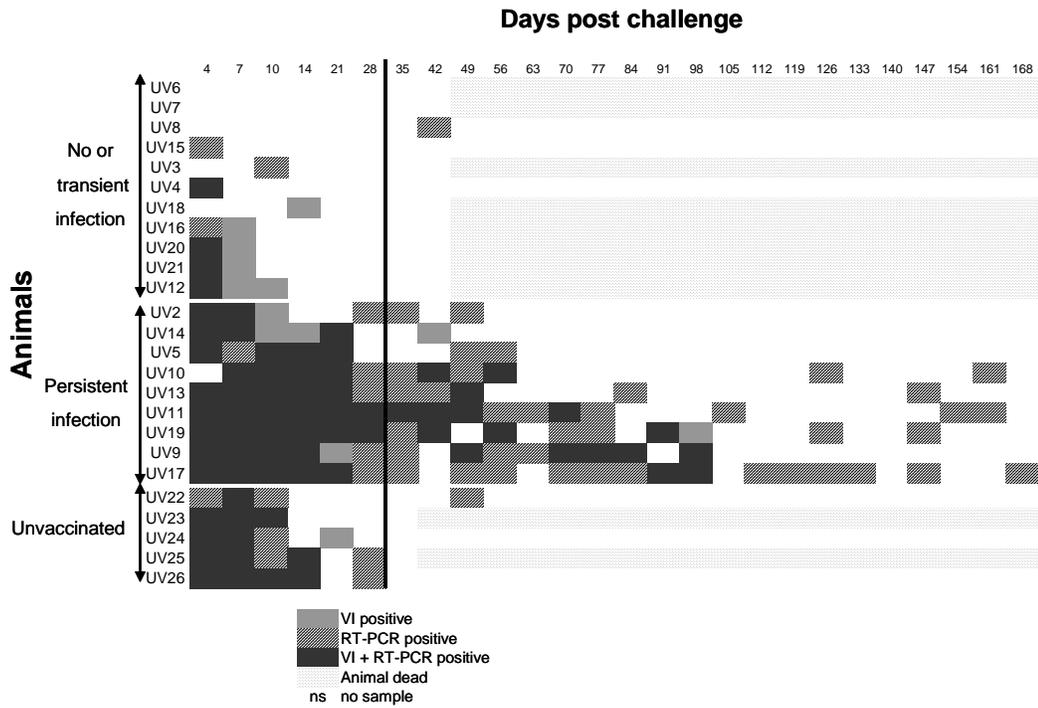


Fig 3. Results of virus isolation and RT-PCR on OP fluids from study 2

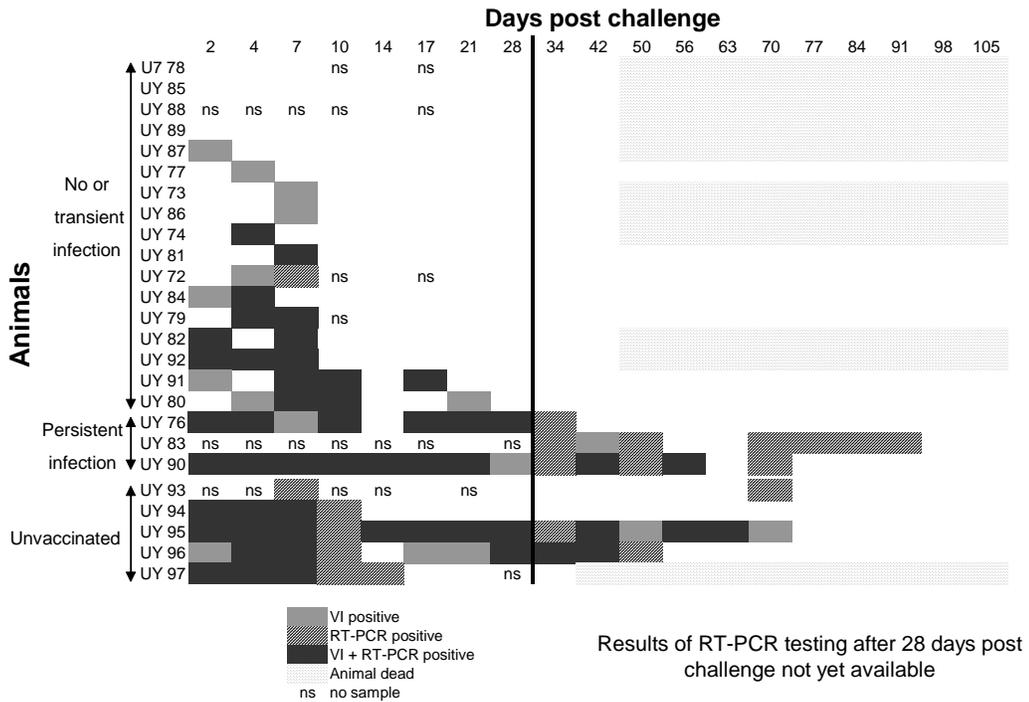


Fig 4. Comparative detection of persistently infected vaccinates from study 1

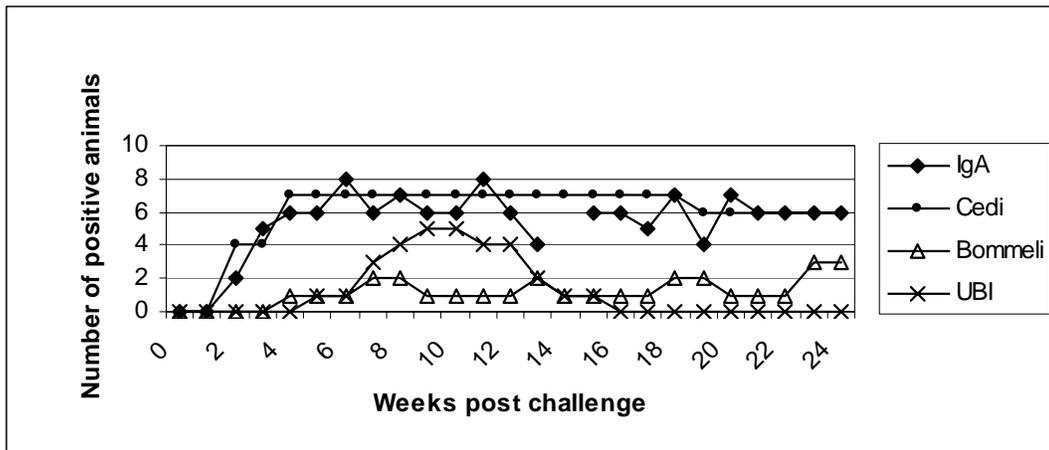
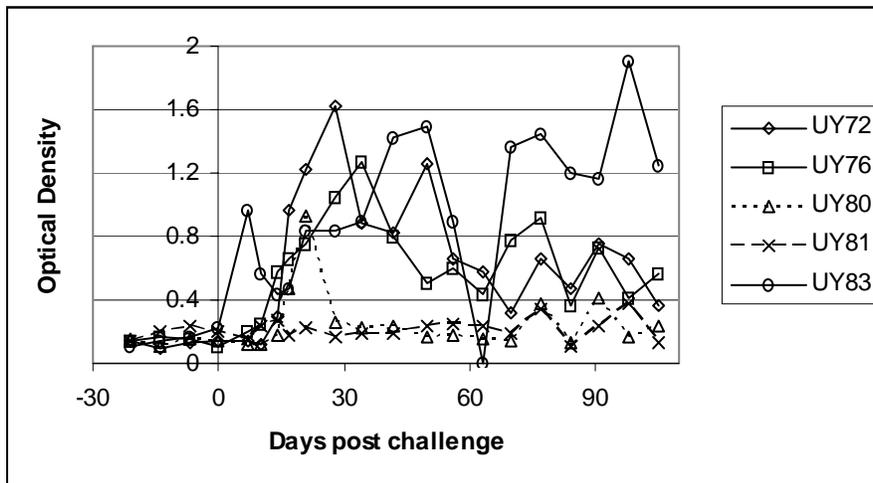


Fig 5. FMDV-specific IgA responses detected in different categories of cattle from study 2

Vaccinated cattle



Non-vaccinated cattle

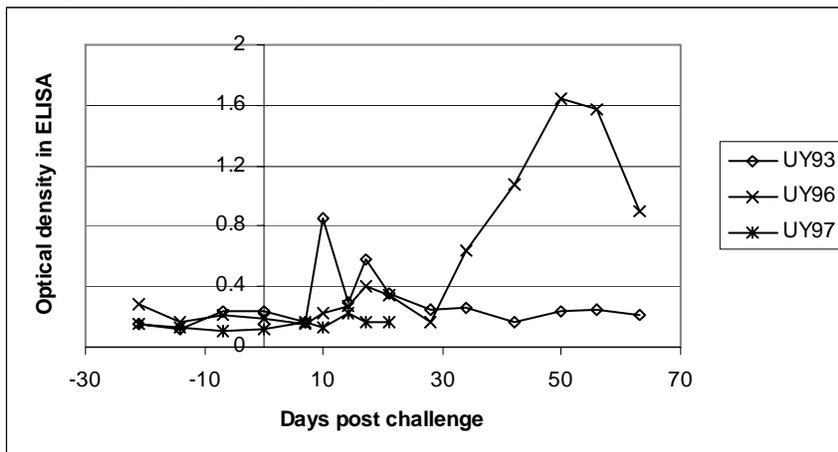
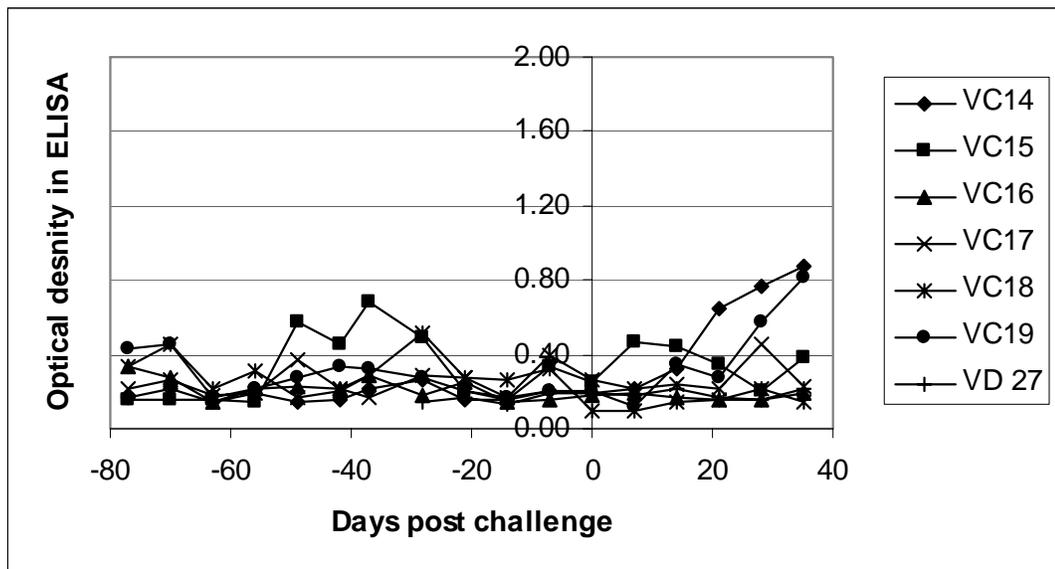


Fig 6. FMDV-specific IgA responses detected in cattle from study 3



Using NSP ELISA (Chekit-FMD-3ABC Bommeli-Intervet) as a Tool for FMDV Serosurveillance in Bulgaria

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Abstract:

Bulgaria has adopted a non-vaccination strategy for the control of FMD since 1993 and is free country from the disease since 1996. For us is deeply important to have a high sensitive and exact laboratory method for early detection of FMDV infection and for serum-surveillance purposes. Therefore Bulgaria has more 300 km. common border with Turkey which use vaccination policy against FMDV in Thrace and a couple of FMD outbreaks caused by more than one and often some exotic FMDV strains each year have been reported in Anatolia. So, each serum sample should be tested minimum against 3 or 4 different serum types of FMDV currently circulating in the neighbouring countries. The commercial 3ABC NSP ELISA tests are available since 2001 on the market. We performed Bommeli-Intervet CHEKIT-FMD-3ABC ELISA test bov-ov in Bulgaria during 2 years period (2002-2003). On this paper we are going to summaries the results of evaluation of the test's sensitivity on the basis of the investigated serum samples. The CHEKIT-FMD-3ABC ELISA was used according to the manufacturers instruction (SOP), accompanied the test-kit 4797 serum samples in 2002 and 5754 serum samples in 2003 have been tested using CHEKIT-FMD-3ABC ELISA Bommeli according to the Bulgarian National monitoring and surveillance FMDV program. Only 4 serum samples from cattle origin have been determined as NSP FMDV antibody positive on the basis of estimation of OD % value and confirming the results using additional NSP ELISA tests. The correlation between the number of non-conclusive and positive evaluated samples have been shown. CHEKIT-FMD-3ABC ELISA Bommeli-Intervet is easy to perform, reproducible and specific. The final result can be obtain in real time and the test can be use with success as a tool in FMDV surveillance programs in FMDV free countries bordering with endemic regions. The non-conclusive serum samples have to be retested using the same or the second confirmation test up to the full determination.

Introduction

Foot-and-mouth disease (FMD) is highly contagious viral and economically devastating disease of cloven-hoofed animals. The causative agent is aphtovirus belonging to the Picornaviridae family for which seven serum types have been described. The detection of antibodies to the nonstructural 3ABC poly protein is the single most reliable indicator of the infection and confirmation of serum conversion of these antibodies is evidence of infection with wild FMD virus (FMDV) (3, 4). Antibodies directed to the capsid proteins of FMDV are induced by both – inactivated (from vaccines) and live viruses (infection, carrier animals) therefore it is not possible to differentiate the origin of the antibodies using routine Liquid Phase (LPBL) or Solid Phase ELISA tests. The non-structural proteins (NSP) of FMDV have received considerable attention in recent years with a search for improved serological tests for FMDV (5). For countries using vaccination in their strategy for the control of FMD outbreaks, it is of a great importance to differentiate post vaccine from post infection derived antibodies in order to discriminate FMDV which is circulated in the field (1). Bulgaria has adopted a non vaccination strategy for the control of FMD since 1993 and has a free country status from the disease since 1996. For us is important to have a high sensitive and exact laboratory method for early detection of FMDV infection and for serum-surveillance needs. Moreover Bulgaria has more than 300 km. common border with Turkey, which use vaccination policy against FMD in Thrace and each year reported of a couple of FMD outbreaks caused by more than one and often some exotic FMD viral strains. There also reports for presence of NSP FMDV serum positives in Thrace as a result of low, but still circulation of wild FMDV in that part of the country (1). The commercial 3ABC NSP ELISA tests are available on the market since 2001. In this paper we present the results of use of Bommeli FMDV 3 ABC NSP ELISA CHEKIT-test (bov-ov) as a tool for FMDV serum – surveillance programs in Bulgaria during 2 years period (2002-2003). We also would like to summary the results of evaluation of the test specificity.

