AGA: EUFMD/RG/03

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 at

Gerzensee, Berne, Switzerland

16-19 September 2003

FOOD AND AGRICULTURE ORGANIZATION OF THE UNITED NATIONS
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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 at Gerzensee, Berne, Switzerland from 16 to 19 September 2003.

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

The acting Chairman of the Research Group, Dr Kris De Clercq, welcomed everybody to the Session and gave the floor to the Chief Veterinary Officer of Switzerland, Dr Hans Weiss. Dr Weiss welcomed the participants to Switzerland and was pleased to note that so many FMD experts could come together in this way. He thanked Dr Griot for organizing the arrangements for the meeting. As a client of laboratories he had to ask himself what he would do if FMD came to Switzerland. Emergency plans required to be tested, and will be tested, and the active contact between laboratories will be very important in peace time to ensure laboratories are better aware and able to respond to emergencies. Research networks for infectious diseases should be a platform for development of new diagnostic tests and vaccines. He wished the Session a very productive meeting.

Dr Griot then welcomed the Research Group to Gerzensee Study Centre, on behalf of the IVI and reminded the Group of the importance of our work for our clients, the veterinary practitioners and farmers, and the importance of communication of our activities and findings.

The Secretary of the EUFMD Commission then thanked the CVO of Switzerland for hosting the meeting and thanked Dr Griot and his staff for their immense efforts in the arrangement of the Session. He added that the site of healthy and contented animals at pasture in the beautiful environment reminded us all of the great progress made in Europe in the last 50 years in FMD control, and the importance of our task to maintain the high health status across our region. He brought the attention of the meeting to the conclusions and recommendations of the General Session of the Commission's member states in April 2003. The work of the Research Group was recognized as very valuable and to be encouraged, but priorities for activities for the biennium 2003-2005 should be identified at the 2003 Research Group Session, to address the major technical issues identified at the General Session. The programme for this Session was set by the discussion of the General Session and the concern of the member states on these issues. He reminded the members that through the 1990s the Research Group had provided leadership and foresight in the technical development of tools for FMD control and the practical implication of their uptake and utilization. The Group had very much taken the international lead in the maintenance of standards in FMD diagnosis and this issue remained as important as ever. He brought the attention of the Session to the fact that in 2004 the Commission will celebrate 50 years of existence and that this biennium will be

an important time to reflect on what has been achieved and how to move forward in our field. Finally, he reminded the Session that the Chair of the Research Group is elected at the Session, and called for nominations. He thanked Dr De Clercq for his immense contribution as Chairman over the past biennium and hoped that the "mountain" of the agenda could be successfully scaled by the assembled international team.

Presentation by Professor Vincenzo Caporale, President of the Scientific Commission on Animal Diseases of the OIE

Professor Caporale reminded the Session that the Scientific Commission was a new title for the former FMD Commission of the OIE, and he outlined the new approach to be taken to address outstanding issues. The OIE certifies the health status of countries and zones for four diseases, FMD, CBPP, BSE and RP. The new approach is to develop ad hoc groups to deal with particular parts of the SCAD terms of reference. The ad hoc groups being developed are for Surveillance Guidelines, Regionalization, zoning and compartmentalization, country recognition (status with respect to disease), vaccine banks, and non-structural protein (NSP) antibody tests for FMD serology. He indicated that surveillance guidelines were required for each of the four diseases where country status is certified, as well as generic surveillance guidelines for terrestrial animal diseases. The surveillance guidelines for FMD need to cover the surveillance standards in the demonstration of freedom from infection in a population regularly vaccinated, in an emergency vaccinated population, and in a non-vaccinated population. The performance of tests at individual and population level was important to determine, for all relevant species. Measures to ensure product safety was also an issue, particularly to identify safe trading practices and commodities for countries that can be applied regardless of FMD status. The safe trade of meat from vaccinated pigs was one situation to be resolved. He suggested that the EUFMD Research Group could play a valuable role in supporting the work of the SCAD ad hoc groups, and that it would be advantageous for the Research Group to work with experts in other regions, such as South America, to develop joint technical papers and opinions.

Adoption of the Agenda

The Chairman proposed that the following Agenda should be adopted:

Item 1 Briefing on relevant research projects
Item 2 Post-vaccinal surveillance – issues, experience, outlook and post-vaccinal surveillance – tests for differentiating infected and vaccinated animals (DIVA)
Item 3 Priority setting for FMD vaccine bank (risk assessment/true prevalence of FMD)
Item 4 Towards virus detection standards – including RT-PCR
Item 5 Diagnostic standards – Reference sera
Item 6 "Rapid diagnostics" for FMD
Item 7 Contingency planning for FMD laboratories
Item 8 Guidelines for air transportation of FMD samples
Item 9 Critical review of inactivation standards
Item 10 Work programme 2003-2005
Item 11 Any other business

Item 12 Open papers on emerging issues/latest developments
Item 13 Presentation and adoption of report

The Agenda was adopted as proposed.

Item 1 – Briefing on relevant research projects and applications supporting FMD research in Europe

Dr Kris De Clercq briefed the session (Appendix 1) on proposals developed and submitted for EC funding in the last year, that had the intention to strengthen the research effort and the network of FMD expertise in Europe. These were:

1. "FMD improCon", a specific research project oriented to support EU policies, that was successful in the first stage of evaluation and is presently under contract negotiations.
2. Reference standards development, that is under consideration by DG-SANCO.
3. European research area network (ERANET) application submitted in June 2003 and which after evaluation may be resubmitted when the eligibility of partners is established, and
4. Project for community and national reference laboratories to strengthen collaboration, which remains to be written in late 2003, with the coordination of the WRL and which should involve FMD and CSF laboratories.

He identified gaps in the European programme to be:

1. Evaluation and other studies relating to contingency planning.
2. Work to develop reference virus isolates/genomes.
3. FMDV survival in animal products.
4. Virus transmission studies, through direct and indirect contact.
5. FMDV vaccine evaluation.
6. New generation marker vaccines.

Discussion

It was strongly suggested that socio-economic aspects that relate to infectious disease control should not be neglected. Further, operational research methods could be very important in the evaluation of contingency plans. The new EU directive, which should shortly be in force throughout the EU, should provide a legal basis for support to resolve some of the technical issues which constrain policy application. The Session greatly appreciated the effort of Dr De Clercq to identify funding options and to coordinate proposals which address the key areas of concern to the Research Group.

Item 2 – Post-vaccinal surveillance (PVS)

I - Issues, experience, outlook

The Secretary of the Commission outlined a number of recent developments relating to surveillance after the use of vaccination in previously free, non-vaccinating countries.

These include the Guidelines for FMD surveillance prepared by the FMD Commission of the OIE, which have been accepted by the International Committee of the OIE in May 2003 but which will be further reviewed and are expected to be revised before May 2004. A difficulty in developing FMD Guidelines has been the lack of definition of an acceptable level of evidence for absence of virus infection in a vaccinated population. He suggested that collaboration with countries such as Uruguay which had conducted significant post-vaccination surveillance could be instructive to the better definition of guidelines and had for this reason invited Dr Andrés Gil to the Session. Further, he indicated that there is some flexibility allowed in the OIE Guidelines that could be beneficial to countries to enable the selection of surveillance strategies are most cost-effective for their epidemiological situation, and where the sampling strategy may be adjusted to compensate for test performances. He indicated that confidence in the surveillance design and sampling to detect previously infected animals was very important, and that quantitative methods to demonstrate confidence in the absence of infection could be very valuable, and for this reason had invited Dr Matthias Greiner to the meeting. Under the articles of the new Directive, tests would be used to classify herds and therefore confidence in the selection of test systems, and the criteria for positive results, will be very important. Issues relating to tests selection need to be resolved, and new methods for test interpretation that could add confidence to the detection of carrier or previously infected animals, should be evaluated. For this reason the potential use of likelihood ratios would be explored at this meeting.

Dr Andrés Gil presented a paper (Appendix 2) on the sero-monitoring for FMD infection following the 2001 type A epidemic in Uruguay.

Dr Nilay Ünal presented the preliminary findings (Appendix 3) of sero-surveillance in Anatolia and Thrace regions of Turkey following the 2003 spring vaccination campaigns.

The importance of PVS is linked to the fact that emergency vaccination around outbreaks could be realized in Europe as a response to FMD outbreaks. Rapid recovery of FMD free status without vaccination will be essential for economical reasons.

Questions are linked to the possibility of virus animal carriers, but also to the definition of notions like “active virus circulation” and “infection freedom”. In this context, little in the way of data on within herd and between herd infection rates, in relation to time-space and to the time-course of outbreaks and vaccination measures, are already available and so, those existing should be very important to analyze.

Dr Matthias Greiner presented a paper (Appendix 4) on novel approaches to the use of surveillance data to demonstrate confidence in the demonstration of freedom from infectious diseases, using the example of CSF surveillance in Denmark. The approach was developed to identify and quantify the role played by “routine surveillance” operations, such as farm visits, abattoir inspections, in the surveillance for CSF, and thereby to reduce the need or cost of structured sampling/sero-surveillance activities. The methodology will be presented during a post ISVEE workshop supported by the OIE and the international EpiLab, in November 2003, in Vina del Mar, Chile. The surveillance activity maybe targeting in different ways populations with higher risks and populations with lower risks of infection, and could be of considerable value to the demonstration of

FMD freedom. Over sampling in the higher risk population must not compromise the probability to sample adequately the lower risk population. A targeted surveillance activity may enhance probability of finding the disease and then reduce the costs of surveillance. This must be relevant also for PVS, with the importance of NSP serology, and of clinical and slaughterhouse surveillance in non-vaccinated sentinel animals.

Recommendations

It is recommended that EUFMD secretariat could approach Uruguay authorities to have the possibility to work with them on their data. A precise knowledge of within herd prevalence and of between herds prevalence from the large serological surveillance surveys realized in this country could be very useful to address the PVS issue, even if the context may be different from the European situation. The whole surveillance system is important, but the serological data are specifically relevant.

In the same way, the serological surveys realized in Turkey could also bring very useful information on this issue. The low percentage of positive results (less than 2 per cent) with the 3ABC test could also be seen as a specificity question, not only as a possibility of a low level of circulation of the virus. Further investigation of the results and the circumstances of the villages which had positive animals is recommended.

The unsatisfactory level of antibodies to structural proteins post vaccination should be investigated by further data analysis.

Methods to quantify confidence in the absence of disease or infection that use non-serological and serological data should be explored for the context of demonstration of FMD freedom in non-vaccination/vaccination scenarios.

II - PVS tests for differentiating infected and vaccinated animals (DIVA)

Two papers on the evaluation of tests for use in PVS were presented, by Dr David Paton (Appendix 5) and Dr Aldo Dekker (Appendix 6). One paper was presented by Dr Michael Collins (Appendix 7) on the use of likelihoods ratios to assist in the interpretation and communication of ELISA data.

Experiments at Pirbright involving contact challenge of vaccinated cattle demonstrated complete clinical protection, but a variable degree of protection against viral replication, with animals either 1) being completely protected from virus replication, 2) supporting transient virus replication, or 3) becoming carriers. No single test detected all of the carriers. Commercially available NSP test kits were not equally effective at detecting infection in vaccinated animals. An in-house test for specific IgA in saliva was the most sensitive approach.

The issue of correct diagnosis in serological tests, and methods to express the probability of correct test result, was explored by Dr Collins. He presented a pilot study of the use of likelihoods ratios (LRs) of a correct result, in the detection of FMD virus challenged animals, using data supplied by the IZS, Brescia. LRs can be expressed at a range of cut-off values, and therefore for each test result, provide an indication of the likelihood of this

result occurring by chance. For this reason, assurance in the likelihood of a correct diagnosis can assist in difficult decisions, such as slaughter of herds containing test positives. The observation that high ELISA values were obtained in most virus challenged, vaccinated animals, with two NSP tests (Bommeli and an in-house assay) and also in tests for antibodies to SPs (the rise in antibodies on challenge) resulted in potentially useful LRs with each of the NSP test, and the use of SP tests. A LR of >100 was observed using the Breschia in-house NSP test for results >40% of the positive control. However, he warned that use of the method would depend on the validity of the experimental data in relation to field exposure of vaccinates, and the results should be considered preliminary findings.

Dr Dekker presented a comparison of 5 DIVA screening/screening plus confirmation tests on a selection of sera from cattle of known status. The ELISAs were obtained from their producer. The results were used to produce ROC curves, and compare the tests using a permutation test. The curve of the Ceditest was almost optimal, and significantly different in ROC curve than those obtained with 4 other tests, and a higher analytical sensitivity for detection of dilutions of the positive serum. For duration of a positive response, the Ceditest and the Aftosa test (from PAHO) were able to detect 16/16 animals at 441 days post-infection, but the specificity of the latter appeared poor. However, with vaccinated calves given intra-nasal virus challenge, the sensitivity of detection was much lower. The infection status (acute infection or carrier status) of these calves was not reported.

Discussion

The possibility to vaccinate herds in emergency and to recover rapidly a FMD free status is linked to serological tests able to differentiate infected from vaccinated animals. The validation of the tests may not require the testing of 300 different animal sera from all FMDV serotypes, and the recovery of FMD free status may not require only serology against NSP. The lowest acceptable prevalence and its accepted confidence value will orientate towards the most appropriate sample size. Possibilities for systematic review and systematic summary (e.g. meta-analysis) of existing evidence should be explored.

Recommendations

It is recommended that the definition of the number of animals required in test validation studies be approached after definition of what would be required in surveillance strategy. To do this, a thorough review should occur of the within-herd prevalence data to be expected.

It is also recommended that comparison of DIVA tests be conducted on the most significant category of animals, those which have been vaccinated, challenged by (preferably) infected animal contact, and shown to have become infected.

Close co-operation between European laboratories and those in other parts of the world is required to achieve this in the shortest time.

SATs genotypes have been less explored than other genotypes in the context of PVS-DIVA. It is recommended that evidence of a problem in detection of SAT infections after vaccination be reviewed before a recommendation on performance of animal studies be made.

It is recommended that a synthesis (meta-analysis) of all the tests be performed so that overall performance, and lack of data, if any, may be realized and completed.

Other ways to interpret test results based on use of different cut-offs and probability calculations should be encouraged as a possible means of identifying infection at herd or individual level.

Item 3 - Priority setting for FMD vaccine bank (risk assessment/true prevalence of FMD)

Dr David Paton presented an overview of FMDV genotype information available to the WRL for 2002-2003, and relevant antigenic characterisation (Appendix 8). Recent trends or events of importance were highlighted. Disparity in continental and regional use of the WRL services was a long term problem leading to relative lack of information from some regions, particularly in Africa and South America. Availability of reference sera for antigenic typing remained an important constraint.

Dr Keith Sumption presented the results of a survey (Appendix 9) of expert opinion on gaps in the global surveillance for circulating FMD virus, and some considerations on the use of livestock population and husbandry systems information to target surveillance. Predictive FMD maps might also assist the targeting of control efforts, including better identification of the need for vaccine antigens.

Dr Mark Thurmond presented in outline a new initiative (Appendix 10) to map FMD risk, using observed FMD data from three countries to develop models for FMD incidence and prevalence that might be adapted to address global information needs.

Dr Marius Gilbert presented an analysis of FMD types O, A and Asia-1 occurrence in time and space in Turkey (Appendix 11). Different spatial-temporal trends for the three types were observed, which may permit prediction of future FMD.

Priority antigens for 2003

It is recognised that we lack information regarding the characteristics and prevalence of FMD types and subtypes in some parts of the world. Furthermore, our systems for matching field isolates to vaccine strains are imperfect. Therefore, a rather conservative approach has to be taken with respect to concluding that certain vaccine strains may no longer be required. Likewise, newly emerged strains may provide locally useful vaccines, but such strains are not necessarily suited to incorporation into international vaccine banks, unless they can be shown to confer a broad coverage of protection.

Data from South America suggest that type A viruses from 2000 and 2001 show a relatively poor match to A24 Cruzeiro and that vaccines based on A Argentina 2001

should also be available. Data from the Middle East suggest that the A22 Iraq vaccine is less useful than previously and that a vaccine based on A Iran 87 would be useful. This could replace the recommendation for inclusion of the related A Saudi Arabia 23/86 vaccine. The Iran 87 vaccine may also provide cover against type A viruses from South East Asia, where the A15 Bangkok vaccine seems less useful. Given the lack of evidence for circulating type C FMD virus, it may be less important to maintain large reserves of the C Noville vaccine strain than previously.

Priority locations from which assisted delivery of isolates to WRL is required

A ranking of priority locations was obtained by analysis of the answers provided by experts to a questionnaire on this subject. The order of priorities obtained was:
1. China
2. Indian subcontinent
3. African horn
4. Africa East

Few or no samples have been submitted to the FMD World Reference Laboratory from these regions in the last three years.

It is instructive to look at which FMD infected countries have the highest populations of susceptible species and are the main exporters of live animals and meat, since these are likely to represent a particular threat. China and India do indeed have some of the largest populations of susceptible species in the world. Countries in sub-Saharan Africa also merit more attention than they have previously been given. A number of projects are now underway to examine in detail the ecosystems in selected countries where FMD is endemic. These studies will seek to assemble information on the location and chronology of FMD outbreaks and on the host species and FMDV serotypes and subtypes involved. This will be analysed in relation to a variety of factors that may contribute to the persistence and spread of the FMD virus such as animal density, husbandry and trading practices.

Principal constraints to sample submission are related either to concerns over the use of the submitted samples and information derived there from or to the cost and effort required relative to the perceived benefit obtained. It was concluded that in the first instance efforts should be made to encourage the submission of more FMD sample materials from Africa, since obtaining information from this region is a relatively high priority and there is a reasonable prospect of improving submissions if resource is targeted here.

Recommendations regarding improved priority setting for vaccine banks

1. Available information on the diversity of circulating FMD viruses and vaccine matching data should be pooled by improving the liaison and exchange of information between regional reference laboratories. A project in the framework of the EU ERA Net will be submitted in the spring of 2004 to take this objective forward. A useful initiative would be to organise meetings of representatives from regional reference laboratories. These could take place every two years. The

World Reference Laboratory will examine the costs and feasibility of organising a first meeting in early 2004.

2. The value of vaccine matching tests on available field isolates should be improved by procuring a more representative supply of vaccine strains and vaccine antisera. This requires closer liaison between vaccine companies and Reference Laboratories. Consideration should also be given to funding reference laboratories to produce their own supplies of reference antisera by Reference Laboratories.

3. Research is needed to improve, standardise and validate in vitro vaccine matching tests. This includes in vivo cross-protection studies and more work to characterise the antigenic sites critical to protection. A new research project under the EU 6th Framework is close to agreement and will support these objectives.

Recommendations regarding improving the estimation of FMD and antigenic type prevalences

1. The submission of more FMDV samples to the World Reference Laboratory should be encouraged to enable genetic and antigenic characterisation studies to be performed. Better liaison with Regional Reference Laboratories may encourage the supply of representative viruses from their collections to the World Reference Laboratory (see recommendation 1 above). Efforts to encourage and subsidise submissions from endemic countries should concentrate initially on targeting resources to countries in sub-Saharan Africa and the horn of Africa where it is likely that financial assistance could have the greatest benefit. The EUFMD Secretariat should co-ordinate such an approach.

2. New research to improve the knowledge of the ecosystems in which FMD is endemic has great potential for identifying the mechanisms by which the virus persists and spreads and thereby to develop risk assessments and new control strategies. The groups should be invited to update the EUFMD on progress in their research in the coming years. The EUFMD Secretariat and the FMD World Reference Laboratory should support these initiatives and help to co-ordinate the activities of different research groups. Discussions and agreement are needed on the extent to which surveillance data received from National Governments by organisations such as international reference laboratories can be made more widely accessible.

Recommendations from the World Reference Laboratory on FMD virus strains to be included in FMDV antigen banks (2003)[1]

The virus strains listed may have equivalents that could be considered as alternatives.

[1] Provisional recommendations at the Session were modified by WRL after further review; O Campos replacing O Lausanne; A Iran 87 moved down to medium; A Saudi 23/86 added as medium priority; A Eritrea 98 and A 87 Argentina moved down to low priority.

High Priority

O Manisa (*covers panasian topotype)*
O BFS or Campos[1]

A24 Cruzeiro
Asia 1 Shamir
A Iran '96
SAT 2 Saudi Arabia

(not in order of importance)

Medium Priority

SAT 2 Zimbabwe
A22 Iraq

SAT 1 South Africa
A Malaysia 97
A Iran 87[1] or A Saudi 23/86[1]
A Argentina 2001
O Taiwan 97 *(pig-adapted strain or Philippine equivalent)*
A Iran '99

(not in order of importance)

Low Priority

C Noville
A15 Bangkok related strain
A Eritrea 98[1]
A 87 Argentina related strain[1]
SAT 2 Kenya
SAT 1 Kenya
SAT 3 Zimbabwe
A Kenya

(not in order of importance)

Item 4 - Towards virus detection standards – including RT-PCR

A paper (Appendix 12) provided by Dr. Wolfgang Philipp and Dr. Heinz Schimmel of IRMM was presented by Dónal Sammin, outlined the requirements of reference system and the steps required in its establishment. For every reference system, both a reference method and reference material are required. A reference material should be commutable (i.e. representative of the normal test analyte) and should be homogenous and stable, giving reproducible results. A reference method should be validated and follow an SOP. Within laboratory variation should be evaluated and all steps should be quality assured.

In the framework of FMD diagnosis, OIE prescribed methods can be taken as reference methods.

Dr. Anton van Loon gave a presentation on the work carried out by QCMD (Appendix 13), formerly the concerted action EU-QCCA. Both the number of programmes which were conducted and the number of participants involved was very impressive. They have shown that it is possible to organize complex proficiency trials and that these can improve laboratory efficiency in diagnosis. They have demonstrated a large difference in analytical sensitivity between laboratories, even when using the same commercially available method. Based on their experience future proficiency panels will use only those dilutions and strains that are clinically relevant as the basis for scoring laboratory performance, although further dilution series and unusual strains will still be included for scientific interest. Dr. van Loon recommended that two PCR processes are performed on each sample; one for agent of interest and the other to evaluate PCR inhibition.

Dr. Kris de Clercq presented a paper (Appendix 14) on current status and deficiencies with regard to FMD virus detection. For FMD virus detection, regardless of the method employed, there are no reference materials available and none of the available methods are suitable for large throughput with high sensitivity, although progress is being made with automated RT-PCR. The current threat of SAT-2 emphasizes the need for diagnostic capability for all seven serotypes.

Conclusion

Reference material is essential in the framework of a QA/QC system and should be produced for virological and serological FMD diagnosis.

Recommendations

1. To establish a proficiency panel for virological testing according to the following timetable:
   - Set up a subgroup to decide on a protocol for preparing a proficiency panel for VI, RT-PCR and ELISA (before January 2004).
   - Distribute proficiency panel for VI, RT-PCR and ELISA to limited group of laboratories (before June 2004).
   - Report the results of testing the proficiency panel for VI, RT-PCR and ELISA (before September 2004).
   - Adopt a protocol for conducting a proficiency test for VI, RT-PCR and ELISA (at the RG session in 2004).
   - Distribute proficiency panel for VI, RT-PCR and ELISA to all national reference laboratories (before June 2005).
   - Report the results of testing the proficiency panel for VI, RT-PCR and ELISA (before September 2005).

2. That the long-term objective should be the development of Reference standards for VI, RT-PCR and ELISA (before 2007).

Item 5 - Diagnostic standards – Reference sera

Dr. David Paton reported on the results of Phase XVII and presented a plan for completion of Phase XVIII (Appendix 15). There is a difference between selection of reference sera and proficiency testing. OIE guidelines state that a strong positive serum, weak positive serum and negative serum are required. Of these, the weak positive serum is the most important. The aims for phase XVIII are:

- Introduction of SPCE
- Preparation and reporting results from secondary standards
- Use of calibrated tests to examine local negative serum panels
- Use of calibrated tests to examine proficiency test panel
- Standardization of internal quality control procedures
- Possibly evaluate new Reference Sera with NSP ELISAs

These aims were approved by the Session.

The session discussed whether reference sera for the SAT-2 serotype should be produced as a matter of urgency for calibration of serological tests for the SAT-2 serotype in the member countries, because of the threat of introduction from Libya, Dr. Have reminded the session that NSP tests can be used for this purpose, as they are not serotype specific. The Group recommended that work continue with the introduction of the SPCE, and encouraged those undertaking potency tests to use the SPCE in parallel to the LPBE to identify levels of response which equate with protection.

Work in progress on the validation of the solid-phase competitive ELISA for FMDV types A, C and Asia 1 was mentioned by Dr Paton and progress in stabilization of the antigen has been made to assist kit development. Further information was subsequently provided on this (Appendix 24).

Conclusion

There is a difference between selection of reference sera and proficiency testing and therefore the two processes should be addressed separately.

The meeting endorsed all objectives of Phase XVIII.

Recommendations

1. The timetable for the completion of Phase XVIII should be:

- Material for testing in the process of Phase XVIII should be distributed before December 2003.
- National Reference Laboratories in countries which do not use vaccination should test sera from at least 1000 non-vaccinated non-infected cattle preferably in the calibrated SPC ELISA. This should be reported to the WRL together with the quality control data, including results on the secondary standards, and the results of the proficiency panel before May 2004.

The report on the results of Phase XVIII should be distributed before September 2004 and discussed at the 2004 RG Session.

Item 6 - Rapid diagnostics for FMD

Prof. José Sánchez-Vizcaíno presented a paper (Appendix 16) on a multiplex RT-PCR combined with restriction enzyme analysis for FMDV, SVDV and VSV detection. For each virus type several isolates were tested with a positive result. A significant result was the fact that the SVDV primers did not detect Coxsackie B5 virus.

Dr. Malik Merza showed information on the development of pen-side test for Rinderpest and FMDV (paper requested but not supplied by author). Validation of the tests is ongoing. Svanova also developed a 3ABC ELISA using a recombinant 3ABC protein from Argentina. The protein was modified in the 3C region to increase stability. Dr. Merza showed initial validation data, which gave promising results. Further validation is necessary, he invited everyone to send positive sera from vaccinated and infected animals from different parts of the world. He mentioned the use of an oligonucleotide ligation assay also known as "padlock" technology as a signal amplification system. In interleukin assays this technique was far more sensitive than conventional technique.

From the discussion on the use of pen-side test two major issues arose. Firstly, the session discussed the performance of pen-side tests compared to laboratory based tests. Secondly, the application and interpretation of pen-side tests were discussed. By whom will the test be performed, and for what purpose will the test be used.

Pen-side tests could be used to:

1. Support early detection of infection in suspect clinical cases (primary/index case).
2. Confirm clinical diagnoses in secondary cases.
3. Rapidly indicate the necessity for additional sampling.
4. Confirm the clinical diagnosis of FMD in the absence of a laboratory infrastructure.

Pen-side tests should only be used by official veterinarian in the course of a disease investigation.

Conclusion

Pen-side tests should be rigorously validated and guidelines should be developed for their application and interpretation.

Recommendations

1. The use of pen-side tests for FMD antigen detection should be encouraged to support the validation of the test with field data.

Item 7 - Contingency planning for FMD laboratories

Dr Dónal Sammin presented the results of a questionnaire survey on the sero-diagnostic capacity of FMD laboratories in EUFMD member countries (Appendix 17). The number of animals tested increased almost 50 fold between 2000 and 2001. With the exception of 4 countries, which did not respond to the questionnaire, all member countries either have a FMD reference laboratory (20 countries) or rely on the service of another member country (7 countries). The monthly testing capacity of member countries as percentage of susceptible animals varies, but with 3 exceptions is less than 1 % and in 8 countries is below 0,1%. In a supplementary questionnaire, information on staff, involvement in contingency planning, types of serological tests performed (mostly LPBE), reagent stocks, production and supply was asked. It was found that existing stocks would often be used up before they could be replaced.

Dr Bernd Haas presented a paper (Appendix 18) on a potential diagnostic test kit / reagent bank for Europe. New control strategies lead to an increased demand for laboratory investigations, especially serological screening. In "peace times" only a limited number of samples is tested, mostly using in-house tests of the national reference laboratories. However, after an outbreak of FMD, laboratories will be expected to quickly scale up their serological screening capacity to levels reaching or exceeding the full capacity of all the countries veterinary laboratories combined. This is a problem especially for countries with a decentralized laboratory structure, where regional laboratories that have no experience with FMD serology would have to take over most of the testing. This will require long lead-in times unless they are supplied with complete, commercially produced test kits. Even if mass serology and FMD diagnosis is performed by the same central laboratory, lack of reagents and test kits could slow down the implementation of disease control measures as well as the lifting of restrictions. However, with a proposed bank, the lead-in time until tested reagents and, preferably, complete tests kits, are delivered in sufficient numbers, could be shortened significantly. The capacity of such a bank should be sufficient to allow a major country to make full use of its testing capacity during the first weeks after an outbreak. Pre-vaccination blood testing carried out in the Netherlands in 2001, as previously shown to be of value in investigation, will require a shorter lead-in time.

Dr François Moutou gave a talk on modelling of FMD epidemic size (Appendix 19). He stated that estimating the size of epidemics is very difficult, not only because of insufficient data, but also because each past outbreak has a very low probability to be seen again and no "average" outbreak exists. The earlier in an outbreak modelling of the epidemic is attempted, the less precise the results will be - whereas the benefits of good predictions would be greatest in the beginning. Important factors to predict the size of an epidemic at an early stage would be the agricultural structure of the affected area, trade pattern, animal density, species involved, age of lesions and the number of aparently "primary" outbreaks.

Conclusions

Serodiagnostic capacity has increased sevenfold since 1995. However 81% of the capacity is concentrated in four countries and three countries reported a monthly capacity equivalent to 1% or greater of the susceptible animal population.

Because of the difficulty to predict the size of outbreaks, modelling is of limited use for diagnostic contingency planning, which should rather try to shorten the lead-in period needed until the full capacity of the countries diagnostic laboratory system can be employed for FMD serology.

Diagnostic test kit / reagent banks are considered an essential part of contingency planning. Without them, the availability of suitable tests could become a limiting factor for the implementation of disease control measures and for the rapid lifting of restrictions once the outbreak appears to be under control.

Validation of NSP tests has now reached a stage, that they could be used for the detection of infection in vaccinated cattle populations as well for the screening of unvaccinated cattle and pig populations (e.g. the situation where there is a lack of suitable type specific testing capacity).

Producers should be involved in the consultation phase before drafting of a plan for a diagnostic test kit / reagent bank in order to define a structure that provides optimum performance for the available budget and avoids the necessity to discard components because their shelf live has expired.

Based on experience and theoretical considerations, it is considered justifiable in emergency situations to carry out serological tests in regional laboratories that do not fully meet the standards for FMD laboratories. However, this applies only to samples from holdings without any clinical signs of FMD. Furthermore, these regional laboratories would need to implement additional biosecurity measures.

Recommendations

Contingency plans for serodiagnosis should prepare the veterinary services and laboratories for large scale serological screening, including identification of the likely lead-in time for such testing.

An European diagnostic test kit / reagent bank should be established in order to ensure the rapid availability of suitable complete test kits, or in cases where such kits are not available, tested reagents in sufficient quantities. This bank should contain test kits for antibodies to NSP suitable for testing of cattle and pig populations. However, serotype-specific test kits / reagents for “anti-structural” antibodies should also be included, because they will still be needed for certain purposes, e.g. testing of small ruminants.

A working group should be established involving EUFMD research group, WRL, SANCO to prepare a recommendation on the structure of a European test kit / reagent bank.
In order to support decisions on which tests should be bought in which quantities for the European diagnostic test kit / reagent bank, a data base should be created and managed by the WRL. This data base should contain the results of validation studies performed with test kits that are ready to be marketed, as well as data on laboratory capacities and epidemiological data which could be expected to support this decision.

The security standards for FMD laboratories[2] should be reviewed. An amendment should be included for laboratories performing only serology on samples from holdings without clinical signs of FMD.

Item 8 – Guidelines for air transportation of FMD samples

Dr Vilmos Pálfi gave an overview (Appendix 20) about the regulations for the transport of infectious dangerous goods by air. He presented information on the most important publications and websites on this matter and emphasized the importance of communication and coordination between shipper and consignee for the safe and reliable arrival of the samples in good conditions. The responsibilities of the shipper, the consignee and the carrier were explained. The lack of knowledge and funds (e.g. for licensed packaging materials) were found to be the most important reasons for problems in sending materials to the WRL.

Conclusions

Selection, collection, packaging and shipping of specimen submitted to the WRL for the diagnosis of vesicular diseases require a very specific knowledge.

Shippers of infectious dangerous goods need proper training in the packaging of materials and the preparation of shipping documents according to the current IATA and ICAO regulations.

The WRL recommends that samples are sent as airfreight and not by a door-to-door courier service in the transportation.

The relevant information on these topics is available, but has to be compiled from a number of separate sources.

Recommendations

A manual containing the principles of selection, collection, packaging and shipping of specimen to the WRL should be compiled. It should include the specific IATA and IAH rules for sending vesicular material to the WRL, including examples of correctly filled out documents and also links to the sources of further information. It should be part of the

[2] Published as an appendix in the Report of the Thirtieth Session of the EUFMD, held in April 1993. Full document will be available in the EUFMD Website.

website of the WRL. EU member states that are required to use couriers should ensure the samples arrive at the authorised agent of the WRL.

The EUFMD Secretariat should alert the member countries to the importance of supporting transportation of samples to the WRL/RRL from countries where FMDV surveillance is currently very limited.

Item 9 – Critical review of inactivation standards

Dr Per Have presented a review (Appendix 21) of methods for describing the effect of temperature and time upon virus survival in products. He summarized the risk reduction measures specified in the new Directive of the EU, under the three phases of an emergency vaccination programme. He highlighted that heat treatments which do not specify a period for heating and cooling cannot be directly compared. The current requirements for heat treatment of meat can be considered to provide a high degree of safety, when applied to low level contaminated products such as meat from vaccinated animals.

Dr Aldo Dekker reviewed the risk analysis process (Appendix 22) which could be applicable to the issue of management of the risk relating to meat and milk from vaccinated herds/animals which test negative by NSP tests, in an emergency vaccination zone. The question of acceptable was considered, since heat treatment of pork or milk can render the products of less value and marketability.

Conclusions

1. Animal products prepared during the waiting period from an emergency vaccination zone before regaining freedom for FMD may constitute a risk of transmitting FMD, depending on species, vaccination status, post-vaccination surveillance and product type, among others. The risk may be linked either to the presence of carriers or recently infected naive animals at risk.

2. The risk of animal products is related to the maximal amount of virus that can be encountered in that particular product. Fresh pork or fresh meat excluding offals from ruminants (carriers or non-carriers) derived from immune, vaccinated animals does not contain infectious virus, whereas fresh meat from recently infected (clinically or subclinically) animals may contain significant amounts of virus.

3. In ruminants, maturation to a controlled pH below 6, deboning and removal of visible lymphatic tissue greatly reduces any viral infectivity. In contrast, not all parts of pig carcasses reach pH below 6, hence thermal treatment is normally applied as a risk reducing step rather than maturation and deboning.

4. Thermal inactivation is widely used to reduce or eliminate microorganisms (bacteria, viruses) in animal products. The kinetics of inactivation can normally be describes as first-order, however, heterogenous populations of varying thermal resistance may exist. The effect of heating is always determined by a combination of time and temperature.

5. The decimal reduction time D and Z-value (heat resistance) of FMDV can be derived from kinetic studies of inactivation rates in relevant products. Such studies are until now lacking or at best incomplete.

Recommendations

1. Commodities (milk, fresh meat and meat products...) that constitute a risk for spreading FMDV should be identified by methods of qualitative risk assessment and a priority list should be established (risk profiling and risk ranking). 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.

2. 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.

3. Until estimates of D and Z have been established, a heat treatment corresponding to 70°C for 1h throughout the product can be used as an interim guideline.