Material and Methods

CHEKIT-FMD-3ABC Bommeli ELISA bov-ov

This ELISA test-kit was used according to the manufacturer's instruction (SOP).

The estimated serum samples were diluted previously 1:100 and added in duplicate to the wells of a 96 well microtitre plates pre-coated with the vector expressed FMD viral 3ABC antigen. The ELISA reaction was made according the SOP. The degree of color that developed was proportional to the amount of antibody complexes on the plate surface and read at 450 nm and reference filter at 492 nm. The final reading for the sample was calculated as follows using the means of the pairs of samples and the median of the 2 positive and 2 negative controls on each plate:

$$\text{Value\%} = \frac{\text{OD sample} - \text{OD neg}}{\text{OD pos} - \text{OD neg}} \times 100$$

Interpretation of the results: if a %OD of less than 20% is negative, 20-30% is ambiguous and greater than 30% is positive.

3 ABC NSP ELISA submitted by WRL FMD Laboratory (WRL – IAH);

3 ABC NSP ELISA submitted by IZSP – Brescia, Italy and

3 ABC NSP ELISA antigen, kindly submitted by CISA Valdeolmos (Spain):

Recombinant 3 ABC polypeptide from FMDV, expressed in *E. coli* was used to perform NSP ELISA. Plates were coated with capture anti 3A NSP protein monoclonal antibodies of FMDV and then the plates have been sensitized by 3ABC NS protein. Detection of antibodies bound to 3ABC in investigated serum samples was performed by addition of anti-sheep or protein – A (for cattle samples) conjugate to horseradish peroxidase (Sigma).

LPHBL FMDV ELISA - WRL FMD Laboratory Pirbright - England:

Monotype LPBL ELISA for O (O-1 Manisa), A (A-5,A-22 and A-24) and ASIA – 1- Samir was used for estimation the type specific FMD antibodies in sera showed positive results in FMDV NSP Bommeli CHEKIT ELISA test (30% cut off). The ELISA tests were performed according the SOP's prepared by the producers. The cut off was established following standard procedures (2).

SERUM SAMPLES:

We made our FMDV serum-surveillance investigations in accordance to the National FMD monitoring programs, approved by the Minister of Agriculture of Bulgaria for 2002 and 2003 years. In 2002 - **4797** serum samples (4293 ovine, 360 caprine and 144 bovine) have been tested. In 2003 - **5754** serum samples have been tested (5435 – ovine, 266 – caprine, 32 - bovine, 15 – from buffaloes, 2 - from deer, 2 – from iah, 2 - from camel).

Results

The CHEKIT Bommeli NSP FMDV ELISA results (4293 from ovine, 360 from caprine and 144 from bovine origin) from serum samples tested during 2002 are shown on Table 1.

The FMDV serum-surveillance program started from 01.04 and finished at 30.11.2002. The 35 sentinel groups of animals were located in a 10-th kilometre border strip zone in Bourgas, Yambol, Haskovo and Kardjali districts of Bulgaria (Map 1.) and bordering with Republic of Turkey and Republic of Greece. In May 2002 when 110 regular samples from (30 cattle, 45 goat and 35 sheep) derived from sentinel village Rezovo (Bourgas District) were negative for presence of antibodies to 3ABC FMDV by NS proteins by ELISA test, while 4 samples from cattle showed positive result by this test. All the samples were retested again with FMDV NS 3ABC Bommeli CHEKIT ELISA test and the same sample from cattle were positive for presence of antibodies against NS proteins of FMDV. The clinical and epidemiological investigations were negative for any evidences for presence of FMDV clinical symptoms or the disease in the village and the surrounding region.

Then we tested the same bovine serum samples positive by Bommeli CHEKIT-FMD-3ABC ELISA using the reagents for detecting NSP FMDV antibodies from three different sources - WRL-IAH Pirbright, England, IZPS – Brescia, Italy and CISA Valdeolmos, Spain with the same result. All 4 positive serum samples by CHEKIT Bommeli were positive for FMDV

antibodies to NS proteins by these 3 ELISAs (Table 2). In that time the suspected animals have been destroyed.

Further investigations using LPBL monotype ELISA were negative and we haven't a success to detect FMDV antibodies to structural FMDV proteins and to determine the type specificity of the antibodies. The additional serological and clinic investigations with the samples and animals of the Rezovo village region showed constant negative results.

The evaluation of the specificity of CHEKI-FMD-3ABC on the basis of distribution of % OD value for 2002 correlated with the NSP serum samples found positive. As shown on Fig 1. only 65 tested samples had range between 20-20% OD or non-conclusive statues. 25 another samples have been evaluated with positive result by CHEKIT-FMD-3ABC ELISA. This is well demonstrated using the distribution of % OD value for the suspected samples from Rezovo cattle, investigated in May 2002 (Fig.2).

In 2003 – 5754 serum samples have been tested (Table 3) using the NSP FMDV ELISA CHEKIT Bommeli. The FMDV surveillance program started again from 01.04.2003 and finished at 30.11.2003. The same 35 sentinel villages were located in a 10-th kilometre border strip zone in Bourgas, Yambol, Haskovo and Kardjali districts of Bulgaria as shown in Map 1.

5694 samples of them were derived from the sentinel animals and in a difference from previous 2002 year they had mainly ovine and caprine origin. All 5754 tested serum samples have been determined as negative for presence of NSP FMDV antibodies. The CHEKIT-FMD-3ABC ELISA OD % value results are shown on Fig 3. The only 5 samples that have a OD% value more than 30% are result of testing additional positive control sera for different purposes and not have the origin from suspected or FMDV positive samples or animals.

Discussion and Conclusions

The virus neutralization test (VNT) and monotype LPHBL ELISA are currently the only prescribed and recommended tests by the O.I.E. for the trade and surveillance. However, these tests require each serum sample to be tested separately for presence of FMDV specific-type antibodies and can not differentiate vaccinated from infected animals. The work with live virus for performance the VNT requires cell culture and virus containment facilities and takes 2-3 days minimum to provide the results.

The evaluation of the specificity of CHEKI-FMD-3ABC ELISA on the basis of distribution of % OD value correlated with the NSP serum samples found positive. Its well shown that only in May 2002, when the suspected animals for the presence of FMDV NSP antibodies have been found and tested in our laboratory the number of samples demonstrated with % OD value as positive or non-conclusive with CHEKI-FMD-3ABC ELISA are grater. Therefore for Bulgaria, which has not enough financial recourses and laboratory staff capacity, and meanwhile is free from the disease for a several years it's easier and chipper to use only one test for FMDV serum-surveillance purposes from the economically point of view.

On the basis of these results and from the experience derived from testing more than 10 000 serum samples from sentinel animas for presence of NSP FMDV antibodies during 2002-2003 we can conclude that CHEKIT-FMD-3ABC ELISA Bommeli can be use with the success as a tool in FMDV serum-surveillance programs in countries free from FMD, using non vaccination practice for the control of FMDV and bordering with regions where FMDV still circulate or still endemic. The test is easy to perform, quicker with reproducible results and with high specificity. Therefore the non-conclusive serum samples for presence of NSP FMDV antibodies have to be retested using the same or the second confirmation test up to their full determination.

Acknowledgements

The authors would like to thank Bommeli Diagnostics Company for technical assistance and calculation of the %OD value for NSP FMD ELISA results during 2002-2003 years made in NRL of Bulgaria accordance to the National FMDV serum-surveillance programs of Bulgaria.

References

Bulut A.N., Cokcalypkan, C. & Aplay, B. 2002. A serosurvey to trace non-structural proteins to FMDV conducted with the sera from Thrace region of Turkey. Report of the

session of the Research Group of the European Commission for the Control of Foot-and-mouth Disease, Cesme, Izmir, Turkey, 17-20 September, Rome: FAO, pp. 87-92.

O.I.E. 2000. *Manual of Standards for diagnosis tests and vaccines*, 4th edition, Chapter 2.1.1

De Diego, M., Brocci, E., Maccay, D. & De Simone F. 1997. The non-structural protein 3ABC of foot-and-mouth disease virus as a diagnostic antigen in ELISA to differentiate infected from vaccinated cattle. *Arch. Virol.* 1997, 142, 2021-2033.

Mackay, D.K.J., Forsyth, M.A., Davies, P.R., Berlinzani, A., Belsham, G.J., Flint, M. & Rayan, M.D. 1998. Differentiating infection from vaccination in foot-and-mouth disease using of panel of recombinant, non-structural proteins in ELISA. *Vaccine*, 16 (5): 446-459.

Schalch, L., D.E. Rebeski, H. Samaras, G. Lozano, B. Thuer & C.Schelp. 2002. Recently generated data with the CHEKIT-FMD-3ABC ELISA kit and methods to monitor the operational performance of a 3ABC ELISA. Report of the session of the Research Group of the European Commission for the Control of Foot-and-mouth Disease, Cesme, Izmir, Turkey, 17-20 September, Rome: FAO, pp. 283-302.

Map1. FMDV sentinel villages 2002-2003

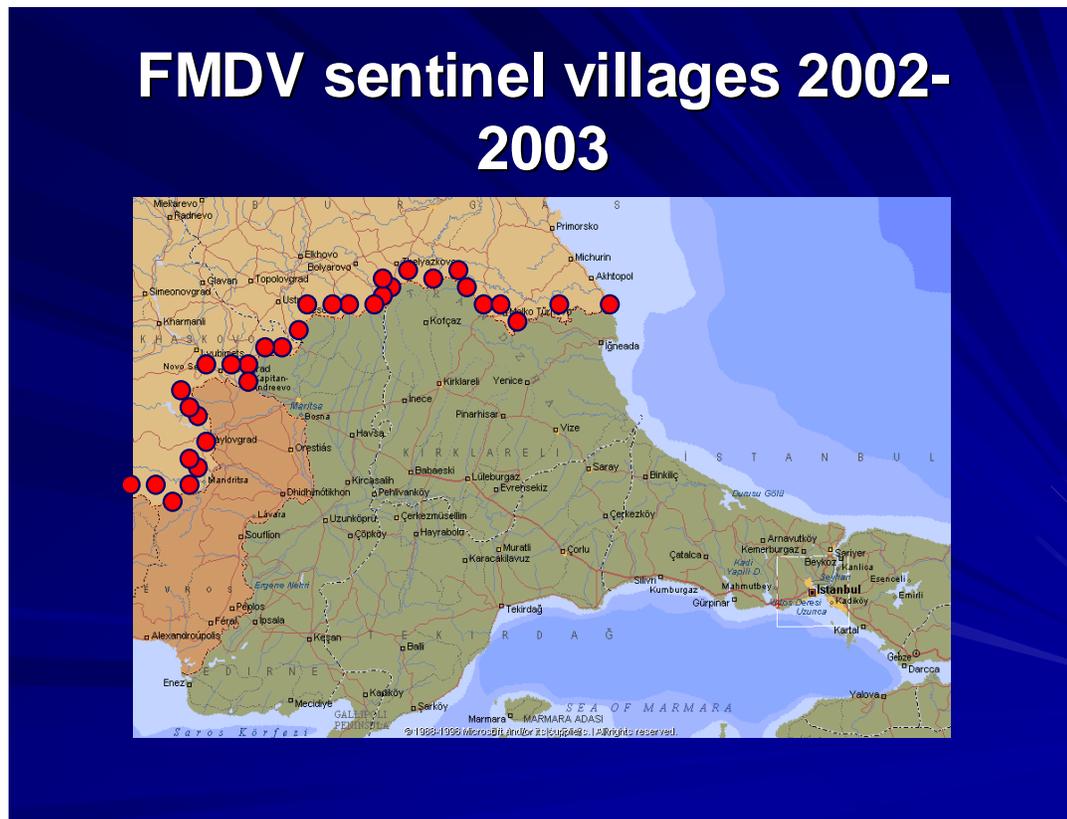


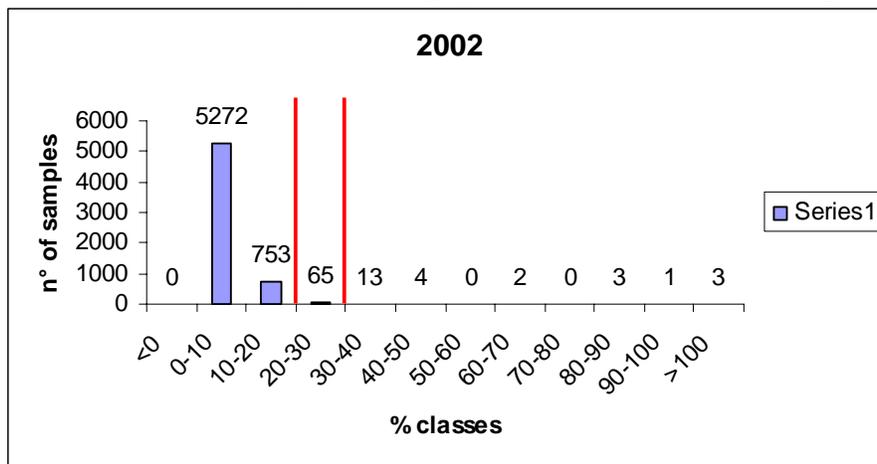
Table 1. Distribution of samples investigated for FMDV antibodies according to the National serum-surveillance programme during 2002

District	Species				NSP FMDV CHEKIT ELISA Result
	Sheep	Goat	Calf	Cattle	
Bourgas	950	175	20	-	NEG
Yambol	1174	40	24	2	NEG
Kardjali	1439	-	-	-	NEG
Haskovo	730	165	20	79	NEG
Total	4293	380	64	81	NEG

Table 2. Results from further investigations of serum samples from cattle positive by CHEKIT-FMD-3ABC Bommeli

Cattle ear tag number	Result from CHEKIT Bommeli	WRL-IAH Pirbright NSP FMDV ELISA	IZSP Brescia NSP FMDV ELISA	CISA Valdeolmos NSP FMDV ELISA	WRL-IAH Pirbright LPBE O (O-1 Manisa)	WRL-IAH Pirbright LPBE A (A-5.A-22, A-24)	WRL-IAH Pirbright LPBE Asia 1
042888	POS	POS	POS	POS	NEG	NEG	NEG
042504	POS	POS	POS	POS	NEG	NEG	NEG
042505	POS	POS	POS	POS	NEG	NEG	NEG
042526	POS	POS	POS	POS	NEG	NEG	NEG

Fig. 1: Distribution of % OD value for 2002 serum samples investigated with CHEKIT-FMD-3ABC ELISA, calculating by the ELISA results from 6115 plate wells.