Item 10 - ACTION PLAN 2003-2005

Progress reports on each of these items will be required at the Closed meeting of the Research group in 2004, and also where indicated below. Underlined person is designated as leader, alternate in italics.

- Assisted delivery for samples from third countries
  Action: EUFMD secretariat (report, each Executive Committee Session)

- Vaccine selection: invite comments from vaccine manufacturers and organise workshop (Jan-Feb 2004) for regional reference laboratories, etc.
  David Paton/ EUFMD secretariat

- Establish guidelines on post-vaccinal surveillance (by April 2004, ahead of OIE in May and Contingency Planning workshop, April 19-23rd 2004) (estimate likely within herd prevalence and definition of minimum requirements for NSP test performance). Interim steps: plan and costs (mid-October). First draft end of November/beginning December. Activities as required.
  François Moutou/Aldo Dekker/ Alf Füssel/Matthias Greiner/Andrés Gil

- Laboratory sero-diagnostic capacity – guidelines (by April 2004)
  EUFMD secretariat

- Phase XVIII WRL → report to RG session 2004 & plan for next phase
  c/o David Paton *(outline plan of John Anderson, WRL)*

- Comparative evaluation of candidate DIVA tests, 1) with sera from experimental infections, with Panaftosa 3ABC ELISA/EITB , deadline 3 months after receipt of kits, and 2) field use in regions with FMD outbreaks, deadline August 2004
  Franco de Simone (E Brocchi)/*Aldo Dekker*/Bernd Haas/ David Paton
  Nilay Unal /Hagai Yadin *(field use in vaccinates +/- clinical FMD; spring)*

- Proficiency panel for virus detection methods (VI, antigen ELISA, RT-PCR)
  → Step 1: limited number of NRLs → report to RG session 2004
  → Review/plan Step 2 = distribution to all NRLs → report to RG session 2005
  c/o David Paton (& staff) + Aldo Dekker/Bernd Haas/Chris Griot/Kris deClerq

- Global FMD surveillance map/models
  Plan: by end of December, 2003
  EUFMD/WRL/FAO/OIE Working group

- Evaluate pen-side tests and develop guidelines
  Plan: by end of December, 2003
  Nilay Unal/ *Hagai Yadin*/EUFMD secretariat *(pilot study on disease outbreak investigation)*

- Working group on biosecurity (serodiagnostic, by Cordoba, April 19-23rd 2004) & high security laboratories (by 11/2004)
  Per Have/José Sanchez-Vizcaíno/Alf Füssel/etc.

- Working group on development of a diagnostic reagent bank)(by Cordoba, April 2004)
  Bernd Haas/Alf Füssel/Kris de Clercq etc.

- Guidelines for sample transport (by Cordoba, April 2004)
  Vilmos Palfi/David Paton/Chris Griot

- WORKSHOP on contingency planning for NRLs (April 2004; Cordoba, Spain) with local organization by José Sanchez-Vizcaíno and attendance by all NRLs. Position papers must be prepared in advance by all working groups.

- Study to assess D-values and Z-values for heat treatment of milk and pork from FMD-infected animals.
  Per Have to draft outline of project (by Jan. 2004) with contribution from Hagai Yadin.

- In 2005 RG group to review vaccine antigens and gaps in sample submissions to reference laboratories (i.e. priority antigens and locations; two-year review).

Item 11 – Any other business

1. *Election of Chairman of the Research Gro*up

   The Secretary indicated that no members had come forward or been nominated for the position of Chairman. He called for nominations from the floor. Dr Haas nominated Dr De Clercq and there were no other nominations. The Session indicated its strong and unanimous support for the continued chairmanship of Dr De Clercq.

2. *Future procedure for the election of Research Group members and Chairperson*

   The Secretary presented a proposal for the election procedure. He indicated that the report of this session would be presented to the Executive Committee in October 2003 and that any new procedures, if adopted, would not come into effect until the General Session of 2005. He proposed that the elected membership would normally remain at 12 persons, and that the representative of the WRL would become an *ex officio* member and thereby invited to each Session. The procedure for election of members was proposed to be through nomination by the Executive Committee of the Commission, of 6 members, with 6 members to be elected on an individual basis. The group of 6 would be voted in as a group at the General Session. The other 6 members would be elected by the General Session and terms of membership would continue to be of 2 years. The proposed terms of membership were discussed and the number of terms that could be served on a continuous basis clarified. Individual members could serve a maximum of 2 terms of 2 years, but there would be no fixed maximum for members nominated by the Executive Committee. The Chairperson of the Group would be elected from any of the members elected by the General Session, and could serve a maximum of 3 terms of 2 years on a continuous basis. Membership of the Group was discussed and was proposed to be restricted to those with internationally recognized expertise of relevance to FMD epidemiology, surveillance and control, particularly in surveillance risk analysis, virus diagnosis, vaccinology and evaluation of control options, where their expertise in the FMD field is applied on a regular basis. The group considered that alternatives should also be considered as well as the above, such as the proposal that the group to be elected following the nomination of the Executive Committee be more than 6.

3. *Open Session 2004*

   The Secretary informed the Group of the offer of Greece to host the 2004 Session, after the option of holding the Session in Canada was no longer possible. The Session would be a very important forum to discuss the progress and results of the work programme agreed in this Session. As 2004 is an important year for the Commission, he considered that it would be appropriate to extend invitations to the Session to a wide geographical region concerned with FMD. The importance of the closed meeting to discuss the work programme was raised, and it was agreed that at least one day at the start of the Session, and a half day at the end, were necessary to achieve the required level of discussion and agreement. It was also agreed that parallel meetings should be avoided, but that pre or post-session workshops could be an effective use of the gathered expertise and opportunity. It was also recommended that the EUFMD

should take steps to ensure that member states should send more than one technical officer and that relevant scientific officers of FMD infected countries should be preferentially encouraged to attend.

4. *Media Officer and information exchange*

The Group discussed the state of information exchange between members and concluded that it would be of very considerable assistance if members were informed of press releases, opinions of members that are reported in the media, publications or reports that would be relevant to the developing policy on FMD diagnosis and control, and events of relevance including mission reports, and meetings planned. It was agreed that in the short term, Dr Griot could act as the focal point to receive and distribute such information, but that a longer term solution through the EUFMD Commission or another institution should be sought. The EUFMD Commission was urged to disseminate information on programme activities to the Research Group wherever possible. The Group was informed that the EUFMD website had been extensively improved in the last year and that it would be very important that some rules were necessary on the circulation of documents of a sensitive nature. For the longer term, it was agreed that research group position papers and answers to frequently asked questions could be a valuable addition to the website.

5. *Location of future meetings*

The gracious offer of Dr Haas to host the closed meeting in 2005 in Germany, following the move of the FMD research to the new location and laboratory to Insel Riems, was gratefully accepted by the Session.

Dr Hagai Yadin offered to host the 2006 open session in Israel. The Group acknowledged the kind offer, and discussed the level of work required to host such a meeting. The success of the previous open Session held in Israel was recalled and Dr Yadin was thanked for his enthusiasm to take on this important role once again.

Item 12 – Open papers on emerging issues / latest developments

Dr Bachmann presented a paper on simulation of FMD in Switzerland for contingency planning (Appendix 23). She described how the InterSpread Plus model was being adapted to the Swiss situation. The issues of spring-autumn animal movements in the Alps were discussed.

Item 13 – Presentation and adoption of the report

The draft report was discussed and changes adopted after discussion. It was agreed the Secretariat would propose introductory texts for several items and distribute for approval.

The meeting closed at 21.46 pm Friday.

Appendix 1

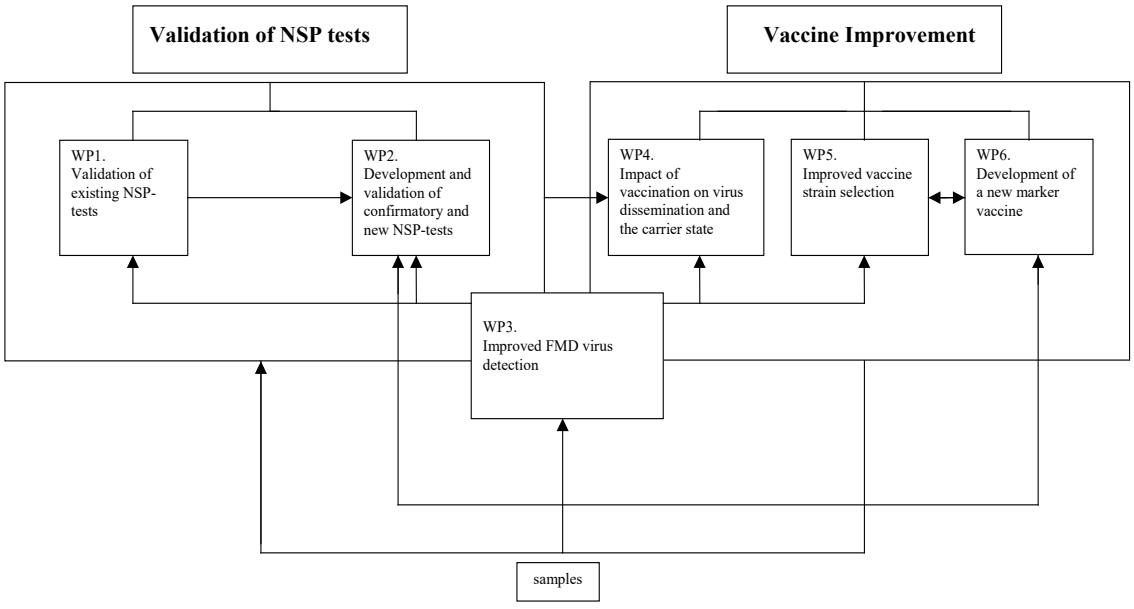
FMD research initiatives and future needs

Kris De Clercq[1] and David Paton[2]

1. Current initiatives:

1.1. Improvement of Foot and Mouth disease control by ethically acceptable methods based on scientifically validated assays and new knowledge on FMD vaccines, including the impact of vaccination. (FMD_ImproCon).

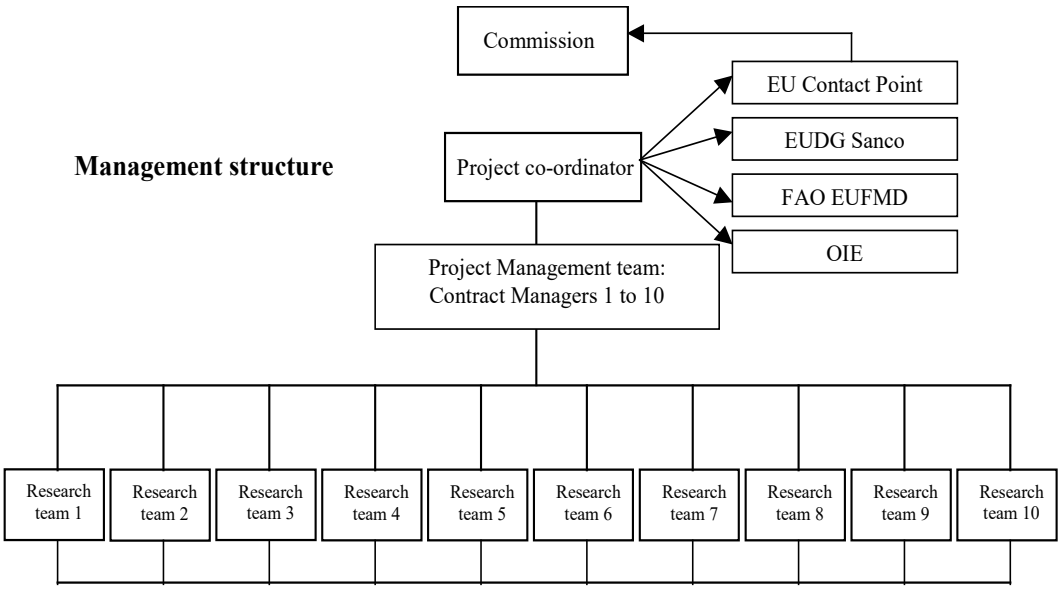
There is a strong desire to reduce reliance on large-scale culling of animals to control future outbreaks of FMD in EU Member States. Therefore, FMD research should address the specific gaps in our knowledge and technological ability with respect to the implementation of a vaccinate-to-live policy. As an alternative to the current FMD control, it is proposed to use emergency vaccination and then to screen for residual infection using tests for antibodies to the non-structural proteins (NSP) of FMD virus. The project is focused on the validation of NSP-based tests. The experimental design will also provide expected outputs in vaccine potency in relation to emergency use and vaccine strain selection.
Project: SIXTH FRAMEWORK PROGRAMME, PRIORITY [SSP/8.1], Policy-oriented Research Priority, STREP project in negotiation round with DG Research / Budget: 2.500.000 Euro, high chance to be granted end 2003.



Workpackages:

1. Validation of existing NSP-tests
 1.1 Preparing calibration tools
 1.2 Diagnostic performance with field samples
 1.3 Validation of diagnostic performance
2. Development and validation of confirmatory and new NSP-tests
 2.1 Peptide NSP-ELISA
 2.2. Biosensor NSP-ELISA
 2.3 Marker ELISA
 2.4 Validation
3. Improved FMD virus detection
 3.1 RCA-ELISA
 3.2 VI: Inadequate growth of the isolate
 3.3 Latent class analysis
4. Impact of vaccination on virus dissemination and the carrier state
 4.1 Transmission experiments with FMDV O UKG 2001
 4.2 Transmission experiments with FMDV O NET 2001
5. Improved vaccine strain selection
 5.1 Cross-protection challenge
 5.2 Determination r-value
 5.3 Antigen profiling
 5.4 Sequencing of vaccine and challenge strains
 5.5 Genetic and antigenetic typing by monitoring in an endemic situation
6. Development of a new marker vaccine
 6.1 Development and evaluation of FMD marker vaccines
 6.2 Mucosal immune responses and dendritic cell targeting of the vaccines.

Management structure



Participants:

1. CODA	Belgium
2. IAH	United Kingdom
3. CIDC-Lelystad	the Netherlands
4. DVI	Denmark
5. BFAV Riems	Germany
6. CISA-INIA	Spain
7. IZSLER	Italy
8. Sap	Turkey
9. AFSSA	France
10. IVI	Switzerland

1.2. Production, storage, control, distribution and replacement of standard sera for the laboratory detection of antibodies against structural and non-structural proteins of the foot-and-mouth disease virus.

The comparison of serological test results among laboratories used for the control of transboundary diseases or for trade purposes must be based on reference standards. The development of weak positive and strong positive reference standards against all serotypes is of the outmost importance.
In December 2002 the Commission adopted a proposal for a new Directive on Community control measures for FMD which reflects the modification made in the OIE Animal Health Code and shifts the emphasis of competing control measures by moving emergency vaccination from a last resort more to the forefront of the control measures.
In the case of vaccination the claim for absence of infection must be supported by testing according to certain guidelines for antibodies against non-structural proteins (NSP). Sufficient purity from such proteins of the vaccines reconstituted for emergency vaccination from antigens stored in the European antigen bank established in accordance with Decision 91/666/EEC is ensured by the manufacturer and as a result of vaccine tests carried out by the Commission.

The aim of the study is to produce, characterise, store, aliquot, distribute and monitor the quality of a panel of reference sera for all serotypes of FMD virus. The reference sera are to be used in the validation and quality control of serological tests for FMD. These tests include assays for the detection of antibodies against both structural and non-structural proteins of the FMD virus.
A secondary objective is to obtain and store as many other samples as may be useful for development and validation of diverse FMD tests.

1.2.1. Vaccination and challenge:
Vaccination and challenge in cattle for the following strains of FMD virus:

A Iran 96	SAT 1 South Africa
A Argentina 2001	SAT 2 Eritrea
O 1 Manisa	SAT 2 Zimbabwe
Asia 1 Shamir	SAT 3 Zimbabwe

On day 21 after vaccination, one vaccinated animal is to be killed and exsanguinated, whilst the three remaining cattle (two vaccinated and one unvaccinated) are to be challenged. On day 28 after challenge, the remaining three cattle are to be killed and exsanguinated.

Vaccination and challenge in pigs for the following strain of FMD virus:

O Taiwan 97 (or Philippine equivalent, pig adapted).

On day 28 after vaccination, three animals are to be killed and exsanguinated, whilst the other nine animals are to be separated into a vaccinated (six animals) and an unvaccinated (three animals) group and then challenged by heel-pad inoculation with 104 TCID50 of the homologous serotype of live FMD virus. On day 28 after challenge, these nine pigs are to be killed and exsanguinated.

1.2.2. Samples:

- Uncoagulated blood samples (weekly).
- Saliva (weekly).
- Probang and nasal swabs (weekly.
- Vesicular epithelium according to availability.
- Complete blood collection at slaughter: a minimum volume of two litres of serum.

1.2.3. Laboratory Tests :

There are four categories of laboratory testing:

1.2.3.1. Preliminary testing

1.2.3.2. Prequalification study of reference sera (bulk material):

At least three different laboratories.

VNT using homologous strain, LPB ELISA, SPcELISA, NSP ELISA.

1.2.3.3. Preparation of pilot batches for selection of candidate sera (pilot study)

Sera serially diluted (6 dilutions) in negative serum: prototype reference standards

Lyophilised: QA/QC.

At least six different laboratories: assays for structural and non-structural FMD antibodies. Collaboration: FAO EUFMD RG, FAO WRL, EU CRL and EC DG.SANCO.

If necessary, a second set of prototype sera should then be distributed and tested.

Statistical and scientific evaluation: compiled in a scientific report.

1.2.3.4. Preparation of candidate reference sera (final lot).

Preparation of reference sera:

- A strong positive serum. This serum should score strongly positive in the relevant test, but within the range of values normally evaluated.
- A weak positive serum. This serum should be a weak one, but should still score consistently positive in the relevant test.
- A cut-off serum. This serum should define the cut-off of the relevant test and as such should score positive at the desired threshold in 50% of cases.

Final verification: a collaborative study with at least 10 laboratories.

1.2.4. Official adoption of reference sera
The reference sera thus qualified needs to be given an official stamp through appropriate adoption by official relevant/representative institutions to ensure their reference status (at least Ph.Eur. Commission and OIE Standard Commission).

1.2.5. Storage of the reference sera: QA/QC
1.2.6. Monitoring of quality of reference sera: QC
1.2.7. Distribution of reference sera
1.2.8. Vigilance: the appropriate use of the standard sera has to be monitored
1.2.9. Property rights, advertising and labeling
1.2.10. Publication of results
1.2.11. Pricing: costs + replacement

Project: SANCO/2003/E2/009, *put forward for tender. To be published by EC.*
Budget: 800.000 Euro; EAGGF-B1 331 *budget*.

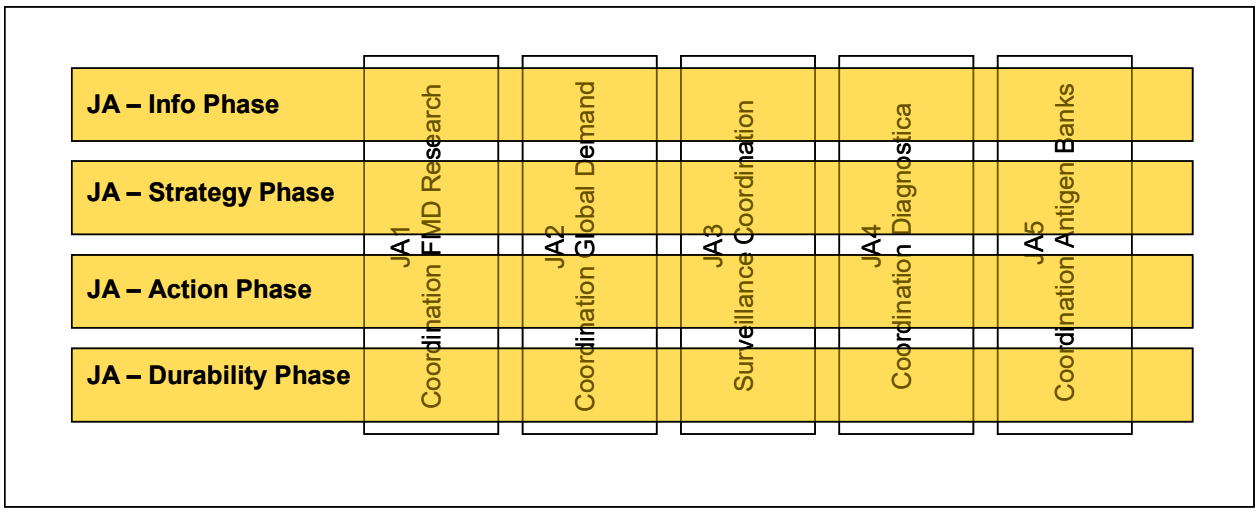
1.3. Responding to the global demand for FMD R&D through coordinated programme development of European reference laboratories and international organisations (CoordiNet-FMD)

Foot-and-mouth disease requires very high levels of coordinated action to prevent entry into free regions and to enable rapid control of the disease upon entry. An improved coordination of research, RTD priority setting and EU policy support tools is urgently needed.

Strategic objectives addressed
1) Increase the co-ordination of European research funding on FMD to address policy requirements at the European level.
2) Identify and address constraints to innovation and participation in FMD research programmes.
3) Develop priority setting processes to guide national and European level RTD identification and evaluation of potential RTD.
4) Develop a process of information sharing at a global level to rapidly identify RTD needs for RTD programme developers and evaluators, and to support European level rapid RTD response to be developed according to risk.
5) Develop strengthened networks in surveillance, diagnostics and vaccine development RTD, to stimulate joint activities and trans-national funding, and to address issues of the necessary European critical mass to provide world leadership.
6) Develop strengthened networks in surveillance, diagnostics and antigen/vaccine banks activities of reference laboratories and bank managers.
7) To characterise world demand for RTD products to 2020 and beyond and to develop processes at international level to stimulate uptake of RTD products for the global marketplace.

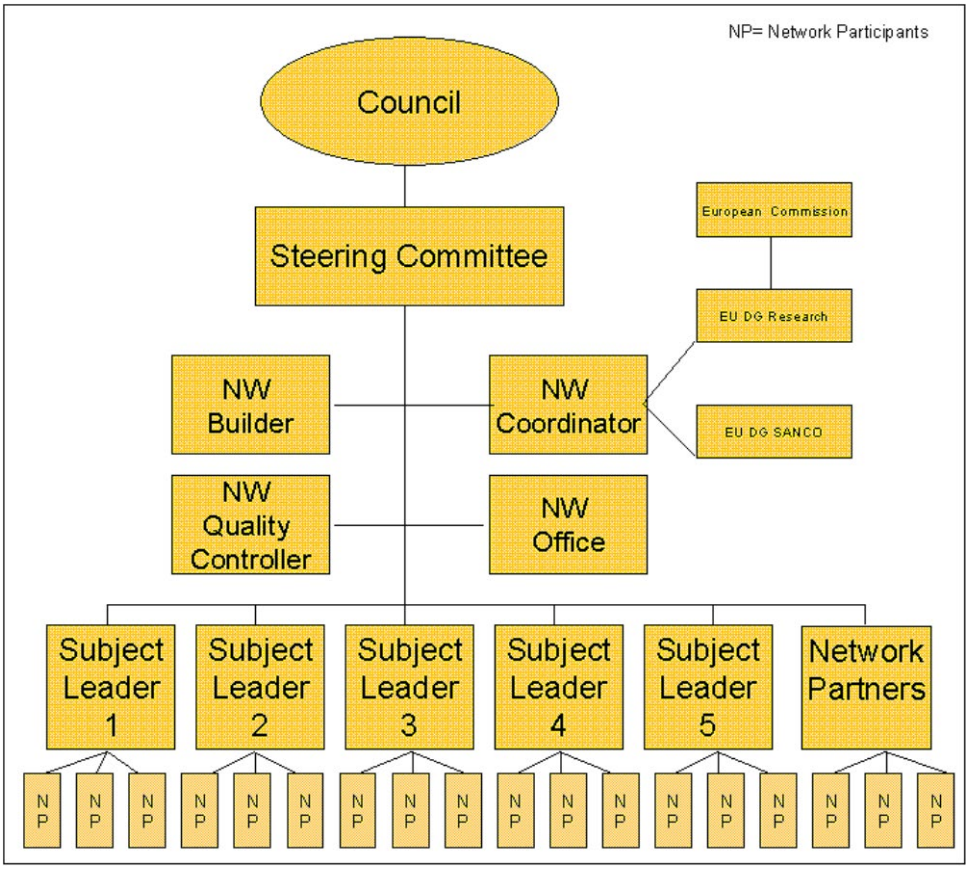
Therefore this project will work on networking trough coordination and collaboration in five major FMD areas:
(1) current FMD research efforts
(2) long term, global demand for FMD research and tools

(3) FMD surveillances
(4) FMD diagnostics
(5) FMD antigen/vaccine banks.



To ensure the achievement of the objectives this Network will be developed along the axis of a maturity model consisting of four levels: Mutual Awareness, Mutual Steering, Research Transformation, Research Convergence.

Management:



Paticipants:

1. CODA Belgium
2. IAH UK
3. FAO
4. Vlerick Belgium
5. IDL UK
6. NCFAD Canada
7. OIE

Project: ERA-NET project to be re-introduced in March 2004 / Budget: 3.000.000 Euro

1.4. Reinforcement of the European networks of community and national reference laboratories and other key research laboratories for infectious diseases included in list-A of the OIE.

Problem description

EU legislation on animal health deals basically with the compulsorily notifiable epizootic diseases (diseases included in the list-A of the OIE), major zoonoses and other diseases which are subject to national control or eradication programmes. Community and national reference laboratories ensure uniformity of testing and provide expert support to the Commission and the Member States. They ensure, through a network, liaison between the national reference laboratories and provide assistance to the Commission. They are key players in developing improved methods of disease control. Disease control measures need to be adapted to the latest scientific development. This would be facilitated by an increase and improvement of the co-ordination of research efforts.

Objective

The objective of the co-ordination action is to reinforce the networks with other relevant research institutions and universities and to provide a platform for discussion of strategies for research in animal health.

2. Further Research Needs:

2.1. Contingency Planning for FMD

A foremost lesson learnt from the FMD outbreaks in 2001 was the need for better contingency planning at all levels. A collaborative project between different European countries to evaluate and promulgate best practices in contingency planning would be highly beneficial.

2.1.1. Establishment of links between national expert groups

These groups are a requirement specified in the new EU FMD Directive.

2.1.2. Establishment of regional laboratory testing capabilities

Local testing centres can reduce test turnaround times and help ease capacity problems for National Reference Laboratories (NRLs). Development of simple procedures with reduced biocontainment needs: rapid small-scale RT-PCR and antigen and antibody detection kits.

2.1.3. Establishment of field-testing capabilities needed for full test validation

2.1.4. Establishment of robotic testing systems for rapid high throughput

Experience from attempts to introduce robotic testing in 2001 suggests that the serological tests need to be modified. New technologies enable all of the test reagents to be mixed simultaneously in so-called homogeneous one-step assays. ELISA methods for the detection of structural and non-structural FMD proteins should be adapted.

2.1.5. Establishment of diagnostic reagent reserves

Manufacture, purchase and manage stocks of key reagents and test kits for distribution to NRLs in an emergency situation.

2.1.6. Establishment of outbreak data control/network systems

New systems to be developed to streamline the flow of information coming from field teams, control centres and testing laboratories.This requires computerisation of all manner of inputs and outputs.

2.1.7. Thermal imaging.

Easy detecting method of high temperature at certain areas of the animal body such as the feet (early clinical sign of FMD).

Project: to be developed. Budget: 3.200.000 Euro.

2.2. Reference viruses, antigens and genomes:

The comparison of virological test results among laboratories used for the control of transboundary diseases or for trade purposes must be based on reference standards. The development of weak positive and strong positive virological reference standards against all serotypes is of the outmost importance.

Project: to be developed. Budget: 1.200.000 Euro.

2.3. FMD virus survival in animal products.

FMD virus survival in animal products especially in pig meat and in milk products from cattle, sheep and goats.

Project: to be developed. Budget: 1.400.000 Euro.

2.4. Transmission of virus trough direct and indirect contact.

Determine the minimum infectious doses for different species and different possible contact materials.

Project: to be developed. Budget: 1.800.000 Euro.

2.5. Vaccine evaluation.

Statistical reliable methods in line with the 3R rule (replacement, reduction, refining of animal experiments).

Project: to be developed. Budget: 2.100.000 Euro.

2.6. Developing an FMD marker vaccine with possibility to distinguish infected from vaccinated animals.

Project: long term project to be developed. Budget: 5.100.000 Euro.

References

Anonymous (2003). Diagnostic techniques and vaccines for foot-and-mouth disease, classical swine fever, avian influenza and some other important OIE List A diseases. Report of the Scientific Committee on Animal Health and Animal Welfare. Adopted 24-25th April 2003.

Appendix 2

Experience in Post-vaccinal Surveillance (PVS) following the 2001 FMD Epidemic in Uruguay

Andrés D. Gil; DV, MS, PhD.
Epidemiological Unit, Veterinary Services, Uruguay agil@mgap.gub.uy

Background

Uruguay is a South American country located in the 36th parallel, between Argentina and Brazil. Its territorial extent is 176,215 Km^2 and it has a human population of 3,145,200. The Uruguayan economy is greatly dependent on the livestock sector having 48,518 farms with 10,598,024 cattle (90% beef and 10% dairy) and 12,084,505 sheep (mostly for wool production).

The history of foot-and-mouth disease (FMD) in Uruguay began in 1870 when it was identified by Bertrand Duprat. In 1961, the Uruguayan Government began planning a control programme. Since 1968, it has been compulsory to vaccinate all cattle three times a year with a hydroxide-based FMD vaccine. As a result of a new eradication programme, an oil-based vaccine has been in use since 1991. As the last outbreak had been in 1990, OIE declared Uruguay free of FMD with vaccination in 1993. After that, Uruguay stopped vaccination of cattle in 1994, and was declared free without vaccination in 1996.

FMD came back to Uruguay in 2000 with an outbreak next to the Brazilian border. This outbreak was eradicated with a stamping-out policy. In 2001, Uruguay had another FMD outbreak near the Argentinian border. The strategy for control of this outbreak began with stamping-out and was later changed to vaccination of the entire cattle population. This last outbreak involved 2,057 farms and was brought under control within 120 days with two rounds of vaccination in that period. After the outbreak, Uruguay vaccinated all cattle at least once a year and young animals at least twice a year.

After the last outbreak the veterinary services started a surveillance programme in the cattle and sheep population. The main activities under national post-vaccination surveillance (PVS) were:

1. A cross-sectional study of both cattle and sheep (September 2001).
2. A study of the evolution of disease in the national cattle population. (February 2002).
3. A study of the evolution of disease in the national sheep population. (May 2002).
4. An evaluation of the national cattle population. (November 2002).

1. A cross-sectional study of cattle and sheep (September 2001)

This study was conducted two weeks after the last affected farm was identified to assess sheep and cattle involvement in the FMD epidemic.

The laboratory diagnostic tools were agar gel immunodiffusion (AGID) in sheep for virus-infection associated antigen (VIAA) and ELISA in cattle for the non-structural protein (NSP) 3B using the test-kit manufactured by United Biomedical Inc. (UBI). The AGID test was used in sheep, because this species is unvaccinated. The specificity of VIAA in unvaccinated sheep is 100% and the sensitivity 92%. The ELISA 3B test was used in cattle, as this was a way to discriminate between vaccinated and infected animals.

Assuming that the FMD prevalence is related to the level of exposure to FMD virus, different prevalences were expected based on the distance between sample farms and outbreak farms. Therefore, sample size was optimized by stratification based on distance from an infected focus. Using the National Farms database (DICOSE) in association with a Geographic Information System (GIS), three strata were defined based on minimum distance from an outbreak farm:

Stratum I included all farms within 5 Km from an outbreak farm.
Stratum II included all farms between 5 and 10 Kms from an outbreak farm.
Stratum III included all farms more than 10 Kms from an outbreak farm.

A total sample size of 210 farms was proposed with 70 farms in each stratum. Within-herd prevalence of NSP antibodies for cattle in seropositive farms was assumed to be 20%, 10%, and 5% for the three strata. Thus, to detect a positive animal with 95% confidence, the within-herd sample sizes for each stratum were 15, 30, and 60 cattle, respectively. The sample size for sheep (n=45) was calculated to detect a positive animal with 90% confidence when there was a within-flock seroprevalence of 5% or more.

Sera were collected from 6,859 cattle on 203 farms and 298 cattle were seropositive by ELISA 3B test. An overall prevalence of 9.3% ± 2.3% was estimated for the Uruguayan cattle population. The prevalences in each stratum were: stratum I 11.1% ± 2.9%, stratum II 2.8% ± 0.8%, and stratum III 2.1% ± 0.9%.

Sera were collected from 6,573 sheep on 114 farms and 63 sheep were VIAA-antibody positive. The National prevalence for VIAA-antibody positive sheep was estimated as 1.1% ± 0.5%. The prevalence in each stratum was estimated as: stratum I 1.7% ± 0.9%, stratum II 0.3% ± 0.1%, and stratum III 1.1% ± 0.9%.

Our data showed an association between distance and seroprevalence in cattle, but no such association was apparent in sheep, possibly because of the unequal size of the strata.

2. Evolution of the disease in the national Cattle population (February 2002)

In February 2002 another serological study was conducted in cattle. In addition to estimating the seroprevalence of NSP antibodies in the cattle population at this time, the aim was to compare this with the seroprevalence estimated in September 2001. The same laboratory test, ELISA 3B (UBI), was again used.

The study design was similar to the September 2001 study, except that on this occasion sampled herds were sub-stratified by production system (as beef or dairy herds) within each geographic strata. The proposed sample size was 230 farms and 8,500 sera. The distribution of the samples was 70 farms in each of stratum I and stratum II and 90 farms in stratum III with 20 dairy farms in each stratum.

The overall prevalence of antibodies to non-structural proteins was estimated as 2.3% ± 0.8% with a breakdown by stratum of: 2.8% (stratum I), 2.4% (stratum II), and 0.6% (stratum III).

Therefore this study demonstrated a large decrease in the seroprevalence of NSP antibodies in the cattle population.

3. Evolution of the disease in the national Sheep population (May 2002)

The aim of this study was to detect with 95% confidence, if 99% of the Uruguayan national sheep flock were free of FMD.

The laboratory screening test again was the AGID for VIAA.

Random sampling was performed in two steps. Firstly, to detect with 95% confidence at least one positive farm if 1% or more of farms have active virus circulation. Secondly, to detect with 95% confidence at least one VIAA-antibody positive animal if within-flock seroprevalence was 5% or more. Sampled flocks (farms) were drawn from 15 strata defined on the basis of flock size and minimum distance from an outbreak farm (Table 1) and 60 sheep (comprised of 30 lambs and 30 adults) were sampled from each flock.

Table 1. Number of flocks sampled, categorised by flock size and distance from nearest outbreak.

Flock size	<5 km	5 a 10 km	>10 km	T O T A L
<100	13	17	20	50
100-199	18	13	13	44
200-499	18	18	15	51
500-1000	21	21	24	66
>1000	44	42	43	129
T O T A L	**114**	**111**	**115**	**340**

18,296 sera were collected from 340 farms between May and August 2002. The estimated overall seroprevalence for VIAA-antibody in the sheep population was 0.16% ± 0,07% and the breakdown by stratum was: 0.23% ± 0.11% in stratum I, 0.08% ± 0.08% in stratum II, and 0.04% ± 0.04% in stratum III.

All lambs were seronegative to VIAA, because they were born after the FMD epidemic and there was no virus activity on these farms. Of the adult sheep tested, 20 were VIAA-antibody positive. These seropositive sheep were located on eight farms, four of which had been outbreak farms and the other four had been next-door to outbreak farms. The farms

with positive animals were monitored but were disregarded when no further evidence of circulating FMD virus was found.