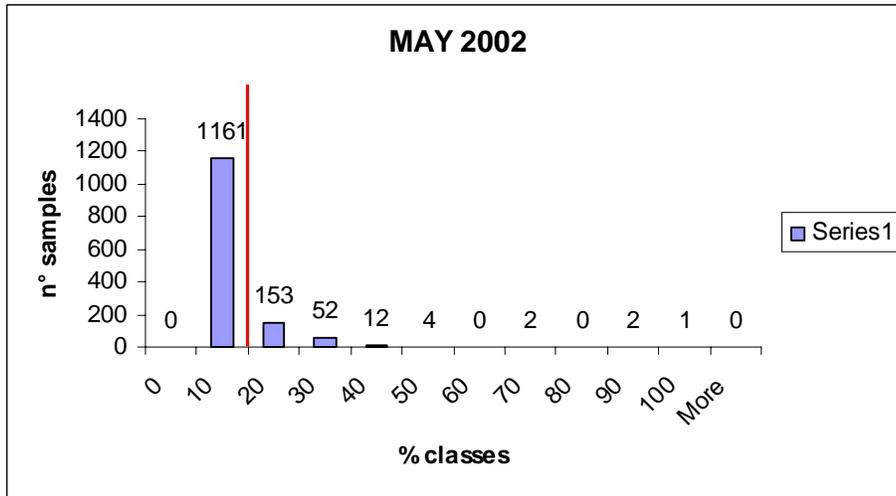
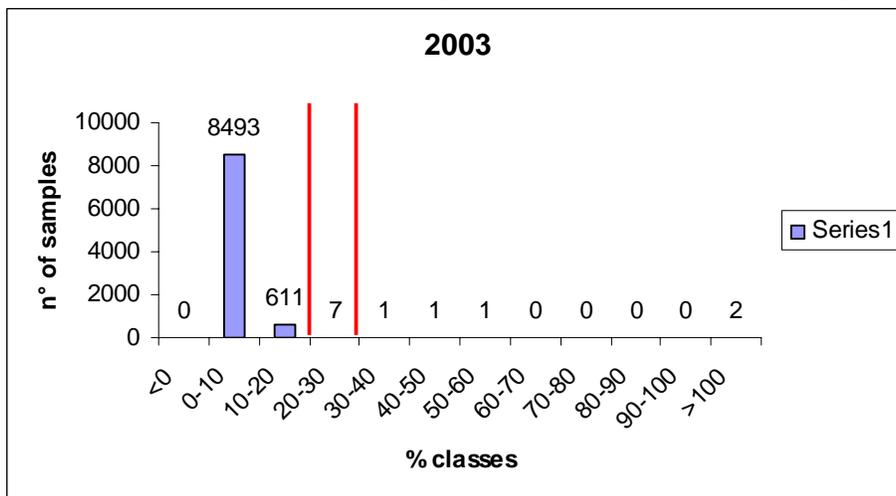


Fig. 2 Distribution of % OD value for May 2002 serum samples investigated with CHEKIT-FMD-3ABC ELISA

Table 3. Distribution of samples investigated for FMDV antibodies according to the National serum-surveillance programme during 2003 and others

District	Species				NSP FMDV CHEKIT ELISA Result
	Goat	Sheep	Wild and circus animals	Trans-border	
Bourgas	96	607	2	12	NEG
Yambol	-	1062	-	2	NEG
Kardjali	170	1174	-	-	NEG
Haskovo	-	2585	-	-	NEG
Blagoevgrad	-	-	19	18	NEG
Sofia	-	7	-	-	NEG
Total	266	5435	21	32	NEG

Fig. 3 Distribution of % OD value for 2003 serum samples investigated with CHEKIT-FMD-3ABC ELISA, calculating by the ELISA results from 9116 plate wells



A serosurvey to measure antibody levels to FMDV and trace antibodies to FMDV NSPs following Spring 2004 FMD vaccination campaign in Turkish Thrace Region

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Introduction

To evaluate protection levels and particularly to investigate infected animal in Turkish Thrace by carrying out FMD NSP ELISA, a serosurvey was carried out. In this serosurveillance a total of 4792 sera were collected and tested by LPB ELISA to measure antibody levels and by FMD NSP ELISA to differentiate infected animals from vaccinated animals. After the serosurvey, field epidemiological investigations were conducted at locations where NSP antibodies were detected in a number of randomly-sampled animals (i.e. where there was "clustering" of NSP seropositive animals), so as to assess the status of NSP antibody positive animals. This paper provides an epidemiological assessment of serosurveillance and follow up surveillance in field condition.

Materials and Methods:

1. LPBE was carried out to measure antibody to FMDV.
2. An FMD NSP ELISA (ready to use kit) was used to detect previously-infected animals.
3. A follow up field investigation was conducted in the case of NSP seropositive animals.
4. Virus isolation was attempted from probang (OP fluid) samples collected during follow up investigations.

Results

As this work is still ongoing, all results will not be available until presented at the meeting.

Results of serosurveillance for FMD and PPR in Evros under a FAO Technical Cooperation project TCP/RER/2903

(Strengthening Active Surveillance for FMD and Other Exotic Diseases in Thrace Region)

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Since the end of 2003 there is a surveillance program of F.A.O. in Thrace region and three countries participate: Greece, Bulgaria and Turkey. Greece and Bulgaria have disease-free status for foot-and-mouth disease (FMD) and peste des petits ruminants (PPR), without vaccination for either virus. The last FMD outbreak in Greece occurred in July 2000 in Evros prefecture. The infecting serotype was Asia 1 and this was also detected during surveillance in the Thrace region of Turkey. Molecular characterisation showed that the virus had most likely come from Turkey. In Turkey FMD outbreaks continued in central and eastern Anatolia, whilst PPR which was first detected in 1999 now seems to be endemic in Anatolia.

EVROS Prefecture – FMD serosurveillance

All the sera samples have been tested for 3 serotypes O, A, ASIA1 by Liquid phase blocking ELISA. 3% were titrated and retested; the results were less than 1/45 and so considered as negative. The surveillance continues and the program will finish when 5,000 to 6,000 samples have been examined.

Veterinary Stations	Bovine	Ovine	Caprine
Alexandroupoli	80	240	-
Ferres	97	180	-
Didimotiho	100	190	40
Kiprinos	85	90	-
Tychero	-	90	-
Soufli	34	250	20
Metaxades	65	70	-
Orestiada	40	225	50
Dikaia	50	50	-
Total	551	1385	110

EVROS Prefecture – PPR serosurveillance

Veterinary stations	Ovine
Alexandroupoli	240
Ferres	180
Didimotiho	190
Kiprinos	90
Tychero	90
Soufli	250
Metaxades	70
Orestiada	225
Dikaia	50
Total	1385

Diagnostic Tools for Epidemiological Surveillance in South America

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Reappearance of FMDV in the FMD-free regions of the Southern-Cone of South-America in 2000-2001 prompted the need to understand the putative origin of these emergencies. Thus, efforts were oriented to identify genetic links among strains, through the constitution of a genetic database by the PAFMDC, including viruses recorded in endemic regions, and during the emergencies. The phylogenetic analysis placed all emergency isolates in a single lineage together with viruses previously circulating in the Southern Cone, or currently appearing in high-risk zones with sporadic disease ("hot spots") of this subregion. No evident link could be established among these isolates and lineages including viruses from the Andean subregion, reflecting two independent production systems and livestock trade circuits.

Further studies were oriented to identify the source of infection. Potentially the episodes could have originated from "hot spots" within the subregion or from persistent infection in areas already declared FMD-free. In this context, serosurveillance of viral circulation through an immunoenzymatic system (screening ELISA-3ABC and confirmatory EITB), developed and validated at PAFMDC, constituted an important adjunct, particularly using the criteria for assessing risk of viral circulation in a population (age stratification, graphic displays, multifactorial analysis, etc), identified after its extensive use in South-America. Follow-up of serosurveys in the Southern-Cone during advanced stages of the eradication programs strongly indicated clearance of viral circulation shortly after last episodes. This was reinforced by the lack of outbreaks in these areas even for many years after suspension of vaccination, indicating that reappearance of disease was not due to a carrier capable of causing an episode. Moreover, the use of this system in longitudinal studies to follow-up viral activity after emergencies, demonstrated clearance of viral circulation shortly after the episodes, again reinforcing the observation that if carriers exist they do not play a significant role for maintenance of FMD, at least under the epidemiological circumstances of the areas studied. Based on these observations, reintroductions of FMD most probably occurred from trade activities involving "hot spots", within the subregion.

Post-vaccinal serosurveillance for FMD: a European perspective on progress and problems

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There has been much debate about the use of the so-called vaccinate-to-live policy for the control of FMD in Europe. According to this approach, spread of the FMD virus from future outbreaks could be controlled by a short period of "emergency" vaccination of surrounding herds, reducing the need for large-scale pre-emptive culling of at-risk animals. Since vaccinated ruminants may become subclinically and persistently infected with FMD virus following challenge exposure, it is necessary to either kill or slaughter under controlled conditions foreseen in the OIE Terrestrial Animal Health Code all vaccinates (vaccinate-to-kill) or to detect and kill or slaughter under controlled conditions all vaccinates that have become persistently infected (vaccinate-to-live), in order to rapidly regain the most favoured trading status of FMD-free without vaccination. The latter approach can be attempted by testing vaccinated animals for the presence of antibodies to certain non-structural proteins (NSP) of FMD virus, which are induced by FMD infection, but not by vaccination with purified vaccines. The numbers of herds and animals to be sampled and tested to be confident that infection has not been missed will depend upon the expected prevalence of subclinical infection amongst and within herds. This in turn will depend upon the manner in which infection is spread and on how vaccination is applied. The sensitivity of the tests used and the size of the herds will also influence the numbers of samples required to be collected and tested.

The new Council Directive 2003/85/EC on FMD takes account of these factors in its provisions for vaccination and for the use of post-vaccination serosurveillance to detect subclinical infection (Anon, 2003). According to the Directive, blood samples should be collected and tested from vaccinated animals and herds within a vaccination zone and from the unvaccinated offspring of vaccinated animals. Either all animals within vaccinated herds must be sampled and tested (Article 56, 3 (b)) or else sufficient numbers must be sampled and tested to enable a 5% prevalence of subclinical infection to be detected with 95% confidence (Annex III, point 2.2). Such sampling is not to take place until at least 30 days after the completion of emergency vaccination. EC legislation, taking into account that the necessary tests are regarded as herd tests and are not suited to verify the status of an individual animal, requires that herds within which at least one confirmed persistently infected animal has been detected must be slaughtered. However, difficulties in predicting the likely prevalence of post-vaccinal, subclinical infection and uncertainty over the performance of NSP tests has cast doubt over the suitability of these proposed sampling regimes.

There are now several commercially available NSP antibody ELISA tests. Under favourable sampling conditions, our estimates are that the sensitivity of these tests for detecting individual persistently infected vaccinated cattle can be as high as 90%, with a 99% specificity. This assumes that the vaccines used have been purified to remove traces of NSP and have been given in an emergency setting involving the application of a single vaccine dose. If all animals can be sampled, at least one animal should score positive to detect a herd. If a test with a 100% sensitivity is used in a herd of 100 animals a prevalence of 1% can be detected, but in a small herd of 10 animals 1 positive animal is equal to a prevalence of 10%. If the sensitivity is lower, e.g. 80%, the chance of missing positive animals is 0.2. To be sure we will detect a farm with 95% confidence the chance of missing animals (0.2^n) should be lower than 0.05. This means the number of positive animals should be at least $2^{(0.2 \log(0.05) = \log(0.05)/\log(0.2) = 1.9)}$. In this case on a large farm (100 animals) a prevalence of 2% can be detected (95% confidence), but on a small farm (10 animals) only a prevalence of 20%.

This therefore sets a limit on the degree of certainty that can be achieved for detecting low levels of persistently infected animals. It has been difficult to obtain reliable information on carrier prevalence under field conditions following vaccine breakdowns and even where available may not be relevant to regimes of husbandry and vaccination intensity that would prevail under European conditions. Suttmoller and Gagero (1965) reported a 50% prevalence at four months after a vaccine trial breakdown in Brazil.

In recent studies in which cattle that had been vaccinated 21 days earlier were exposed to five days of direct contact with unvaccinated, infected cattle, nine out of 20 vaccinates became persistently infected with FMDV giving a prevalence of 45% (Cox et al., in press). On the one hand, this challenge was more even and severe than is likely under field conditions, but on the other hand field vaccination may be less reliable and in a field situation, challenge may occur before so much time has elapsed for the development of post-vaccination immunity. Therefore, scenarios can be envisaged in which the prevalence of carriers within a vaccinated population could be greater or smaller than 45%.

Measures can be taken to mitigate the risks from missing subclinical infections in vaccinated populations. If ring vaccination is used, then the animal population outside of the vaccination zone will have a higher susceptibility to FMD virus infection. Therefore, the EU Directive requires a buffer zone to be established around a vaccination zone and for animals to not be moved out of the vaccination zone until FMD-free status has been attained, which will be at least six months after the last outbreak or last vaccination, whichever is the longest. It would also be prudent to avoid the introduction of unvaccinated animals to vaccinated herds for the same time interval.

Another problem for post-vaccination serosurveillance is that testing large numbers of samples will lead to many false positive test results; on average at least one false positive result can be expected every time a herd of 100 or more animals is tested with a method that has a 99% specificity. No confirmatory tests have been introduced in European laboratories to verify whether or not such results are specific. However, recent findings suggest that some of the different 3ABC NSP ELISA tests do not score the same sera as false positive and therefore providing they are of sufficient sensitivity, they may be used to confirm each others results. Other solutions may be to analyse the test results on a herd-basis and to look for evidence of multiple seroconversions or sub-threshold increases in antibody levels that could be indicative of genuine infection. Another option is to retest all positive samples without changing the assay and then to resample and retest animals that continue to score positive, although this will marginally reduce the overall sensitivity of the assay. At the time of resampling reactors, additional blood collections should be made from neighbouring animals for delayed seroconversion that could have been missed at the first sampling. Herds testing negative at the second sampling would be considered uninfected, whilst herds showing an increased level of seroconversion would have to be culled. Individual reactors that remain seropositive would be culled. Based on the mathematics described above you need at least two positive animals to detect at least one with 95% confidence. If you cull only the one that reacted positive, you probably miss one carrier animal that was not NSP positive. The risk associated with carriers missed by NSP testing should therefore be quantified and compared to the risk related to the currently accepted non-vaccination policy in which clinically affected and at risk herds are destroyed along with those identified as infected by other means.