The conclusion of this study was that 99% or more of the sheep flock were free of FMD. The VIAA-antibodies detected had persisted since the 2001 FMD epidemic.

4. Evaluation of National Cattle population (November 2002)

The aim of this study was to verify the cattle health situation of Uruguay with respect to FMD and to demonstrate with 95% confidence, that 99% or more of the farms do not have virus activity.

The ELISA 3B (UBI) was used as screening test, and the ELISA 3A (UBI) as a confirmatory test for FMD. In accordance with UBI claims for these tests and with this testing strategy we expected a sensitivity of 100% and a specificity of 99.3%.

The study was conducted between November 2002 and February 2003. The design featured random sampling stratified by production system (as either dairy or beef). From a proposed sample size of 396 farms, 377 farms were sampled comprising 118 dairy farms and 259 beef farms. In each farm 30 cows, 15 calves and 15 steers were randomly selected. This design aimed to detect a positive herd, with 95% confidence, if the prevalence of positive herds was more than 1% and if the within-herd seroprevalence in positive herds was 5% or more.

No farms were excluded from this study, such that 76 (20%) of the sampled farms had been FMD outbreak farms and 72 (19%) had been neighbouring an outbreak farm. 18,698 cattle were sampled (5,284 dairy cattle and 13,414 beef cattle). The sample breakdown by category of animal was: 11,038 cows, 4,427 calves, and 3,233 steers.

The sample coverage was close to 100%, because update of the national database is compulsory every year. The participation was also excellent; the difference between the proposed number of farms (396) and the actual number sampled (377) is explained by some farms not having cattle at the time they were visited.

The overall estimate of seroprevalence based on the ELISA 3B test was 1.98% ± 0.26% and the distribution by category was: 2.72% ± 0.41% for cows, 0.11% ± 0.05% for calves, and 2.14 ± 0.44% for steers. After confirmation by ELISA 3A test the overall prevalence estimate was revised to 0.65% ± 0.11% and the distribution by category was: 0.84% ± 0.17% for cows, 0.00% for calves, and 0.85 ± 0.37 for steers. Farms with positives animals were monitored over time and were disregarded when further clinical and serological studies showed no evidence of virus activity. The positive results could only be explained by lack of specificity of the test and it was concluded that the cattle population in Uruguay were free of FMD.

A Serosurveillance to Determine the Prevalance of Antibodies against Structural (SP) and Non-structural Proteins (NSP) of FMDV Following the Spring-2003 FMD Vaccination Campaign in Turkey

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Introduction

- **For the spring 2003 vaccination campaign, two different serosurveys were conducted in Turkey:**

  - ***1st: Anatolian serosurveillance***

  - **The trivalent FMD vaccine (O1 Manisa, A96 Iran and Asia1) produced by Sap Institute was used in the Anatolian region of Turkey.**

  - **A serosurvey was carried out after this vaccination to assess vaccination policy and monitor situation of general antibody surveillance in Anatolian part of Turkey.**

- *2nd: Turkish Thrace Serosurveillance*
- **The trivalent FMD vaccine (O1 Manisa, A96 Iran and Asia1) donated by the EU was used in Turkish Thrace including the Anatolian part of Istanbul and Çanakkale provinces**
- **After this campaign a serosurvey was performed in Thrace**
- **-to assess immunity level and**
- **monitor antibody to non-structural proteins (NSP).**

This presentation indicates these two surveillance results

1. ANATOLIAN SURVEY

Materials And Methods

1. Test Sera

•A total of 200 villages and 24 large ruminants from each village were selected and sera were collected at 30 days post vaccination (dpv),

•Sera were collected from three different age groups:

- 04-12 months; once vaccinated or without vaccinated animals
- 12-24 months; once or second time vaccinated animals
-- >24 months: more than twice vaccinated animals group

2. *Virus strains*

•FMD inactivated virus types O_1 Manisa, A_{96} Iran and Asia-1 were used as control virus in this study.

3. *Liquid-phase blocking ELISA*

•The liquid-phase blocking ELISA was carried out as described by Hamblin et al. (1986a).

Results

- To evaluate the post-vaccination antibody levels,
- a total of 4765 sera from cattle were tested by the LPB ELISA at a single dilution of 1:100, which was accepted as the protective level.

FMD Serotypes	Positive	**%**	Negative	**%**
O	3701	**78**	1064	**22**
A	3390	**71**	1375	**29**
ASIA-1	3541	**74**	1224	**26**

Table 1: Cumulative results of the sera collected from the cattle in Anatolian part at 30 days postvaccination

	4765					
	0-1 (1603)		**1-2 (1516)**		**> 2(1646)**	
FMD Types	+	**%**	+	**%**	+	**%**
O	1095	**68**	1191	**78**	1415	**86**
A	996	**62**	1097	**72**	1297	**79**
Asia-1	1066	**66**	1144	**75**	1331	**81**

Table 2: LPB-ELISA results of the sera collected from cattle in Anatolian part, 30 days postvaccination: Distribution by ages (+)=positive, %= percentage of positivity

Analysing data from epidemiological units individually

FMD TYPES	O	A	ASIA-1
COUNT	199	199	199
AVARAGE	19	17	18
MAX	24	24	24
MIN	0	0	0
STD DEV.	5	5	6
COVARIATION	28	30	33

Table.3. Data generated from analysis of results for each serotype in terms of number of positivity and negativity in epidemiological units.

Discussion

- Protective antibody levels of sera collected at day 30 against types O, A and Asia-1 were 78%, 71% and 74% respectively.
- These results showed that the protection rates were quite sufficient during the first month of vaccination.
- Although the protection rates were acceptable, significant variations were observed between individual units (Table.3).
- When the least protected units were removed (16 units), the protection rates were much better.

- The reason for this variaton:
- The animals in some units were vaccinated for the first time for this serosurveillance,
- Vaccination failures,
- Sampling errors.

2. TURKISH THRACE SEROSURVEILLANCE

- ***Materials And Methods***

1. ***Test Sera***

1.a.

- A total of 200 villages and 24 large ruminants from each village were selected and sera were collected at 60 days post vaccination (dpv).
  - Sera were collected from three different age groups like Anatolian survey.

1.b.

- *Same amount sera were used to test the antibody against NSP of FMD virus*

Additionally for this:

- To evaluate the protective level of the vaccine in the field experimentally, 60 seronegative cattle were vaccinated and were bled sequentially at days 28 and 120 and were tested by LPB-ELISA.

2. Virus strains

FMD inactivated virus types O_1 Manisa, A_{96} Iran and Asia-1 were used as control virus in this study.

3. *Liquid-phase blocking ELISA*

The liquid-phase blocking ELISA was carried out as described by Hamblin et al. (1986a).

Results

- To determine the post-vaccination antibody levels
- A total of 4768 sera from cattle were tested by the LPB ELISA at a single dilution of 1:100.

	Positive	%	Negative	%
O	2452	**51**	2316	**49**
A	2595	**54**	2173	**46**
ASIA-1	2765	**58**	2003	**42**

Table 1: Cumulative results of the sera collected from the animals at 60 days postvaccination

	Large Ruminants (2400)				Small Ruminants (2368)			
FMD Serotypes	**Positive**	**%**	**Negative**	**%**	**Positive**	**%**	**Negative**	**%**
O	1389	**58**	1011	**42**	1063	**45**	1305	**55**
A	1495	**62**	905	**38**	1100	**46**	1268	**54**
ASIA-1	1577	**66**	823	**34**	1188	**51**	1180	**49**

Table 2: LPB-ELISA results of the sera collected from the animals at 60 days postvaccination: Distribution of results by animal species

	04-12*(1547)		12-24*(1609)		>24*(1605)	
	Positive	**%**	**Positive**	**%**	**Positive**	**%**
O	517	**33**	855	**53**	1076	**67**
A	571	**36**	906	**56**	1113	**69**
ASIA-1	563	**36**	986	**61**	1211	**75**

Table 3: Distribution by ages.(*)=month (+)=positive, %= percentage of positivity

	Large Ruminants (2400)						Small Ruminants (2368)					
	04-12*(791)		**12-24*(800)**		**>24*(805)**		**04-12*(756)**		**12-24*(809)**		**>24*(800)**	
FMD Serotypes	Positive	**%**	Positive	**%**	Positive	**%**	Positive	**%**	Positive	**%**	Positive	**%**
O	290	**36**	504	**63**	593	**73**	227	**30**	351	**43**	483	**60**
A	331	**41**	559	**69**	603	**74**	240	**31**	347	**42**	510	**63**
ASIA-1	315	**39**	582	**72**	678	**84**	248	**32**	404	**49**	533	**66**

Table 4: Distribution by animal species and animal ages. (*)= month, (+)=positive, %= percentage of positivity

Table.5 LPBE results of experimentally vaccinated cattle at 28 and 120 days

FMD TYPES	28 DAYS (60 SERA)	%	120 DAYS (44 SERA)	%
O	57*	**95**	5	**10**
A	58	**97**	10	**23**
ASIA-1	57	**95**	7	**14**

(*)= number of positive sera, %= percentage of positive sera

Table.6. Analysing data from epidemiological units individually

	CATTLE			SMALL RUMİNANTS		
FMD TYPES	**O**	**A**	**ASIA**	**O**	**A**	**ASIA**
COUNT	**100**	**100**	**100**	**100**	**100**	**100**
AVARAGE	**14**	**15**	**16**	**11**	**11**	**12**
MAX	**24**	**24**	**23**	**23**	**24**	**23**
MIN	**2**	**6**	**6**	**1**	**1**	**0**
STD DEV.	**4**	**4**	**4**	**5**	**5**	**5**
COVARIATION	**32**	**28**	**24**	**47**	**48**	**45**

Discussion

- A total of 4768 cattle and small ruminants were bled at day 60 of vaccination and 51%, 54% and 58% protection rate (as a cumulative) were determined for types O, A and Asia-1 respectively.

- Although the results of cattle were higher compared to those of sheep, better protection rates should have been obtained following 2 months post-vaccination.

- These differences between the protection rates of cattle and sheep can be explained by the vaccination scheme (twice annually for cattle, once a year for sheep).

- Other significant differences were detected at distribution of ages.
- As it can be seen in tables 3 and 4,
  - protection rate of cattle >2 years of age was 73%, 78% and 84% for type O,A and Asia-1 respectively.
  - However protection rates of young animals and also small ruminants were much lower compared to those of regularly vaccinated older animals.

- These results cannot be explained only by animal species and age differences.
- This decrease was also determined in experimentally vaccinated animals (table.5.).

- When individual units were examined, great variation was observed (table 6).

- As a conclusion, unexpectedly low immunity levels were observed at 60 days postvaccination.

2.b. 3 ABC ELISA RESULTS

Sera, which were collected for Thrace serosurvey, were examined to trace for antibody to FMD non-structural proteins by Bommeli Checkit 3 ABC ELISA kits.

	Number of sera tested	Positive	%
Cattle	**2400**	**45**	**1.87**
Small Rumin.	**2368**	**17**	**0.7**
Total	**4768**	**62**	**1.30**

•The percentage of NSP positive animals was 1.3 % in total, 1,87 % for cattle and 0,7 % for small ruminants.

•Majority of the positive animals were from Istanbul and Çanakkale provinces. Only few animals were found as positive in other provinces.

•The results were comparable with the results of previous NSP surveys conducted in this region, although number of sera were much higher in this survey (4768 in this survey, 1310 previous survey)

•All animals, which were detected as positive, were over two years old.

•No FMD outbreaks were detected for more than two years in this region.

•These results showed that the possibility of active virus circulation in Thrace has been very low.

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•Vaccine, which was used in Thrace vaccination campaign, was donated by EU.

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Appendix 4

Demonstrating freedom from disease using multiple complex data sources
A proposed standardised methodology and case study

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Evan Sergeant[5] and other members of the International EpiLab research team
on Disease Freedom

Scientific Summary

The Agreement on Sanitary and Phytosanitary Measures (SPS agreement) of the World Trade Organisation requires that, in international trade, measures taken to protect animal, plant or human health should be based on scientific principles and not maintained in the absence of sufficient evidence. Countries support such measures by using science-based risk analysis, which in turn demands science-based assessment of the disease status (free or infected) of each of the trading partners. Traditionally, national disease status has been determined using structured cross-sectional surveys, which are generally difficult and expensive to implement. On-going surveillance may also be assessed by expert panels, but there are no accepted methods for quantifying either confidence in the surveillance process, or the probability of national disease freedom demonstrated thereby. This report presents a proposed framework and detailed methods for quantitative assessment of complex surveillance data from multiple sources, and an illustrative case study using evidence from three surveillance systems to demonstrate Denmark's freedom from classical swine fever.

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Note about the case study

This EpiLab project focussed on classical swine fever. Nevertheless, the authors believe that the methodology is not specific to the disease. The materials shown will demonstrate an approach, which -- after necessary modifications -- as also suitable for the surveillance of FMD.

Framework for analysis

The scenario tree is proposed as the modelling format for analysis of surveillance systems under a null hypothesis of the country being infected at a level equal to or greater than specified design prevalences. A scenario tree is developed to represent all known significant factors influencing the probability that a unit in an infected population will be detected as infected. The conditional probabilities associated with each limb of the tree are then multiplied together to give the overall probability of each limb's outcome, and these are summed for all branches with positive outcomes to give the probability that the whole surveillance process will have a positive outcome for a randomly chosen population unit, given that infection is present in the country (the system unit sensitivity).

Independence and clustering models are described for analysis. Under the independence model, overall system sensitivity of detection is derived directly from system unit sensitivity, as the probability that one or more of the independent units processed would have positive surveillance outcomes, given an infected country. Under the clustering model, animals (and disease) are assumed to cluster in groups, and surveillance system sensitivity is calculated taking this into account, by stepwise aggregation of sensitivity at each grouping level in the tree.

Surveillance processes give either complete or incomplete coverage of the population, and the sensitivity of a process with incomplete coverage must be adjusted for its representativeness of the population. This is achieved through calculation and use of a sensitivity ratio for the process; the ratio of its sensitivity to that of a truly representative surveillance process.

The surveillance process's sensitivity, P(³1 positive unit | country infected), is the confidence level for the statistical test of the null hypothesis. If one has a prior estimate of P(country is free of disease), one can then use Bayesian inference to calculate a posterior estimate of this probability, given the negative surveillance results.
Where multiple surveillance systems are available, the results of the analysis of each (whether they be survey-based or the result of scenario tree analysis) may be combined to produce an overall estimate of the confidence of the combined surveillance system.

While this research has developed the framework for a practical methodology to analyse complex surveillance data sources, it has also identified a number of areas of further research which would enhance the methodology. These include 1) standardised, transparent and acceptable methods for eliciting expert opinion, 2) methods to adjust the value of information based on the time of collection, and 3) methods to account for the lack of independence between surveillance systems when calculating the combined confidence that surveillance systems provide.

Case study

The methodology described above was used to analyse three different surveillance systems that provide evidence of Danish freedom from classical swine fever. The surveillance systems examined were:

1) A structured CSF sero-surveillance system, based on the collection of blood samples at abattoirs. Sampling was targeted at adult animals, with differential sampling pressures for boars compared to sows, and for South Jutland compared to the rest of the country;
2) Abattoir inspections (ante-mortem and post-mortem) routinely carried out at all abattoirs, primarily for food safety purposes; and
3) Clinical surveillance based on farmer observation, and routine visits by veterinarians to farms.

Each surveillance system was modelled using separate scenario trees, and estimates of the system confidence generated using stochastic modelling. Data sources used in the analysis included the Central Husbandry Register database, results of serological analysis of blood samples, abattoir slaughter records, and the VetStat drug prescription database (used as a proxy for veterinary visits). A number of parameters in each model were provided either by an expert informant, or through educated guesses.

Analyses were performed using a number of different design prevalence combinations, to examine the impact of the assumptions under the null hypothesis. In addition, for each surveillance system, a parallel analysis was conducted based on a hypothetical fully representative system using the same surveillance approach. For instance, in the case of sero-surveillance, this involved conceptually sampling from the farm population (rather than targeted sampling from the abattoir population). For meat inspections, it was based on the theoretical examination of animals selected from the farm population.

The results of analysis indicated that (not surprisingly) the estimated system sensitivity (or equivalently, confidence in the surveillance system) was very sensitive to the design prevalence assumptions under the null hypothesis. When reduced to a common period of one month's worth of surveillance, and based on those values used in the study, the sensitivity of the sero-surveillance system was estimated as 26.37% with a 5th to 95th percentile range of 23.44% to 27.87%. The sensitivity for the meat inspection system was 67.80% (39.46% to 90.34%) and for the clinical surveillance system was 93.80% (90.77% to 96.43%).

The sensitivity ratio is the ratio of the sensitivity of the actual system, to the sensitivity of a theoretical fully representative system. It indicates the effect of targeting the system, and indicates if a system is more or less effective than random selection. The sensitivity ratio for the sero-surveillance system was 3.73, for the meat inspection system was 0.998 and for the clinical surveillance system was 0.991. This indicates that the sero-surveillance system was very well targeted and much more efficient that simple population sampling. The other two systems were essentially equivalent to representative population sampling.

The combined sensitivity of the three surveillance systems was calculated providing a monthly confidence of 98.53%. If surveillance data over the period of one year were considered, the confidence would increase to essentially 100% (1 – (1´10-22)).

The strength of evidence for freedom from CSF is undeniable, and sensitivity analysis shows that even if the confidence in one or more systems is greatly overestimated, the annual confidence in the combined surveillance system well exceeds international requirements. Nevertheless, it is recommended that further research be undertaken in this area, including the use of more formal methods to generate estimates from expert opinion, and the application of a proposed methodology to account for the lack of independence between surveillance systems.

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Appendix 5

Detection of FMD Infection in Vaccinated Animals

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The purpose of this paper is to briefly review the state of development of tests for the detection of FMD infection in vaccinated animals and the guidelines that are available for their validation and ultimate use. A brief update will be given on studies to develop and evaluate such tests at IAH-Pirbright, in connection to a recent vaccine-challenge experiment.

The most direct method to detect FMD infection in vaccinated animals is to look for the FMD virus. However, the acute phase of virus replication may be transient and in the absence of clinical signs, the chance of detecting this is small. The proportion of vaccinated ruminants that go on to become persistently infected is variable as is the duration of virus carriage. Persistently infected animals (sometimes referred to as "carriers") can be identified by virus isolation or RT-PCR tests on oropharyngeal fluid (probang) samples, but the method has a low diagnostic sensitivity (Haas and Sorensen, 2002). Studies in South America found that virus could only be detected intermittently from serial oropharyngeal fluid samples (47% positive) collected from 78 persistently infected animals that scored consistently seropositive in tests for antibody to non-structural proteins (NSPs) (Bergmann, personnel communication). Another serious limitation of oropharyngeal fluid testing and subsequent virological tests is the difficulty of carrying out both procedures rapidly on a large scale. Tests for virus on oropharyngeal fluids can be carried out to try to confirm the status of animals identified as potentially persistently infected by serological examinations. However, only a positive result adds much weight to the diagnosis because of the high likelihood of achieving a false negative result. Furthermore, if serological tests are required to be used on a herd-basis, then herds testing positive should have all of their animals tested by the confirmatory method, and not just the individual seropositive animals.

There has been considerable progress in the establishment of commercially available serological tests for antibodies to NSPs (sometimes referred to as FMD marker tests). The OIE Diagnostic Manual recommends the use of the South American 3ABC "PANAFTOSA" ELISA and confirmatory western blot technique (enzyme-linked immuno-electrotransfer blot or EITB) (OIE, 2000; Bergmann et al., 2000). This system has been used on by far the largest number of samples, albeit mostly from cattle and is the approach that the OIE's ad hoc working group have recently recommended to be considered as the reference (or index) method to which others should be compared (OIE 2002). These tests are considered to have been validated in cattle and to have a high diagnostic sensitivity and specificity (OIE, 2002). The validation data presented to the OIE ad hoc working group included a very large number of results from serial samples collected from persistently infected cattle. However, these included field sera that had

been pre-selected on the basis of reactivity in the test under evaluation or came from experimental animals that had been infected without prior vaccination. There are questions over the availability of the test to laboratories in Europe and over the ease of interpretation of the western blot technique. Preliminary studies carried out by European laboratories have not confirmed the high specificity of the 3ABC "PANAFTOSA" ELISA test reported in South America (Dekker, Haas and De Clercq, personal communication).

There are two commercially available tests for antibodies to FMD NSPs (Fig 1). One is the FMD-CHEKIT-ELISA from Bommeli (Schalch et al, 2002) and the other is the UBI FMDV NSP ELISA (Liu et al., 2002). Both of these are indirect ELISAs requiring different conjugates (and therefore different kits) for the examination of sera from ruminants and pigs. Both tests are easy and quick to perform, although both involve a pre-dilution step in the preparation of the test serum, which significantly adds to the work involved when testing very large sample numbers. Whereas the Bommeli test employs a recombinant 3ABC antigen directly coated to the ELISA plate, the UBI test employs a 3B peptide. UBI can also supply two additional peptide-based tests which can be used to confirm the result obtained with the 3B indirect ELISA. One is a blocking test, in which sera reacting in the 3B test are incubated with free 3B peptide. The other is an indirect ELISA with a 3A peptide coated the wells of the ELISA plates. Another test that is expected to become commercially available is the Ceditest FMDV-NS from Cedi Diagnostics. This is a blocking ELISA based on the method of Sorensen (Fig 1; Chung et al., 2002). Data pooled from different European laboratories on testing a rather small number of 15-75 persistently infected cattle that had been experimentally inoculated with FMDV after vaccination suggested that the sensitivity of detecting such animals was approximately 70%, 80% and 90%, using the UBI, Bommeli and Sorensen methods respectively. It should be emphasised that not all sera were tested with all of the assays and the data on the Sorensen test relates to the original method and not the newly produced test kits from Cedi Diagnostics.

Other NSP tests under development include additional 3ABC ELISAs, other peptide-based ELISAs using 3B (Grunmach et al., 2000) or 2B (Inoue, personal communication) and 3D-based ELISAs. Alternative methods to detect FMD infection, post-vaccination, include the detection of specific IgA in oral secretions and the use of multiple antigens presented in a different format to that used in the western blot, for example some form of micro-array. To date, rather variable results have been obtained using different methods of IgA analysis, but only small numbers of samples have been examined. It would also be worthwhile to evaluate cytokine and cell mediated responses as a basis for specifically detecting the immune response due to infection. Pen-side versions of tests for the detection of infection-specific responses would also be very useful and various methods are under development.

Despite considerable interest in better FMD marker tests, the numbers of recent publications on the subject are few. Those involved in the development and evaluation of tests for the detection of FMD infection in vaccinated animals are frequently asked why validation of new tests is rather slow. Three contributory factors are (1) the requirement

for a very high degree of certainty in the information on test performance, (2) the complexity of the different scenarios for test use that have to be evaluated and (3) the lack of sera from known vaccinated and infected animals.

In May 2002, the OIE approved a new set of guidelines that expands on the chapter in the OIE Diagnostic Manual and the paper of Jacobson (1998). This defines a validated assay as a method that "consistently provides test results that identify animals as positive or negative for a particular analyte or reaction which, by inference, accurately predicts the infection status of the animal with a predetermined degree of statistical certainty". The stages of assay development and validation are described in some detail and examination of these reveals a number of critical issues for NSP validation. Firstly, there is a requirement to know the purpose of testing and the expected prevalence of the infection to be detected. This is needed in order to calculate the sensitivity and specificity requirements for any tests. However, for FMD NSP tests, there is a lack of information on the expected within-herd prevalence of persistently infected animals in post-vaccination populations. Consequently, requirements for sensitivity and specificity have not been accurately determined. Other problematic issues are the lack of reference standard sera, the lack of a readily available index method and the difficulty of fulfilling the requirement to test sera obtained from at least 300 individual persistently infected animals. The International Atomic Energy Association (IAEA) has also been involved in establishing requirements for the validation of NSP tests (Crowther, 2001) and held a workshop to review progress on this subject in March 2002.

Some guidance on the usage of NSP tests is available in the OIE Terrestrial Animal Health Code (2003) (http://www.oie.int/eng/normes/MCode/A_00157.htm) and in the new EU FMD Directive (Anonymous, 2003). The OIE code establishes the criteria for returning to a FMD-free status, of which two of the available options require NSP serosurveillance, i.e. (1) Recovery of FMD-free status without vaccination, after at least 6 months, by stamping out, emergency vaccination and post-vaccinal serosurveillance. (2) Recovery of FMD-free status with vaccination, after at least 18 months, by vaccination plus post-vaccinal NSP serosurveillance. Further guidance is to be found in the OIE Preliminary Guidelines for the establishment or the regaining of recognition for a FMD-free country or region (with or without vaccination). However, it is not always clear which guidelines are applicable to which control and surveillance scenarios. The EU Directive sets out three phases to vaccination: (1) from the start of vaccination until 30 days after the last vaccination. (2) From 30 days after vaccination until the completion of the serosurveillance. (3) From the completion of serosurveillance until the restoration of the FMD-free status. However, the within herd sampling criteria appear ambiguous with alternative options to sample at a level to detect 5% of infection with 95% confidence or to sample all vaccinated animals and their unvaccinated offspring in all vaccinated herds (in all vaccinated herds).

Provisional results are available from a recently conducted comparison, carried out at IAH-Pirbright, on tests for detection of infection in vaccinated cattle. The study involved samples obtained from 20 "O" Manisa vaccinated and 5 unvaccinated cattle. These were challenged at 3 weeks post vaccination, by 5 day contact with 5 "O" UKG 2001 needle-

challenged donor cattle. Unvaccinated and donor cattle were removed after 5 days. All challenged cattle were kept for 28 days and 15 challenged cattle were kept for approximately 6 months. A summary of the results is presented in Table 1. All of the unvaccinated cattle developed FMD. Although none of the vaccinated cattle showed any clinical signs of FMD, nor any FMDV viraemia, they could be divided into three categories on the basis of virological tests carried out on post-challenge oropharyngeal fluid samples. One group of 5 animals appeared not to have been infected (Group 1). A second group of 6 animals showed a transient infection persisting up to 12 days post challenge (Group 2). A third group of 9 animals became persistently infected with FMDV (Group 3). Blood samples collected from all of these animals for up to 91 days post challenge were tested by the Ceditest FMDV-NS ELISA, the UBI FMDV 3B peptide NSP ELISA and the Bommeli CHEKIT-FMD-3ABC ELISA. A fourth serological NSP test used was an indirect ELISA with a 2B peptide (Inoue, personal communication). Finally, saliva samples collected at the same time points as the blood sampling were analysed by ELISA for FMDV structural protein-specific IgA antibody.

Most of the assays were effective at detecting infection in the unvaccinated cattle. One animal was largely missed by the UBI test whilst salivary IgA levels waned to undetectable levels in some of these cattle within 3 weeks of infection. Most of the samples collected from the cattle in Group 1 scored negative in most of the tests. No reactivity was seen at all with the UBI and Cedi Diagnostics tests, whilst the most reactivity was seen with the IgA saliva test. Only one animal in Group 2 was kept beyond 28 daps post challenge. None of the samples collected from this group were positive in the UBI or 2B tests, whereas some samples were positive in the Bommeli and Cedi Diagnostics tests. Many samples were positive by the IgA saliva test with a peak of reactivity at 16 days post infection and a subsequent decline thereafter. All 9 animals in Group 3 (the important persistently infected group) were monitored beyond 91 days post challenge. The order of sensitivity of detection of infection in these animals was:

1. IgA Saliva (beyond 21 days post challenge, detected 8/9 animals most of the time).
2. Cedi Diagnostics test (beyond 28 days post challenge, detected 7/9 animals)
3. 2B Peptide ELISA (maximal detection of 6/9 animals at 42-63 days post challenge)
4. UBI ELISA (maximal detection of 6/9 animals at 63 and 70 days post challenge)
5. Bommeli ELISA (maximal detection of 3/9 animals).

Different tests missed different animals and no one test detected all of them.

It is impossible to recreate all possible scenarios of FMD exposure experimentally. These results demonstrate that there is considerable variation between vaccinated cattle in their responses to a high level of contact challenge. Such differences are likely also to be seen under field conditions. Further experiments to mimic field exposure are also needed in sheep and pigs. The findings confirm that 100% detection of persistently infected animals is difficult to achieve and that there are considerable differences in the performance of different assays. The IgA test and the 2B peptide test both merit further development.

Acknowledgement

Prototype Ceditest FMDV-NS kits were supplied by Cedi Diagnostics. Dr Toru Inoue provided the 2B peptide. Many colleagues at Pirbright Laboratory participated in the planning and/or execution of the animal and laboratory experiments reported here.

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Fig 1. Schematic comparison of three FMD NSP ELISA kit methods, with key differences between tests underlined.

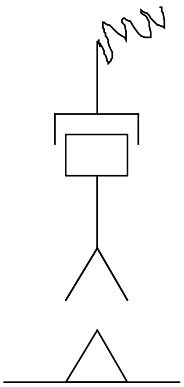
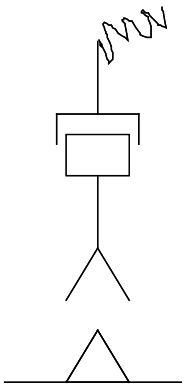
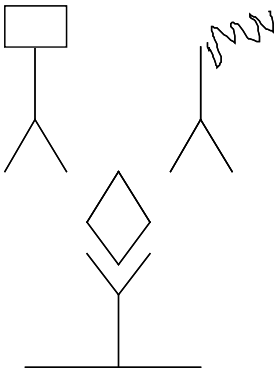
Bommeli	UBI	Cedi Diagnostics
CHEKIT-FMD-3ABC	FMDV NSP ELISA	Ceditest FMDV-NS
		
Indirect ELISA	Indirect ELISA	Blocking ELISA
Species specific kits	Species specific kits	One kit for all species
Serum predilution step	Serum predilution step	No serum predilution step
Takes 3 hrs	Takes 2.5 hrs	Takes overnight or 4 hrs
+ve / -ve control serum	+ve / -ve control serum	+ve / -ve control serum
Result rel. to +ve control	Result rel. to +ve control	Result rel. to +ve control
Ambiguous range	No ambiguous range	No ambiguous range

Table 1. Summary results for vaccine challenge study in which vaccinated or unvaccinated cattle were challenged by contact with FMDV inoculated donor cattle

- All 5 unvaccinated controls showed severe clinical disease (more severe than after needle challenge)
  - Virus was isolated from oropharyngeal fluid samples and blood
  - 2 possible persistently infected animals (positive by RT-PCR only at 28 or 42 dpc)
  - 3 animals retained for several months post challenge
  - Consistent responses in most tests for infection

- All 20 vaccinates clinically protected
  - No viraemias detected
  - 5 animals: no oropharyngeal virus isolated post challenge
  - 6 animals: probang virus isolated sporadically up to 12 dpc
  - 9 animals became persistently infected
  - 12 animals retained for several months post challenge
  - Considerable variation in responses to different tests for infection

Appendix 6

Comparison of commercial ELISAs for antibodies against FMDV non-structural proteins

A. Dekker, P. Moonen, E.M. v.d. Linde

Introduction

The 2001 foot-and-mouth disease (FMD) outbreaks in Europe have made clear that using emergency vaccination can shorten the duration of the outbreak. Until recently in the OIE code there was a big gap between the time needed to regain the status free of FMD without vaccination when using only stamping out or stamping out supported by emergency vaccination. For exporting countries this was a negative incentive for using emergency vaccination in case of an outbreak, unless culling all vaccinated animals was considered an option. The availability of tests that can differentiate between vaccinated and infected animals (DIVA principle) made it possible to reconsider the OIE code. In the current code therefore the use of DIVA tests has been implemented and the gap in time needed to regain the status of free of FMD without vaccination between using only stamping out, or stamping out supported by emergency vaccination has been greatly reduced. Currently there are several commercial DIVA tests for FMD available, some of them sold as screening test, others as confirmatory test. In the current study we compare 5 of these tests on a selected set of positive and negative sera from cattle.

Materials and Methods

Cattle sera negative for antibodies against non-structural proteins of FMD virus (FMDV) were obtained from a slaughterhouse (n=464), a BHV-1 field study (n=264), non-vaccinated cattle in animal experiments (n=210) before infection and from vaccinated cattle in animal experiments (n=29) before infection. Sera from various infection experiments using various strains (A, O, C and Asia-1 isolates) were used to validate the performance after infection (table 1).

Four screening and two confirmatory ELISAs for antibodies against non-structural proteins of FMDV were obtained from their producer (Table 1). In each plate we included a positive control serum and 7 serial dilutions of this control made in negative serum.

Table 1: Sera used in the study

	Screening				Confirmatory	
	UBI 3B	Aftosa	Bommeli	Ceditest	3B+NB	3A
Total number cattle sera	1522	1018	914	1517	1517	1520
Non-infected cattle	951	685	343	953	948	950
Non-infected vaccinated cattle	29	17	29	29	29	29
Intradermal infected cattle 1 to 20 dpi	217	217	217	215	218	218
Intradermal infected cattle 21 to 180 dpi	63	63	63	62	63	63
Intradermal infected cattle over 180 dpi	48	48	48	48	48	48
Sentinel (from 2 months after infection)	5	5	5	4	5	5
Intranasally inoculated cattle	112	0	112	111	110	111
Contact cattle	126	0	126	124	125	125

Using the 63 sera collected from cattle 21 to 180 after infection (type A and O) and 284 (Bommeli), 667 (Aftosa), 953 (Ceditest) or 948 (UBI 3B, 3B + neutralisation buffer (NB) and 3A) sera from non-infected cattle we produced ROC curves. The data were analysed using the computer programme CMDT (Briesofsky, 1999), which determined the cut-off at which sensitivity and specificity were equal, and statistically compared the curves using a permutation test (Venkatraman and Begg, 1996). The standardised cut-off was used to determine the ability of the tests to detect antibodies against non-structural proteins early and late after infection.

Using logistic regression we determined the moment 50% of the infected cattle was positive using 396 - 398 sera from cattle used in infection experiments collected between 0 and 28 days after infection. To determine the endurance of the antibody response we tested sera collected up to 665 days after infection from 16-17 cattle infected with FMD type A/TUR/14/98. To check the performance of the different tests in intranasally inoculated cattle, we selected 38 sera from calves, vaccinated and non-vaccinated, 21 to 71 days after inoculation with a low dose of FMD isolate O/NET/1/2001.

Results

Figure 1 shows the upper left corner of the ROC curves. The curve of the Ceditest is almost optimal, with almost 100% sensitivity at 100% specificity. At the time we produced the results of the UBI 3B + NB confirmation test, we also obtained results of this test without neutralisation buffer. These data were also analysed, but showed that the UBI 3B test in this instance was less sensitive but a bit more specific at the same cut-off (not shown in figure 1). In table 2 the results of the statistical comparison shows also that repeating the UBI 3B ELISA produces a significantly lower ROC curve, when comparing it to all other tests. The UBI 3B ELISA with neutralisation buffer (3B + NB) produces a significantly lower ROC curve when compared to the UBI 3B, 3A or Ceditest ELISA. The Ceditest ELISA, however, produces a significantly higher ROC curve when compared with any other test (table 2).