Evidently, the confidence with which available NSP tests can be expected to detect persistently infected vaccinated animals will be strongly linked to herd size. Since it is hard to predict the likely prevalence of infection in vaccinated herds, an approach for large herds could be to begin by sampling sufficient animals to detect a low prevalence of infection with 95% confidence and then to relax the stringency of sampling if warranted by the initial results. Small herds are more difficult to deal with. One could (1) accept the risk associated with a potentially inadequate level of sampling, (2) use only a vaccination-to-kill policy in small herds, (3) have additional biosecurity restrictions on small herds after an outbreak is over or (4) avoid vaccinating such herds in the first place. This last solution is attractive on a number of grounds including the fact that small herds generally pose a relatively low risk to neighbours and are not therefore a high priority for vaccination. Secondly, it is extremely important to implement emergency vaccination as quickly as possible in order to reach a critical proportion of vaccinates in the susceptible population, whereas vaccinating small herds is slow on a per capita basis. However, it can be anticipated that such a policy would be unacceptable to the owners of small herds and therefore politically difficult to implement.

Vaccinate-to-live in pigs, potentially an attractive option following the adoption of the proposed amendments to the OIE Terrestrial Animal Health Code, may be less troublesome for NSP-based exit testing. Firstly carriers do not occur. Secondly, large herd sizes mean that low level virus circulation can be detected with confidence by NSP serosurveillance, even if test sensitivity is low. Specificity however becomes a greater problem, but since pigs are kept in pens, one could discriminate between false positives and true reactors by whether or not test positive results are spatially clustered.

Taking account of technical progress and recognising the difficulty of proving the absence of a low level of persistently infected animals within a vaccinated population, the OIE, at its General Session in May 2004, has altered the requirements for countries using long-term vaccination programmes for FMD control. In this situation, vaccination takes place in the entire country or geographic zone and is applied continuously. Instead of being required to prove absence of persistently infected animals, the veterinary authorities in such countries will be required to show that virus is no longer circulating and will then gain the trading status of FMD-free with vaccination. Countries that are FMD-free with vaccination cannot easily export live ruminants due to the risk of some being persistently infected. So far the EU requires that meat derived from such animals must be de-boned, matured and pH-controlled before being exported, however the OIE recently modified its rules to allow such meat without restrictions..

To prove absence of virus circulation by serosurveillance is much easier than to prove absence of infection since no definite decision needs to be taken as to whether NSP reactors are genuine or not; it will suffice for herds with NSP seroreactors to be resampled and retested to show absence of seroconversion. The OIE is still finalising its FMD surveillance guidelines and an Epidemiology subgroup was formed to discuss this issue in June 2004. More detailed guidance has been provided on the follow-up procedures necessary when NSP reactors are discovered in vaccinated animals, but this guidance does not apply to the situation envisaged in Europe – i.e. emergency vaccination and then rapid recovery of the status of FMD-free without vaccination.

In conclusion, problems associated with the imperfect specificity of NSP tests may be overcome by a resampling and retesting programme, but the requirement to use serosurveillance to prove complete absence of any persistently infected cattle in a vaccinated population cannot be met. However, the risk associated with a low level of undetected carriers will be low. This risk needs to be better quantified and compared to the risks of other control policies, including the previously accepted one of non-vaccination. The revision of the OIE guidelines for serosurveillance requirements in countries wishing to attain the status of FMD-free with vaccination and the transposition of the new EC Directive on FMD with its effects also on imports might conceivably lead European countries to include in their contingency planning an approach to disease control in which emergency vaccination could be followed by serosurveillance to detect virus circulation rather than infection (including detection of all carriers).

However, there is currently no provision in the OIE code for a country that was previously FMD-free without vaccination to use emergency vaccination to help control an outbreak and then move rapidly to the status of FMD-free with vaccination; only countries that were FMD-free with vaccination in the first place can regain this status within 6 or 18 months, depending on the scenario.

Therefore, following an outbreak of FMD in a previously free-without vaccination country or zone there is the imperative need to regain free status followed by a decision whether this status will in future be maintained with or without regular prophylactic vaccination. While this situation may hypothetically become relevant also for certain peripheral parts of the Community where the FMD situation in a neighbour country deteriorates and intervention by the Community on the territory of that country would not be admitted, the disadvantages including difficulties in maintaining an adequate separation of livestock and their products within the two zones and possible trade disincentives for the zone which was free without vaccination (including the rest of the European Community) still make this a questionable option. Furthermore, such an approach is not in line with EU legislation.

References

Anon (2003). Council Directive 2003/85/EC on Community measures for the control of foot-and-mouth disease repealing Directive 85/511/EEC and Decisions 89/531/EEC and 96/665/EEC and amending Directive 92/46/EEC. Official Journal of the European Union L306, Volume 46, 22 November 2003.

Cox, SJ, Voyce, C, Parida, S, Reid, SM, Hamblin, PA, Paton, DJ & Barnett, PV (In press) Protection against direct contact challenge following emergency FMD vaccination of cattle and the effect on virus excretion from the oropharynx. Vaccine.

Sutmoller P & Gaggero A (1965) Foot and mouth disease virus carriers. Veterinary Record 77, 968-969.

Authors Conclusions

- Problems associated with the imperfect specificity of NSP tests may be overcome by a resampling and retesting programme.
- It will not be possible to prove complete absence of infection (including detection of every carrier) by serosurveillance and this problem will be greatest in small herds.

Authors Recommendations

- More detailed guidelines are required for use of NSP serosurveillance in support of emergency vaccination-to-live policies in countries currently FMD-free without vaccination.
- Consideration could be given to use of an approach to disease control in which emergency vaccination in previously FMD-free countries could be followed by serosurveillance to detect virus circulation rather than infection (including detection of all carriers).

Knowledge Management and Systems Interoperability in Animal Health

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Abstract

The changing patterns in the spread and distribution of major infectious animal diseases, old and new, which pose a serious impact on animal and human health and welfare as well as human livelihoods, make animal health a matter of global public concern.

Wherever such diseases occur, they should be recognised promptly and be dealt with expeditiously. A major prerequisite is that knowledge about such high impact animal diseases and about good practices for their management be globally shared. Such knowledge should be instantly available to the global professional community, in developed as well as developing countries.

The spread of the Pan-Asian FMD Type O strain, which culminated in serious outbreaks in Europe, notably the UK in 2001, demonstrated the global risk that FMD constitutes.

During the 2001 FMD epidemic in the UK, there was extensive use of the internet, e.g. for accessing programs such as AVIS FMD, which at the height of the outbreak recorded over 8 million visits from users worldwide. But there was only partial interoperability of emergency management systems, and fragmented use of other IT tools, such as GIS and mathematical models.

Since 2001, a critical threshold has been crossed in the cost of information, which now enables all stakeholders, even in the most economically challenged of settings, to enjoy affordable access to expert knowledge, even at broadband speed, in both office and field settings. But there is not yet a coherent theoretical basis on which the strategic role of ICT, and in particular knowledge management and interoperable communications, in preventing, preparing for and responding to animal disease outbreaks or in the progressive control of such diseases (in areas where they are still endemic) can be planned, despite the growing recognition that effective responses to high impact diseases in particular are knowledge- and expertise-driven. Using FMD as an example, the paper sets out a possible model of a fully interoperable management systems design for use in the animal health setting, thereby indicating one way to harness the power, bandwidth and portability of ICT in disease surveillance, prevention and management.

In conclusion, the paper advocates interoperability as the enabling condition for a holistic disease management approach, using knowledge management systems to promote best practices, with biosecurity as the underlying driver.

Introduction

Marshall McLuhan famously predicted that the effect of television would be to make the world into a Global Village¹. He also declared "the medium is the message". In retrospect, he was right to see that a change in the dominant information and communications technology would completely alter the world, but the medium that has brought about the global village is not television, but the internet.

Few threats to the well-being of the global village, especially its inhabitants and livestock, are greater than transboundary animal disease (TAD). But we are not yet skilled in approaching TADs from a global village perspective. At this seminal point in the evolution of the EUFMD Commission, this paper sets out a possible way forward for the Commission in harnessing the power of the internet to the prevention of, and protection from, disease threats to the "village".

The Internet

What has the internet changed? Perhaps the greatest impact of the internet is to fundamentally alter how access to knowledge and expertise is managed. In effect, it reverses the centralising tendency of university and library-based knowledge cultures by pushing access to knowledge out to the periphery, to the site of need. In this process some of the key barriers to access are lowered or removed:

1. **cost:** economists correctly focus on the "cost of information" as a key determinant of power and status. Now the internet opens up at least the theoretical possibility that anyone can access knowledge for the cost of a local phone call.
2. **time:** a website is open 24/7. It can be visited without leaving the office or home.
3. **distance:** there is no distance over the internet between the enquirer and the expert.

¹ Marshall McLuhan, Quentin Fiore and Jerome Agel, *War and Peace in the Global Village*, (1969)

The mere fact that these variables are undergoing such profound change does not, however, guarantee that only good and benefit will follow. Sites are vulnerable to hacking, and viral attacks; even simple power outages can render the system unusable, and there are constant security concerns with any open system, which means that a secure intranet may be the appropriate environment for the system described in this paper. Equally, if the lowering of barriers merely provides more people with cheaper access to inaccurate or, worse, maliciously mendacious information or data, the results are orders of magnitude worse not better than the current status quo. So the new challenges are to maintain the quality, accuracy and utility of the knowledge and expertise to which the internet (or secure intranet) grants access, and to do so in a manner that promotes the overall well-being of the global village.

What is interoperability?

Interoperability is a term commonly used by first responders (fire services, police, ambulance) in emergency planning and preparedness to describe the capacity of their communications system to talk to each other. It can equally be applied to describe the enabling condition of the internet, the fact that communications use a common language (HTML) and common communications protocols, meaning that at a certain level messages can flow from almost any computer or computer-like device to almost any other. The penalty paid for such high levels of interoperability however, can be severe, whether manifest in the form of virus attacks or spam, making oversight and security key issues in any fully interoperable system.

Interoperability failures were graphically and tragically demonstrated in two seminal events of 2001. During the 9/11 attack on New York it became clear that the radio and other communications systems of the first responders – police, fire, emergency services – were not able to talk to each other. Desperate attempts to patch together mobile phones ensued, but clearly to little avail. As one report indicated: “it was a bad time to be handing out business cards when the towers were on fire”. Since that time, considerable efforts have been made to bring systems together; but as well as the technology issues, culture plays a critical role in making the goal of interoperability achievable. Will different working groups, with often widely varying practices and discourses, be prepared to work together when for so long they have worked apart, even pursuing forms of rivalry, or turf wars?

In the UK 2001 FMD outbreak, interoperability was also an issue. Problems manifested themselves both at the technical level, in such matters as the incompatibility of computing systems and the lack of transparency across databases, but also at the cultural level, where groups such as veterinarians were suddenly required to work on the one hand with the military and on the other with mathematical modellers and scientists with an operational research rather than life science background. Those directly involved are perhaps best able to judge how severe the impact was of such problems. Harmonisation and interoperability of systems are likely to be the major determinants in the design of FMD contingency plans and the testing of those plans through simulation exercises.

Interoperability and the Global Village

If interoperability at the technology level is the goal for the Global Village, the balance sheet of benefit and penalty is quite mixed – the open standard of email and HTML permits the low cost, speedy communications of email and Instant Messaging, but also the plague of spam - the balance sheet, seems to the authors of this paper to be unequivocally positive at the cultural, or operational, levels. In reaching towards a definition of interoperability that suits the needs of those in charge of preventing and managing TADs, the term must be moved beyond meeting the requirements of “first responder” cultures, to include a capacity to manage and deploy coordinated expertise to the point of need, in a manner well demonstrated during 2002-03 by international collaboration on SARS.

This appropriation of terminology is similar in nature to, and to an extent inspired by, the migration of the term “biosecurity” from a process of preventing infectious agents leaving a secure facility to a goal encompassing the safety and security of the world’s supply of food and other key natural resources. So what are the dependencies for achieving interoperability at a cultural or operational level? They may be summarised as:

- Common discourse
- Common standards
- Common values
- Common good.

While clearly a village is in one way a loose coalition of individual and family interests, at a higher level it expresses a transparent value system for a whole tribe. It is this transparent value system which needs to underpin the practical and operational goal of creating a knowledge management system for FMD that meets the goal of interoperability.

The lack of a common value system was one cause of turbulence in the UK 2001 outbreak. It became rapidly clear that the general public was directly and emotionally involved in the outbreak, to an extent that no one had predicted. In respect of socio-economic impact, it was a secondary shock to realise that the recreational and tourism economy of the countryside and its amenity value to city dwellers had suffered as much from FMD as the farming and food industries, and in some senses more. While farmers were compensated for stock losses, guest houses and tea rooms had no way of recouping lost business. Public sympathy started to shift away from farmers to other parts of the rural economy.

The outbreak as a whole consolidated a widespread perception that something was seriously wrong in the fundamental relationship between people, especially in the developed world, and the animals they used to feed and clothe them. Restoring this relationship, practically, ethically and even spiritually, would be as important to future success in TAD management as progress in science and technology, vital as that is. As a result, the interoperability model proposed in this paper also assumes that only a "holistic" approach to TADs, which by nature engages with all stakeholder interests and uses a multi-disciplinary methodology, is likely to succeed. In other words, "extrinsic" drivers, such as stakeholder attitude, are now as potent as the "intrinsic" change drivers with which animal health professionals are grappling. The theory for this approach is well articulated in the "Farm to Fork" or "Stable to Table" methodologies, and do not need rehearsal here.

The Infectious Diseases Paradigm

During the last decade or so there have been three major paradigm shifts in animal health and the impact of animal health on human welfare and health:

- An increasing frequency of serious outbreaks of high impact, transboundary animal diseases such as foot-and-mouth diseases (FMD), classical swine fever (CSF), peste des petits ruminants (PPR) and avian influenza (AI).
- The emergence of either new diseases (such as BSE and Nipah) or old diseases with altered virulence (such as avian influenza transmission to humans in East Asia).
- The increasingly frequent emergence and re-emergence of zoonotic and food-borne diseases with international scope and global economic impact.

These change drivers are themselves consequences of the rapid changes in the farming systems (especially the emergency of large-scale, centralised industrial farming and processing conglomerates), the increasing demand for livestock products which is fuelling what has been termed "the livestock revolution", rapid and distant movement of humans and animals, climatic changes and the globalisation of trade and movement of livestock products.

In common with many commercially driven revolutions, the "livestock revolution" has witnessed increased specialisation in production and management processes, coupled with the emergence of ever more specific domains of expertise. These are relatively typical signs of a "second generation" business process, such as automobile production went through in the so-called Deming revolution. Out of this has come an emphasis on productivity, a secure supply chain, and just-in-time logistics, geared to meet the demands of the world's major retail supermarkets, such as Walmart, Carrefour, Aldi and Tesco. On the production side, this represents a quite extraordinary achievement, and the condition of interoperability is met.

But a casualty of this process has often been a capacity to look at animal production and welfare, disease prevention and management from a holistic point of view. This has meant that a major change in approach to the management of the consumption side has been forced on the "middle men", primarily the supermarket chains. Now both public opinion and regulatory pressure is moving the livestock revolution into much more of a "third generation" approach, where sustainability and traceability have taken centre stage, and the consumer wants to know from the label everything necessary to judge the quality and ethical acceptability of the product. Traceability is a manifestation of the cultural approach to interoperability, and it draws its strength and validation from the level of personal accountability now expected of producers. For example, the UK supermarket chain Waitrose now publishes pictures of the

local suppliers it uses immediately above their produce. If your meat tastes bad you can see whom to blame.

This trend towards personal accountability is one of many symptoms of the pressure in the market place to “act local”, however much global production methods are in use. At a wider level, personalisation of production values is now being brought into line with personalisation of taste and consumption values, nowhere more evident than in the ICT industries. Paradoxically, while unified GSM telephony standards make it possible to use the same phone anywhere on the planet, even the ring tone of that same phone has to be totally personalised and responsive to rapid and unpredictable changes in consumer behaviour. Animal health professionals and the current regulatory systems will need to cope with the same paradoxical pressures.

In one sense, they are at an advantage over ICT colleagues. At their best, animal management practices, even in high input systems, have been personalised, and their owners have practised a holistic approach, probably without applying that term to their practice. In some ways the advocacy of a holistic approach to disease prevention is more of a reversion than a revolution. What has changed however, is that the personalisation process now extends to indirect stakeholders in animal production – the end consumers - rather than just farmers and the markets where they sell their stock. The most direct impact is obviously on the trend for ever more informative, and honest, labelling of foods; but because the nexus between our health and our diet is also growing ever tighter, there is also a new moral edge to the issue, bringing traditional bulk foodstuffs such as salt and sugar into the same moral twilight zone as alcohol and tobacco. In effect, interoperability is the practical and operational outcome of an effective, holistic, knowledge-driven approach, where the determining condition is now less the enabling function of interoperability – to enable all of us to talk to each other – but rather its content function, that we also understand more clearly what we are actually talking about.

Think Global: Act Local

The factors underlying the “livestock revolution”, globalisation of trade in livestock commodities and changes in climatic patterns have led the UK Royal Society to conclude that the spread of transboundary animal diseases has changed from the previous pattern of episodic “natural spread” into adjacent countries and regions, to a pattern in which disease (e.g. FMD or Classical swine fever) jumps long distances to infect countries and regions distant from endemic areas². Thus, ***animal diseases have become a one-world problem requiring a one-world solution.***

Foot-and-mouth disease (FMD) is now acknowledged as the prime example of the one-world problem requiring one-world solution. With respect to the three main facets of the new paradigm of animal infectious diseases, we now truly live in the “Global Village”.

As an axiom, therefore, this paper rests on the proposition: **Think Global, Act Local**. To be effective locally, however, we need a mechanism for efficiently accessing, deploying and validating global expert knowledge at the point of need, the point of use and thereby the point of action.

We also contend that the management of the new animal health paradigm should be viewed not merely in terms of technical or veterinary interventions, but more and more in terms that treat the goal of animal and human welfare and health, i.e. the impact of the livestock sector on human livelihoods and well-being.

New Science Tools and Opportunities

The contemporary sciences which offer hope for the new paradigm can be grouped into four categories:

- Molecular Biology
- Quantitative epidemiology
- Risk Analysis, and
- Information and Communication technologies (ICT).

² Infectious Diseases in Livestock. London, The Royal Society, 2002 Policy Document, 15/02. ISBN 085403579 6
http://www.royalsoc.ac.uk/templates/search/websearch.cfm?mainpage=/inquiry/commissioned_papers.htm

Of these, ICT and possibly risk analysis are the only ones which are not a natural succession from the traditional approaches. Consequently, their potential may well be undervalued in the management of high impact diseases like FMD.

The Potential for Information and Communication Technologies

The tools that now exist, or that could readily be developed, for ICT include:

- Mobile phones, with cameras
- Satellite telephony and data management
- GIS/ GPS
- Real time data streams
- The Internet
- Dedicated devices integrating surveillance, diagnostics and communications.

The challenge is to develop an objective-driven strategy that recognises that knowledge management has to be “people-centred”, has clear processes for capturing expert knowledge and transforming it into information that is transmissible to, and useable by, persons of different levels of competence and requirements. Therefore, in developing an effective knowledge management package there will be six key steps:

- Experience and expertise at an individual level
- Institutional knowledge capture and transfer/ preservation of “at risk” knowledge.
- Validation
- Interpretation
- Communication
- Concerted action.

While each step is necessary in its own terms, the validity of the system as a whole depends on combining all six into a “virtuous circle”, through which the lessons of a given concerted action get digested into individual and institutional competence on an ongoing basis.

Interoperability

It is premature perhaps to offer a formalised definition of interoperability commensurate with the holistic vision we have described. But the concept of interoperability may be represented schematically, as shown below, as a means of moving towards such a definition. Interoperability has a number of obvious enabling conditions or critical dependencies:

- Systems connectivity/ transparency
- Shared meta data
- Single data dictionary
- Common data interchange standards
- Security
- Cross-system SOPs
- Trained users.

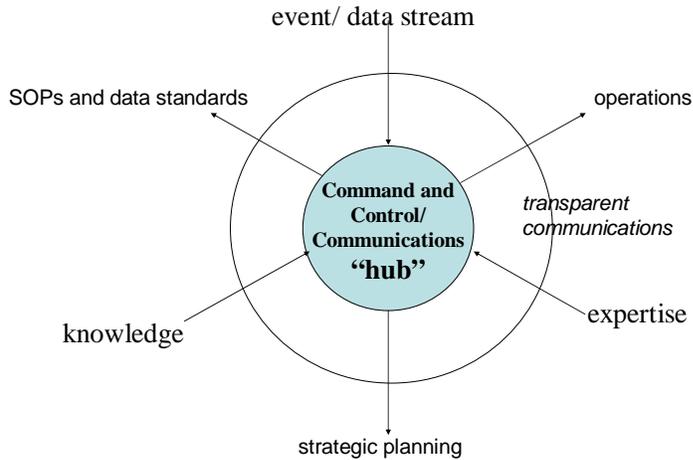
But these enabling conditions merely set the stage for the higher level of interoperability that we believe is of the essence in effective TAD prevention and control, a level we have described as “cultural”. In such a model there are three key types of input that interoperability can make transparent to all direct and indirect stakeholders. These may be characterised as:

1. Event and data stream
2. Knowledge flow
3. Expertise.

These inputs are processed in a “hub”, which may in practice be fully virtual and distributed, but is probably best operated with a significant degree of centralised control, and lead to three critical outputs which enable effective intervention and management:

1. Strategic Planning
2. Standard Operating Procedures (SOPs) and data standards
3. Operations

Interoperability

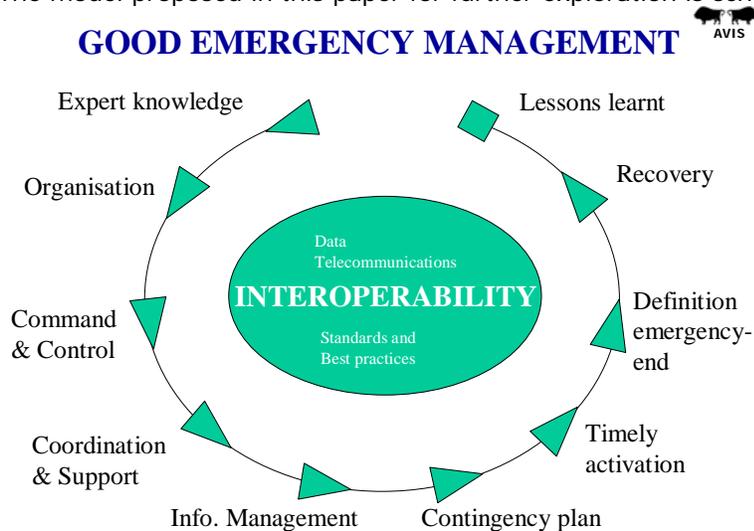


A Way Forward Using the AVIS FMD Program Model

The 2001 FMD outbreaks demonstrated significant advances in the use of information and communications technologies (ICT). GIS systems were applied to mapping and monitoring the outbreak, and there was extensive use of the internet, e.g. for accessing programs such as AVIS FMD, in the education and support of both professionals and the public (over 8 million users of AVIS FMD worldwide during the outbreak itself).

Since 2001, a critical threshold has been crossed in the cost of information, which now enables all stakeholders, even in the most economically challenged of settings, to enjoy affordable access to expert knowledge, even at broadband speed, in both office and field settings. But there is not yet a coherent theoretical basis on which the strategic role of ICT, and in particular knowledge management, in preventing and responding to animal disease outbreaks can be planned, despite the growing recognition that effective responses to high impact diseases in particular are knowledge- and expertise-driven.

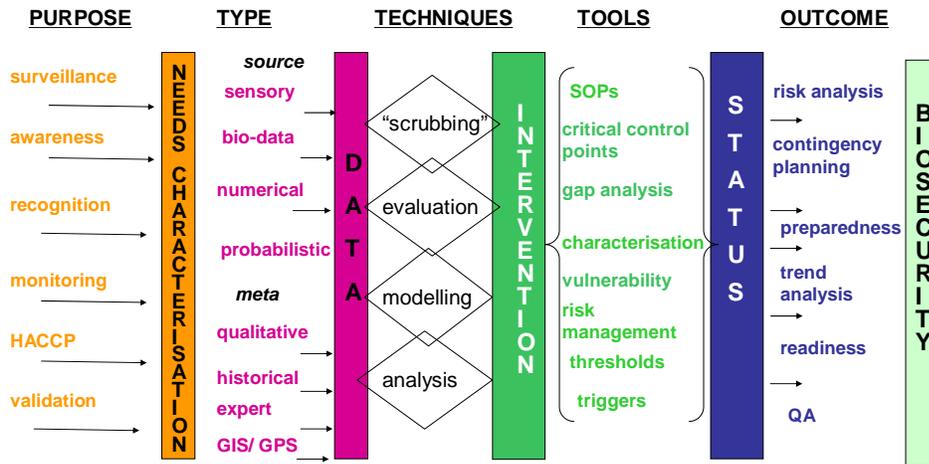
The model proposed in this paper for further exploration is schematised as follows:



Event and Data Streams

The critical dependency for effective command and control, once interoperability is achievable, is a fast, accurate event and data stream flowing into the communications hub. Using FMD as an example, the diagram below sets out a possible model of types of knowledge that are necessary for use in the animal health setting, so harnessing the power, bandwidth and portability of ICT in disease surveillance, prevention and management.

Data Typology & Interoperability Model: 1.2



The model is “cylindrical” & continuous

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A defining condition of this model, not surprisingly, is complexity. or hurricanes. Because of this complexity the most critical requirement is that a multidisciplinary expert group is given the tools and the span of control necessary to deal with the problem, tools which are built to manage complexity to a point where actions can nevertheless be coherently executed.

Complexity

The key challenge at the “hub” is dealing with complexity. It is the characteristic of complexity which perhaps most clearly sets biological events apart from other naturally or maliciously occurring crises, such as fires or hurricanes. Given that the only response possible to complexity is a multidisciplinary one, the obvious secondary challenge facing command and control is one of communications across operating cultures. In the “heat and smoke” of a live event, communications failures and conflicts within the response teams can be symptomatic of a failure to cope with complex environments as much as “turf wars”. As a first step in rectifying this problem, at a national level the various systems used by key stakeholders must converge and become transparent. But in due course, dictated by the intrinsically transboundary nature of many disease threats, the authors of this paper see great potential for benefit in development of highly expert, highly integrated supranational response teams capable of dealing quickly and effectively with such events. The highly distinguished achievements of the EUFMD Commission, founded over fifty years ago on such a vision of supranational collaboration, suggest that it is best placed to act as the point of focus for such considerations in the future.

Systems Solution

The event and data stream model proposed above derives in part from cybernetic theory, which proposes that in any management system the flow of work, and of the information that supports and drives that work, be as far as possible contributory to as smooth and “frictionless” an operational process as possible. Communications conflict, whether at the hardware, software or user level may be the first and most deadly site of friction, and even breakdown. And at the heart of many communications conflicts are less “intrinsic” factors, such as the phone not working, than the “extrinsic” factors, where people refuse to talk to each other.

In the cybernetic approach, the goal, however idealised, is to promote a “virtuous circle” of activity, where one event leads naturally and logically to the next, and where the incentive to perform well is aligned at each level. Using initially a HACCP derived analytical approach, it was relatively straightforward to detect points of weakness in current or recent historic systems, such as the inability of one laboratory’s computer network to access another’s (for example for “security” reasons), or the inability to move data sets from one system to another because either the database design or the electronic data interchange (EDI) standards necessary for such transfers were not in place.

If this theoretical approach is to translate successfully into operational practice, the paper also proposes that a trend which is beginning to emerge in veterinary services in response to the need to demonstrate higher levels of preparedness and readiness for emergency situations may need some careful scrutiny and modification. This trend is to follow a classical “incident command system” (ICS) model, derived partly from the military and partly from management practices in regard to natural disasters, such as wildfires.

There are two very striking benefits from following this model: 1. at the level of people-to-people communications, having unified command and control and a single communications systems is essential; and 2. at the level of data capture and exchange having a single, unified data dictionary, transparent architectures and common interchange standards can only promote swifter more accurate and more flexible responses. But there is also a significant weakness: a TAD outbreak is not the same as a fire; and the strategic skill set required to manage an outbreak is very different from that of responding to an acute natural disaster, however similar some of the tactical aspects may look. But if the animal health community does not come up with its own, disease-specific, model of how to respond, then the gap will be filled for it by a generic solution. Perhaps the EUFMD Commission will be willing to take leadership in such a strategic planning exercise, since by virtue of its scope and constitution whatever it can achieve for FMD will, *mutatis mutandis*, be highly transferable to other TADs.