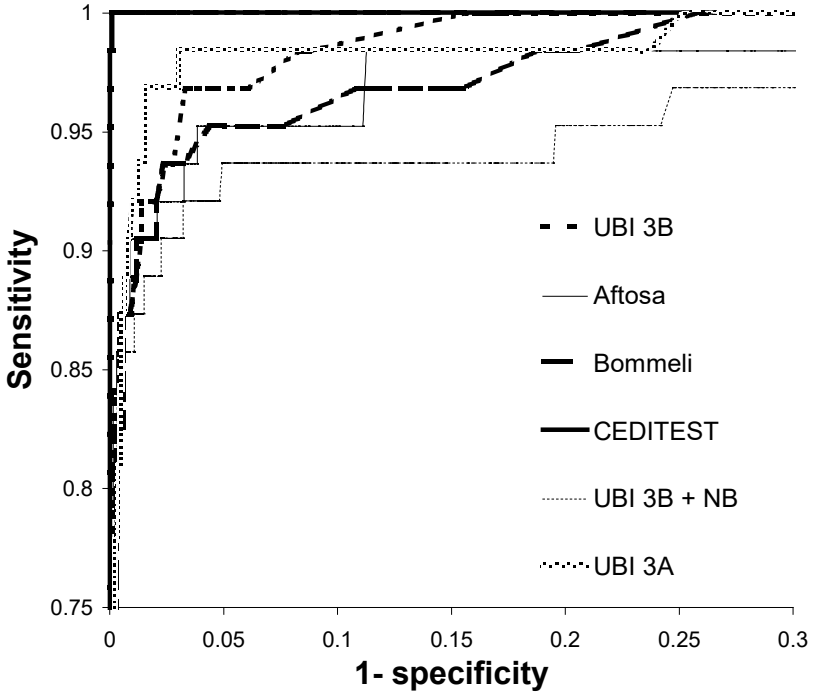


Figure 1: The upper left corner of the ROC plane with the 6 different tests.

Table 2: P-value using a permutation test, i.e. the chance of finding these curves if the two curves were actually the same (the null hypothesis that both curves are similar).

	UBI 3B	UBI 3B rep	Aftosa	Bommeli	Ceditest	UBI 3B + NB
UBI 3B repeated	**0.000**					
Aftosa	0.580	**0.001**				
Bommeli	0.396	**0.000**	0.908			
Ceditest	**0.001**	**0.000**	**0.002**	**0.005**		
UBI 3B + NB	**0.016**	**0.000**	0.123	0.302	**0.000**	
UBI 3A	0.587	**0.002**	0.194	0.104	**0.001**	**0.022**

Using the computer programme CMDT we determined the cut-off at which the sensitivity and specificity were equal, the results are shown in table 3. Using these standardised cut-offs we were able to compare the different tests for detection of antibodies early and late after infection.

Analysis of the data of the positive control serum showed that the Aftosa and Bommeli test had the lowest analytical sensitivity, UBI 3B and 3A ELISA had a little bit higher analytical sensitivity, but the Ceditest was consistently positive at a 2 to 4 fold higher dilution. A very low variation coefficient (range 0.54 - 2.4 %) was found in the Ceditest ELISA for the dilution of the positive control serum where the test scored positive (> 60.7%). In the other tests this variation coefficient was always more than 10% even up to 42%.

Table 3: Cut-off for the different tests when sensitivity and specificity are equal

	Cut-off	Sensitivity and Specificity
UBI 3B	0.098	0.97
UBI 3B repeated	0.102	0.87
Aftosa	1.495	0.95
Bommeli	0.108	0.95
Ceditest	60.69	1.00
UBI 3B + NB	15.23	0.94
UBI 3A	0.197	0.97

Based on sera from various FMD potency tests using various A, O, C and Asia-1 types of FMD we determined the day at which 50% of the cattle responded positive (table 4). Both the Bommeli test as well as the Ceditest is able to detect infection in 50% of the cattle almost within a week after infection.

Table 4: Day after infection at which 50% of the cattle responded positive

	D_{50}	SE	Number
UBI 3B	8.6	0.4	398
UBI 3B repeated	10.4	0.6	397
Aftosa	10.7	0.8	398
Bommeli	7.1	0.4	398
Ceditest	7.1	0.2	396
UBI 3B + NB	9.3	0.4	397
UBI 3A	9.3	0.4	396

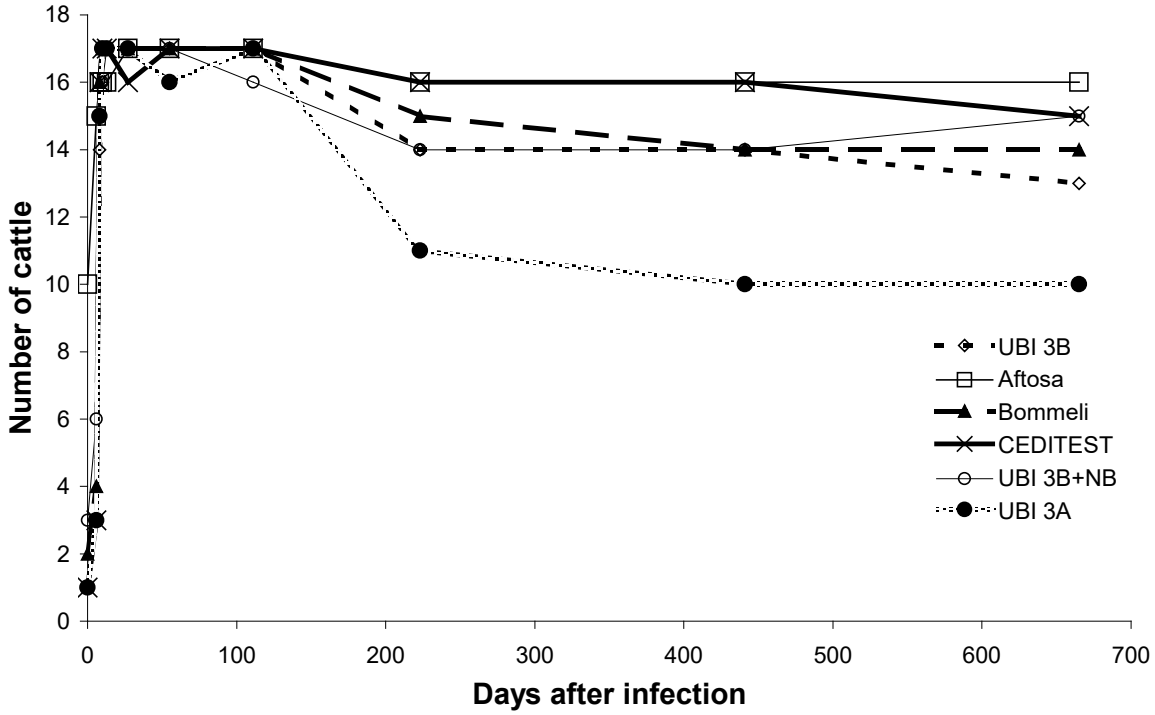


Figure 2: Comparison of the endurance of detection of infected cattle.

Figure 2 shows that both the Aftosa test as well as the Ceditest was able to detect all 16 infected cattle present at 441 days. At 665 days after infection one of the sera had a percentage inhibition of 60% in the Ceditest ELISA, and was therefore just negative at the cut-off we selected. Figure 2 also illustrates the poor specificity of the Aftosa test, already 10 cattle are positive at day 0.

Table 5: Number of cattle reaction positively in the test. Using 38 sera from calves (26 non-vaccinated, 12 vaccinated) inoculated with FMD isolate O/NET/1/2001 collected between 21 and 71 after inoculation.

	Non-vaccinated	Vaccinated	Total
UBI 3B	24	1	25
UBI 3B repeated	24	2	26
Bommeli	21	4	25
Ceditest	21	5	26
UBI 3B + NB	21	4	25
UBI 3A	21	5	26

The UBI 3B ELISA tests the highest number of non-vaccinated cattle inoculated with O/NET/1/2001 positive (table 5), whereas the highest number of vaccinated and inoculated cattle are positive in the Ceditest and the UBI 3A ELISA.

Conclusions

The new OIE code states that regaining freedom of FMD without vaccination a country may use emergency vaccination, but has to either cull all the vaccinated animals or use serological tests that detect antibodies against non-structural proteins. To be able to use these tests, the characteristics have to be evaluated, and alternative tests to confirm a positive result are necessary. The work presented in this paper is an extension of the work described by Moonen et al. (2003). Three new ELISAs, one for screening and two for confirmation, have become available recently and were added to the study of Moonen et al. (2003). Using the same set of cattle sera we compared all the tests for sensitivity, specificity, analytical sensitivity and the ability to detect antibodies early and late after infection. A low number of sera were available from calves intranasally inoculated with FMD isolate O/NET/1/2001, and these sera were used to see whether the results obtained with cattle infected intradermally in tongue are representative for cattle infected by another route.

Using a limited set of positive and a large set of negative sera we produced ROC curves to be able to compare the several tests. As found previously (Moonen et al., 2003) the UBI, Aftosa and Bommeli test produced similar results. The UBI 3A ELISA produced an ROC curve comparable to that of the 3B ELISA of the same producer, based on this result the 3A ELISA could also have been selected as screening test. This is in contrast to the results of Shen et al. (1999) who found cross-reactive responses in sera from vaccinated animals. The UBI 3B ELISA with neutralisation buffer produced a significantly less optimal results when compared to the UBI 3A and 3B ELISA, whereas the Ceditest ELISA produced significantly better results than all other tests.

The Ceditest ELISA not only produced a better ROC curve, but also had the lowest variation coefficient when testing dilutions of a positive control in each plate. This shows that the Ceditest ELISA is very reliable and robust. The Ceditest ELISA also had a better analytical sensitivity and detected antibodies earlier in infection compared to most other tests. The endurance of detection was the highest with the Aftosa test, which detected all sera positive till the end of the experiment. The fact that only 16 cattle were positive after 111 days after infection was caused by the fact that one cow had died due to torsion in the colon.

Based on the results of cattle intradermally infected in the tongue, the Ceditest ELISA performs superior compared to the other tests. The question remains whether these sera are representative for the situation in the field. For this reason we included some sera from recent experiments in which calves were intranasally inoculated with FMD isolate O/NET/1/2001. From the 38 sera collected between 21 and 71 days after inoculation only 25 to 26 reacted positively in the tests. The sensitivity for detecting intranasally inoculated calves was between 65.8% (95% CI: 63.9 - 67.4%) and 68.4% (95% CI: 66.5 - 70.0%). However, in the 12 calves that had been vaccinated two weeks before inoculation, only 1 to 5 calves were positive in the tests, resulting in sensitivity below 50%. This indicates that most tests have difficulty to detect infection in vaccinated cattle when inoculated intranasally with a low virus dose. The question remains whether these calves were really infected. Perhaps the immunity of the calves prevented virus growth. In the 26 non-vaccinated calves, however, the tests also find 2 - 5 calves negative. All these non-vaccinated calves had shown a four-fold increase in neutralising antibodies when tested in the neutralisation test using O Manissa. The sera found negative in one of the ELISAs mostly had low ($< 2\ ^{10}\log$) neutralising antibody titres, but all titres were above the titre of the OIE cut-off reference serum and several even higher than the OIE low positive reference serum (Moonen et al., 2000). This result shows that even infection

in non-structural proteins. Much more sera have to be tested from vaccinated and non-vaccinated animals infected by other routes than injection.

Although the results of this comparison shows that all screening tests have a high sensitivity and specificity, more work should be done on the ability of these tests to detect infection in a field situation, or animal experiments similar to the field situation. In addition validation and comparison of different test on sera from sheep, goats and pigs should be performed.

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Appendix 7

Likelihood ratio analysis of FMD ELISA data provided by IZS, Brescia

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Abstract
Evidence-based medicine encourages the use of quantitative diagnostic test results to estimate probability of a particular diagnosis. Likelihood ratios (LR) are among the best tools for extracting the most value from a diagnostic test result. They utilize the magnitude of a test results to estimate the odds that an animal with a particular test result actually has the disease in question. LRs are based on actual data from well-characterized cases and controls. FMD ELISA results using structural (serotype-specific) and non-structural proteins (NSP) were analyzed to determine likelihood ratios for each assay using multiple result ranges comparing results on animals vaccinated and challenged with FMD virus to those only vaccinated for the disease. The calculated likelihood ratios thus reflect the odds an animal with the given level of ELISA result is FMD-infected versus vaccinated. All data were provided by Dr. Emiliana Brocchi (Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy). The NPS FMD ELISAs had higher LRs than did ELISAs to structural proteins. Using existing cut-off values the Brescia NSP ELISA had a higher LR than did the Bommeli NSP ELISA using existing definitions of a positive test.

Introduction

The principal goal of a diagnostic test is to help practitioners increase the probability of a correct diagnosis. Predictive values are useful in this regard but require the estimation of disease prevalence (i.e. pre-test probability of disease) in the population {Vecchio 1966 36 /id}. Food-producing animals exist in numerous discrete populations (herds or flocks), and the prevalence of disease can differ greatly among them. Infection prevalence significantly affects predictive values (PV), i.e., the positive predictive value (PPV) of tests is low when prevalence is low and the negative predictive value (NPV) is low when disease prevalence is high, thus the PV of tests can vary significantly among herds.

Evidence-Based Medicine (EBM) is the scrupulous, explicit and judicious use of the best evidence available in making decisions about the care of individual patients. The practice of EBM means integrating clinical expertise with the best available clinical evidence from systematic diagnostic research {Sackett, Strauss, et al. 2000 2629 /id}. The likelihood ratio (LR) is one example of external clinical evidence and a powerful tool in EBM. LRs give the same information as PVs but can be used independent of pretest disease prevalence {Giocoli 2000 2617 /id}.

The purpose of the study was to define LRs for five FMD ELISAs using data provided by Dr. Emiliana Brocchi (Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy).

Methods

Serum samples. Sera from 176 cattle vaccinated for FMD were evaluated. The sera originated from 5 countries and animals had been vaccinated with one of 4 different vaccines. Sera were collected 12-28 days post-vaccination. Sera from 89 cattle vaccinated for FMD and then challenged (hereafter simply referred to as the challenged group) originated from 3 countries. Results from all challenged cattle were considered in the analysis regardless of whether they had been challenged with the same or a different FMD strain than the one they were vaccinated with. For more specific information on the samples readers should consult Dr. Brocchi.

Assays. The author has no first-hand knowledge of the assays. Interested readers should contact Dr. Emiliana Brocchi (Instituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Brescia, Italy) for specific information if needed.

Data manipulations. Vaccinated animals sampled >28 days post-vaccination were excluded (n=4). Two cattle with high pre-vaccination serum antibody levels were excluded (n=2). Results reported as ranges, e.g., 14-23, were averaged. Results reported as < or > were converted to whole number as follows:

<0% = 0.0
<10% = 1
>270 = 300
>640 = 700

Data analysis. Frequency distributions for ELISA results on sera from the vaccinated and challenged cattle were graphed and result ranges selected by visual examination of the data to provide rational numbers of animals in each range. For each results range the percentage of cattle in each group (vaccinate or challenged) was calculated. LRs for each range were calculated as:
% challenged cattle / % vaccinated cattle.

Results

Bommeli NSP ELISA (NSP-1).

Results Range (% positive)	% Cattle Sera in Range		Challenged/Vaccinated LR
	Vaccinated	Challenged	
0.00 – 0.49	53.4	6.7	0.13
0.50 – 9.99	26.1	4.5	0.17
10.00 – 19.99	13.6	7.8	0.58
20.00 – 29.99	5.1	5.6	1.10
>30.00	1.7	75.3	44.16

Brescia NSP ELISA (NSP-2).

Results Range (% positive)	% Cattle Sera in Range		Challenged/Vaccinated LR
	Vaccinated	Challenged	
0.00 – 4.9	18.8	0.0	0.00
5.0 – 9.99	79.0	11.2	0.14
10.00 – 14.99	1.1	9.0	8.13
15.00 – 39.99	0.6	23.6	42.71
>40.00	0.6	56.2	101.69

ELISA for antibody to structural proteins (FMD serotype-O).

Results Range (% positive)	% Cattle Sera in Range		Challenged/Vaccinated LR
	Vaccinated	Challenged	
0 – 150	71.8	49.1	0.68
150 - 199	14.5	15.1	1.04
200 – 299	11.3	5.7	0.50
300 – 399	1.6	7.6	4.68
>400	0.8	22.6	28.08

ELISA for antibody to structural proteins (FMD serotype-A).

Results Range (% positive)	% Cattle Sera in Range		Challenged/Vaccinated LR
	Vaccinated	Challenged	
0 - 99	57.1	37.7	0.66
100 - 199	21.4	22.6	1.06
200 - 299	19.8	1.9	0.10
>**300**	1.6	37.7	23.77

ELISA for antibody to structural proteins (FMD serotype-C).

Results Range (% positive)	% Cattle Sera in Range		Challenged/Vaccinated LR
	Vaccinated	Challenged	
0 – 99	69.0	35.8	0.52
100 - 199	11.1	17.0	1.53
200 – 299	11.9	17.0	1.43
300 – 499	6.35	1.9	0.03
>500	1.6	28.3	17.83

Discussion

A perfect serological test would discriminate between FMD-vaccinated and FMD-infected cattle with no errors. Unfortunately, there are no perfect tests and even for very well-designed tests the biology of host response to infection causes results to vary among animals. Consequently, determination of whether an animal is vaccinated or infected based on serology alone is a probabilistic determination. Dichotomous interpretation of tests, i.e., positive/negative, that produce results on a continuous scale causes some of the most valuable information produced by the test to be discarded, namely the magnitude of the result. The magnitude of ELISA results (OD values or transformed OD values, such as percentage positive) is generally a direct measure of the concentration of antibodies in the sample. This, in turn, may be related to the likelihood of disease or infection in the animal, expressed as a likelihood ratio: for each range of test result, the percentage of animals with the target infection divided by that of an appropriate control group. The strength of a relationship between magnitude of ELISA result and probability of infection or disease can only be determined using large numbers of well characterized samples. The samples must originate from animals that reflect the "real world" situation.

The data for this preliminary analysis of likelihood ratios for FMD ELISAs was provided to the author who can not provide specifics about the details of vaccination or challenge of the animals providing serum samples nor the performance of the assays. The data set is small and findings can be considered preliminary at best. Any extrapolation of results to the field situation is dependent on whether the status of the vaccinates and challenged animals reflects a true field situation.

Not surprisingly, for all ELISAs evaluated, higher ELISA results were seen more often in challenged than vaccinated animals. This is reflected in the LRs for each assay. If the ELISA that best discriminates vaccinated from challenged cattle is the one that generates the sharpest change in LR with increasing ELISA result range and the highest LRs at the highest ELISA range, then the Brescia NSP ELISA appears to be the best of the assays evaluated.

Appendix 8

WRL report: FMD global situation
Proposed priority antigens for 2003-2004
Where are the information gaps?

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The conjectured status of FMD globally is illustrated in Fig 1 and the cumulative report of FMD samples analysed at the World Reference Laboratory (WRL) is shown as Table 1. OIE FMD report listings for 2003 to date have involved Argentina, Bolivia, Botswana, Hong Kong, Libya, Malawi, Paraguay, South Africa, United Arab Emirates and Zimbabwe.

Fig. 2 shows the relationships between recently isolated FMD type O viruses and various reference strains. In 2002 & 2003, the PanAsia strain continues to persist in some countries (e.g. Turkey, Iran and Lebanon) and has now been detected in Afghanistan. The newly identified Ind2001 strain (probably a derivative of PanAsia viruses of the late 1990's) previously identified in India, Iran, Bahrain, United Arab Emirates Oman, Saudi Arabia and the Palestinian Autonomous Territories, has now been found in Pakistan (2002 & 2003). Another lineage, more closely related to O1/Manisa and some Indian vaccine strains, has also been found in Pakistan in 2003; this virus was first detected in Pakistan in 1998 and appears to have changed little in the intervening years. Viruses belonging to a lineage distinct from both PanAsia and Ind2001 were found in Turkey in 2000 and 2002; these were related to viruses isolated in Iran in 1997.

In the Far East viruses of the Cathay topotype continue to be isolated from the Philippines and Hong Kong, while in Vietnam both viruses belonging to the the Cathay topotype and the ME-SA topotype (PanAsia strain) and continue to co-circulate. In Nepal two lineages were found, one belonging to the PanAsia strain and the other related to an isolate from Bhutan in 2002 (these are more closely related to the Ind2001 strain than to the PanAsia strain). Two distinct lineages were present in Bhutan in 2002, one as just mentioned related to the Ind2001 strain and the other possibly part of the PanAsia strain.

Fig. 3 shows the relationships between recently isolated FMD type A viruses and various reference strains. It is evident that, since 1999, at least 4 to 5 main genetic lineages have been present in Iran. This appears to contrast with surrounding countries where only one or two lineages have been detected (i.e. Turkey, 2; Iraq, 1; Pakistan, 1). However, a lesser number of samples have been submitted from these countries. Nothing is known about the situation with type A in Afghanistan since no samples containing that serotype have been received since 1975. Multiple lineages circulate in India; however, these appear to be distinct from those in Pakistan, Iran, Iraq and Turkey (data not shown). It is interesting that the Iran99 strain has been detected in Turkey for the first time since 1999 (S. Aktas

and U. Parlak, personal communication, 2003). The single isolate received from Bhutan is related to Indian type A viruses from the mid-1990's.

Fig 4 shows the relationship between a recent isolate from Libya and other SAT 2 viruses, showing the closest match to viruses from Cameroon in 2002, Saudi Arabia in 2000 and Eritrea in 1998.

An analysis of FMD sample submissions to the WRL by region is shown as Table 2. It can be seen that there is a relatively good rate of submission from some parts of the Middle East and South-East Asia, but that submissions from elsewhere are very sporadic. In some regions, such as South America and India, regional reference laboratories undertake considerable analysis of their own.

Difficulties with vaccine matching tests have arisen over the availability and consistency of post vaccinal typing sera, discrepancies between different testing methods and a lack of cross-protection data to underpin the in vitro results. Vaccine potency, as well as antigenic match is also of great importance in vaccine selection. A lack of available post vaccinal antiserum prevented the WRL from conducting matching tests between the recent SAT 2 FMD virus isolated from Libya and the vaccine strains most likely to confer protection based on genetic considerations (see above). Based on WRL and/or S American data, the type A viruses from Argentina and Brazil in 2000 and 2001 showed limited cross-reaction with A24 Cruzeiro. Type A viruses of Middle Eastern origin isolated at WRL in recent years have shown a great diversity both genetically and antigenically. A Iran 96 appeared to be an antigenically appropriate vaccine strain for many viruses of Turkish and Iraqi origin. Other viruses from Iran and Syria were more poorly matched to A Iran 96 and also often showed a poor match to the A22 Iraq vaccine strain. In some cases, better matches were obtained by ELISA and VNT using the vaccine strain A Iran 87, which also showed a good match to some Type A viruses from Malaysia and Thailand.

There have been difficulties in obtaining Market Authorisation for use of vaccine antigens stored in the International Vaccine Bank at Pirbright. The UK authority, Defra, is therefore establishing a new bank of antigen stocks which are being potency tested at the WRL.

In order to improve the process of vaccine selection, more should be done to make use of already available information by increasing the co-operation and collaboration between different regional reference laboratories, vaccine manufacturers and the WRL. More resources are needed to carry out more testing on available viruses and steps should be taken to improve the availability and consistency of post vaccinal antisera. Cross-protection studies are required to validate in vitro testing methods and research is also needed to better define the epitopes critical for protection. In order to improve the coverage of samples submitted to the WRL, steps should be taken to promote exchanges between regional reference laboratories and to target sample collection efforts to regions where surveillance information is sparse.

Table 1.

FMD WRL
Cumulative Report for January-August 2003

Country	No. of				FMD virus serotypes				SVD virus	NVD
	samples	O	A	C	SAT 1	SAT 2	SAT 3	Asia 1	(a)	(b)
AFGHANISTAN	57	8	-	-	-	-	-	-	-	49
BHUTAN	21	2	1	-	-	-	-	-	-	18
BOTSWANA	20	-	-	-	-	-	-	-	-	20
HONG KONG	7	3	-	-	-	-	-	-	-	4
IRAN	45	21	11	-	-	-	-	-	-	13
ISRAEL (PAT)	1	1	-	-	-	-	-	-	-	-
ITALY	45	-	-	-	-	-	-	-	45	-
LEBANON	4	4	-	-	-	-	-	-	-	-
LIBYA	10	-	-	-	-	2	-	-	-	8
NEPAL	6	5	-	-	-	-	-	-	-	1
PAKISTAN	44**	18	10	-	-	-	-	7	-	10
PHILIPPINES	23	9	-	-	-	-	-	-	-	14
TURKEY	10	4	3	-	-	-	-	-	-	3
UNITED ARAB EMIRATES	3	3	-	-	-	-	-	-	-	-
VIETNAM	8	8	-	-	-	-	-	-	-	-
TOTAL	**304****	**86**	**25**	**-**	**-**	**2**	**-**	**7**	**45**	**140**
*	Institute for Animal Health, Pirbright Laboratory, Woking, Surrey GU24 0NF									
(a)	Swine vesicular disease virus									
(b)	no foot-and-mouth disease, swine vesicular disease or vesicular stomatitis virus									
	detected									
**	One sample from Pakistan contained a mixture of foot-and-mouth disease virus types									
	O and A									
60 out of 98 positive samples tested as original suspension (Jan-Jun) were typed by enzyme-linked immunosorbent assay										
(61%) and the remainder (39%) were typed following cell culture passage										

Table 2.

Viruses submitted to WRL 2001-2003 by region

	2001		2002		2003		2001-3	
	Viruses	Countries	Viruses	Countries	Viruses	Countries	Viruses	Countries
South America	9	3	2	1	0	0	11	4
Europe	11*	4	0	0	0	0	11*	4
Middle East	117	11	58	8	44	4	219	15
Indian Subcontinent	6	2	45	2	51	4	102	4
South East Asia	27	5	36	4	17	2	80	6
China/Taiwan/HK/Korea/Japan	12	1	7	2	3	1	22	2
Africa North	0	0	0	0	2	1	2	1
Africa West	15	3	1	1	0	0	16	4
Africa Central	0	0	0	0	0	0	0	0
Africa East	2	1	12	2	0	0	14	2
Africa South	0	0	9	2	0	0	9	2

**Excluding UK*

Fig 1.

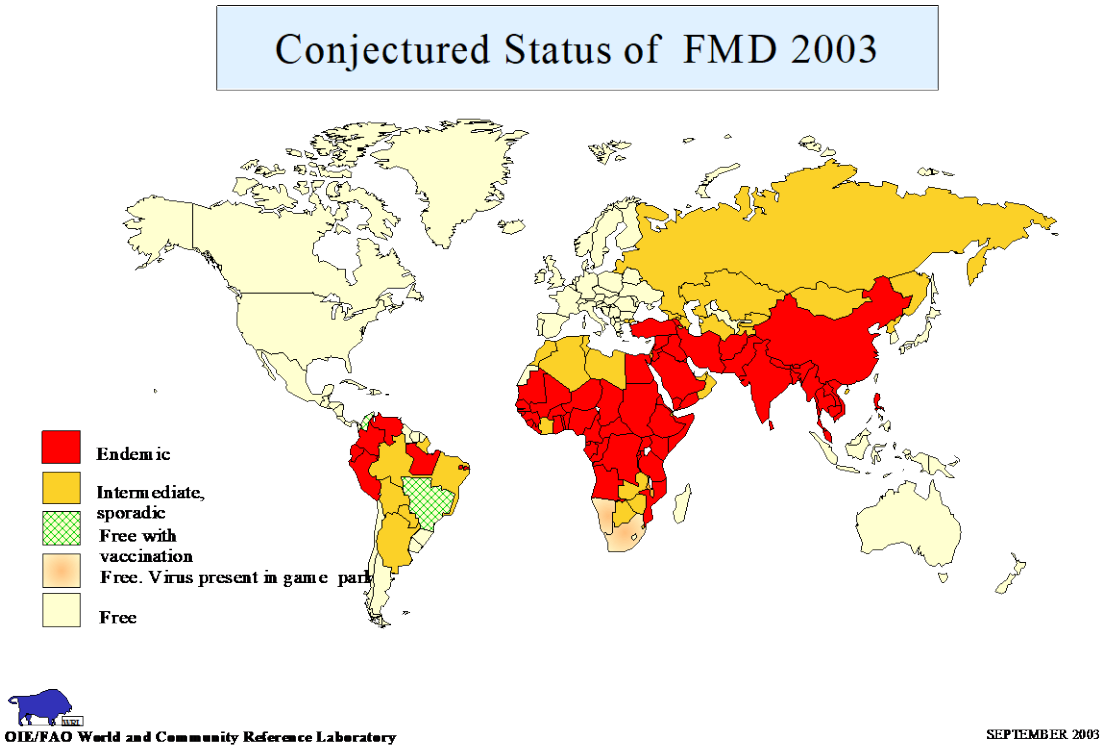

Conjectured Status of FMD 2003
Endemic
Intermediate, sporadic
Free with vaccination
Free. Virus present in game park
Free
OIE/FAO World and Community Reference Laboratory
SEPTEMBER 2003

Fig 2.

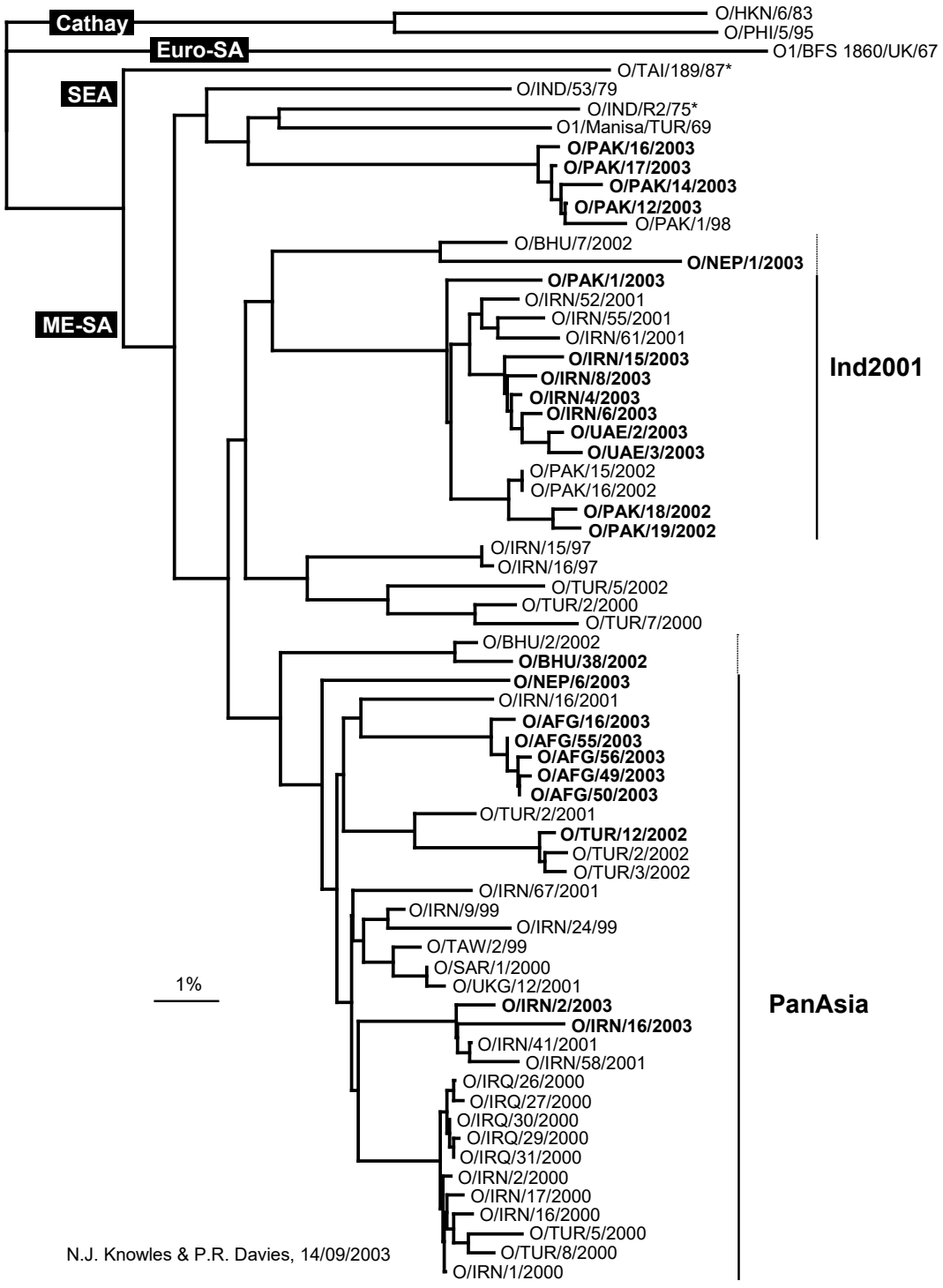


Fig. 1. Neighbor-joining tree based on a comparison of VP1 nt 1-639 showing the relationships between recently isolated FMD type O viruses and reference strains. Viruses received in 2003 are shown in bold. *, not WRLFMD reference numbers.

Fig 3.