The Pivotal Role of the EUFMD and Similar Regional Organisations in Knowledge Management

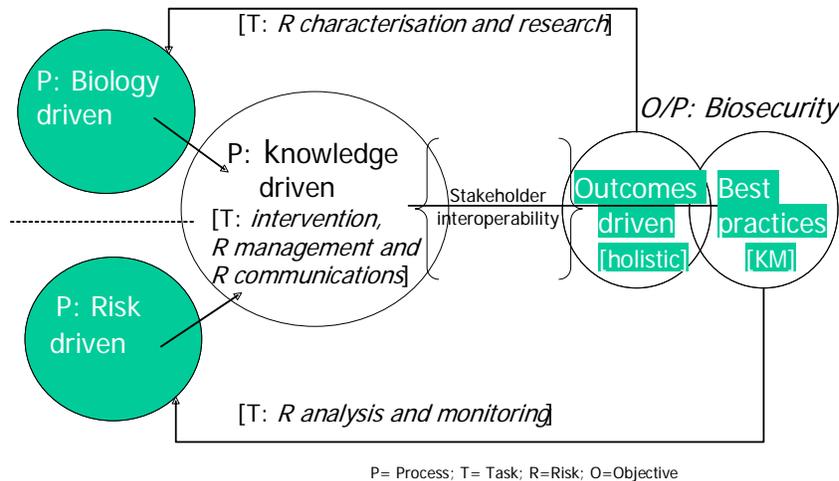
In dealing with the global animal health problems we need to advance the concept that was conceived some 50 years ago when some European nations decided to set up a semi-autonomous FMD Commission within the framework of the operations of an international organisation, i.e. FAO of United Nations. In the new Global Village, a key role of the EUFMD Commission, in terms of Knowledge and Disease Risk Management, must incorporate the concept of an inter-operability hub focusing on:

- Transparent communications technology
- Consistent key terms and common definitions
- System wide SOPs
- Best available practices
- Consolidated body of knowledge and experience
- Centralised, expert led, coordination [command and control]

Managing Animal Health by Biosecurity

The new animal health paradigm must also imply a new paradigm in animal health management with a unifying driver. We propose that such a driver is **BIOSECURITY**.

Management by biosecurity



FAO defines biosecurity for agricultural systems as:

“[...] a strategic and integrated approach that encompasses the policy and regulatory frameworks (including instruments and activities) that analyse and manage risks in the sectors of food safety, animal life and health and plant life and health, including associated environmental risk. Biosecurity covers the introduction of plant pests, animal pests and diseases, and zoonoses, the introduction and release of genetically modified organisms (GMOs) and their products, and the introduction and management of invasive alien species and genotypes. Biosecurity is a holistic concept of direct relevance to the sustainability of agriculture, food safety, and the protection of the environment, including biodiversity”.

To take into account the holistic concept advocated by FAO, an outcomes-driven management system based on best practices is hereby advocated:

What is harder to model, as it is not yet fully instantiated anywhere in the world, is a systems model that genuinely supports multidisciplinary working. This goal may be sufficiently difficult to achieve that at this point may better be described as a vision. But in that the EUFMD Commission was set up as a model of collaborative, multidisciplinary research undertaken to meet a vision of a disease free Europe, at this fiftieth anniversary moment embracing a vision of systems and cultural interoperability may be a prudent and inspiring move.

Policy and science of FMD control: the stakeholders' contribution to decision making. A call for Integrated Animal Disease Management

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Abstract

Effective control of foot-and-mouth disease (FMD) - prevention, surveillance and response - requires integrated animal disease management as a cooperative effort between stakeholders, scientists and decision makers, at all levels: local, national, regional and international. This paper suggests a process and outlines specific critical issues that need to be addressed in order to best use the science and technology that is available now and to develop new technologies that will lead to significant improvements. The overall objective is not to allow the disease or the disease control measures to damage, violate or destroy public health, the environment, or the economy, or to allow politics to drive disease control policies at the expense of the ethical relationship between man and animals. Critical issues of prevention, surveillance and response policies are examined, and specific recommendations are made to reduce the risk or effect of natural and deliberate introductions. For prevention: a) rapid portable diagnostics and provision of vaccines to control and eradicate the reservoirs of disease and b) alerts, leading to increased controls at borders, animal movement restrictions and biosecurity on farms. For surveillance: a) reporting of unusual symptoms, rapid diagnostics and identification of patterns, b) enhanced role of GIS linked to an IT system, and c) collection, storage and sharing of disease information. For response policies: a) the role and implementation of stamping out and of vaccination, and b) simulation exercises with stakeholder participation. For all aspects of FMD control, consideration should be given to: a) the composition, responsibilities and role of the balanced, permanently operational Expert Group in EU member states as specified in the EU FMD Directive, b) establishment of a balanced, permanently operational European Expert Group, and c) establishment of both a European and an International FMD Task Force. Stakeholders need access to accurate, up-to-date, unbiased information about the science of disease control, how the technologies work and can be used, and an assurance that the technologies best fit for the required purpose will be used. Researchers need to work together to avoid duplications and gaps in their research and to recognize the benefit of new, and sometimes innovative, technologies. They also need feedback from stakeholders on the acceptability and best use of the technologies. A process to achieve these goals through an EU funded collaborative research project will be described.

Introduction

Stakeholders are defined as all those who have a direct or indirect interest in FMD due to the impact of the disease on their or their animals' lives. This involves a cross-section of the population, ranging from those with extensive knowledge of livestock to those who have little or no knowledge or education concerning the complexity of the issues. The recommendations in this paper are based on information gathered from open meetings, private consultations and extensive internet communications, with a wide and geographically dispersed range of stakeholders, scientists and policy makers that began in February 2001 when FMD was identified in the UK. The views of stakeholders must be taken seriously because their cooperation is essential in the implementation of an effective and ethical scientific disease control process and because they represent large and diverse political pressure groups. They are increasingly vocal, often with little consensus amongst the varying interests they represent, and need to be persuaded that the control measures are appropriate, proportionate and necessary. If they say that certain measures are unacceptable, the policy makers must take their concerns into account. In order for control policies to be undertaken in a cooperative manner, it is imperative that stakeholders have access to the most objective and accurate information about the underlying science and that the implications are clearly explained and open to effective consultations and debate. Consultation exercises must be easy to access, genuine and transparent.

The role of scientists is central in the process to determine:

- How we can use the science and technology that is available now in rapid, effective, proportionate and acceptable control.
- How we can develop new technologies and new approaches using these technologies to improve control.

The overall objective is **not** to allow the disease or the disease control measures to:

- Damage, violate or destroy public health, the environment, or the economy, or

- Allow politics or trade issues to drive disease control policies at the expense of the ethical relationship between man and animals.

The desired objectives should be subject to examination and agreement well before an outbreak: *“Control strategies might seek to minimize various quantities, such as total animal loss, duration of the epidemic (which is currently the main objective in England and Wales[DEFRA, 2004]), regional spread, financial loss (to several economic sectors), or animal suffering.”* (Haydon, Kao and Kitching, 2004). Furthermore, control measures and associated regulations must be planned and implemented with sensitivity to local and regional traditions and practices (Crispin, Roger, O’Hare *et al.*, 2001).

No apologies are made for concentrating on FMD in the UK in 2001, since this epidemic was the largest emergency exercise for FMD in a developed country. Lessons must be learned so that there is no repetition of the errors that were made (Royal Society, 2002). In particular, there is a consensus amongst stakeholders that:

- We will not carry out the mass slaughter of healthy animals.
- We will not incur the financial and social costs to the livestock industry, tourism and the rural economy, or cause unnecessary loss of genetic diversity.

This might also reduce the risk of a deliberate introduction. F.P. Horn and R.G. Breeze (2004): *“Deliberate attack is not about dead cows, swine or sheep; it is about economic harm and terrorizing the public....The public will not accept mass slaughter ... nor is such slaughter necessary any longer. Our starting point must be that we will not get into a situation in which slaughter of millions of animals is necessary: this is what terrorists want to see.”*

Discussion

How should we control foot and mouth disease? There are key questions and recommendations related to the three main areas of control: prevention, surveillance and response that need to be addressed.

Prevention

It is the responsibility of national governments to take all reasonable steps to prevent entry of disease, including:

- Rapid portable diagnostics, especially to identify countries and regions posing a risk.
- Vaccines to control/eradicate reservoirs of disease
- Increased continual import surveillance and controls
- Development of an alert system at borders and to inform livestock keepers
- Animal movement restrictions based on sound science
- Biosecurity, particularly at farm level, based on sound science

Proposal: The establishment of an International Task Force for surveillance and for vaccination (on request), with funding for the purchase of vaccine, especially in developing countries. Since the reagents for real-time RT PCR assays have a shelf life of only about 12 months, the creation of diagnostic test kit banks should be considered. International good-will and cooperation is needed to identify, control and eradicate reservoirs of disease.

Proposal: Nationally and regionally, we need inter-agency cooperation to reduce risk of entry of disease at borders, and a higher level of international cooperation to reduce illegal meat imports. The measures must be appropriate to the source of risk, whether from innocent tourists, criminal meat trade, or deliberate introduction. In the case of tourism and trade, when entry is from a country or region with FMD, then passengers and cargo must be subject to increased inspection. Using modern technology, such risks can be identified before the plane or cargo ship has landed.

To further reduce the risk from tourists innocently introducing disease by importing meat products, all persons entering the EU should sign a declaration that they have no meat products in their possession. Further measures are often taken, but is there scientific evidence to support their effectiveness? It is often recommended that a sufficient number of sniffer dogs should circulate at all ports of entry, including all terminals. This should be regarded as a means of preventing illegal entry of meat and meat products, but it is not an effective means of detecting pathogens.

In some countries, arriving passengers must declare whether they have been in contact with farm animals in the previous week. If the answer is affirmative, sometimes they are required to sign a declaration that they will not be in contact with farm animals for at least three days, and they are often separated for customs officials to inspect, clean and disinfect shoes. Is this separation from livestock

necessary? Is FMD virus really transmitted on the soles of shoes? It should be a simple experiment to take washings from muddy shoes that have been on FMD infected premises at varying intervals, and then test for the presence of the virus. Bartley, Donnelly and Anderson (2002) have reviewed FMD virus survival in animal excretions and on fomites.

General measures recommended to reduce the risk of introduction from all causes:

- Up-to-date detection technology, including air sampling, should be operating in cargo holds and at baggage inspection, and possibly on aircraft traveling from FMD infected countries and regions. Depending on the level required, this might involve technology to detect aromatics which identify putrescence that would indicate the presence of meat products and/or technology to detection pathogens, including FMDV.
- Amnesty bins should be provided at all ports of entry for disposal of animal products.
- Penalties must be sufficiently severe to act as a deterrent.

Surveillance

It is the responsibility of national governments to participate in an international surveillance system that can identify patterns of disease in time and space and anticipate hazards and threats. The purpose of surveillance, and a measure of its success, is its ability to provide answers to these five questions:

1. *Where is FMD in the world?* We need a comprehensive international surveillance system that will catalog historical events, and identify endemic and new outbreaks, with mapping and virus typing information. To achieve this goal, attention needs to be given to how disease information is obtained and how it is handled:

- What information needs to be gathered, and how detailed should it be?
- Is the information up to the minute, as recent as possible?
- Who reports disease? CVOs, military, private diagnostic labs, private companies (e.g. those involved in agribusiness)?
- Who keeps the information? Regional or international centres? Most acceptable is a neutral, international organization (OIE/FAO).
- Who has access to the information and how is the information distributed? How are issues of confidentiality, trade, and prevention of panic, addressed? Should there be varying levels of distribution and varying levels of detail of information?
- What information do stakeholders, scientists and policy makers, need to know? As an example, R.G. Breeze (personal communication, 2004) suggests: *"we need to know the complete genetic sequence of every FMD virus (including vaccine viruses) circulating in the world today and to keep these data current (we also need to go back historically in the archives to sequence past viruses). ... We should link the sequences to geography in an attempt to map distribution of fingerprinted viruses..."*

2. *Is it coming here?* The international surveillance system must have the capability to identify emerging patterns in time and space, and act as a warning system to inform decisions at regional, national and local levels that can be taken to prevent entry and spread of disease. Regional and global commercial flows, as well as travel patterns, must be taken into account, and used to consider preparation of appropriate vaccines, as well as if and when to raise the levels of surveillance at borders. Possible responses need to be agreed, for example if an incoming aircraft has been identified as contaminated with FMDV or other pathogens, should it be held in quarantine for inspection?

There is an urgent need for active liaison between the national and international veterinary agencies with their counterparts in the police, Interpol, and trade to actively monitor and share information on the illegal trades in meat and bush-meat, which present major diseases risks.

3. *Is it here now?* The rapid identification of disease requires both the cooperation of the livestock sector and the effective use of a continuous real time active national surveillance programme, including the use of appropriate permanent, targeted (e.g. milk collection, livestock markets) and portable rapid diagnostic devices.

A formal, active FMD surveillance methodology is described by Bates, Thurmond, Hietala *et al.* (2003), including:

- New technology for use in cost-effective mass screening and environmental testing;
- Embedding FMD surveillance in existing mass-screening systems for endemic diseases;
- Strategic targeting of high-risk animals and locations; and

- Strategic use of specimens submitted for routine diagnostic testing.

It is essential that the data is not skewed or artificially unbalanced in its collection, especially if the data will be used to determine current and future control measures.

4. *If it is here now, where will it be next?* Geographic information systems coupled with knowledge of meteorological conditions, of individual animal identification, and of animal movement or nomadic patterns can be used in conjunction with satellite tracking systems to enable models of disease to be more accurately predicted in real time. These should remain under veterinary control to ensure that control systems are realistic and to avoid some of the misconceptions that may arise from modellers unfamiliar with animal disease patterns (Taylor, 2003; Honhold, Taylor, Wingfield *et al.*, 2004; Kitching 2004).

M.E. Hugh-Jones (personal communication, 2004) explains: *“GIS have a definite place in modelling and prediction and accurate situation reports, but their most important function is to accurately plot where cases have occurred, where field diagnostic kits have confirmed cases, and where suspect cases have been found not to be affected. When GPS chips are incorporated into handheld data loggers, these sites can be recorded automatically without the risk of error. Too many farms in 2001 were identified by map reference numbers and slaughter initiated, or attempted, in spite of owner claims that the stock were not affected; in each case the slaughter team had the right map reference but the wrong map, or vice versa. Nobody needs such lethal and expensive mistakes.”*

F.P. Horn and R.G. Breeze (2004): *“Once the presence of FMD is confirmed, as part of an Internet-based Command and Control system, continuous real-time surveillance must be employed to define the extent of the problem around the initial detection and to predict and track the progress of infection through the national agricultural commerce streams.”*

5. *If it was here, has it gone?* What is role of serology? Is it really necessary to slaughter sero-positive animals, if no significant carrier state can be shown to exist? This is a good example of further information being necessary in order for a sound scientific policy to be implemented.

Response

Stamping out. It is generally accepted by stakeholders that effective control requires the slaughter of infected animals, although in some countries and regions, FMD infected animals are not slaughtered, but are kept isolated until recovered. Acceptability of the policy of stamping out would be much greater if it is accompanied by credible and scientifically sound measures to ensure that only those animals that are infected are slaughtered and that every reasonable attempt is made to prevent the spread of disease and thus minimize the number of animals slaughtered. To this end, what is required is rapid identification of disease, rapid restriction of animal movements, rapid slaughter of animals that are infected, and, if these measures are considered insufficient to control the disease, then a rapidly implemented programme of vaccination.

Rapid identification of disease. Rapid identification of the introduction of a foreign animal disease relies on a combined strategy: a continuous monitoring programme using state of the art detection and tracking technology, as described above, and the cooperation of livestock keepers to look for and report suspicious symptoms.

The front line – livestock keepers and veterinary practitioners. Arguably, the most important factor rests with livestock keepers' ability to recognize suspicious symptoms and, once recognized, their willingness to report the suspicious symptoms. This requires a high degree of trust that the authorities will respond in a fair and proportionate manner. Following the experience of the UK FMD 2001 outbreak, it has become apparent that trust has broken down, and in some cases this lack of trust has extended from the livestock keepers to their veterinarians. Countries where this breakdown has not occurred should take care to maintain this essential trust, and countries, like the UK, where the trust has been lost, need to take the initiative to win back this trust (Crispin, Roger, O'Hare *et al.*, 2002).

Fundamental to maintaining or winning trust is:

- An effective distribution of information
- An effective consultation process that is easy to access, genuine and transparent

and confidence that:

- Local large animal veterinary expertise is available and that there is government support to enhance/maintain a strong veterinary service

- Animals will be inspected and tested rapidly and at minimal expense
- Identification of disease will not result in financial loss to the livestock keeper
- Neighbouring holdings will not be subject to unnecessary restrictions
- Livestock keepers will be kept informed of the local disease situation
- The government will respect and, not marginalize, agriculture in general, and all sectors involved in agriculture – from agribusiness to part-time farmers and the keepers of companion animals, and the large numbers of varied and important sectors in between. This is vital, since disease can appear in any animal, regardless of how it is kept.
- Consideration will be given to the characteristics of livestock management systems and of the species, which may have differing susceptibility and transmission risks (Kitching 2002; Wernery and Kaaden, 2004).
- Sound science and the best technologies will be used in all aspects of FMD control
- Regulations are necessary, proportionate, and appropriately targeted
- Stakeholder concerns will be taken into consideration by the regulatory authorities
- Systems will be put in place in “peacetime”, ready to deal effectively with an emergency.

Proposal: Stakeholders should be brought into the process of determining disease control policy. Where national governments fail to take a lead, the agriculture sector should be allowed to work jointly with government in control measures. Whether under the control of government or the livestock sector, national programmes should be established to train livestock keepers to identify disease, maintain effective biosecurity and to vaccinate. The livestock sector should consider working together to establish a system of veterinary insurance, where models from other countries should be considered.

Use of on-farm and near-farm rapid diagnostic tests. A variety of diagnostic tools can play a role in FMD control, depending on the purpose for which they will be used:

Those that are ready and available for use now, should be included in current contingency plans. What is meant by ready for use? At the October 2004 meeting of the USAHA/AAVLD Epidemiology Committee meeting on diagnostic validation, a USDA representative stated that validation only indicates that the testing procedures have complied with specific guidelines, and that validation does not refer to the quality or fitness for purpose of the test. The Smartcyclor real-time RT PCR assays are considered a valuable tool and have already been distributed throughout the US National Animal Health Laboratory Network. Depending on the situation, especially to rule out false positives, confirming tests can be used.

Those that are in development, should be designed for specific purposes. For example, penside dipstick tests can be useful in decisions related to the lifting of isolation and movement restrictions, while portable PCR devices can be linked through wireless internet for real time remote expert management in a crisis, avoiding the difficulties that can arise from the need to transport samples rapidly to a laboratory.

Vaccination. There are a number of key issues that need to be addressed before an outbreak occurs. These include some logistical and fiscal difficulties, including the number of serotypes to consider, location of depots, time to prepare vaccine stocks, time to implement vaccine use, distribution of vaccine, etc.

R.G. Breeze (personal communication, 2004) suggests best vaccine match based on genetic sequencing, but stresses that speed of response is crucial: conventional vaccine selection may allow administration of vaccine to start at about day 8 of an outbreak, but we need to be able to respond more rapidly and to implement vaccine use if or as required by day 4 or 5 of an outbreak. However, Kitching (2002) points out that depending on the method of introduction of infection and of the species at risk, the speed of response need not always involve slaughter and/or vaccination. Kitching and Hughes (2002) observe that recovering sheep pose little threat to other animals.

Stakeholders should contribute to decisions on issues such as:

- Who provides the advice and who makes the decision to vaccinate?
- Who decides on the vaccination strategy?
- If there is disagreement, should a European Expert Group be established as the final and rapid arbiter? Such a group might also be of assistance to member states on request.
- If sufficient vaccine is not available to protect all animals, which animals will be vaccinated? Pre-registration, in a simple format, should be considered.
- What is the goal of vaccination? In a country normally free from FMD, long term immunization is not necessary. What is needed is a fast-acting vaccine that will pass through the system in about

three months. In an FMD endemic country, on the other hand, a single vaccine that can be given at birth and remain effective for a long period would be preferred.

- The livestock industry needs assurance that meat and milk products from vaccinates will be acceptable and (possibly) that live animals, subject to testing, can be exported. Consideration should be given to a new approach to export regulations based on more extensive testing; this would have the added advantage of providing more detailed international information on the geographic and temporal distribution of strains.
- Consideration should be given to the creation of an EU Task Force (working closely with EU vaccine banks) to rapidly lead a vaccination programme within the EU and, if requested, overseas.
- What is the risk from carrier animals? Can “healthy carriers” be identified by PCR tests? If carriers are not considered a significant risk, is it necessary to slaughter antibody positive animals?
- How to ensure that national governments apply regulations sensibly and do not over-regulate? (For example, in the UK, it has not been made clear that post vaccination, meat for UK consumption would not require treatment, only meat for export).

FMD Control Measures

Expert Group. FMD prevention and control is complicated, and needs a truly balanced expert group, with in-built checks and balances, to ensure that no one consideration or interest predominates (including political interests and unnecessary restrictions used as a trade barrier). Cooperation is essential, between scientists, between agencies, and at all levels, national and international. The role of the Expert Group should be clearly defined. The role of other groups that may be appointed, e.g. as the UK has done, to “challenge” the Expert Group, should be examined so that there are no conflicts which may cause serious delays in a time of emergency.

Composition of Expert Group. The expertise required to fulfill the responsibilities specified in Article 78.1-3 of the EU FMD Directive (D. Paton, personal communication, 2003) involves expertise in:

- FMD diagnosis
- Vaccination as a control measure
- Vaccine production
- Logistics of disease control, including military and emergency management engineers
- International developments of relevant new technologies, in diagnostics, surveillance, electronic ID, vaccines and anti-virals, and robotics (for lab tests)
- The workings of the state veterinary service
- Animal husbandry practices, including commercial and non-commercial, breeds at risk, minor breeds, companion animals and wildlife, with sensitivity to local and traditional practices
- Epidemiology of veterinary infectious diseases
- Epidemiological modelling and cost-benefit prediction
- Risk assessment and risk management
- Legal matters relating to disease control
- Environmental controls relating to carcass disposal
- General rural affairs, including tourism

This demonstrates the wide influence that control measures for diseases such as FMD have on the economy of any country and emphasizes the importance of the establishment of this Expert Group as an independent body.

Consideration should be given to the provision of a system to create sub-groups or panels on an *ad hoc* basis to deal with issues that may require more in-depth consideration. An example of such a sub-group of independent scientific input might be to provide a transparent process for the selection of diagnostic assays, perhaps making more transparent the increasing role played by patent agreements. This could be an effective mechanism to broaden the input from the normally small (one person?) composition of the Expert Group on a particular issue, and might provide a better approach than the establishment of an outside group to “challenge” the Expert Group.

Proposal: The establishment of a balanced, permanently operational European Expert Group, with both the new EU FMD reference lab and EUFMD/FAO as important members, and to act as the final arbiter if there is a complaint about a national Expert Group or the control policy of a member state, and to be available to all member states for advice on request.

Emergency simulation exercises must include a broad range of stakeholders, including the livestock sector, military and emergency management engineers, in order to increase the levels of preparedness

for a future outbreak. A standard platform for communication between all agencies should be in place. Control policies cut across agencies, whatever their name, that deal with: agriculture, food, rural development, tourism, environment (e.g. water supply), security, trade and international relations.

Dissemination of information that is accurate, up-to-date, objective and comprehensive. A major initiative in furthering this objective will be the forthcoming EU-funded FMD and CSF Coordination Action, which is scheduled to begin January 2005. Coordinated by IAH-Pirbright, the project's objective is to avoid duplication and gaps in research, and partners include the key players: the EU reference labs, DG-Sanco, OIE, and EUFMD/FAO. The author of this paper will be the principal officer of the project's Central Network Resource, responsible for dissemination of information amongst the partners and with stakeholders in a two-way exchange. The goal will not be to achieve a consensus, but to allow for flexible decision making based on informed discussions and effective research without stifling innovation. A long-term objective is to establish a European Animal Health Association as a forum for exchange of information leading to collaborative resolutions, amongst scientists, policy makers and stakeholders.

Conclusions

Integrated animal disease management enhances disease control policy by the involvement of all stakeholders in the decision making process, allowing co-ownership of policy and encouraging collaborative and acceptable strategies and applications of technologies. It requires critical issues to be addressed and the implementation of realistic, practical and often innovative scientific advances in diagnostics, epidemiology, prevention and control, as part of the continuing preparation to deal with future outbreaks of FMD. The outlines for stakeholder involvement and a command structure within the EU are suggested. The dissemination and discussion of accurate, up-to-date, objective and comprehensive scientific knowledge is described and a consultation process suggested. Review of transboundary disease continues to be an area of increasing importance, within the EU and globally, as altering disease patterns are monitored. Continued vigilance and rapid resolution are a necessary part of integrated animal disease management. A two-way flow of information should be encouraged between those who develop the control technologies and those who use them. Users need to understand what technologies are available now, what technologies are in development, and how they can be effectively used to help in disease control. Researchers need to have feedback on how their technologies will be used on the ground and what would be most useful for future development. Processes are suggested below to ensure that decisions are based on the best and most appropriate advice.

Recommendations

- Implementation of effective national FMD Expert Groups according to the specifications of the EU FMD Directive, and enhanced to include the creation of *ad hoc* sub-groups or panels as required.
- Creation of a European FMD Expert Group that is available to all member states for advice on request.
- Creation of a European and International FMD Task Force for assistance with surveillance and vaccination on request, involving both diagnostic test kit banks and vaccine banks.
- Discussions leading to the creation of a European Animal Health Association involving stakeholders, scientists and regulators, with power to propose and vote on resolutions.

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Amongst the many who encouraged us and patiently helped us to understand the complexities of FMD control: research scientists and veterinarians, especially Paul Kitching, Martin Hugh-Jones, Roger Breeze, John Crowther, David Paton, Keith Sumption, and Sheila Crispin, as well as farming representatives and people whose concerns motivated them to search for and disseminate information, including John Thorley, Karen Conyngham, Alan Beat and Mary Critchley.

References

Bartley, L.M., Donnelly, C.A. & Anderson, R.M. (2002) Review of foot-and-mouth disease virus survival in animal excretions and on fomites. *Vet. Rec.*, 151: 667-669.

Bates, T, Thurmond, M.C., Hietala, S.K., Venkateswaran, K.S., Wilson, T.M., Colston Jr., B.W., Treves, J.E. & Milanovich, F.P. 2003. Surveillance for detection of foot-and-mouth disease. *J Am Vet Med Assoc.*, 223(5): 609-616.

Crispin, S.M., Roger, P.A., O'Hare, H. & Binns, S.H. 2002. The 2001 foot and mouth disease epidemic in the United Kingdom: animal welfare perspectives. *Rev. sci. tech. Off. int. Epiz.*, 21(3): 877-883. (also available at www.oie.int/eng/publicat/rt/2103/5.4.Crispin.pdf)

DEFRA. 2004. *Foot and mouth disease – contingency plan.* (available at www.defra.gov.uk/footandmouth/contingency/index.htm)

Haydon, D.T., Kao, R.R. & Kitching, R.P. 2004. The UK foot-and-mouth disease outbreak – the aftermath. *Nat Rev Microbiol.*, 2(8): 675-681.

Honhold, N., Taylor, N.M., Wingfield, A., Einshoj, P., Middlemiss, C., Eppink, L., Wroth, R., Mansley, L.M. 2004. Evaluation of the application of veterinary judgement in the pre-emptive cull of contiguous premises during the epidemic of foot-and mouth disease in Cumbria in 2001. *Vet. Rec.*, 155: 349-355.

Horn, F.P. & Breeze, R.G. 2004. *U.S. Agricultural and Food Security: Who Will Provide the Leadership?* (available at www.humanitarian.net/biodefense/fazdc/usaha_fadp.html)

Kitching, R.P. 2002. Future research on foot and mouth disease. *Rev. sci. tech. Off. int. Epiz.*, 21(3): 885-889. (also available at www.oie.int/eng/publicat/rt/2103/5.5.Kitchingfuture.pdf)

Kitching, R.P. 2004. Predictive models and FMD: the emperor's new clothes? *Vet Journal* 167: 127-128.

Kitching, R.P. & Hughes, G.J. 2002. Clinical variation in foot and mouth disease: sheep and goats. *Rev. sci. tech. Off. int. Epiz.*, 21(3): 505-512. (also available at www.oie.int/eng/publicat/rt/2103/1.8%20Kitchingsheep.pdf)

Royal Society. 2002. *Inquiry into infectious diseases in livestock.* London. The Royal Society. (available at www.royalsoc.ac.uk/inquiry/intro.htm)

Taylor, N. 2003. *Review of the use of models in informing disease control policy development and adjustment.* London, Department of Environment, Food and Rural Affairs. (available at www.defra.gov.uk/science/Publications/2003/UseofmodelsinDiseaseControlPolicy.pdf)

Wernery, U. & Kaaden, O.-R. 2004. Foot-and-mouth disease in camelids: a review. *Vet Journal*, 168: 134-142.

POSTER SESSION

Chairperson: Dr Nilay UNAL
 Rapporteur : Dr Stéphane ZIENTARA
 Dr Georgi GEORGIEV

At the Open Session of the EUFMD Research Group Meeting 17 posters were presented. These posters deal with

- Epidemiology (1)
- FMDV structure (1)
- FMD vaccines (2)
- Pathogenesis (4)
- Diagnosis (molecular diagnosis-7)
 (serological diagnosis-2)

Recommendations of the Poster Session

The reporting group would like to underline the fact that the decision of the FMD Research Group to organise such a poster session was a good initiative.