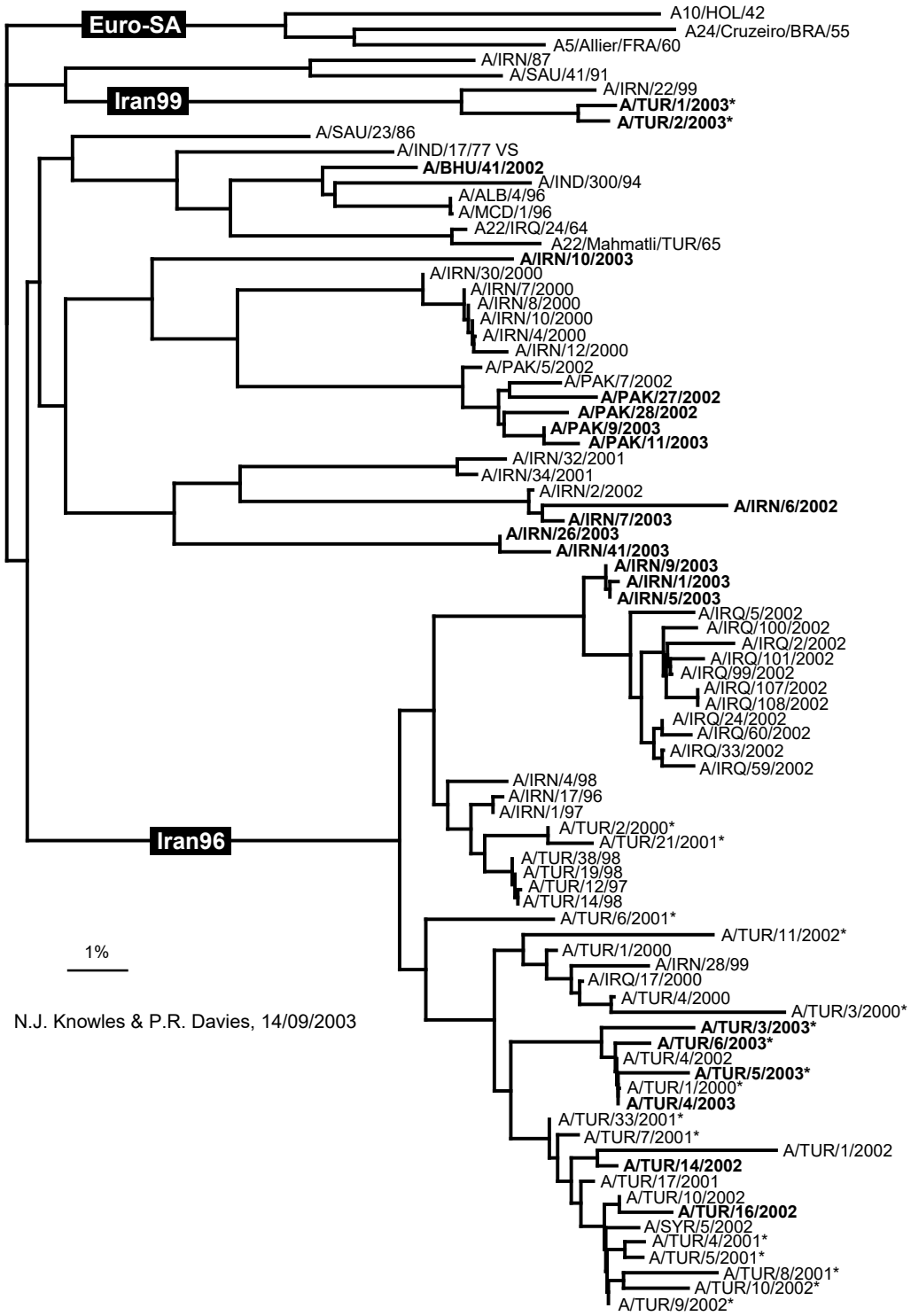
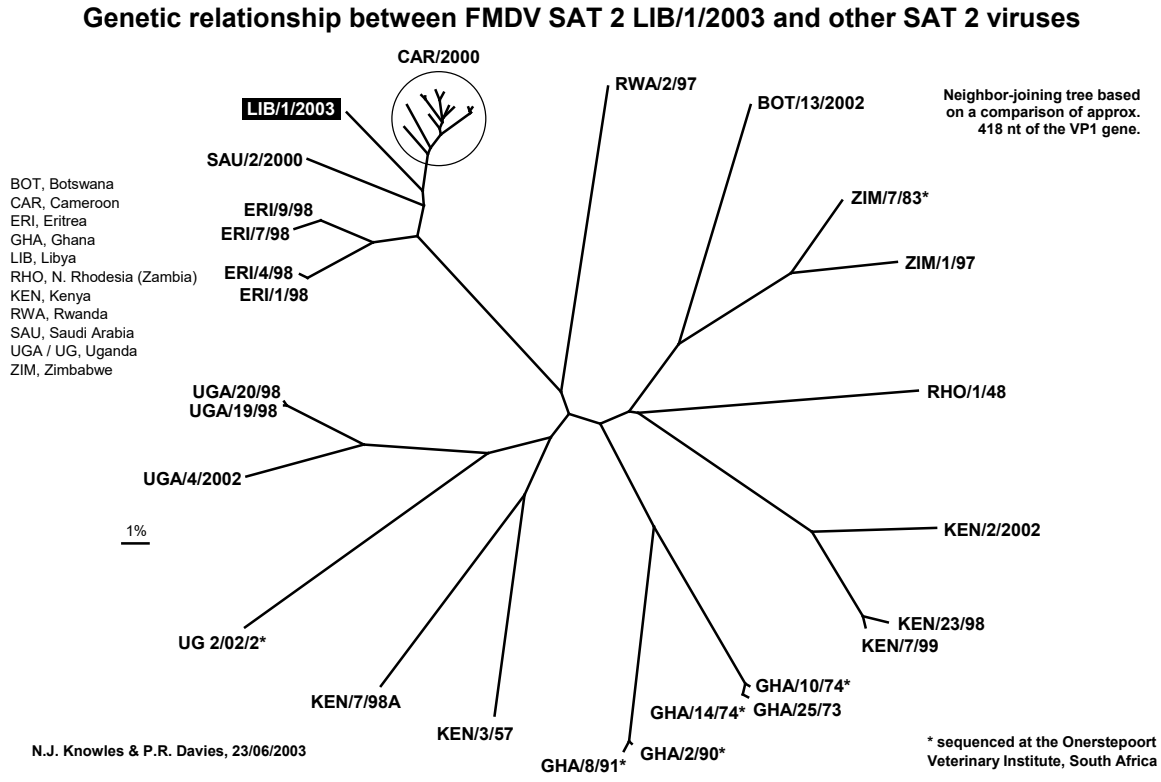


Fig. 2. Neighbor-joining tree based on a comparison of VP1 nt 469-639 showing the relationships between recently isolated FMD type A viruses and reference strains. Viruses received in 2003 are shown in bold. *, sequences provided by Sinan Aktas and Ünal Parlak, FMD Institute, Ankara, Turkey (note: these are not WRLFMD reference numbers).

Fig 4.


Genetic relationship between FMDV SAT 2 LIB/1/2003 and other SAT 2 viruses
CAR/2000
RWA/2/97
BOT/13/2002
LIB/1/2003
SAU/2/2000
ERI/9/98
ERI/7/98
ERI/4/98
ERI/1/98
ZIM/7/83*
ZIM/1/97
RHO/1/48
UGA/20/98
UGA/19/98
UGA/4/2002
1%
KEN/2/2002
KEN/23/98
KEN/7/99
UG 2/02/2*
KEN/7/98A
KEN/3/57
GHA/10/74*
GHA/14/74*
GHA/25/73
GHA/8/91*
GHA/2/90*
Neighbor-joining tree based on a comparison of approx. 418 nt of the VP1 gene.
BOT, Botswana
CAR, Cameroon
ERI, Eritrea
GHA, Ghana
LIB, Libya
RHO, N. Rhodesia (Zambia)
KEN, Kenya
RWA, Rwanda
SAU, Saudi Arabia
UGA / UG, Uganda
ZIM, Zimbabwe
N.J. Knowles & P.R. Davies, 23/06/2003
* sequenced at the Onerstepoort Veterinary Institute, South Africa

Appendix 9

Survey on expert opinion on priority regions for improving FMD virus submission to Regional/World Reference Laboratories

Keith J. Sumption, Secretary, European Commission for the Control of Foot-and-Mouth Disease, Animal Health Service, FAO, Rome

Summary
Priority regions for improving our information on circulating virus strains/types were perceived as China, the Indian sub continental region, and the Horn of Africa. Constraints to the submission of samples differed between these regions; for the Chinese region these were perceived as concern over the use of information or upon the health status (essentially political concerns), whereas in Africa the constraints were perceived as the lack of value of typing to the originator in relation to cost and effort involved. Lack of submission of samples to national laboratories was a problem of several regions. In the short term, efforts to support laboratories to submit samples to reference laboratories could be effective in parts of Africa, and may assist establishing the value of virus typing in regions where vaccination is used. Surprisingly, respondents did not perceive lack of strain typing in the Sahelian eco-system to be an issue, despite significant animal populations and proximity.

Background
The relative richness of FMDV sequence and virus characterisation data presented in the reports of the WRL to the EUFMD Commission, or other meetings, tends to obscure the extent of the poverty of information from other regions. It will be important to address these information gaps to assist in risk assessment for Europe, including the selection of appropriate antigens, In addition the phylogenetic studies should assist in identification of transmission hot spots/reservoirs and routes of spread. This survey was conducted in order to elicit supportive information to assist priority setting of EUFMD efforts to improve the timely supply of virus characterisation/typing information from endemic areas considered to represent a risk to the European region, following four recommendations of the 35th General Session of the EUFMD Commission relating to this issue:

1. That the EUFMD should organise an ad hoc group in close collaboration with OIE to investigate the factors contributing to under-reporting and also practical means of improving global surveillance, prioritised according to the areas of highest perceived risk to member countries.
2. That EUFMD should support the timely supply of representative field samples of FMD virus to regional diagnostic laboratories and to the WRL from areas lacking the means to supply.
3. That the Commission should explore how risk assessment approaches could be used to target both information gathering by the EUFMD and surveillance efforts into areas of higher risk and/or higher epidemiological uncertainty in respect of FMD.
4. The OIE, FAO and EUFMD should strongly encourage the increased submission of field samples to regional diagnostic laboratories and to the World Reference Laboratory and also investigate incentives towards this end.

The workload of the WRL should also be continuously monitored to ensure that the resources available are adequate for the tasks involved.

Methods

A questionnaire was sent by e-mail to the members of the EUFMD Research Group, to OIE and FAO FMD Reference Laboratories, to staff members of FAO and OIE, to the three FMD vaccine producers in Europe and to one in Argentina, and to fifteen FMD experts who have acted as consultants on FMD control for FAO and have a wide international experience.

The first question required the respondents to indicate, from the list of regions provided, the 5 most significant/important areas where FMD virus typing information is weakest, in other words, the most significant gaps in world surveillance for circulating virus strains/antigenic types. The second question requested that respondent to indicate the countries, or regions within countries, where support and efforts to collect and type virus isolates should be focussed, for the three regions considered most significant in terms of weak surveillance, identified in question 1. The third question concerned the perceived constraints to submission of specimens to RRL and the WRL, in the priority country/region indicated in question 1, where surveillance was considered to be weakest. Respondents were asked to score the reasons provided in a check list, or state other reasons.

Results

Twenty four responses were received (24/45) in time to compile this report. Four responses were received from three of the vaccine producers, from three reference laboratories (BVI, OVI and ARRIAH), three from FAO and OIE staff members, and from 8 international experts.

The response to Question 1 was inconsistent, with many of the respondents choosing to score multiple regions with the same level of significance, rather than selecting 5 regions. For this reason, the responses were analysed in two ways. Tables 1 and 2 indicate the frequency of citation of regions as the “most significant” (score of 1) or in the two most significant categories (scores of 1 or 2).

“China” received the most citations as the most significant region of weakness in surveillance, with the “Indian subcontinental region” (Indian-SCR) in second position. This was consistent for EUFMD Research group members, and non-members (all others consulted). When the number of citations as most or second most significant were totalled, “China” and the Indian-SCR were again consistently in first and second position, although East Africa tied with the Indian-SCR for non-Research Group respondents. Apart from “China” and the Indian SCR, third and fourth placed regions were usually on the African continent. The “horn of Africa” region was third mostly frequently cited as most significant, with East Africa, Central Africa, West Africa and the Arabian Gulf region tying for fourth position, ahead of other regions in the near east/west Asia, southern Africa, south-east Asia or Latin America. Research group members differed from non-members in placing higher importance on the Arabian Gulf region and Central Asia (the “stans”).

Given the inconsistency in scoring method between respondents, the scores for regions were compared using a citation index calculated for each region,

incorporating a correction to responses involving multiple entries of the same score. Higher indexes result from a combination of frequency of citation and from the higher significance of the score for each region. Citation indexes were compared between EUFMD Research Group and non-Research group (Figure 1), as a percentage of the total citation index to correct for the different number of respondents in each group. The results support the ranking of China and Indian-SCR as most significant regions of weakness, but also suggest a higher importance is placed on north/eastern African regions by EUFMD research group members than by others.

Regarding countries or regions in the most significant category for gaps in surveillance (question 2), "China" was cited most often (10 times), with one respondent indicating south and western parts of the P.R. of China should be the focus of attention. India was cited three times, and thereafter a large number of countries and some regions were cited, with only Somalia receiving more than one citation. Considering Yemen, Ethiopia and Eritrea were also cited, this region can be considered of significant concern.

The scoring of perceived constraints to the submission of specimens to international reference laboratories differed between regions. For African countries, the major constraints were, in order of decreasing importance, perceived to lack of importance of virus typing to the originator, cost and effort to transport, and low submission of samples and lack of containers; use of the information and impact on health status were less important. For "China", the latter two were perceived as the most important constraints (Figure 2). For the group of countries from India to near-east, including the CIS countries, the constraints were perceived to be similar to those in Africa. An interesting amount of supplementary information on constraints was supplied. Obligations and politics relating to the Regional Reference Laboratory, problems relating to air transport, lack of training and management skills, and lack of return for effort involved, were each mentioned twice. Conflict between provincial and national authorities in reporting outbreaks, relating to potential in-country restrictions, was an interesting issue raised that is perhaps under recognised at the international level.

Discussion

The lack of surveillance information from many parts of the world that are not considered free of FMD presents a major problem for risk assessments relating to illegally traded ruminant products, particularly where human migration from such countries is significant. The ranking of "China" and "India" as the first and second most significant weak spots in global surveillance was not surprising, given the population size of susceptible species; China ranks first in number of pigs (414 million) , first in small ruminants (290 million) with India second (181 million), and third (128 million) in terms of large ruminants behind Brazil (173 million) and India (313 million). Sequence analysis of FMDV isolated from neighbouring regions also supports the hypothesis that continuous virus circulation in both countries provides the source for outbreaks in neighbouring countries – both have been labelled as "dripping taps". In both countries facilities and expertise to conduct FMDV characterisation exist and timely analysis and publication of trends in virus circulation and emergence would assist in building international confidence -and preparedness – and would assist in developing national control strategies. Political barriers to the export of virus specimens to the WRL, are clearly perceived as a constraint. It is not obvious how the EUFMD Commission can influence this situation. As the advantages

to the countries concerned to export virus specimens for typing is not obvious, alternatives to improve the timely supply of essential information must be sought. Support for international networking of FMDV scientists from these countries and agreements on rights of access to global sequence databanks might assist early provision of virus sequence information.

Concern over the weakness in surveillance for circulating FMDV in Africa was indicated by the high scoring for several African regions. Although the importance attached to regions did depend to some extent on the location of the respondent, the general response indicates a high concern for the weakness of surveillance information for the Horn of Africa/East Africa region. The constraints to submission of samples from this region appear to be related, the lack of importance placed on the issue in relation to cost, effort and lack of return to the originator. This suggests the problems could be amenable to specific ,targeted inputs to support laboratories, or possibly NGOs or individuals, to source and supply epithelial samples from confirmed or suspect cases, at relatively low cost. Such support might also assist in developing a long term interest and experience in the epidemiology of FMDV in the region, if support to acquire epidemiological information is also given. The results of the survey do show some surprising differences from a simple reading of animal population data for FMD infected countries. It is noticeable that Sudan, with one of the largest susceptible animal populations in Africa (50 million and 38 million small and large ruminants, respectively), was not suggested as a weak spot for surveillance. All the more surprising given the recent SAT2 outbreak in Libya, the source of which is uncertain but may relate to animals moved from neighbouring countries, or possibly the Horn of Africa, and the long standing animal trade with Egypt. Similarly, Nigeria, with a major susceptible animal population (45 million small and 20 million large ruminants) was also not cited in the responses, or Bangladesh, with 35 million and 25 million small and large ruminants, respectively. One possibility is that the lack of surveillance information over time from the region as well as the country concerned, results in lack of a threshold level of information to trigger awareness of the potential virus circulation; we fail even to perceive what may be the situation. In some of these countries, short term input to obtain information and specimens could greatly clarify the existing situation. If it is assumed that high animal densities present a risk for FMD circulation then animal density maps could be used to highlight potential risk zones or regions. Further, indicators of animal mobility or contact could be used to modify or refine the risk prediction. Figure 3 is a predicted cattle density map for Eurasia and Africa; the very high densities in parts of India and Pakistan are clear and support the high score of these regions in the survey, as does the moderate cattle densities in Ethiopia and parts of East Africa. High cattle ownership per unit human population is a feature of pastoralist societies, but may also occur in small holder dairy systems in highly productive agro-ecological zones. The regions of the Sahel and East Africa in Figure 4 are mostly pastoralist zones, where high herd size and high mobility are the norm; taken together with the bovine density map, one can postulate the dark areas would be prime candidates for high virus transmission rate and risk of FMDV circulation. If this is the case, it is surprising that almost all of the countries in the Sahelian region with significant cattle density were not suggested by respondents in the survey as weak spots for virus surveillance. Several of these potential hot spots for virus circulation occur close to the borders with Libya and Egypt.

In conclusion, the survey results suggest that different approaches will be needed to improve the supply of virus isolates or FMDV typing information for different regions. Short term targeted support for African laboratories might overcome constraints to virus submission from parts of this region. The survey identified significant level of concern on lack of information on the virus types circulating in north-eastern Africa. The lack of identification of a surveillance issue in Sahelian ecosystem was surprising in view of the animal population, and husbandry systems in this region and may reflect a general lack of information emanating from this region.

Tables

Table 1 - Regions indicated as "most significant" for weakness in FMDV type information. Ranking was determined by the total of citations of region as score 1 in question 1 (numbers of citations in brackets).

Rank	All	Research group	Non-Research group
1	China (12)	China (4)	China (8)
2	Indian-SCR (6)	Indian-SCR (3)	Indian-SCR (3) Africa-Horn (3)
3	Africa-Horn (5)	Africa-Horn (2), East Africa (2), Arabian Gulf (2), "Central Asia-the stans" (2)	Central Africa (2), West Africa (2), Africa-SADC (2)
4	East Africa (3), Central Africa (3), West Africa (3), Arabian Gulf (3)	4 regions (1 vote)	7 regions (1 vote)

Table 2 - Regions indicated as "most significant" or "next most significant" for weakness in FMDV type information. Ranking was determined by the total of citations of region with score 1 or 2 in question 1 (numbers of citations in brackets).

Rank	All	Research group	Non-Research group
1	China (17)	China (7)	China (11)
2	Indian-SCR (10)	Indian-SCR (3)	Indian-SCR (7), East Africa (7)
3	East Africa (9)	Africa-Horn (2), Central Africa (2), Arabian Gulf (2), "Central Asia-the stans" (2)	Africa-Horn (5),
4	Central Africa (8) Central Asia – the stans (8)	5 regions with 1 vote	Central Africa (4)
5	Africa –Horn (7)		5 regions with 3 votes

Figures

Figure 1. Significant regions for the world for increasing FMDV surveillance; research group and non-research group responses as a percentage of the total citation index for each group.

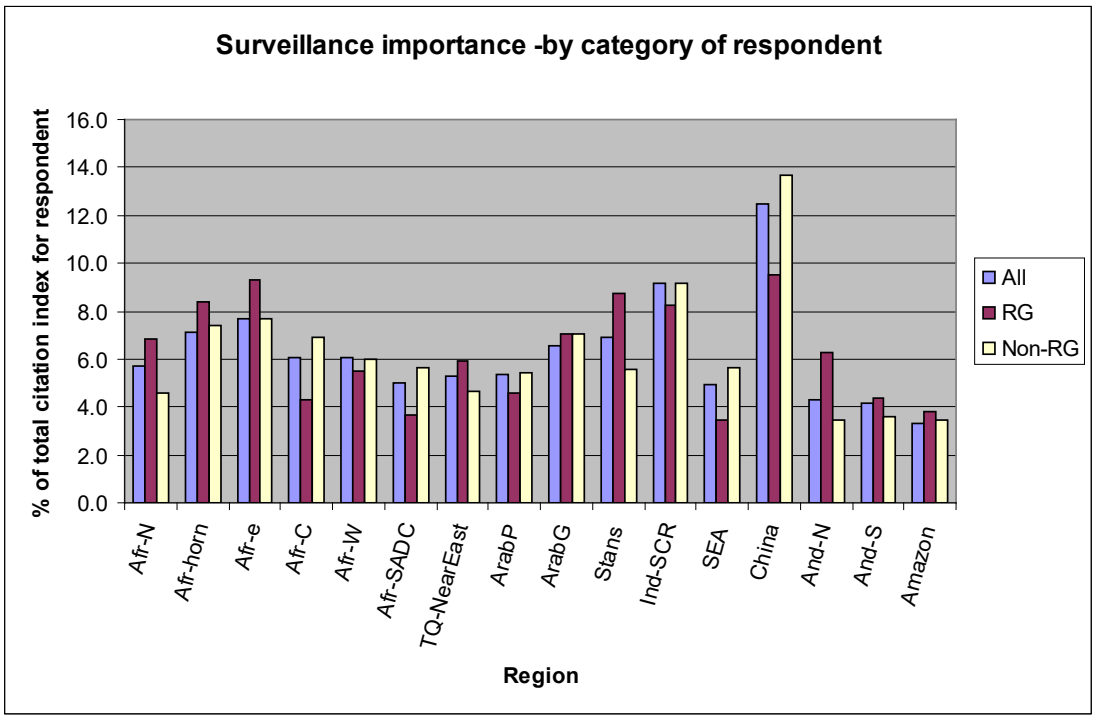


Figure 2. Perceived constraints to specimen submission –question 3.
The average score given to each form of constraint is illustrated, for the responses relating to Africa and China. Countries in the ""Other" category were mostly in India subcontinent and Central Asia.

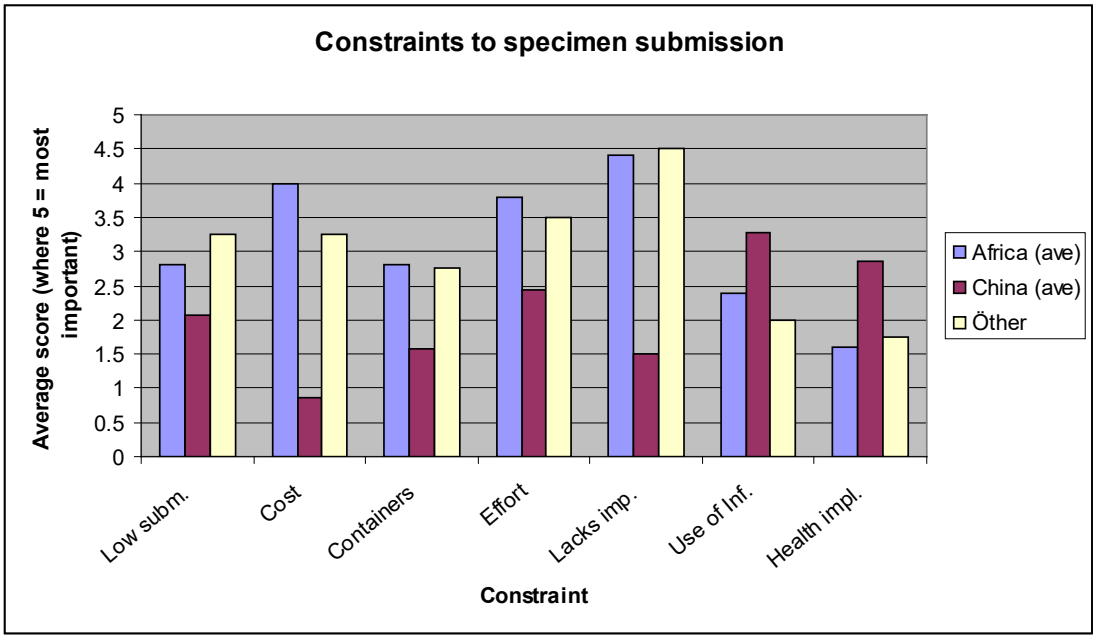


Figure 3. Bovine density map for Eurasia and Africa (FAO Livestock Atlas series)

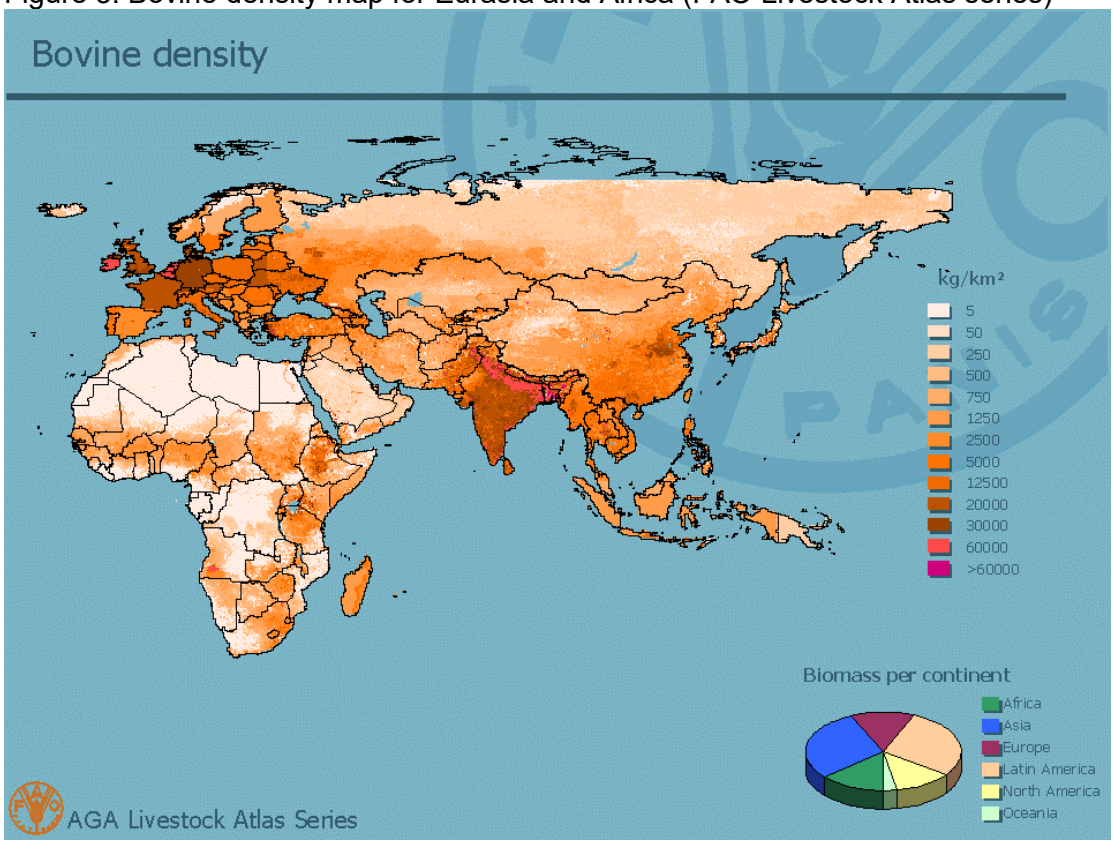
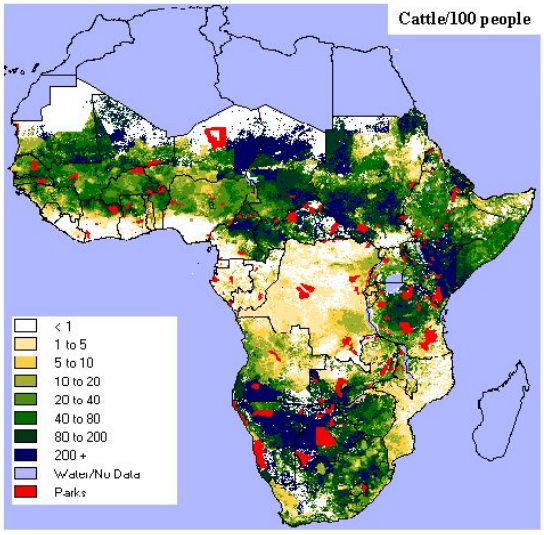


Figure 4. Rate of cattle to human population in sub-Saharan Africa (FAO Livestock Atlas Series)



Appendix 10

Initiatives in FMD risk mapping- approaches for estimating prevalence and incidence of disease in the context of global surveillance

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The purpose of this report is to introduce our new project on FMD modeling for global surveillance in three prototype countries. An objective of the presentation is to foster collaboration with international agencies and with individuals who are interested in FMD surveillance globally, as well as in the specific countries being studied.

Although the U.S. has maintained FMD-free status since 1929, the risk of FMD entering the country has increased substantially in the past several years. Moreover, should the U.S. acquire FMD, the livestock marketing infrastructure and management practices will likely promote very rapid and widespread dissemination of FMD throughout the country. In a study modeling FMD spread in three California counties, the magnitude of an hypothetical FMD epidemic was projected to be substantially –greater than that experienced in 2001 by the U.K.,[1] in part because of the considerable inter-herd[2] and intra-herd transmission[3] likely on the large, intensively managed dairy herds. It has become increasingly critical for national biosecurity, therefore, that FMD surveillance systems be developed both to detect the virus after it enters the country (*post facto* surveillance) and to predict or forecast the risk of FMD entering the country (global, preemptive risk surveillance).[4,5]

Last month we began a 'proof of principle' study of surveillance modeling to forecast FMD globally. The study is supported by the U.S. Armed Forces Medical Intelligence Center (AFMIC). The three prototype countries selected are Afghanistan, Pakistan, and Iran. The objectives of the initial stage of the project generally are to identify and capture information both directly relevant and remotely related to FMD for each country and to develop models to assess FMD incidence and prevalence and movement or change in directionality of geographic distributions -in each country.

General approach

We hope to obtain information relevant to FMD and specific times, locations, and serotype and strain type from the web or other electronic sources, including OIE and the FMD World Reference Laboratory, Pirbright, England. Information unavailable from electronic sources is being sought through officials and individuals within each country

and from organizations, such as FAO. Surrogate data that may serve as predictors or covariates for FMD also will be obtained, where possible. Examples of these kinds of data include geographic animal census information, meteorological data, soil data, transportation routes, regional distributions of ethnic groups, vaccination rates and serotypes, control programs, government funding for FMD, and number of government veterinarians assigned to FMD. We are formally seeking help in obtaining these kinds of data. In collaboration with the WRL in Pirbright, we are planning to develop, as part of the project, a public website for FMD that will provide cross-referenced information about FMD historically for all countries and, for some time to come, on a real-time basis.

It is recognized that realistically prevalence and incidence are not likely going to be estimated with any degree of accuracy, given the data available for most countries. Thus, part of our effort will be to explore how well surrogate data, such as gross national product dedicated to animal health, number of government veterinarians, provincial animal density, forage type, etc. might improve the predictability of FMD being in a particular country or in specific region of a country, based on available case data from OIE and the WRL. We are accessing remote sensed data to examine the feasibility of including other types of covariate data, such as soil moisture and vegetation.

Prevalence densities will be approximated temporally and spatially within countries, using GIS information, spatial modeling methods, and Bayesian approaches. We will be considering Bayesian approaches and survival and Markov chain Monte Carlo methods to characterize the conditional probability, for example, of a region or country having a new case of FMD in a specified time period.

Current status

We have begun characterizing FMD case data mainly for Pakistan between 1996 and 2000, which are the most recent data for which case location was reported. A few examples of some of the preliminary analyses are presented here. The annual distribution of the first outbreaks reported and the mean geographic centers of the outbreaks for each year are shown in Figure 1, in which over 50% of all outbreaks were reported where the larger points are indicated. The sequential changes in mean center suggests a general annual southwesterly movement of outbreaks. Figure 2 shows the estimated kernel density of FMD outbreaks (outbreak cases/km^2), considering all reported outbreaks between 1996 and 2000. We recognize the data will represent an under-reporting of cases, and, for now, we assume proportionally similar under-reporting throughout the country. Thus, even thought the density values might be considerably under-estimated, the densities would provide a general sense of the relative distribution of outbreaks. Availability of animal population data would permit a crude estimation of prevalence and incidence for these areas. Figure 3 presents the distribution of outbreaks relative to transportation arteries and utility grid lines, which typically parallel roads. The province-specific FMD densities for neighboring India and for Iran are shown in figure 4; we do not yet have data for Afghanistan.

Summary

We are interested in developing collaborative efforts aimed at improving the ability to predict when and where FMD can be found in the world. In particular, we are requesting assistance in obtaining information about FMD cases identified by date and location, animal density and movement, and remote sensed data for these three countries. Other surrogate data are of interest as well. Examples, would include number of veterinarians assigned to FMD in a country, the annual amount budgeted for border checks of animals or animal products, and the number of FMD vaccine doses used per year. If you have any interest in this project, or you know someone who might be, please contact Dr. Mark Thurmond, or any of the other investigators listed, by email.

Acknowledgements

We gratefully acknowledge a grant from the Armed Forces Medical Intelligence Agency (AFMIC) and support from the U.S. Department of Agriculture (USDA/CSREES).

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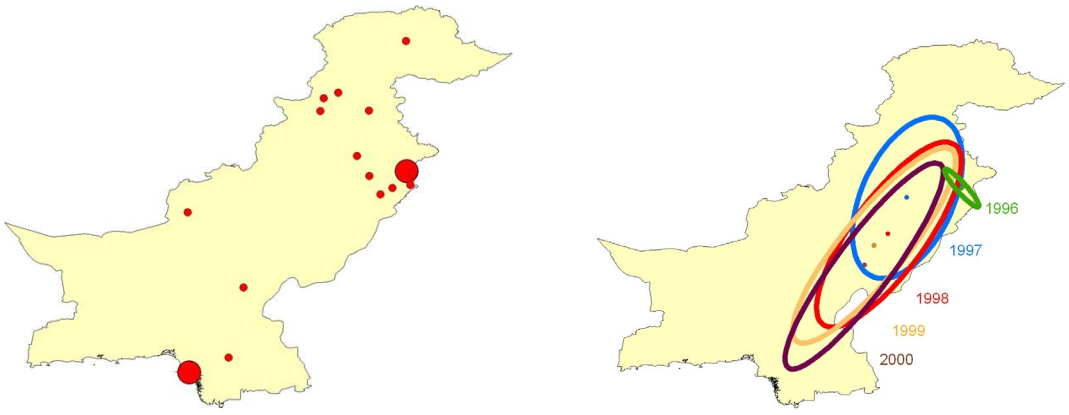


Figure 1. Distribution of FMD cases reported by Pakistan 1996-2000 (left), where at least the 50% of the outbreaks were reported where the larger dots are indicated, and geographic mean centers of outbreaks for each year, where the major axis of the ellipse would indicate directional change of a mean center (right).

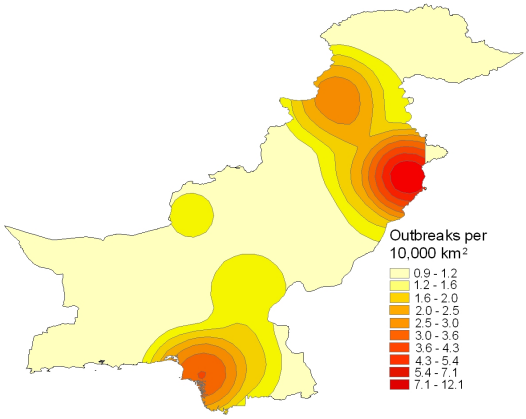
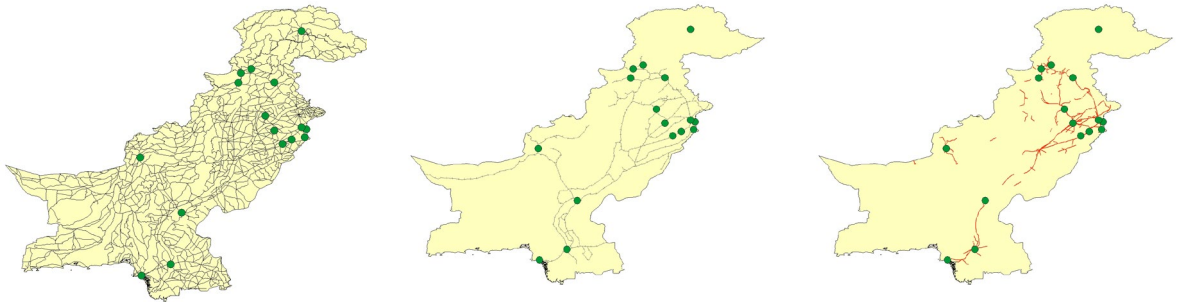


Figure 2. Kernel density estimates of FMD outbreak density for outbreaks reported by Pakistan 1996-2000.



Roads **Railroads** **Electricity grid lines**

Figure 3. Distribution of FMD outbreaks reported by Pakistan 1996-2000 and proximity to roads, railroads, and electric utility lines

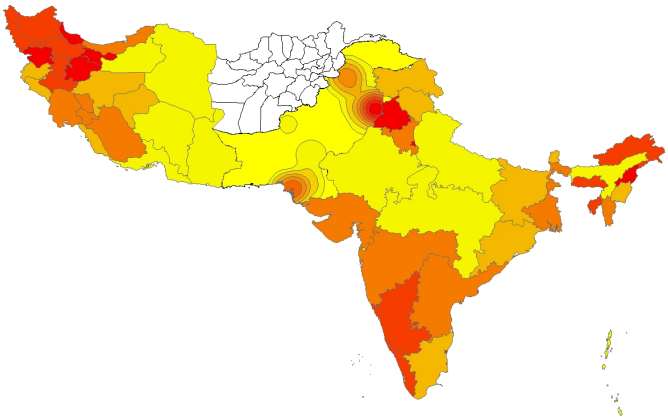


Figure 4. Province-specific FMD densities for Iran and India, and kernel density for reported outbreaks from Pakistan. FMD data for Afghanistan have not been obtained.

Appendix 11

Pilot study on FMD risk mapping in the Eurasian ruminant street: focus on Turkey

Gilbert, M., Mohammed, H, Tufan, M., Aktas, S. and Slingenbergh, J.