On the basis of the presented posters in this session the reporting group makes following recommendations:

- necessity to reinforce the surveillance of FMD around the world in particular in Africa
- develop studies on the structure of the FMDV proteins in order to better understand their fonctions and develop antiviral molecules
- develop reagents (recombinant antibodies or monoclonal antibodies) for diagnosis or studies on pathogenesis
- develop and reinforce studies on the pathogenesis of FMD and the interactions virus\cells
- develop studies on the immune response against FMDV in susceptible species
- develop studies on the molecular diagnosis of FMDV (real-time PCR, loop mediated amplification, rolling circle amplification)
- develop tools for rapid and reliable diagnostic method for serological diagnosis of FMD
- necessity of comparative studies, validation and standardisation new diagnostic methods

Summary of the posters presented

Identification of FMDV replication in cells within the foot and tongue epithelia

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This study deals with identification of FMDV replication sites by in situ hybridisation in pigs.

In situ hybridization (ISH) has been used to detect FMDV RNA in tissues from infected pigs. A digoxigenin-labelled RNA probe corresponding to a coding part of the RNA-dependent RNA polymerase (3D) genomic region was prepared.

Results indicate that the basal cell appears to be the cell type demonstrating the highest signal for the detection of the FMDV positive sense RNA in both tongue and foot epithelium. The detection FMDV positive sense RNA showed very strong signal in basal cells (especially in foot lesions). Mouth lesions showed in general less signals than in foot lesions. Although the *stratum spinosum* cells show more signs of cytolysis than the basal cells, the FMDV RNA signal in the *stratum spinosum* cells was more diffuse and less concentrated. These results are strongly suggesting that the basal cells could be the early replication site of FMDV in vivo.

Laser Micro-Dissection studies of FMDV infection in pigs

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The objective of this study was to isolate and quantify foot-and-mouth disease virus (FMDV) in the different epithelium cell-types in order to observe potential differences in FMDV RNA distribution in specific tissues.

Laser Micro-Dissection (LMD) was carried out on frozen sections from selected tissues of infected pigs. After RNA extraction the samples were tested for FMDV and 18S ribosomal RNA (as a RNA marker) by real time quantitative RT-PCR.

The data suggest that there is more virus detected in the epithelium of skin, especially the stratum basal cells, than in tongue and soft palate. The differences in virus level in epithelia may be a contributing factor to the formation of vesicles in certain tissues.

The pathogenesis of FMD in young lambs

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This poster presents the first study on the pathogenesis of FMD in young lambs using quantitative PCR and in situ hybridization (ISH).

The pathogenesis of FMD was characterised by measuring viral RNA levels in daily serum and nasal swab samples and in post-mortem tissue samples, measured by quantitative RT-PCR.

FMDV in lambs initially dermatotropic, can become myocardiotropic and myotropic with fatal results.

FMDV RNA detection in pig samples by conventional and Real Time RT-PCR methods

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The main aim of the present study was to assess the suitability of different RT-PCR protocols for the FMDV RNA detection and/or quantification in samples collected from pigs experimentally infected.

FMDV RNA was detected in blood samples of infected pigs only until 3 d.p.i. The biopsy samples were all positive, only kidney samples were negative. The quantification of viral RNA in these samples, indicated that the lymph nodes have the greater viral RNA concentration, while the liver are the samples with less quantity of FMDV.

The results obtained in this study suggest that pharyngeal swabs are samples more suitable to perform the FMD diagnosis at least during the first 10 days of infection.

Immunogenicity and protection conferred by synthetic peptides that reproduce B and T epitopes of FMDV in pig

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The authors analysed the immunogenic potential conferred in pigs afforded by 3 synthetic peptides which included B (sites A and D) and T (protein 3A) cell epitopes of FMDV, and the protection induced after a challenge with an homologous strain.

Significant neutralization antibodies and lymphoproliferative responses were detected in the three groups of pigs immunised.

The result show that this peptide TB maintain the immunostimulatory ability of B- and T-cell components. Although the vaccination protocol followed with these primers were not enough to prevent the FMD, the clinical findings and the low detection of viral RNA in swabs of the immunised pigs are consistent with a partial protection and with a restricted viral transmission by the respiratory via.

Diagnostic differences between real-time RT-PCR and virus isolation tests on cattle probang samples following infection with FMDV

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This report highlights an elevated incidence of mismatches between Real Time PCR and virus isolation with probang samples taken between 6 and 21 days post-infection (dpi) from experimentally infected cattle.

For the period 1 to 5 dpi there was a 5% mismatch between PCR and virus isolation tests. For the period 6 to 21 dpi, about 77% (52/68) of the samples were positive by RT-PCR compared to about 29% (20/68) by virus isolation. In 50% (34/68) of samples the RT-PCR and virus isolation results did not agree. The greatest disagreement of 75% (12/16) was on 7 dpi.

The possible technical and biological reasons for these discrepancies between real-time RT-PCR and virus isolation such as implications of the quality of probang samples, the cell cultures, automated extraction, the presence of antibodies and stage of the disease are discussed.

A novel, but simple molecular method for detection of FMDV

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This study describes loop mediated amplification (LAMP) which is a novel technique that amplifies specific nucleotide sequences under isothermal conditions. Unlike PCR, a denatured template is not required and the amplified product of LAMP can be visualised with the naked eye.

LAMP may prove a cost-effective method for the detection of FMDV genomes at the pen-side, in the field and by developing countries.

Towards the construction of an O₁ Manisa chimeric vaccine

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The first stage in the construction of a vaccine with improved marker characteristics, based on the widely used vaccine strain, O₁ Manisa is described.

A full-length cDNA clone of O₁ Manisa was constructed using an exchange-cassette strategy based on an existing A₁₂ full-length cDNA clone.

After this step, the major antigenic site, the G-H loop of VP1 (residues 132-159), will be replaced with the corresponding region of alternative serotypes (A₂₄ Cruzeiro and C₃ Resende) in order to enhance the marker characteristics of the vaccine.

Production of monoclonal antibodies against the three SAT serotypes of FMDV, helpful as standardised reagents in diagnostic assays

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The authors have developed panels of MAbs specific for each of the three SAT serotypes.

FMDV SAT 1: out of 35 MAbs raised against type SAT 1, strain Botswana 1/68, 27 neutralise virus infectivity; 10 of them do not recognise in WB isolated viral proteins, suggesting they identify conformation-dependent epitopes, while 17 are WB-positive and presumably react with VP1. *FMDV SAT 2:* 22 MAbs were raised against type SAT 2, strain Zimbabwe 5/81. Five MAbs with neutralising activity recognise a linear epitope in WB, 13 further neutralising MAbs, negative in WB, detect conformational epitopes. Out of 5 non-neutralising MAbs, 3 react as conformation-dependent MAbs, while one clearly positive in WB recognised a linear epitope, presumably located on VP2. *FMDV SAT 3:* only 2 weak positive MAbs specific for type SAT 3 were produced. All MAbs from the three panels are type-specific, except one that cross-reacts with the seven FMDV serotypes.

Sets of MAbs specific for FMDV type SAT 1 and SAT 2 are available.

Analysis of possible genetic differences in selected tissues from cattle with acute and persistent FMDV infection

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This paper explores the primary structure of some areas of the genome to gauge its variability in tissues from several disease stages.

Cattle were exposed to FMDV type O UKG/34/2001 and tissue samples from epithelial lesions during the acute stage and the dorsal soft palate of carrier animals were collected.

Parts of the IRES, leader, 2B, 2C, 3C and 3D genomic areas derived from the epithelium and nasopharyngeal tissues were sequenced.

Some genetic variability was seen in the virus from different stages of disease. Whole genomic sequencing of these RNAs is currently underway and will allow a broader assessment of the viral genetic heterogeneity within infected animals.

A ring-test for the laboratory detection of FMDV by RT-PCR and virus isolation

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The paper describes organisation the ring test which will provide valuable comparative data on the relative performance of virus isolation (VI) and the different RT-PCR methods.

The IAH, Pirbright Laboratory will co-ordinate a proficiency panel for FMDV virological testing.

Purification of RNA dependent RNA polymerase and its predicted three-dimensional structure

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The authors describe the purification of FMDV RNA dependent RNA polymerase, 3D protein in order to determine the predicted three-dimensional structure.

The 3D protein was purified by affinity chromatography with Ni Sepharose.

In near future the authors have planned to determine the structure of the crystallized 3D protein by X ray analysis.

Analytical validation of One-Step real-time RT-PCR for FMDV RNA

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This poster describes the use of a real-time Reverse Transcriptase PCR (real-time RT-PCR) for the detection of serial dilutions of FMDV RNA, purified from the supernatant of virus productions on cell cultures.

Viral RNA from 82 different strains (29 strains for serotype A, 10 for serotype Asia and 43 for serotype O) was extracted and Real-Time RT-PCR was performed.

This work shows that the discussed technique is able to detect a broad range of FMDV subtypes and serotypes.

Production of a single-chain variable fragment antibody against foot-and-mouth disease virus

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This poster describes the construction of a scFv antibody from the murine monoclonal antibody (mAb) 16A11, specific for foot and mouth disease virus (FMDV) O/SKR/2000, and its active form produced in *Escherichia coli*.

The sequencing results showed that the V_H gene of constructed scFv was composed of germline VH1S6-DFL16.2-JH2 and gremlin VK1-135-JK1 for the V_L gene and the expressed scFv protein (about 30kDa) was detected on SDS-PAGE and immunoblotting. ELISA results showed that scFv retained almost the same antigen affinity and specificity as its parent mAb.

The scFv antibody against FMDV has been successfully constructed and produced, which lays the foundation for further study and applications.

The Development of an indirect ELISA for the detection of antibodies to the non-structural protein 3ABC of the Foot and Mouth Disease virus; The use of a polyclonal conjugate that allows a multi-species detection of antibodies

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This communication presents an indirect ELISA for the detection of antibodies to the 3ABC antigen of FMDV in the sera of Bovine, Swine, Ovine and Caprine utilising a newly devised polyclonal conjugate.

The bovine sera showed, as expected, a sensitivity and specificity of 100% using a PP cut-off value of 49.7. When testing the sheep sera, the ELISA showed a sensitivity of 89.2 and a specificity of 100% at a cut-off PP of 30. When testing the swine sera a sensitivity of 95.8 and a specificity of 100% were obtained at a cut-off PP of 30.

By using the test system presented in this communication, i.e. using the 3ABC protein as a coating antigen and a multi-species conjugate as the detection antibody one can overcome the problems of variations in the antibody response to the NS proteins.

Complexity of foot-and-mouth disease outbreaks in Kenya

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This paper provides a description of the viral diversity and epidemiological complexity of FMD in Kenya.

Five FMDV serotypes (O, A, C, SAT 1 and SAT 2) were recorded in outbreaks in various parts of Kenya in the year 2003 and the first half of the year 2004. Serotype O caused majority outbreaks followed by SAT 2, SAT 1 and A while serotype C was responsible for only one outbreak. Vaccine strains show varying immunological relationships to field strains.

These studies have indicated that FMDV serotypes in Kenya belong to different lineages and genotypes. Serotype SAT 2 exhibits the highest intratypic variation. This is complicated by the fact that the extent of transmission of FMD between wildlife and domestic livestock has not been investigated in any detail.

Rolling Circle Amplification for detection of FMDV

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The authors describe the use of the rolling circle amplification technique (RCA) for the detection of FMDV. RCA was shown to allow detection of cDNA, prepared from viral RNA which was purified from the supernatant of virus productions on cell cultures.

First, the RCA technique was optimized on a synthesized template with the exact sequence of a 29 bp region in the 3D gene of the FMDV RNA. In a next step, RCA was optimized on double stranded cDNA generated from the viral RNA after a reverse transcription step which was followed by normal PCR amplification.

Starting from purified FMDV RNA, RCA allows detection after cDNA generation of the target sequence. In future experiments, serial dilutions of the purified RNA will be tested. Moreover, RCA will be optimized either with the viral RNA directly as a template, or after a single reverse transcription step. Finally, the technique will be validated on spotted saliva and field samples.

Feedback from group of participants from the “European State Veterinary Services”

Most significant technical questions/areas of work in need of attention:

1. International Surveillance and Early Detection
 - a. - of threats to the country through legal and illegal trade, as well as windborne infection
 - b. - of disease once it is established in the country
2. Laboratory Testing and Supporting On-Farm Diagnostics:
 - a. Rapid confirmatory tests to identify the virus type and suitable vaccine strains
 - b. Tests to support the vet on the farm in reaching a diagnosis
3. Vaccines (vaccinate to live being the chosen policy):
 - a. Surveillance to support the strains kept in the vaccine bank
 - b. Post-vaccination surveillance for cattle, sheep, goats and pigs identifying infection in vaccinated livestock
 - c. Independent evaluation of vaccine strategies for all species
4. Epidemiology
 - a. Improve the understanding of the role of the carrier animal in the epidemiology
 - b. The role of milk and milk tankers in the spread
5. Modeling
 - a. Predicting the outcome from various control strategies (movement restrictions, culling, vaccination)
 - b. Modeling of air-borne spread of virus from infected premises
6. Cost-benefit analysis
 - a. Predicting the outcome of disease control strategies on the local and national economics taking on board socio-economic issues

Easing the two-way communication with the Executive Committee:

- Broaden the audience by including section on outbreak management, contingency planning, etc.
- Identify senior veterinary officers from VS to participate in Open Session
- Organize presentations by target group (risk manager, lab manager, scientists)
- Indexation of presentations with key-words
- Include Executive (“CVO”) Summary or statement of relevance in presentations
- Opt for epidemiological/management focus of relevant presentations
- Identify drivers of demand, e.g. Directive or OIE code changes, lessons learned, bio-terrorism contingency
- Two-way exchange of itemized/prioritized “wish lists” between RG and EC
- Two-way exchange of itemized/prioritized action lists between RG and EC

**Feedback from Middle East and North and East Africa Group
(Participants from Egypt, Ethiopia, Iran, Kenya, Lebanon, Morocco, Yemen)**

Considering that:

- Animal movement within the Middle East region could involve movement of animals from South and South-East Asia and also from East Africa. Both regions are considered harbouring regions for FMD viruses.
- Some countries found difficulties in sending samples to WRL.
- Previous studies revealed that FMDV serotypes have a distinct topotype in Africa and Middle East as well as a higher level of intratypic variation within each serotype.
- WE AND YOU DO KNOW THAT WE ARE NOT PART OF EUROPE, BUT FMDV DOESN'T.

We recommend:

1. For the region to contribute to the FMD control, surveillance network including laboratories within the region in order to have an effective early warning system is highly needed.
2. To encourage the research within the region and to facilitate the information exchange through workshops, meetings within the region and also participation in the international meetings and workshops.
3. A further detailed study on the evaluation of available vaccines for most developing countries of Africa and Middle East on the basis of strict geographical clustering of independent evolving virus population should be encouraged.
4. Building up national and regional expertise in the field of FMD epidemiology, diagnosis and vaccine production should be considered as a priority.
5. To encourage use of high quality vaccines, and also diagnostic kits
6. Establishment of regional reference laboratory is highly needed.

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