Summary

Turkey is important for the understanding of the spatial epidemiology of Foot and Mouth Disease (FMD) because of its geographical location, forming a bridge in the 'Eurasian ruminant street' stretching from southern Asia to Mediterranean Europe. FMD remains endemic in parts of Turkey where new strains (e.g. Asian topotype O during the mid nineties, type Asia 1 in 1999) occasionally arrive from the countries bordering to the east. Apart from the dairy cattle in the proximity of urban agglomerations in the western part of Turkey most of the ruminant livestock populations are located in the eastern part of the country. As a consequence, the main animal movement is from east to west and, similarly, so is the pattern of pathogen spread.

FMD outbreaks in Turkey are routinely recorded by the General Directorate Protection and Control (GDPC) under the Ministry of Agricultural and Rural Affairs (MARA). Serosurveillance data on type O, A, and Asia-1 are collated at province-level. These FMD records may be matched to available data sources on livestock distribution and movement (MARA and also Wint 2003, Livestock Geography Atlas, FAO), in turn influenced by the above geographical features of the demand and production of meat and dairy products. Thus, the geospatial discrepancies in production and demand help to explain livestock movement and, with it, the propagation of FMD virus.

Metapopulation ecology is a useful conceptual framework for the analysis and modelling of spatial and temporal patterns of diseases (see Grenfell & Harwood 1997). It assumes that the population is made of individual sub-populations connected by dispersal. In this framework, the presence or absence of an organism in an area is mostly determined by the dynamic within the area that may lead to local extinction (e.g. demographic, environmental stochasticity), and the recolonisation relies on individuals coming from occupied areas connected by dispersal (Hanski & Gilpin 1997). In spatial epidemiology, this would translate into a probability of disease persistence in an area, and in a probability of new infection in areas were the disease is absent. If we apply this concept to FMD epidemiology, one can assume firstly that disease persistence in an area would be dependent on the local health management, animal husbandry and other features of the production environment, and secondly that new infection would be a two-scale process: direct contact plus short to medium range windborne contagion between herds sharing the same grazing areas, and long-distance contagion aided by the transportation of animals or infectious material from infected areas. In the present context, long-distance transportation involving surface transport of live animals is believed to be instigated by the commercial food chains, linking production surplus with high demand centres.

A preliminary step prior to develop models is to assess if the observed spatial and temporal patterns of disease distribution are compatible with the assumptions underlying the metapopulation approach. Firstly, if the disease persists in time, then a significant correlation

between the disease status at year n, and disease status at year n+1 should be observed, i.e. temporal autocorrelation. Similarly, in presence of spatial contagion, a correlation between the status of province n, and the status of the neighbour provinces is expected, i.e. spatial autocorrelation. The first step, is thus to explore temporal and spatial autocorrelation in disease status. The correlogram is a plot that describe the decrease in correlation (increase of the 1-ρ(h) function) as a function of the distance or duration between observations. Typically, it is a rising curve (Figure 1), having low values (high r) for observations separated by short distances (short period of time), and that increase up to a distance (or duration) upon which no correlation is observed between observations (see Rossi et. al. 1992 for a detailed description of the use of correlograms in ecological modelling).

The temporal correlograms of FMD outbreaks in Turkey throughout 1990-2002 is shown in Figure 2. The correlograms have been treated separately for the period 1990-2002, and for the period 1997-2002 because the rather versatile Asia topotype O is believed to have entered Turkey in the course of 1996. In fact, FMD type O showed a strong temporal autocorrelation over long period of time (1-4 years), which means that observations separated by several years are significantly correlated. In contrast, FMD type A showed very weak sign of temporal autocorrelation, indicating that the number of outbreaks at each year is nearly independent of past outbreaks. In type Asia-1, there is an indication of a correlation for observations separated by 1 (one year to the next), or 3 years for the period between the beginning and the end of the outbreak. Spatial correlograms (Fig. 3 a, b, c) of the sum of FMD outbreaks during 1990-2002 and 1997-2002 respectively indicated a significant autocorrelation in FMD type A up to a distance of 2 decimal degrees (approx. 175 km), up to a slightly shorter distance in FMD type O (note the difference in spatial pattern between the 1990-2002 period and 1997-2002 period), and very few signs of spatial autocorrelation in FMD Asia-1. Modelling the spatial autocorrelation in the sum of outbreaks allow to derive spatial interpolation using a method known as kriging (Isaaks & Srivastava 1989). This method was used to map the interpolated sum of outbreaks of FMD type A, type O, and Asia-1 throughout 1997-2002 (Fig. 4 a,b,c). These maps allow visualising the distribution of hotspots located mainly in Ankara (Type A, O, Asia-1), Erzurum (type A, O, Asia-1), Sivas (Type O, and to a lesser extent, type A), and Kayseri (type A). Given that the above clumping of outbreaks by serotype negates the existences of individual topotypes, each behaving distinctly differently in geographical terms, it could well be that the hotspots shown do not so much form predilection sites for different serotypes as well as indications for the likely places any type of infectious disease pathogen would spread to.

In summary, the exploratory analysis of spatial and temporal patterns in FMD outbreaks in Turkey over the 1997-2002 period show that evidences of disease persistence pattern (as indicated by significant temporal autocorrelation), of disease contagion (as indicated by spatial autocorrelation), and of the effect of long-distance contagion (as suggested by hotspots located in areas with high consumption and trade) are present at various degrees in the data, and allow developing the metapopulation model integrating these three main factors (persistence, short and long distance contagion).

This model is under development, and would eventually assist the prediction of disease spread given that FMD risk appears a function of past and present distribution of disease status, which relates in turn to long-distance movements of animals, triggered by geospatial discrepancies in production and demand of animal protein commodities. Of course, this approach may also be explored for other countries in the region. In fact, work is in progress for the analysis of FMD in Iran.

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Figure 1. Typical correlogram of spatially (temporally)-structured data, starting at high correlation (low value) for observation separated by short distance (period of time), and reaching the plateau of 1 for observations separated by high distances (period of time).

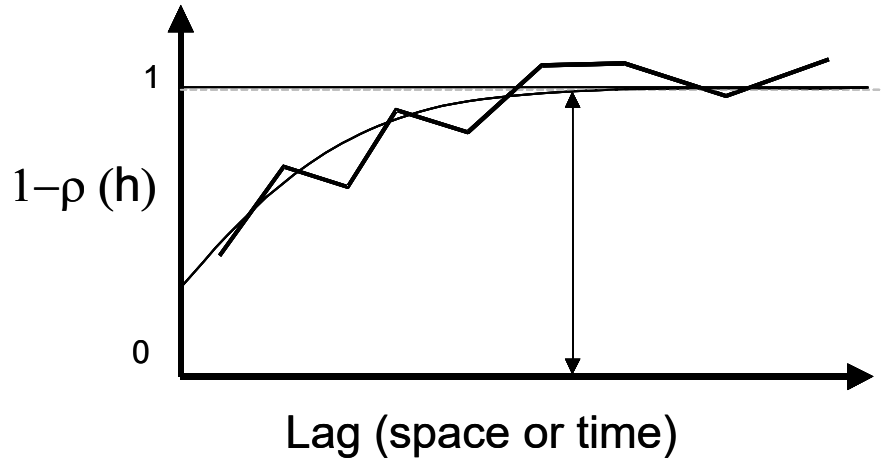


Figure 2. Temporal correlograms of FMD disease outbreaks in Turkey grouped by different serotypes and analysis period.

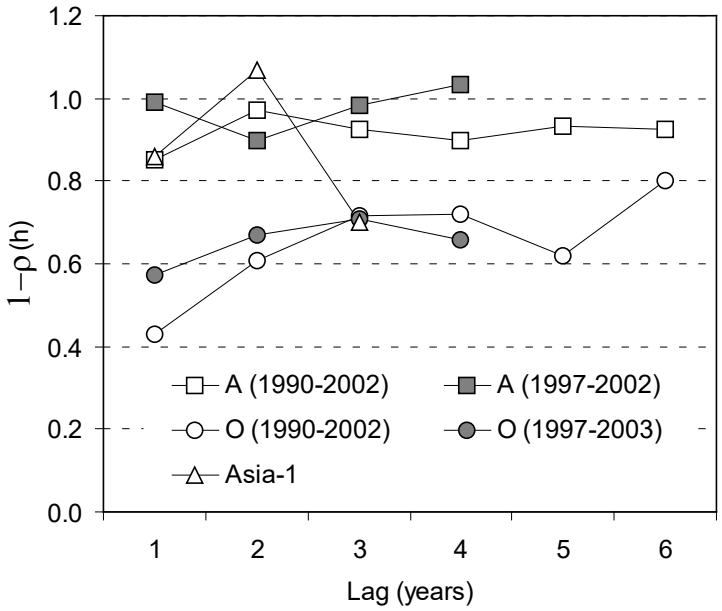


Figure 3. Spatial correlograms of the sum of FMD disease outbreaks in Turkey grouped by different serotypes and analysis period.

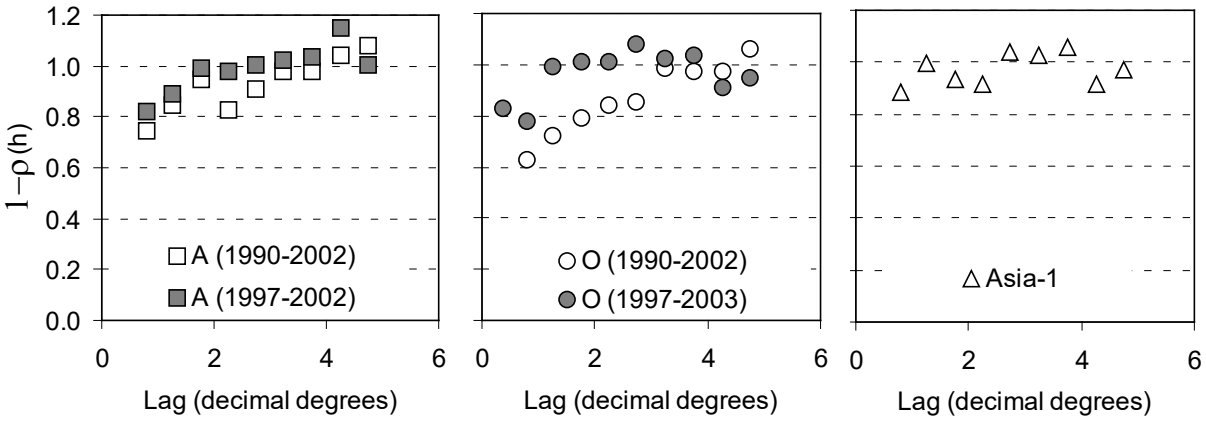
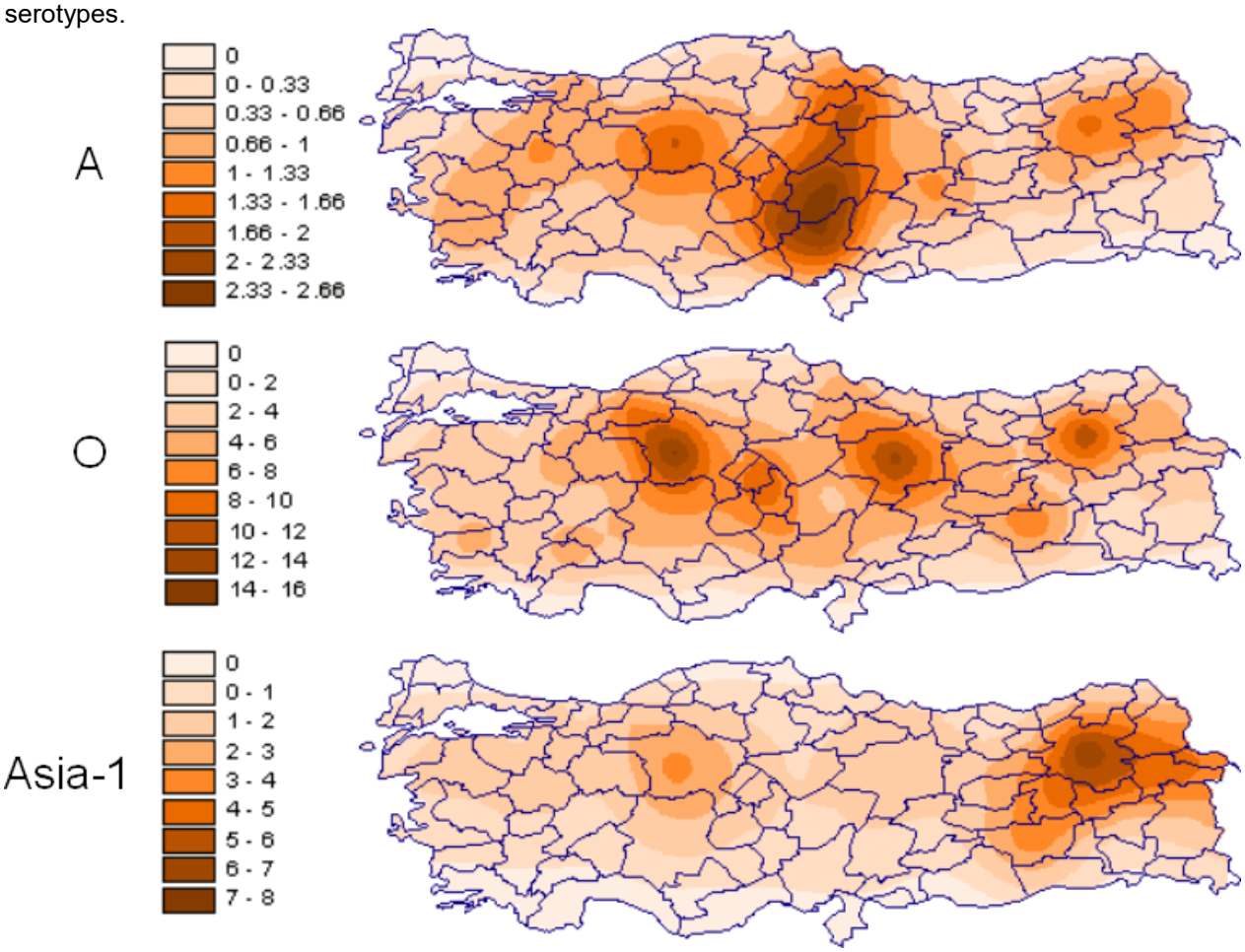


Figure 4. Sum of FMD outbreaks throughout 1997-2002 interpolated by ordinary kriging, grouped by serotypes.



Appendix 12

The development of nucleic acid based reference standards

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Reference materials (RM) are basically designed for the calibration of instrumentation or a measurement procedure, the verification of trueness and precison of a specific measurement procedure, or to assign a value to in house quality assurance materials. RM are necessary to develop and to validate (new) analytical measurement procedures and to verify the correct application of a (new) analytical measurement procedures. Hence, reference materials are a means of support for the precise definition of legal limits and a means to control the correct implementation of regulatory obligations. Their development, production and certification are described in great detail in multiple guides (e.g. ISO Guide 34 and ISO Guide 35) giving clear indications of the critical steps in this highly complex process. Referring measurements to a common reference method or material leads, if designed appropriately, to an improvement of a measurement results.

It is of major interest for the EU to support the development of RM that allow the necessary comparability and traceability for the implementation and the monitoring of European legislation. The Institute for Reference Materials and Measurements of the European Commission has a large experience in the development, certification, maintenance and distribution of RM. These materials belong to several categories of application, like environmental RM, materials for industrial production processes, RM certified for chemical or nuclear analysis and others.

Which are the most important general criteria to consider in the development of a suitable reference system?

Generally, reference systems consist of reference methods and reference materials. All available relevant expertise should be at the basis of the design of any reference system. Apart from the application of basic metrological principles, further knowledge on underlying biological principles, analyte properties and matrix influence must be kept in mind to allow the development of a commutable standard.

The certified property of a standard should ideally be traceable either to a SI unit or to other internationally accepted units.

The measurement procedure needs to be well understood to include as many contributions as possible to describe an uncertainty, which could be determined through an assessment by measurements (Type A) or through estimations based on long term experience (Type B).

A reference measurement procedure generally requires a validated method and a standard operating procedure to be followed strictly. All measurements that affect

results must be traceable to SI or to other internationally accepted units. Method performance studies require homogeneous and stable validation samples containing the analyte at defined levels. Characterisation measurements must be carried out under reproducible conditions to possibly identify within laboratory variation. All measurement procedures should be under the control of a quality assurance system.

Referring measurements to a common reference method or material leads generally to an improvement of a measurement results.

The certified value of a RM and its uncertainty have to be representative for a complete batch and therefore homogeneity and stability of the material must be properly assessed.

The material properties could be certified with respect to a SI unit or another accepted unit using a primary measurement method, or through an interlaboratory comparison. For the latter, one should consider using different methods with independent calibrations to reduce the probability that all laboratories produce the same systematic error.

The RM should be commutable, i.e. the ability of material to show inter-assay properties comparable to routine samples analysed by the assay.

Finally, a highly structured and co-ordinated approach involving all relevant stakeholders is required to setting up reference systems. This can be achieved only on the basis of international co-operation. The assignment of responsible reference laboratories might be appropriate in order to ensure proper execution of complex reference methods requiring highly skilled laboratories.

IRMM started some years ago with the development of new 'biotechnology related' RMs, in a first instance for the detection and quantification of genetically modified organisms (GMO) in food. These materials consist of a freeze - dried stabilised powder of e.g. seeds of GM plants in powder of seeds of non - GM plants and are designated for use in PCR based methods. As a consequence, and basically due to the challenges encountered with these powders, IRMM currently develops a new generation of nucleic acids based RM. These include so far plasmids, linear PCR products or genomic DNA for the detection and quantification of GMO and the detection of food pathogens. In addition, several RM based on dried genomic DNA of the major bacterial food pathogens for a qualitative detection in PCR are currently certified by IRMM.

Challenges related to the development of a reference system for the molecular detection of FMD virus

The low virus load in samples and the cultivation period makes it an attractive goal to use sensitive molecular techniques to accelerate the diagnosis for the presence of FMD virus in a sample.

The development of a RNA based standard for the reliable (qualitative) detection of the FMD virus is certainly a major challenge and does not totally meet our experience in developing DNA calibrators for the quantification of a specific GMO content using real-time quantitative PCR methods. But many challenges encountered in the GMO

RM development apply to any approach for the preparation of nucleic acid based standards.

However, the following points should be considered:

1. The source of the amplicon, intended use, definition of the analyte
Is it to develop a DNA standard (viral cDNA, linearised or in circular plasmid) and to correlate it to a viral RNA concentration or is it preferable to develop stable RNA molecules as direct calibrators for reverse transcription measurements. Should it be a strain specific or generic molecule for general detection? Ideally, the material should be commutable for all detection methods under discussion.

2. The extraction procedure
Matrix effects and extraction effects can have an important impact on subsequent analysis in molecular amplification methods. A validated extraction method is generally recommended.

3. Stability and storage
The stability of the material at a defined temperature in defined environment must be monitored and assessed. The water content, saturation with inert gas, light sensitivity and other parameters must be included in a stability assessment. The material should be stored at centralised and supervised facilities and the material should be dispatched in an agreed manner meeting all requirements to guarantee stability and hence the validity of the certificate.

4. Homogeneity
The standard must be homogenous within a single unit and between different units. The measurement repeatability influences the minimum heterogeneity that can be detected. The homogeneity should therefore be determined with a method creating the lowest possible coefficient of variation. The homogeneity assessment is especially important and complex when preparations (e.g. independent standards for different strains) are mixed (e.g. DNA in DNA).

5. Matrix effects
Any known matrix effects must be considered when choosing applicable detection methods. The reference material must be commutable for any chosen method.

6. Production and production control, recovery
Nucleic acids bind to different matrices. The type of recipient has an influence on the recovery of stabilised, dried DNA and leads thus to enormous variation in subsequent measurements. Any other manipulation step during the production must be monitored with control measurement, preferably with independent primary methods. Degradation is one of the major problems to consider.

7. Ring trials
Co-ordinated ring trials could be designed for the validation of a standard operating procedure of a reference method to be implemented or for the assignment of a value to a commutable reference material.

8. Data treatment

All data should be stored in a central file and the results should be discussed in the presence of all participants. Outliers must not be removed on statistical ground only or if simply no technical reason can be identified. The value for certification can be calculated after techncial scrutiny of the data, provided the quality of the remaining data allow that.

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EXTERNAL QUALITY CONTROL OF MOLECULAR DIAGNOSTIC METHODS

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EXTERNAL QUALITY CONTROL ?

- Integral part of a laboratory quality management system
- Detection of weak spots in performance
- Improvement of reliability of results, gives confidence
- Comparison of performance with other laboratories
- Provision of uniform reference level when International Standards are lacking
- Expensive, but saves money

External Quality Control in Europe : 1998

- No uniform regulatory system for medical laboratories across Europe
- Most countries have QC organisations for external proficiency testing (NEQAS, INSTAND, EQUALIS, SKMM etc.)
- Differences in approaches, but regular proficiency testing mainly focused on (virus)culture and serology.
- Lack of International Standards or reference reagents for molecular testing
- High rate of false-positives (> 20 %); sensitivity often unclear
- European Union Concerted Action for Quality Control (EU-QCCA)of Molecular Methods in Diagnostic Virology : Start in March 1998
- Endorsement by European Society for Clinical Virology and major diagnostic industries (ILC)

European Union Concerted Action for Quality Control of Molecular Diagnosis in Diagnostic Microbiology (EU-QCCA)

AIM

- To establish an external quality control programme for assessment and evaluation of existing and evolving nucleic acid amplification procedures in diagnostic virology

STRATEGY

- Preparation and distribution of proficiency panels
- Organisation of (virtual)post-distribution workshops
- Production of reference materials e.g.‘run controls’

EU-QCCA PROFICIENCY PANELS

- Purpose : to help labs to determine their own performance
- Composition determined by QCCA Working Groups
- Consist of 8 - 12 samples
- Include different sero/genotypes at various concentrations
- Preferably inactivated and freeze-dried to improve stability and facilitate shipment
- Reporting time 4 - 6 weeks
- Accompanied by questionnaire on technical details
- Results of distribution published in detailed reports and scientific publications

EU-QCCA Proficiency Panels : Number of Participants

Agent	1998/99	2000	2001
Enteroviruses	63	52	79
HSV	71	75	86
CMV	-	-	98
HBV	46	62	-
HCV	63	91	-
HIV	58	76	-
Chlamydia trachomatis	-	105	-
TB	-	-	(80)

Enteroviruses

Viral Diagnosis

- most frequent cause of aseptic meningitis
- virus culture (CSF) increasingly replaced by NAT's
- NAT'S mostly in-house developed
- sensitivity and specificity often unknown

Research

- Association reported with chronic conditions as ALS, chronic fatigue syndrome, post-polio syndrome

Enterovirus proficiency panels

- Panels consisting of 12 samples
- Include various serotypes / genogroups (prototypes/ clinical isolates)
- Include dilution series (PBS/ 5% FBS)
- Consist of inactivated, freeze-dried culture supernatants
- Production at UMC Utrecht; freeze drying at SVM
- Predistribution testing at 2 reference laboratories
- Shipment at ambient temperature, non-hazardous goods
- Each panel accompanied by technical questionnaire

Distribution characteristics

	1998/99	2000	2001
Participating laboratories	63	52	79
Number of countries	17	15	19
Reporting laboratories	59	46	68
Reported datasets	70	50	81
Evaluable datasets	69	47	78

FIRST EV PANEL: COMPOSITION AND RESULTS

Code	Virus	Titre		Reference labs		Participants (n=70)
		TCID50/ml	Geq/ml*	1	2	No positive
A01	EC9	10^4	10^7	+	+	69
A02	EC9	10^2	10^4	+	+	60
A03	Neg	-	-	-	-	2
A04	EC22	$3.2x10^4$	N.D.	-	-	3
A07	Neg	-	-	-	-	3
A08	PV2	$1.6x10^3$	10^4	+	+	64

* : Based on experimental QPCR for CB3 (P. Muir)

FIRST EV PANEL: COMPOSITION AND RESULTS

Code	Virus	Titre		Reference labs		Participants (n=70)
		TCID50/ml	Geq/ml	1	2	No positive[$]
A05	CA9	$4x10^{2}$	10^{7}	+	+	69
A12	CA9	$4x10^{2}$	10^{7}	+	+	69
A06	CA9	40	10^{6}	+	+	63 (62)
A09	CA9	4	10^{5}	+	+	55 (54)
A10	CA9	$4x10^{-1}$	10^{4}	-	+	30 (28)
A11	CA9	$4x10^{-2}$	10^{3}	-	-	10 (8)

* : Based on experimental QPCR for CB3 (P. Muir)
$: Between brackets: No of correct dilution series

EV FIRST PANEL: QUALITATIVE ANALYSIS

Type of sample	Code	No of correct data sets (n=70)
Strong positive	A01, 05, 06, 08, 12	59 (84%)
Positive	A02, 09	52 (74%)
Weak-positive*	A10, 11	31 (44%)
Negative	A03, 07	66 (94%)
Strong positive, positive, negative	see above	46 (66%)

*: one or both samples

1st EV PANEL: SCORE VS. TYPE OF ASSAY

SCORE	Type of PCR				
	Single (n=15)	Semi-nested (n=11)	Nested (n=26)	Roche (n=16)	TOTAL (n=68)
11	1	1	4	1	7
10	3	2	11	2	18
9	4	2	5	9	20
8	-	3	-	-	3
7	3	1	3	3	10
<7	4	2	3	1	10
Median	8-9	8-9	10	9	9

PERFORMANCE SCORES ON QCMD EV-03

ASSAY	n	10- 11	9	8	7	<7	MEAN
Single PCR	20	10	3	-	2	5	8.5
Semi-nested PCR	11	5	-	2	1	3	7.9
Nested PCR	25	11	7	1	2	4	8.5
Real-Time PCR	6	2	1	-	-	3	6.8
NASBA	3	1	1	-	-	1	7.0
Other commercial	11	8	2	-	-	1	9.4

SCORE VS EXTRACTION METHOD : EV- A PANEL

Extraction method	N	Score (points)		
		≤ 8	9	≥ 10
Chaotropic agent	10	I	ΓΓI	ΦI I I I I
Silica extraction	6	ΓΓΦI	Γ	I
Proteinase K	2	Γ		Γ
QIAmp Viral RNA	14	Γ	ΓΦΦI	ΓΓΦI I I I I I
Trizol reagent	5	ΦΦ		I I I
TRI reagent	4	ΦΦI I		
RNAzolB	3	ΓI		Γ
High pure isolation	3	Γ	I	I

Γ : single PCR Φ: semi-nested PCR I : nested PCR

SCORE VS REVERSE TRANSCRIPTASE: EV- A PANEL

RT Enzyme	N	Score (points)		
		≤ 8	9	≥ 10
AMV	15	ΓΓΓΦΦΦ	Φ|	ΓΦ| | |
MMLV	25	ΓΦΦ| |	ΓΓΓΦ| |	ΓΓΦ| | | | | | | | |

Γ : single PCR Φ: semi-nested PCR | : nested PCR

SCORE VS DETECTION METHOD : EV - A PANEL

Detection Method	N	Score (points)		
		≤ 8	9	≥10
Gelelectro-phoresis	35	ΓΓΦΦΦΦΦ | | | | |	ΦΦ| |	ΓΦΦΦ| | | | | | | | | | |
Hybridisation	16	ΓΓΓΓΓΦ	ΓΓΓΓ|	ΓΓΓ| |

Γ : single PCR Φ: semi-nested PCR | : nested PCR

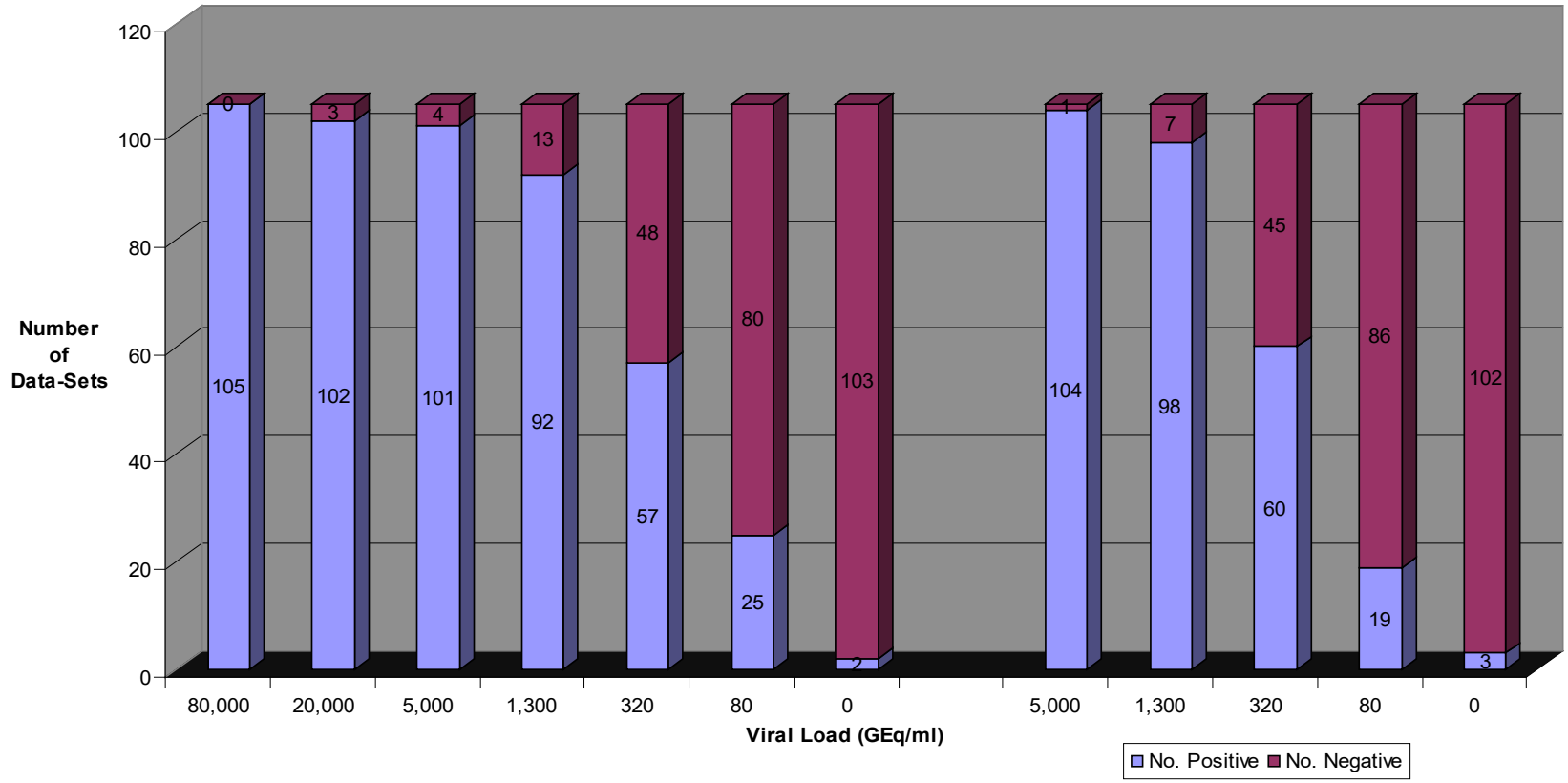
QCMD Proficiency Testing : False-Positivity rate

Agent	% In-house assays	1998/99	2000	2001
Enteroviruses	76 – 95	3.6%	4.0%	1.2%
Herpes simplex virus	89 – 95	4.8%	8.1%	5.8%
Human immunodeficiency virus	8 – 11	2.4%	1.5%	-
Hepatitis B virus	39 – 44	5.3%	1.4%	-
Hepatitis C virus	17 – 24	1.2%	0.9%	-
Cytomegalovirus	72	-	-	2.4%
Chlamydia trachomatis	9	-	-	1.0%

SCORE VS TYPE OF ASSAY: HIV

Assay	Panel 1 No.	Panel 1 Score 8	Panel 1 Score 7	Panel 1 Score ≤6	Panel 2 No.	Panel 2 Score 8	Panel 2 Score 7	Panel 2 Score ≤6
Commercial								
bDNA	*7*	4	1	2	*8*	8	-	-
NASBA	*7*	0	4	3	*8*	1	2	5
RT-PCR	*23*	9	11	3	*24*	9	6	9
	19	17	2	-	*32*	27	3	2
Hybrid Capture II	*-*	-	-	-	*1*	-	-	1
In-house								
Qualitative	*5*	2	2	1	*6*	1	2	3
Quantitative	*2*	-	1	1	*-*	-	-	-

Participant Results on First QCCA Cytomegalovirus Proficiency Panel- Qualitative (n = 105)



QCMD - CMV01: Scores on Qualitative Results

ASSAY	n	12-11	10	9	8	7	<7
Qualitative							
• in-house	57	9	9	19	12	6	2
• commercial	14	3	1	4	5	1	0
Quantitative*							
• in-house	17	7	6	1	1	1	1
• commercial	12	1	2	4	3	2	0

* : Quantitative results were converted to pos/neg results before scoring

PROBLEMS IN PROFICIENCY TESTING OF MOLECULAR DIAGNOSTIC ASSAYS: EXPERIENCE EU-QCCA

- Lack of international standard preparations
- Logistics : shipment / payments
- Inactivation/freeze drying (f.e. HBV, HCV)
- Panel samples to resemble clinical material (EV, HSV)
- Timely, high quality reporting to participants
- Achieving required level of quality/professionalism/QC system
- Costs

European Union Concerted Action for Quality Control of Nucleic Acid Amplification in Diagnostic Virology

STATUS (August 2001)

- Third year of program: EU sponsoring would stop by September 2001; at the time, no suitable EU program identified for sponsoring (5th FP: QL, Growth; DG-SANCO)
- Request for continuation (ESCV, ESCMID, Industry)
- Need for further expansion of the program and increase in professionalism
- Business plan prepared, including grants from Industry
- QCMD : Quality Control *for* Molecular Diagnosis

QCMD STRATEGY

- Expand distributions : more pathogens and applications i.e. detection, quantitation, genotyping and sequencing, and more countries
- Develop International Standards and Working Reagents, partly in collaboration with other organisations
- Set up an interactive web-site to present and promote the programme, to collect, publish and discuss results of distributions, and to provide a forum for technical and scientific discussions (www.qcmd.org)
- Present the programme and its results at scientific meetings, in scientific journals and to accreditation bodies etc.

REQUIREMENTS FOR AN EXTERNAL QUALITY ASSESMENT ORGANISATION

- **Independency** : a not-for-profit organisation without commercial, financial or professional conflicting interests

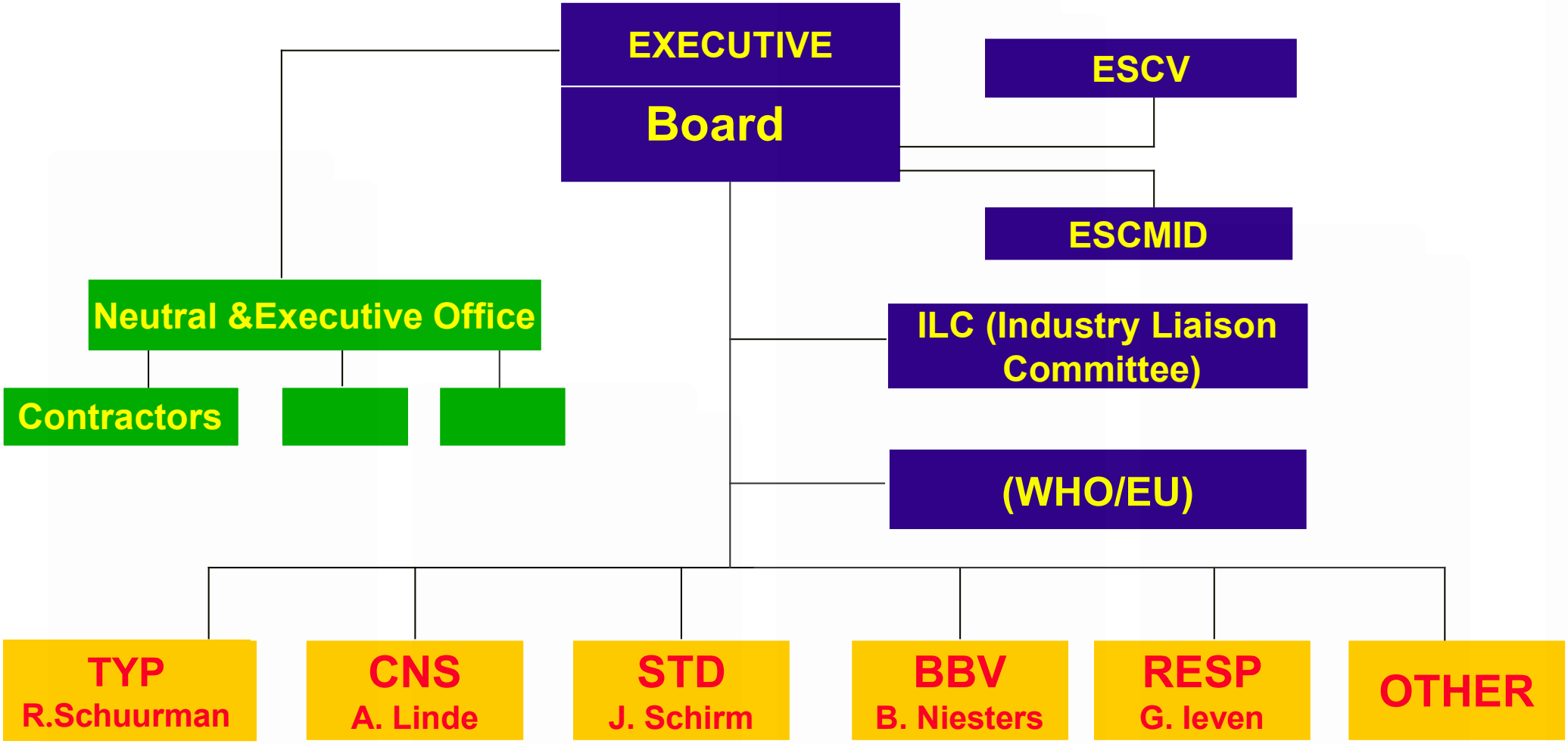
- **Transparancy** : clear structure of organisation and of responsibilities

- **Reliability** : confidence in independent judgement

- **Respectability** : organisation managed by experts in the field

- **Confidentiality** : establishment of a neutral office

- **Quality** : quality management system, compliance with international guidelines/directives (ISO, CEN, EU)

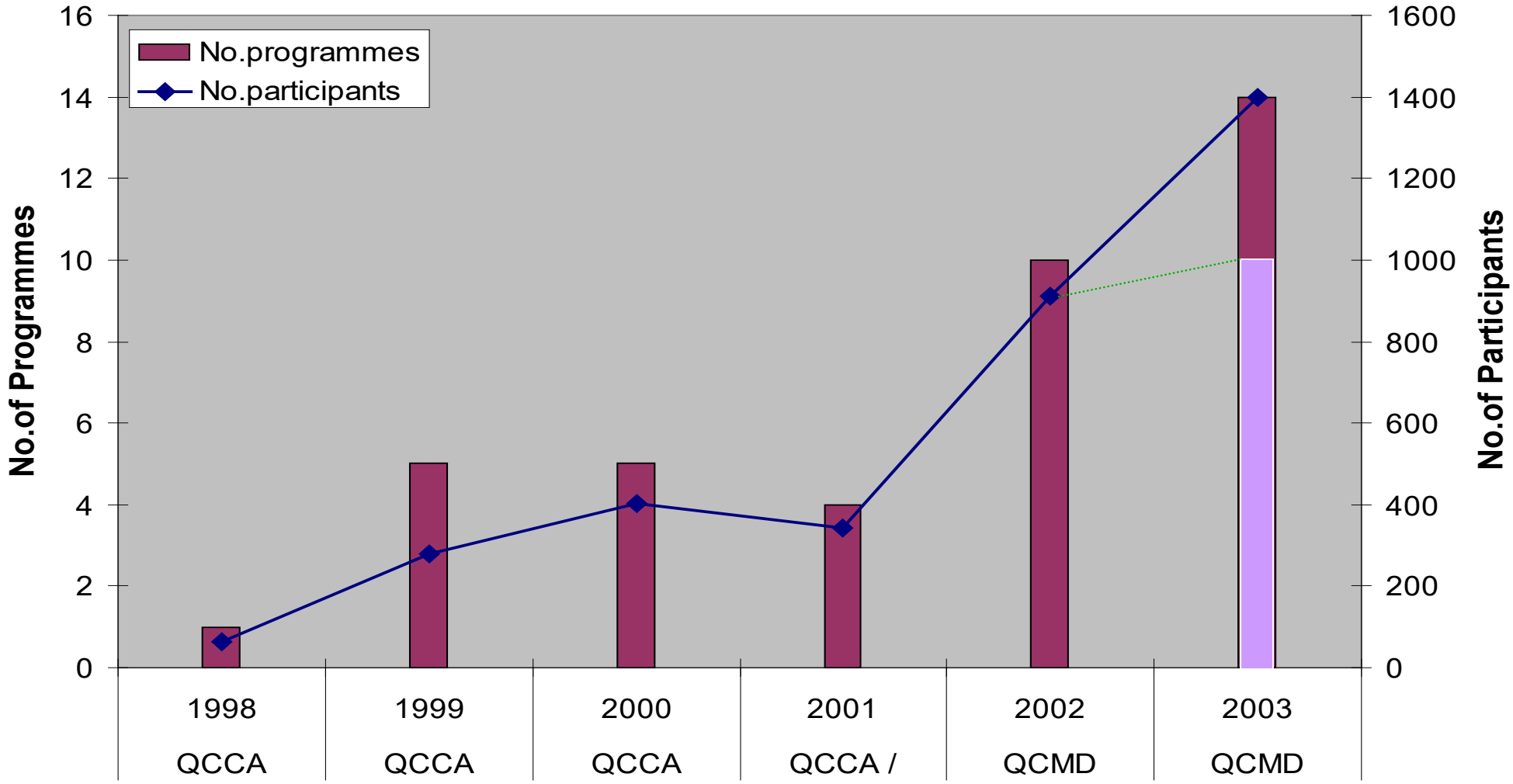

EXECUTIVE
Board
ESCV
ESCMID
Neutral &Executive Office
Contractors
ILC (Industry Liaison Committee)
(WHO/EU)
TYP
R.Schuurman
CNS
A. Linde
STD
J. Schirm
BBV
B. Niesters
RESP
G. Ieven
OTHER

Quality Control *for* Molecular Diagnostics

WWW.QCMD.ORG

EXECUTIVE	:	Graham Cleator, Paul Klapper, Jim Reid, Anton M. van Loon (chair)
OFFICE	:	Paul Wallace
BOARD	:	Executive members Chairs WP's Representatives ILC, ESCV, ESCMID, major collaborating institutions

Annual Program & Participant numbers



An International QC Programme

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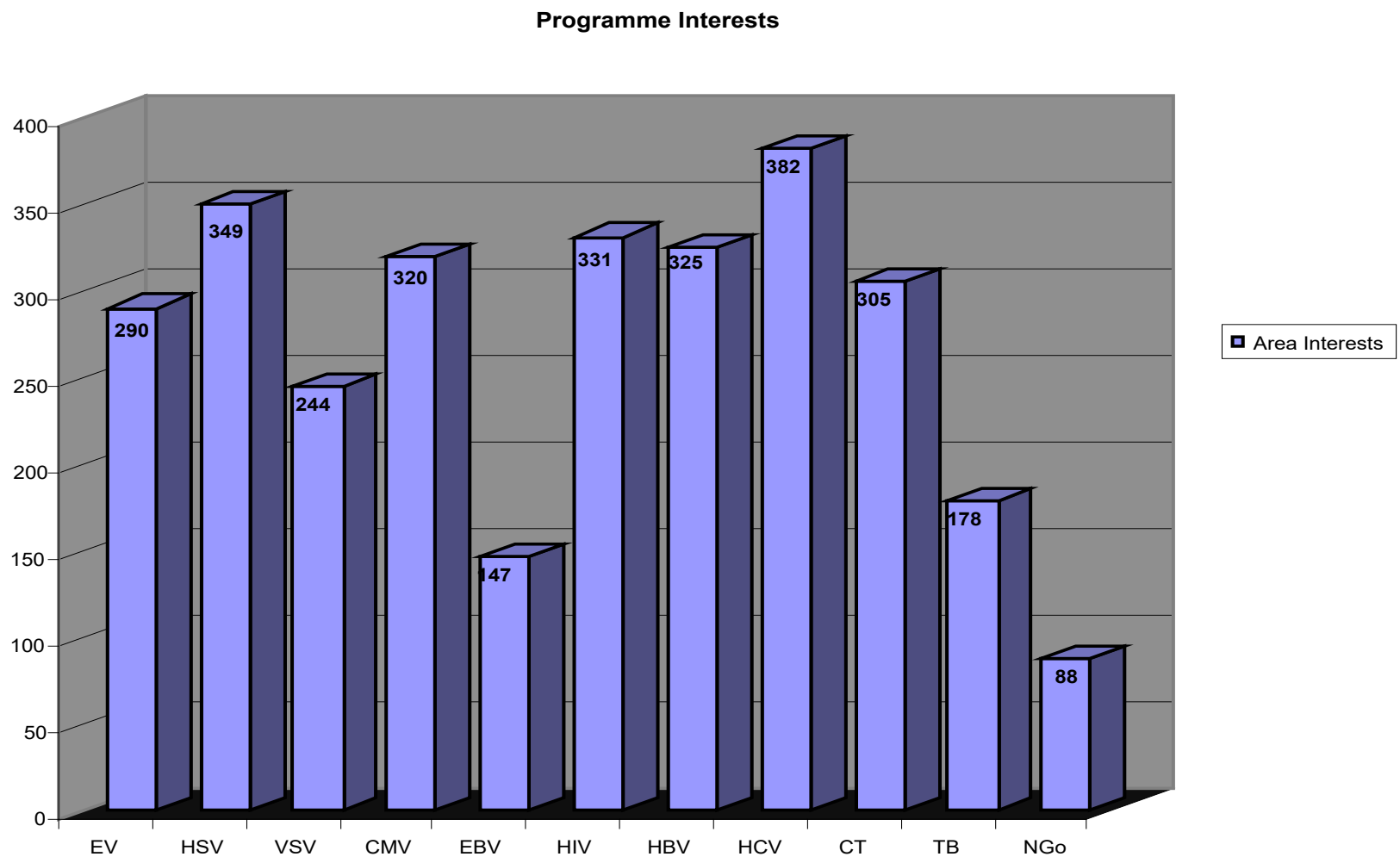
QUALITY CONTROL for MOLECULAR DIAGNOSTICS



The QCMD programme is organised in collaboration with the European Society for Clinical Virology and the European Society for Clinical Microbiology and Infectious Diseases.

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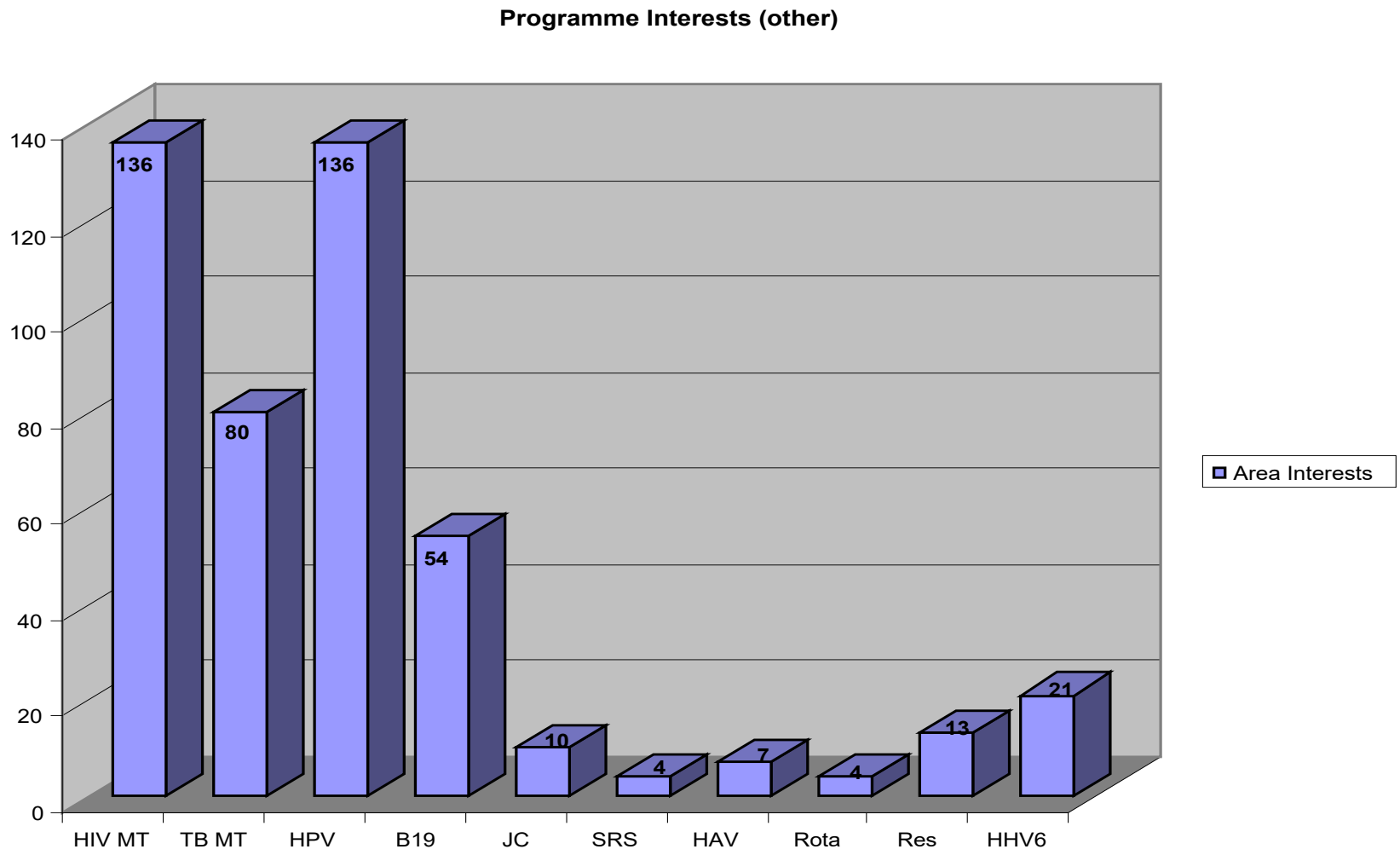
www.qcmd.org

QUALITY CONTROL for MOLECULAR DIAGNOSTICS



The QCMD programme is organised in collaboration with the European Society for Clinical Virology and the European Society for Clinical Microbiology and Infectious Diseases.

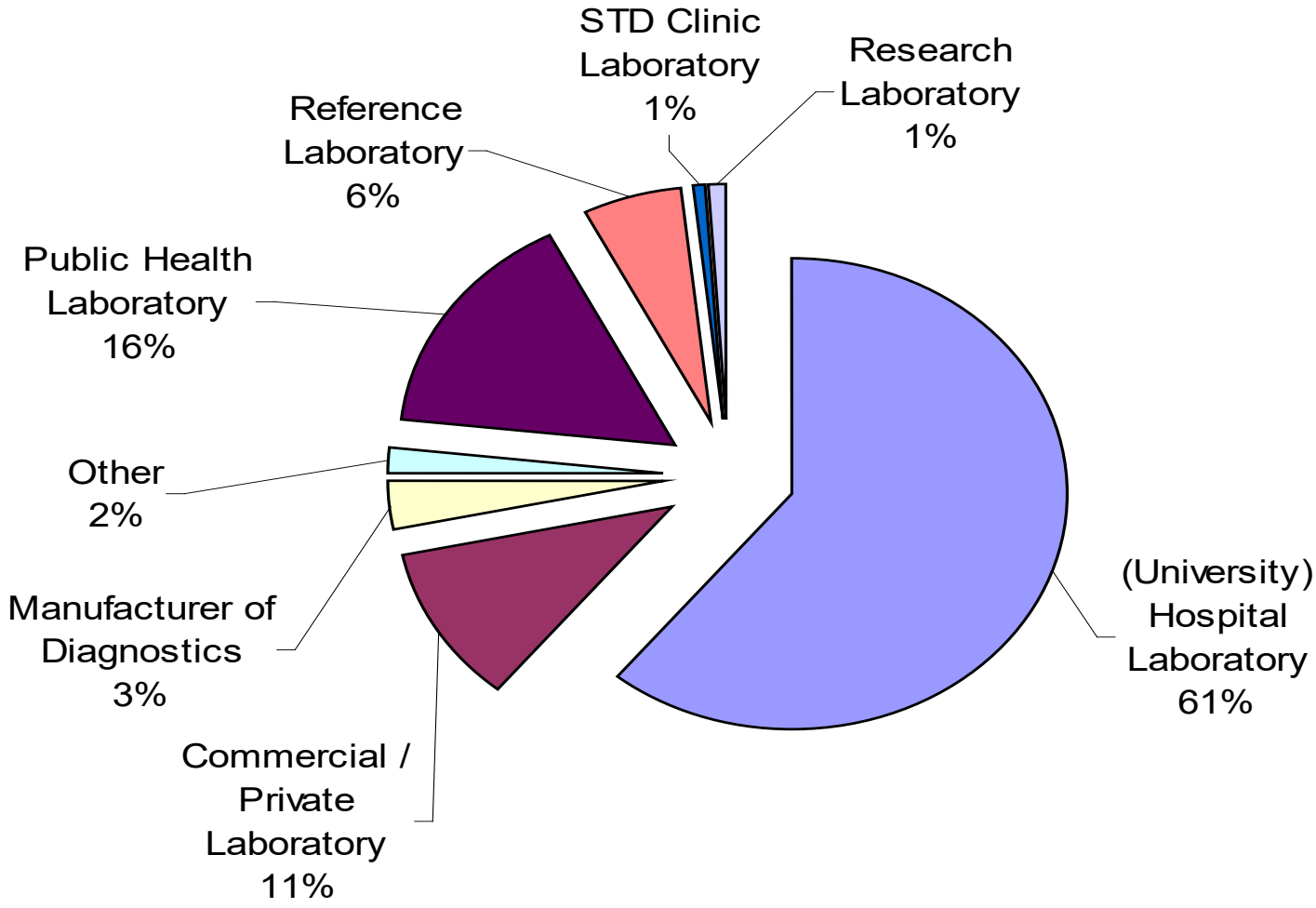
The number of Participants for each distribution / year:

Programme	1998	1999	2000	2001	2002	2003
Enterovirus	63	52	x	77	105	101
Herpes simplex virus	x	68	73	86	102	103
Human immunodefiency virus	x	54	77	x	69	90
Hepatitis B virus	x	45	61	x	73	79
Hepatitis C virus	x	59	88	x	83	92
HCV genotype	x	x	x	x	x	60*
Chlamydia trachomatis	x	x	105	x	119	122
Mycobacterium tuberculosis	x	x	x	82	86	+
Cytomegalovirus	x	x	x	98	96	70*
HIV resistance genotyping	x	x	x	x	102	+
Epstein Barr virus	x	x	x	x	75	+
Varicella zoster virus	x	x	x	x	x	91
Neisseria gonorrhoeae	x	x	x	x	x	+
Legionella pneumophila	x	x	x +	x	x	+

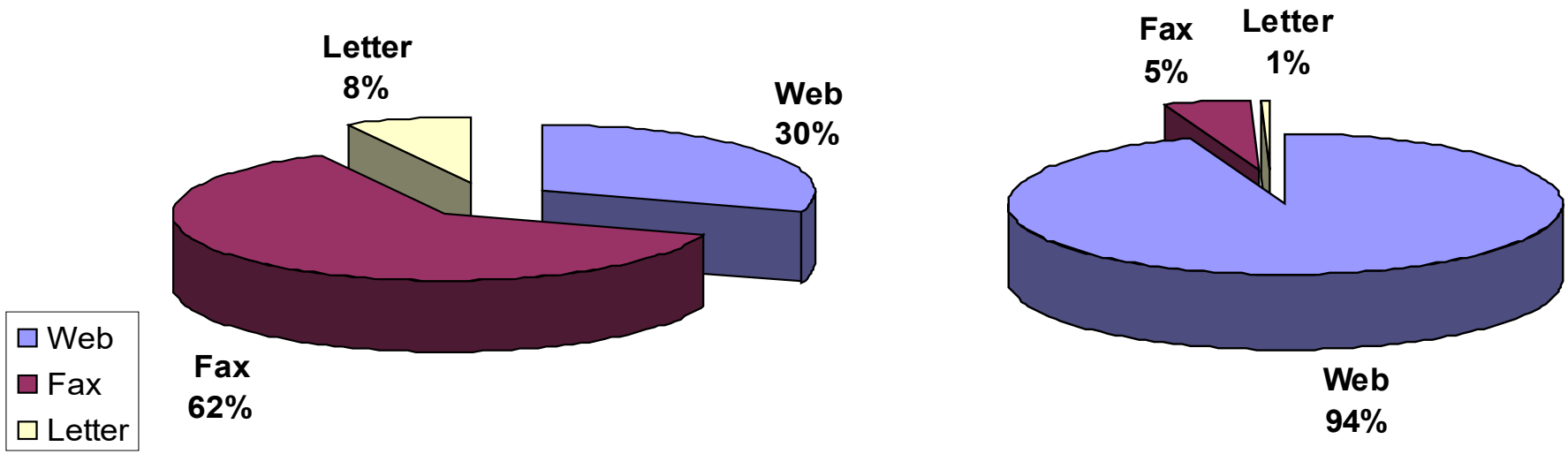
Summary of the 2002 Molecular QC programmes for Virology and Microbiology

Programme Type	Distribution Date	No. Participants	No. data Sets	Total Tests	% correct Total test	% equivocal Total test	% False +ve From total Negative Samples	% False -ve From total positive Samples	% Commercial assays
CMV	11/05/2002	89	105	1260	81.6	1.0	2.9	20.3	81.0
CT	03/18/2002	111	116	1160	66.8	0.9	0.6	46.0	87.9
EBV	12/17/2002	61	67	603	80.6	0.0	3.0	19.0	29.9
EV	06/10/2002	93	100	1200	79.0	1.6	5.5	27.7	7.0
HBV	05/20/2002	70	97	776	89.2	0.1	2.1	11.9	51.5
HCV	05/20/2002	80	124	992	91.8	0.1	0.0	9.2	88.0
HIV	05/20/2002	64	90	720	84.8	0.6	0.0	14.6	90.0
HSV	07/03/2002	98	109	1308	87.8	0.9	0.9	14.8	9.2
MT	11/05/2002	74	82	984	79.7	2.1	4.1	22.9	65.9

Major Participating Laboratory types:



Changes in Reporting types from 2002 to 2003



Summary and Conclusions

- An external QC organisation was established in 1998 (EU-QCCA) with partial funding by EU
- Management by experts from the field
- Fourteen distributions : 7 agents, 45-105 laboratories/distribution
- Large variation in sensitivity, mainly with in-house methods
- False-positivity rates between 1 - 8%; need for further improvement
- EU-QCCA succeeded by QCMD (Sept. 2001)
- Same approach with increase in quality/professionalism and dedicated, full-time employees; compliance with guidelines (ISO, CEN, EU)
- Commitment from ESCV, ESCMID and major diagnostic industry
- Expansion of program to include more targets, applications, and also more laboratories in more countries

Appendix 14

Foot-and-mouth disease virus detection based on a diagnostic method or an integrated quality controlled strategy

Kris De Clercq
CODA-VAR, Section Development of Diagnostic Tools for epizootic diseases,
Ukkel, Belgium

Foot-and-mouth disease virus (FMDV) detection used for national and international FMD control must be based on the diagnostic methods described in the OIE Manual of standards for diagnostic tests and vaccines (4th edition, 2000; http://www.oie.int/eng/normes/mmanual/A_00022.htm). The manual describes 4 identification methods: (i) virus isolation, (ii) antigen-ELISA, (iii) complement fixation test (CFT), (iv) nucleic acid recognition methods.

Virus isolation

Virus isolation (VI) remains the ultimate proof of the presence of live FMDV. Primary cells like bovine thyroid cells (Snowdon, 1966) or lamb kidney cells (House and House, 1989) are very sensitive but laborious to maintain. The quality can differ with each cell batch and requires therefore a well established quality assurance/quality control (QA/QC) system to guarantee a 24/24h availability of cells with the same sensitivity. Cell lines are easier to cultivate. Mostly pig cell lines are used such as IB-RS-2, PK15 or SK6 or a baby hamster kidney cell line (BHK-21). The cell line used, must be sensitive enough to isolate FMDV from samples coming from different species. Pig cell lines were not always suitable for isolation of FMDV coming from goats or sheep excreting sometimes very low amounts of virus (Bouma et al., 2001). The BHK-21T cell line seems to be less species dependant (Ahl, 2000).

Well established low positive controls are essential within a QA/QC system to proof that each cell batch is highly sensitive. These low positive controls must be calibrated to international accepted standards which are unfortunately not available. An international ring test is urgently needed for virus detection. This would increase the confidence and allow mutual recognition of results. It would also fill in a big gap in the framework of accreditation.

A small proportion of samples may give negative results for infectivity but positive results by ELISA or RT-PCR (Alexandersen et al., 2003). Exact figures for sensitivity and specificity can hardly or not be found, although years of experience with VI. However, these figures are essential to determine the positive predictive value of the test result, should be use to calculate the sample rate in surveillance campaigns and brought into account for calculating the true prevalence of FMDV.

Complement fixation test

The CFT has been of great value in the past in many FMD laboratories. The CFT is serotype dependant and requires a good practical knowledge of anti-complement reactions. Near Europe it is still used nowadays in some Transcaucasian countries and e.g. in regional laboratories in Iran (Mahmoudan et al., 2003). CFT could be replaced by the more sensitive antigen-ELISA (Roeder and LeBlanc Smith, 1987) but the availability of an ELISA reader,

ELISA plates and reagents are essential factors. The necessity of using a more sensitive test depends on the phase of the FMD control campaign.

Antigen detection

The antigen (Ag)-ELISA as described by Ferris and Dawson (1988) will identify the serotype of the FMDV present. In some laboratories however the test is limited to some serotypes (classical A/O/C and sometimes Asia1). A pan-serotype antibody would be very useful.

As for all tests the quality of the result depends in the first place on the quality of the sample. Although the sensitivity of the test is lower than VI, the success rate is high for samples from cattle and pigs. For sheep the percentage of samples directly yielding a positive ELISA result is lower (Alexandersen et al., 2003). A sample with a negative ELISA result requires further examination in highly susceptible cell cultures.

Each ELISA plate should contain the necessary low positive controls. As for VI there is a lack of international standards and the same remarks accounts. Also for Ag-ELISA it is difficult to find figures concerning the test performance. As for many tests the validation stopped with determining the analytical sensitivity (until which dilution the virus can be found) and specificity (cross reactivity). Although more and more laboratories claim to work within an accreditation system, hardly anything is known about the number of false positives and negatives based on a reliable number of samples. It must be stressed that the require numbers of positives needed for validation is very difficult to get for diagnostics dealing with exotic diseases.

To check the presence of FMDV in sheep flocks or in vaccinated cattle with low excretion of virus the number of samples must be relatively high to be statistical meaningful. Probang sampling is highly unpractical on large scale. Mouth or nose swabs are an alternative (Callens et al., 1998) and could provide a large number of samples (which in turn could be the bottle neck). A high throughput test such as ELISA is then acquired but the sensitivity of the ELISA should be improved as the amount of virus in those swabs will be too low. This increase can be obtained by using rolling circle amplification (RCA) ELISA (Schweitzer et al., 2000).

A pen-side test is in fact based on the same reaction between antibody-antigen and often revealed by chromatography technology and maybe in future by biosensors. For antibody detection a pen-side test was already brought on the market before the UK outbreak in 2001 by Genesis Diagnostics. Several companies and the WRL (Reid et al., 2001) are working on this kind of tests. The latter was based on a pan-serotype monoclonal antibody for capture of the antigen.

Pen-side tests do not acquire a well organized and safe sample transport, have the advantage of the short time before a result is known and can easily be repeated. They must be very robust and therefore a full validation is absolutely essential.

Pen-side tests bring in the problem of the user and the interpretation of the results. Will all potential users be aware of the nature of the samples to be taken and how? What about a negative result: case closed? What about a positive result: will all users declare their results or will the animal transport activity the night before an outbreak (high risk period 1 – Crauwels et al., 2001) increase even more than is already the case now. If pens-side tests are used by

accredited people: will permanent training be assured even in absence of an FMD outbreak for many years? How will continuous validation be organized?

The Ab Microarray is designed to be used as a screening tool. It is a clear high-throughput bench method. A wide range of different Mabs is immobilized on glass surface. In the field of screening proteomics the technique already exist to immobilize over 500 Mabs on a surface of 1 cm2. The result is read by a fluorescent image reader. The most difficult point in the microarray platform is the data analysis. Often software is needed for the interpretation of the result. A full validation of the test and the data analysis is of the outmost importance. For FMD virus-screening, an Ab microarray using Mabs developed for Ag profiling and reacting each a little bit different and covering a very wide range of FMDVs could be developed.

Nucleic acid recognition methods

Polymerase chain reaction (PCR).

During the recent (2003) avian influenza outbreaks in Belgium the presence of the virus was confirmed and an outbreak declared on the basis of a PCR result only. Also during the classical swine fever epidemic in the UK in 2000 PCR declaration of outbreaks was done on the basis of RT-PCR results (D. Paton, personal communication, 2002). In the field of FMD the discussion is still going on whether a positive RT-PCR really means that live virus is present. Thanks to this discussion the possibility exists for a country of not declaring an outbreak and meanwhile clearing out the possible dangerous suspect.

Various procedures are developed: conventional RT-PCR (Rodriguez et al, 1992), RT-PCR ELISA (Callens et al., 1998), nested RT-PCR (Moss and Haas, 1999), real time RT-PCR (Reid et al., 2002). Some developed methods could distinguish among the serotypes (Vangrysperre and De Clercq, 1996; Callens and De Clercq, 1997; Reid et al, 1998). These tests are at least as sensitive as VI and clearly faster. But what is the added value if the result is only taking into account when it is complemented by VI. Of course also here we must admit that there is not one method for which the number of false positives and negatives is described. Reid et al. (2002) claim that the tests could be used to avoid a second passage for secondary herds. To use RT-PCR as high throughput expensive automates are necessary (Reid et al., 2003) and even then ‘only’ 90 samples a day could be managed which is too little for a statistical virus screening.

A portable PCR is developed as a kind of pen-side test (Hearps et al., 2002). The same remarks made for the pen-side antigen detection could be made here.

The question of including valuable controls is very relevant. Some teams use plasmids, others synthetic cDNA or RNA. The latter is the only one covering the whole chain from RNA isolation till PCR product. International agreement and standardization would be very welcome.

DNA microarrays

DNA microarrays are microscopic glass slides on which cDNA fragments are spotted. The cDNA fragments can hybridize with viral genome if present in the sample. It has been developed for subtyping of influenza virus (Li et al., 2001). These results open interesting

perspectives in the generalization of the use of this high throughput technology of microarrays to the study of FMDV epidemiology.

Integrated quality controlled strategy

All above mentioned diagnostics are often evaluated as a pure laboratory stand alone method. Seldom FMDV detection is seen as a complex of different elements in which each step is important. The weakest element in the chain determines the strength of the chain. Whether new diagnostic methods brings in an added value or not depends on what exactly we want to achieve and if that new method fill in the gap. One of the main gaps is full validation of tests with determining the test performance and an accompanying continuous QA/QC system. Although very important for reliability and sampling strategies it is rarely studied. The majority of institutes performing FMDV diagnosis are research institutes. Research money is not available for validation activities and the research boards of these institutes do not consider these activities as high level research. Researcher themselves find it often boring and certainly not innovating. It demands a lot of work before it can be published and is often not accepted by high qualified scientific journals. Money must be made available for validation studies.

FMD diagnosis should be seen as a complete system (contingency plan) that has to be managed very well. First the strategy has to be defined with the goals on short and long terms, with the weaknesses and the strong elements. It should be clearly defined where investments are needed to obtain the goals and which elements can be left out. Some new developments are nice to have but are not essential to the strategy. Once the strategy is clearly defined it should be applied but must be continuously checked. A quality control of the strategy must be implemented. When elements are out of line actions must be taken and if necessary the strategy adapted.

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Appendix 15

An update on progress with the FAO Collaborative Studies for FMD Serology Standardisation, Phases XVII and XVIII

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Introduction and Methods

Reference sera are needed to help develop, validate and standardise serological tests. Greater emphasis on good laboratory practice has led to an increasing recognition of their importance and their lack of availability for FMD non-structural protein (NSP) antibody tests has hampered the introduction of these assays and the development and introduction of vaccinate-to-live policies (Anon, 2003). Difficulties in establishing FMD reference sera are due to the fact that the disease can affect different host species and the existence of multiple antigenic serotypes and subtypes of the FMD virus. Moreover, serology is used for different purposes, including the detection of previous or persistent infection in vaccinated or unvaccinated animal populations and the evaluation of post vaccinal immunity. The threshold for the cut-off between negative and positive samples may also differ according to whether serology is used to detect infection at a herd level or for certification of individual animals. Finally, there are limited biosecure animal facilities in which experiments can be performed to create the different categories of reference sera that are needed.

The OIE recommends the establishment of strong, weak and negative reference sera (Anon, 1998). Of these, by far the most important is the weak positive serum, which should be consistently positive, but not a strong positive. It is used to check that the sensitivity of a given test is at least moderate and to prepare secondary and tertiary standards as internal controls to monitor ongoing test performance. The OIE does not specifically recommend that a cut-off serum should be established, although the value of such a serum is that it provides a more stringent measure of test sensitivity and can be used to calculate units of antibody (Table 1).

The Phase XVII project has followed a similar pattern to previous exercises. The primary aim has been to establish and distribute FMD reference sera for each of several serotypes of FMD. For each serotype of FMD, the four reference sera have been a strong, weak, cut-off and negative serum. The sera have been produced by experimental vaccination or infection of cattle and have been distributed either frozen or freeze dried after various treatments to inactivate any live virus and after inocuity testing to confirm that this has been effective. This process is rather complex and a simplified approach would be to distribute only strong positive serum and a large volume of negative serum. The strong positive serum would have to go through the full range of treatments and testing to verify inocuity and would be supplied freeze dried. However, the bulk negative serum could be supplied directly, never having entered high security facilities. Recipients of these two sera would receive instructions for making their own dilutions of the positive serum in the negative one and report on their results. This would allow great flexibility

in adjusting the dilutions on the basis of preliminary results, without the need for repeated and very expensive distributions of fresh prediluted samples.

During the Phase XVII study, candidate reference sera for serotypes O, A and Asia 1 were distributed twice to 9 laboratories. The origin of these sera are shown in Table 2. Test results from the first distribution were presented and discussed at the Research Group meeting in Izmir (Paton et al., 2002). It was agreed to strengthen the weak positive sera and accordingly, a revised panel was distributed to the same number of laboratories. For each serotype, a single strong positive serum was distributed, along with four candidate weak positive reference sera representing a range of slightly different dilutions. The negative serum was not redistributed.

Results

The results are presented in Tables (3-12). NSP ELISA results are only presented for the type O sera, as the other sera derived from vaccinated rather than infected animals.

Type O Reference Sera. The strong positive serum was scored consistently positive in all tests, including those for NSP antibodies. The strongest weak positive candidate serum (#1) also scored positive in all tests. Candidate weak positive serum #3 scored closest to the cut-off level of 1/45 by VNT and 1/90 by liquid phase blocking ELISA (LPBE). In the SPCE and NSP ELISAs, the serum was less consistently positive.

Type A Reference Sera. The strong positive serum was scored positive in most tests, except for some VNTs where a heterologous virus was used. The strongest weak positive candidate serum (#1) also scored positive in most tests, except for VNT with heterologous virus. Candidate weak positive serum #3 scored closest to the cut-off level of 1/45 by VNT and 1/90 by liquid phase blocking ELISA (LPBE). However, there was considerable variation in test results by VNT. In the SPCE the serum was borderline positive.

Type Asia 1 Reference Sera. The strong positive serum was scored consistently positive in all tests, although with considerable variation in titre by VNT. Similarly, the weak positive candidate sera scored inconsistently by VNT. However, by LPBE and SPCE, Weak serum #2 was appropriate for use as a weak positive serum and weak positive #3 as a cut-off serum.

Discussion

The Phase XVII study has now achieved its aim of establishing strong, weak, cut-off and negative reference sera for FMDV serotypes O, A and Asia 1. The weak positive sera selected for reference standards are #1 and #3 (serotypes O and A) and #2 and #3 (serotype Asia 1).

Phase XVIII has as its aims: 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 paper presented at this meeting by Anderson et al) in addition to serotype O (Paiba et al., in press). 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.

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Table 1. Example calculations of units of antibody relative to results obtained for a cut-off serum in a virus neutralisation test.

e.g.1 cut-off serum = 1/16 → 1 unit
test serum = 1/32 → 2 units
test serum = 1/8 → 0.5 units

e.g.2 cut-off serum = 1/8 → 1 unit
test serum = 1/32 → 4 units
test serum = 1/8 → 1 unit

Table 2. Origin of reference sera

Cattle immunised by	**Serum collected at**	**Dilution of original to prepare ref sera**				
		Strong	**Weak#1**	**Weak#2**	**Weak#3**	**Weak#4**
Infection with O SKR 2000	34 dpc*	1/2	1/10	1/42		
Vaccination with A Iran 96	39 dpv**	1/2	1/24	1/90		
Vaccination with Asia 1 Shamir	47 dpv**	1/2	1/18	1/80		

* days post challenge
** days post vaccination

Table 3. VNT results for serotype O

	O SKR Strong +v	O SKR Weak 1	O SKR Weak 2	O SKR Weak 3	O SKR Weak 4	Virus used
	Titre	Titre	Titre	Titre	Titre	(strain)
WRL	**1/355**	**1/118**	**1/102**	**1/45**	**1/24**	O SKR
LAB 1	**1/250**	**1/173**	**1/216**	**1/74**	**1/86**	O SKR
LAB 2	**1024/512**	**512/256**	**1/256**	**256/128**	**1/128**	O Manisa
LAB 3	**1/141**	**1/58**	**1/46**	**1/14**	**<1/16**	O SKR
LAB 4	**1/178**	**1/64**	**1/64**	**1/16**	**1/8**	O Manisa
LAB 5	**1/75**	**1/53**	**1/53**	**1/22**	**1/22**	O Manisa
LAB 6	**1/512**	**1/91**	**1/181**	**1/45**	**1/45**	O SKR
LAB 9	**1/338**	**1/294**	**1/195**	**1/56**	**1/23**	O UK 31/01

Table 4. VNT results for serotype A

	A Iran '96	**A Iran '96**	**A Iran '96**	**A Iran '96**	**A Iran '96**	**Virus**
	Strong +v	**Weak 1**	**Weak 2**	**Weak 3**	**Weak 4**	**used**
	Titre	Titre	Titre	Titre	Titre	(strain)
WRL	**1/447**	**1/141**	**1/90**	**1/50**	**1/32**	A Iran 96
LAB 1	**1/1024**	**1/218**	**1/280**	**1/146**	**1/134**	A Iran 96
LAB 2	**1/256**	*	*	**1/16**	*	A22 IRAQ
LAB 3	**1/2042**	**1/162**	**1/91**	**1/58**	**1/91**	A IRAN 96
LAB 4	**1/64**	**1/2.8**	**1/2.8**	**1/2.8**	**1/2.8**	A22
LAB 5	**1/32**	**1/19**	**1/10**	**1/8**	**1/11**	A22
LAB 6	**1/128**	**1/32**	**1/45**	**1/32**	**1/45**	A IRAN
LAB 9	**1/1778**	**1/316**	**1/285**	**1/216**	**1/223**	A Iran 96

Table 5. VNT results for serotype Asia 1

	As 1 Sh Strong +v	As 1 Sh Weak 1	As 1 Sh Weak 2	As 1 Sh Weak 3	As 1 Sh Weak 4	Virus used
	Titre	Titre	Titre	Titre	Titre	(strain)
WRL	**1/389**	**1/138**	**1/90**	**1/32**	**1/27**	As1 Sham
LAB 1	**1/1144**	**1/336**	**1/384**	**1/96**	**1/84**	As1 Shamir
LAB 3	**1/159**	**1/41**	**1/32**	**1/11**	**1/14**	As1 Shamir
LAB 4	**1/128**	**1/22**	**1/16**	**1/16**	**1/8**	SAU 32/92
LAB 5	**1/64**	**1/19**	**1/16**	**1/16**	**1/16**	As1 Shamir
LAB 6	**1/128**	**1/64**	**1/45**	**1/64**	**1/32**	ASIA 1
LAB 9	**1/635**	**1/198**	**1/190**	**1/77**	**1/70**	NEP 29/97

Table 6. LPBE results for serotype O

	O SKR	O SKR	O SKR	O SKR	O SKR	Virus
	Strong +v	Weak 1	Weak 2	Weak 3	Weak 4	used
	Titre	Titre	Titre	Titre	Titre	(strain)
WRL	**1/1202**	**1/457**	**1/427**	**1/107**	**1/98**	O SKR
LAB 2	**1/640**	**1/360**	**1/180**	**1/45**	**1/45**	O Manisa
LAB 4	**103/87**	**95/77**	**92/72**	**61/48**	**51/41**	O Manisa
LAB 5	*	*	*	*	*	O LAUS*
LAB 6	**1/1490**	**1/593**	**1/595**	**1/166**	**1/104**	O SKR

Table 7. LPBE results for serotype A

	A Iran '96	A Iran '96	A Iran '96	A Iran '96	A Iran '96	Virus
	Strong +v	Weak 1	Weak 2	Weak 3	Weak 4	used
	Titre	Titre	Titre	Titre	Titre	(strain)
WRL	**1/2048**	**1/362**	**1/181**	**1/128**	**1/128**	A Iran 96
LAB 2	**1/180**	*	*	*	*	A22 IRAQ
LAB 6	**1/563**	**1/83**	**1/72**	**1/55**	**1/51**	A IRAN

Table 8. LPBE results for serotype Asia 1

	As 1 Sh Strong +v	As 1 Sh Weak 1	As 1 Sh Weak 2	As 1 Sh Weak 3	As 1 Sh Weak 4	Virus used
	Titre	Titre	Titre	Titre	Titre	(strain)
WRL	**>1/2048**	**1/1122**	**1/794**	**1/256**	**1/224**	As1 Sham
LAB 6	**1/1071**	**1/293**	**1/266**	**1/74**	**1/54**	ASIA 1

Table 9. SPCE results for serotype O

	O SKR Strong +v % Inhibition	O SKR Weak 1 % Inhibition	O SKR Weak 2 % Inhibition	O SKR Weak 3 % Inhibition	O SKR Weak 4 % Inhibition	Virus used (strain)	Notes
WRL	**92**	**87**	**86**	**71**	**70**	O Manisa	
LAB 1	**57**	**41**	**39**	**17**	**13**	O Manisa	
LAB 3	**87**	**81**	**79**	**65**	**61**		100-"Remaining %OD"
LAB 4	**76**	**52**	**48**	**29**	**27**	O Manisa?	
LAB 6	**81**	**60**	**64**	**32**	**44**	Unstated	1/10 final diln
LAB 7	**104**	**87**	**90**	**72**	**67**	O Manisa	
LAB 9	**92**	**85**	**85**	**65**	**51**	O Manisa	In house Competitive

Table 10. SPCE results for serotype A

	A Iran '96 Strong +v	A Iran '96 Weak 1	A Iran '96 Weak 2	A Iran '96 Weak 3	A Iran '96 Weak 4	Virus used	Notes
	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	(strain)	
WRL	**92**	**49**	**50**	**47**	**39**	A22 IRAQ	
LAB 1	**85**	**59**	**55**	**47**	**43**	A Iran 96	
LAB 3	**85**	**55**	**53**	**44**	**36**		100-"Remaining %OD"
LAB 7	**99**	**77**	**68**	**70**	**62**	A Iran 96	
LAB 9	**95**	**94**	**94**	**93**	**93**	A Iran 96	In house Competitive

Table 11. SPCE results for serotype Asia 1

	As 1 Sh Strong +v	As 1 Sh Weak 1	As 1 Sh Weak 2	As 1 Sh Weak 3	As 1 Sh Weak 4	Virus used	Notes
	% Inhibition	% Inhibition	% Inhibition	% Inhibition	% Inhibition	(strain)	
WRL	**98**	**92**	**90**	**69**	**63**	As1 Shamir	
LAB 1	**90**	**89**	**89**	**73**	**69**	As1 Shamir	
LAB 3	**91**	**90**	**89**	**88**	**87**		100-"Remaining %OD"
LAB 7	**94**	**84**	**80**	**55**	**56**	As1 Shamir	
LAB 9	**96**	**95**	**95**	**89**	**87**	NEP 29/97	In house Competitive

Table 12. NSP serology results

	O SKR	O SKR	O SKR	O SKR	O SKR	NSP	Notes
	Strong +v	Weak 1	Weak 2	Weak 3	Weak 4	used	
	%Inh/pos	%Inh/pos	%Inh/pos	%Inh/pos	%Inh/pos		
WRL	135,110,110	64,52,52	48,43,44	12,10,12	12,16,15	3ABC	Bommeli kit (old version)
LAB 5	POS	POS	POS	AMBIG	NEG	3ABC	
LAB 9	+++/POS	+++/POS	+++/POS	+/NEG	+/NEG	3ABC	Brescia/Intervet diagnosis (1/100)

Appendix 16

Rapid and differential diagnosis of Foot-and-Mouth disease virus (FMDV), swine vesicular disease virus (SVDV) and vesicular stomatitis virus (VSV) by one-step multiplex RT-PCR assay in clinical samples

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A novel highly sensitive and specific gel-based one-step hot-start multiplex RT-PCR has been developed and standardized for rapid diagnosis of OIE List A vesicular diseases in clinical samples. The test allows a simultaneous and differential detection of Foot-and-mouth disease virus (FMDV, the seven serotypes), Swine vesicular disease (SVDV) and Vesicular stomatitis virus (VSV, the two main serotypes) which may cause undistinguished clinical symptoms and lesions in infected pigs.

The method uses three primer sets, one specific for each virus (FMDV, SVDV or VSV), amplifying DNA fragments different in length, that allows a gel-based differential detection. Primer sets were selected in highly conserved viral genome regions to be compatible in a multiplex RT-PCR assay. Specific primer set for FMDV amplifying a fragment of 275 bp in all the seven FMD viral serotypes A, O, C; Sat1, Sat2, Sat3 and Asia1, was selected in the 3D gene coding region: FMD-B (Saiz et al, 2003) and a newly designed FMD-C. For SVDV, a previously described primer set SS4/SA2 (Nuñez et al., 1998) was selected, defining an amplicon of 154 bp. A new VSV-1/VSV-2 primer pair specific for VSV was designed in the L gene region that amplifies both Indiana-1 and New Jersey serotypes, delimiting an amplicon of 110 bp.

Total RNA was extracted from samples using a unique extraction method, the commercial *Tripure Isolation Reagent (Roche Molecular Biochemicals)*, following manufacturer's instructions. For the specificity assays using DNA viruses, viral genome was purified using the *High Pure PCR Template Preparation Kit (Roche Molecular Biochemicals).*

Optimal conditions for the vesicular multiplex RT-PCR assay were established and a hot start amplification was performed using the three specific primer sets (FMD-B/C, SS4/SA2, VSV-1/2) in a multiplex format in a single step, single tube reaction. Reverse transcription-amplification was carried out using MuMLV reverse transcriptase and Taq Gold polymerase *(Applied Biosystems).*

In order to determine the detection limit of the test, ten-fold serial dilutions, in serum, of infected cell cultures with each of the following viruses were used in the multiplex RT-PCR assay: FMDV A(A_{22} Iraq) , O (O_1 Manissa/Turkey)and C (C_1 Noville) and VSV Indiana-1 and New Jersey serotypes grown in BHK-21, as well as SVDV (UK-72) grown in IBRS-2. A sensitivity of 0.2 $TCID_{50}$, 0.02 $TCID_{50}$ and 0.07 $TCID_{50}$ were reached for each of the FMDV A, O and C serotypes respectively in the multiplex RT-PCR. Moreover, 0.126 $TCID_{50}$ was the detection limit for SVDV and a sensitivity of 6.4 $TCID_{50}$ was achieved after analysis of VSV Indiana-1 serotype as well as 0.7 $TCID_{50}$ was the corresponding detection limit in the case of New Jersey serotype using the multiplex format.

The specificity of the multiplex RT-PCR reaction was tested using seventeen FMDV isolates representatives of the seven serotypes A, O, C, Sat 1, Sat 2, Sat 3 and Asia 1, eleven SVDV strains and VSV Indiana-1 and New Jersey reference strains, as well as Coxsackievirus B5 and other domestic animal related viruses (CSFV, BDV, PCV-I, PCV-II, BVDV, PRRSV, ASFV, ADV, MCFV, BTV). In addition, nucleic acids from porcine whole blood, serum, swabs and tissue samples from healthy pigs, and several non-infected cell lines were employed in the specificity test. Multiplex RT-PCR was observed to be positive for all the FMDV, SVDV and VSV isolates obtaining the expected specific amplified product in each case (fig.1 and 2), while no positive products were found in the RT-PCR when other related viruses, cell lines or non-infected porcine tissues samples were assayed (data not shown).

Specificity of the amplicons was confirmed by restriction endonuclease digestion using AhdI (Saiz et al., 2003) and/or BsrFI for FMDV, ClaI for SVDV and BsmaI and BsaJI for VSV.

In order to assess the validity of the method in clinical specimens, samples from pigs experimentally inoculated with FMDV, SVDV or VSV were used. All the *in vivo* experimentations were performed in the BSL-3 animal facilities at CISA, Valdeolmos. Pigs were inoculated with one of the following viruses: A_{22} Iraq, O UK-2001 or C_1 Noville FMDV isolates; UK-72 SVDV strain; and Indiana-1 and New Jersey VSV serotypes. EDTA-blood, serum, nasal and pharyngeal swabs, faecal faeces (in the case of SVDV), vesicular epithelium and tissues samples were collected at different days and analysed by multiplex RT-PCR. Specific positive amplification product was observed in each case according to the inoculated virus.

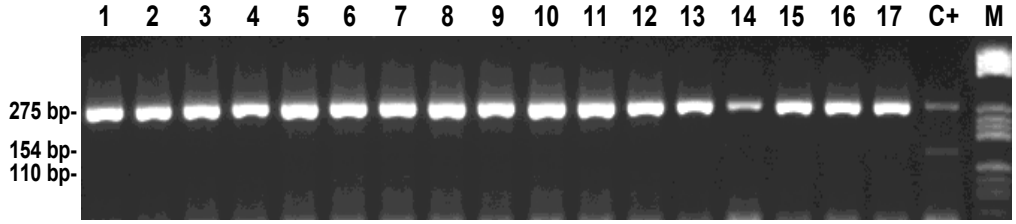

Fig 1. Detection of FMDV strains by Multiplex RT-PCR assay: A: **1:** A22 Iraq (IRQ24/64); **2:** A Pehuajo, Argentina/92; **3:** A24 Cruzeiro, Brazil 3/55; **4:** A5 Allier, France/60; O: **5:** O1 Manisa/Turkey/69; **6:** O Algeria 1/99; **7:** O1 BFS 1860/UK/67; **8:** O1 Kaufbern, Germany/67; **9:** O1 Campos, Brazil 1/58; **10:** O1 Caseros, Argentina 2/67; C: **11:** C1 Spa70 (CS-8 Sta Pau); **12:** C1 Noville, Switzerland/65; **13:** C3 Argentina/85; SAT1: **14:** RV 11/37 Rhodesia; SAT2: **15:** RHO 1/48, Rhodesia; SAT3: **16:** RV 7/34, Rhodesia; Asia1: **17:** LEB/88, Lebanon. C+: Multiplex RT-PCR positive control.M: Molecular Weight Marker V. *(Roche Molecular Biochemicals).*

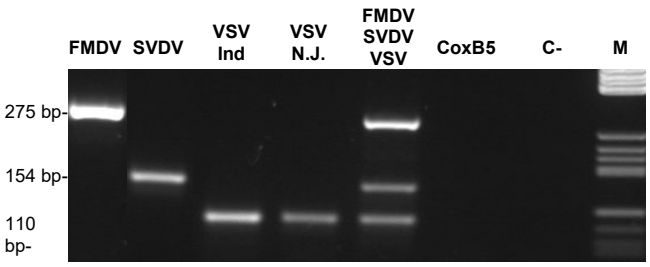

Fig.2. Simultaneous and differential detection of FMDV (O serotype), SVDV (UK-72 strain) and VSV (Indiana and New Jersey serotypes) in multiplex RT-PCR assay. CoxB: Coxsackie B5 virus; C-: Negative multiplex RT-PCR control. M: Molecular weight marker V (Roche Mol. Biochem.)

The multiplex test showed to be a simple, economical and reliable tool for rapid diagnosis of vesicular diseases in clinical samples, spending less than six hours in obtaining the results and it can be used even in the cases of an hypothetical co-infection.

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Appendix 17

Serodiagnostic capacity of FMD National reference laboratories in EUFMD member countries

Dónal Sammin, EUFMD Secretariat

Introduction

The results of a questionnaire survey on the diagnostic capacity of FMD laboratories in EUFMD members countries was presented at the 35th General Session of EUFMD, Rome April 9-11, 2003 (Sammin, 2003). During the discussion which followed, a number of issues were raised. Firstly, as to whether current FMD serodiagnostic capacity was adequate to meet the likely needs for serosurveillance in a future outbreak situation and secondly, if stocks of kits, reagents and equipment were adequate and could be re-supplied speedily to meet the needs of large-scale serosurveillance. The Session recommended that a follow-up questionnaire survey be conducted to assess current stocks of reagents and equipment for serosurveillance and that the level of diagnostic activity for FMD in member countries should be reviewed on an annual basis and discussed by the Research Group.

Materials and methods

The initial questionnaire, circulated to Chief Veterinary Officers (CVOs) of EUFMD member countries in February 2003, consisted of two parts, the first attempting to gauge requirements for FMD laboratory diagnosis and the second addressing FMD laboratory capacity in member countries. Assurances were given that replies to the questionnaire would be treated confidentially and therefore an alphabetic code was assigned to each member country in order of their reply. A supplementary questionnaire on serodiagnostic capacity was addressed to a contact person (designated by the CVO) in the FMD National Reference Laboratory (NRL) of each member country in August 2003. This paper draws from the results of both questionnaires.

Results

Initial CVO Questionnaire

Response rate

Twenty-seven of 33 (82%) member countries replied to the initial questionnaire (including two late replies received after the report was prepared for the General Session).

Q3 How many animals in your country were tested for FMD antibody during 2000, 2001 and 2002?

Eighteen countries tested suspect animals for FMD antibody in each of the three years whereas only two countries did not have any animals tested for FMD antibody during the three year period. A total of **75429** animals were tested for FMD antibody during 2000 (57.6% cattle; 30.7% small ruminants; 11.1% pigs and 0.7% other species), **3674142** animals were tested during 2001 (4.5% cattle; 91.0% small ruminants; 1.8% pigs and 2.7% other species) and **226464** animals were tested during 2002 (26.2% cattle; 60.8% small ruminants; 12.6% pigs and 0.4% other species). The total number of animals in each country tested for FMD antibody during 2000, 2001 and 2002 is given in **Table 1.** The number of animals

tested is also expressed as a percentage of the susceptible farmed animal species in each country (using 2001 population data for cattle, small ruminants and pigs derived from FAOSTAT http://apps.fao.org/default.htm).

Q4 Are there designated laboratory facilities for FMD diagnosis in your country?
*If **NO** where are samples from suspect animals sent for diagnosis?*
Twenty respondents have a National Reference Laboratory for FMD (**Table 1**). Two member countries have regional centres capable of FMD sero-surveillance in addition to a centralised testing facility. Of the seven countries without facilities, three rely on the world reference laboratory (Pirbright, UK), three on the Danish Veterinary Institute, Lindholm and one on CODA-CERVA-VAR, Belgium. Two member countries with limited diagnostic capabilities (serological testing only) also avail of the world reference laboratory for some of their diagnostic requirements and one member avails of IZS, Brescia.
A further two countries responding to the supplementary questionnaire also have an NRL. Four member countries did not respond to either questionnaire and their current diagnostic capacity is uncertain.

Q9 What serological tests are used in your country for FMD diagnosis?
Each of the 22 NRLs can perform at least one of the OIE-prescibed methods for detection of FMD antibody. Eighteen laboratories can perform the liquid phase blocking ELISA (LPBE), 15 can perform virus neutralisation tests and four laboratories can perform the solid phase competition ELISA (SPCE) developed at the WRL.

Six laboratories have an alternative ELISA for detection of antibody to structural proteins of FMDV. Five laboratories have the Cedi-diagnostics type-O specific test and two have developed an alternative ELISAs in-house. A monoclonal-antibody based competitive ELISA has been developed at Brescia and a solid phase blocking ELISA at Lindholm.

Thirteen laboratories have an ELISA for antibody to non-structural proteins (NSP) of FMD virus; ten have the Bommeli (CHEKIT-FMD-3ABC) ELISA, two have the UBI (FMDV-NS-EIA) ELISA and two have developed an NSP ELISA in-house (Brescia 3ABC ELISA and Lindholm competitive 3ABC ELISA).

Q10 What is the maximum number of serum samples that could be tested per month in your country for antibodies to FMD virus with present laboratory resources?
Sero-diagnostic capacities of FMD laboratories in member countries are given in **Table 2**. The total sero-diagnostic capacity of EUFMD member countries (if the testing capacity for all available methods are added together) exceeds 1.3 million sera per month. Considering only those methods (virus neutralisation, LPBE and SPCE) prescribed by the Office International des Epizooties for the purposes of international trade, serodiagnostic capacity exceeds 0.95 million sera per month. The combined testing capacity of six laboratories with ELISAs for antibodies to non-structural proteins of FMD virus exceeds 0.6 million sera per month (whilst a further seven laboratories which have an NSP ELISA did not state their testing capacity). The discrepancy in these figures arises because one country has the capacity to test up to 480,000 sera per month by either SPCE or NSP ELISA through a network of regional testing centres (following a lead-in time of ten weeks).

Serodiagnostic capacity is summarised by categorising NRLs according to the actual numbers of sera that can be tested per month (**Table 3**) and as a percentage of the susceptible livestock population that this represents (**Table 4**).

Q11 How many serum samples were tested in your country for antibodies to FMD virus during 2000, 2001 and 2002?

The total number of sera tested by each available method across the member countries in three successive years (2000-2002) is given in **Table 5**. As can be seen from this table the SPCE developed at WRL and other solid phase ELISAs for antibodies to structural proteins of FMD virus were used to achieve most of the increase in serological activity in 2001.

Supplementary NRL Questionnaire

Response rate

Eighteen of 22 (82%) NRLs replied to the supplementary questionnaire.

Q1 How many staff are involved in FMD-related diagnostic and research activity?
Q2 Are all diagnostic/research staff trained in serodiagnostic methods?
Q3 Is there a reserve of technical staff that could be called upon in the event of an emergency?

The numbers of staff trained in serodiagnostic methods in each NRL and a breakdown of the staff numbers according to qualification (where provided) is given in **Table 6**. Fifteen respondents stated that there was a reserve of trained staff which could be called upon in the event of emergency.

Q4 Has the NRL been consulted in the formulation of National contingency plans for dealing with an FMD outbreak?
Q5 If YES, did the plans include contingencies for large scale serosurveillance?

Sixteen NRLs have been consulted in the preparation of national contingency plans, and in the case of 11 NRLs, provisions have been made in those contingency plans for large-scale serosurveillance. The provisions, where specified, incuded the availability of a reserve of trained staff (n = 4), preparation of reagents in-house (n = 3), decentralisation of screening tests to regional laboratories (n =2), arrangement for the supply of extra test-kits (n = 1), employment of extra technical staff (n = 1) and recent purchase of robotic equipment for ELISA (n = 1).

Q6 How many sera per day could be tested for antibodies to FMD virus (structural proteins) with present laboratory resources?

Serodiagnostic capacity was compared to the numbers of staff trained in serodiagnostic methods in each NRL and specifically to the number of technicians (**Table 6**). The extent of inter-laboratory variation in the number of sera tested per technician per day (56-1833) presumably represents differences in both the serological methods used and the level of automation.

Q7 Does the NRL have serological tests for detection of antibodies to FMD virus other than the virus neutralisation test and liquid phase blocking ELISA?

(*see* **Table2** *and replies above to* **Q9** *from initial questionnaire*)

Q8 Were these alternative serological tests developed and produced in-house?

If ***NO****, please provide details of the supplier (source of the kit/reagents) for each test*
(*see replies above to* **Q9** *from initial questionnaire*)

Q9 Are positive control sera (secondary reference standards) produced in-house?
*If **NO**, from where is positive control serum obtained?*
Ten NRLs produce secondary standards (control sera) in-house, six rely on supply of secondary standards from the WRL and two have an alternative source (including one NRL relying exclusively on standards supplied with commercially-derived test-kits) (**Table 7**).

***Q10 For each of the ELISA-based serological tests available in your laboratory, please provide the following information: (a)** The number of sera that could be tested with the kits/reagents available in the NRL at the present time and **(b)** The lead-in time for supply of further kits and reagents if existing stock had to be replaced*

For those NRLs which detailed the number of sera that could be tested with available kits/reagents (n =14), the total reserve held is sufficient to test approximately 1 million sera. However, four NRLs hold 89% of this reserve and for each type of ELISA (LPBE, alternative ELISA for antibodies to structural proteins of FMD virus, NSP ELISA), the number of sera which could be tested with available kits/reagents varies greatly between laboratories (**Table 7)**.

One NRL, although not specifying existing reserves, stated that the supply of reagents was unlikely to be a limiting factor in large-scale serosurveillance. However this NRL would require a lead-in time of at least 2-3 months to replace existing reserves. For the other NRLs the lead-in time for resupply of kits/reagents was estimated to be in the range of 6 to 30 days for the LPBE (mean of 20 days), 14 to 30 days for alternative SP ELISAs (mean of 21 days) and 3 to 56 days for NSP ELISA (mean of 21 days).

For each NRL where sufficient data was provided (n = 13), the number of sera that could be tested for SP antibody with available kits/reagents was divided by the number of sera that could be tested per day to estimate the number of days uninterrupted testing which could be performed with existing reserves (**Table 7**). For eight of these NRLs available kits/reagents would be used up within 20 days of full capacity testing (i.e. within the time required to replace stocks).

Q11 If the NRL had to increase the level of serological testing beyond the maximum capacity estimated at present (and test large numbers of sera by NSP test) what would be the most important limiting factors?
Ranked in order of priority by NRLs, both the supply of test kits and reagents and the availability of trained staff were identified as the most significant limiting factors in attempting to increase serodiagnostic activity (**Table 8**). The supply of control sera was also identified as a significant limiting factor by many laboratories.

Apart from the limiting factors listed in the questionnaire, two NRLs identified logistical problems with respect to sample handling, result reporting and integration of information from multiple test centres as potential limiting factors for large scale serosurveillance.

Q12 If EUFMD were to organise a workshop on contingency planning in NRLs for large scale serosurveillance....prioritise the issues which should be considered
Possible subject areas for a workshop were ranked in order of priority (**Table 9**). A majority of NRLs ranked arrangements for the supply of test kits and reagents (n = 12) as one of their top priorities followed by the standardisation and supply of control sera (n = 8) and arrangements for staff-training (n = 7).

More than one NRL requires help in each of the areas listed in the questionnaire and most NRLs require help with the standardisation and supply of control sera (n = 12). On a more positive note, at least one NRL could provide help in each of the areas identified.

Points for discussion

- 29 of the 33 member countries replied to one or both questionnaires. 22 of these countries have a National Reference Laboratory for FMD. The capacity of the other four member countries to test for FMD antibody is unknown.

- Serodiagnostic capacity of member countries has increased almost sevenfold since 1995, the last occasion on which a survey of diagnostic facilities was conducted by EUFMD (Leforban, 1995). At that time the total capacity of NRLs was 190,000 sera per month and the capacity for semi-automated serological testing was 110,000 sera/month (as compared with the present day capacity of approximately 1.3 million sera per month, 90 *per cent* of which is by ELISA testing). In 1995, four laboratories had a testing capacity of more than 20,000 sera per month and a further four laboratories had a capacity of 10,000 sera per month. Whilst there has been a significant increase in sero-diagnostic capacity since 1995, 81 *per cent* of present testing capacity is concentrated in four NRLs and only three countries have a monthly testing capacity relative to national herd/flock size on the scale likely to be required for large-scale serological screening. Furthermore, if emergency vaccination were employed and serology was to be used to differentiate vaccinates from infected animals a greater testing capacity for antibodies to NSPs would be required. A number of questions need to be addressed:

  Is serodiagnostic capacity in EUFMD member countries adequate?
  How quickly can this capacity be scaled up in the event of an FMD outbreak?
  Is contingency planning for large scale serosurveillance adequate?
  Which of the factors limiting large scale serosurveillance can be addressed in advance of an FMD outbreak?

- Only four countries use the solid phase competition ELISA (SPCE) developed at the WRL despite the fact that this ELISA, is now accepted by OIE for the purposes of international trade. It would probably be easier to scale up testing capacity in the event of an emergency with the SPCE than with the liquid phase blocking ELISA (LPBE).

- A critical consideration is the availability and supply of diagnostic reagents and reference sera during a crisis. Although in discussion of FMD diagnostic capacity in 1995 it was agreed that there was no need to bank reagents for large-scale screening, at the more recent 67th executive committee (2002), it was suggested that reference sera and a bank of reagents should be prepared for a crisis situation. Only two of the sixteen laboratories performing the LPBE can generate the required reagents in-house. Most NRLs estimated that LPBE test kits could be resupplied within 14-30 days of a crisis situation. However, IAH, Pirbright (the most likely source of the necessary reagents for other NRLs) has stated that it would take 2-3 months to replace their existing reserves.

- ***Implications of EU draft directive:*** The new draft directive requires national laboratories (NLs) to be equipped and staffed for large-scale sero-surveillance. It makes provision for

the establishment of a community reference laboratory (CRL) and formalises the relationship between NLs and this CRL with respect to information exchange, staff-training, research and development, quality assessment and standardisation.

- ***Ongoing collection of data from FMD diagnostic laboratories by the EUFMD secretariat.*** A system for recording and reporting the level of submissions to FMD laboratories on a yearly basis is required. Issues of confidentiality and security of information must be addressed.

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CODE	Livestock population (x 10^6) [1]	NRL[2]	2000 Number of sera tested[3]	2000 Sera as % livestock[4]	2001 Number of sera tested[3]	2001 Sera as % livestock[4]	2002 Number of sera tested[3]	2002 Sera as % livestock[4]
A	**0.6**	NO	*0*	0.000	*2*	0.000	*0*	0.000
B	**5.3**	YES	*2991*	0.056	*2621*	0.050	*2762*	0.052
C	**53.1**	YES	*581*	0.001	*3109282*	**5.851**	*117669*	**0.221**
D	**2.3**	YES	*17316*	**0.752**	*7184*	**0.312**	*19891*	**0.863**
E	**28.0**	YES	*2044*	0.007	*33924*	**0.121**	*2684*	0.010
F	**42.6**	YES	*278*	0.001	*11654*	0.027	*186*	0.000
G	**3.8**	NO	*0*	0.000	*1*	0.000	*0*	0.000
H	**3.6**	YES	*14*	0.000	*214*	0.006	*12*	0.000
I	**2.4**	NO	*37*	0.002	*5*	0.000	*0*	0.000
J	**5.0**	YES	*5563*	**0.110**	*1889*	0.037	*4817*	0.096
K	**1.1**	YES	*0*	0.000	*0*	0.000	*0*	0.000
L	**NA**	NO	*0*	0.000	*0*	0.000	*0*	0.000
M	**55.5**	YES	*0*	0.000	*62729*	**0.113**	*12934*	0.023
N	**46.5**	YES	*4235*	0.009	*8396*	0.018	*13160*	0.028
O	**18.6**	YES	*2271*	0.012	*203363*	**1.091**	*7194*	0.039
P	**15.9**	NO	*22676*	**0.143**	*18835*	**0.119**	*25299*	**0.159**
Q	**14.7**	YES	*317*	0.002	*3645*	0.025	*606*	0.004
R	**10.8**	YES	*126*	0.001	*8655*	0.080	*1*	0.000
S	**45.6**	YES	*0*	0.000	*7070*	0.015	*1*	0.000
T	**6.0**	YES	*305*	0.005	*1647*	0.027	*1305*	0.022
U	**1.0**	YES	*4461*	**0.450**	*682*	0.069	*570*	0.057
V	**16.5**	NO	*0*	0.000	*184030*	**1.116**	*0*	0.000
W	**4.0**	YES	*7*	0.000	*178*	0.004	*3005*	0.075
X	**0.1**	NO	*0*	0.000	*0*	0.000	*0*	0.000
Y	**1.2**	YES	*2617*	**0.215**	*1504*	**0.124**	*691*	0.057
Z	**1.6**	YES	*945*	0.058	*1486*	0.091	*2267*	**0.138**
AA	**23.2**	YES	*8645*	0.037	*5146*	0.022	*11410*	0.049

[1]Susceptible livestock population = cattle, small ruminants and pigs from FAOSTAT, 2001
[2]National Reference Laboratory for FMD
[3]Total number of sera tested for FMD antibody each year
[4]Percentage of susceptible livestock population tested for FMD antibody (>0.1% in bold font)

Table 1 FMD serodiagnostic activity in EUFMD member countries 2000-2002

CODE	VN[1]	LPBE	SP ELISA[2]	NSP ELISA[3]	Sera per month[4]	Relative serodiagnostic capacity[5]
B	60000	20000	40000	40000	160000	3.02
C	10000	8000	**32000**	32000		
			480000*		562000	1.06
D	-	2000	-	-	2000	0.09
E	500		10000	10000	20500	0.07
F	✓	15000	✓	✓	15000	0.04
H	900	3200	-	7500	11600	0.32
J	✓	1000	-	500	1500	0.03
K	-	1440	-	-	1440	0.13
M	3000	10000	-	20000	33000	0.06
N	300	1500	-	2000	3800	0.01
O	50000	-	120000	-	170000	0.91
Q	1600	-	80000	✓	81600	0.56
R	5000	-	**8000**	✓	13000	0.12
S	2400	4800	✓	✓	7200	0.02
T	800	1900	-	-	2700	0.04
U	400	1000	-	-	1400	0.14
W	-	180000	-	-	180000	4.51
Y	-	12000	-	-	12000	0.99
Z	-	1000	-	✓	1000	0.06
AA	800	15000	10000	15000	40800	0.18
AB	-	✓	-	✓	-	-
AC	-	✓	-	-	-	-
	135700	279840	780000*	607000*	1320540	

[1]Virus neutralisation
[2]ELISA other than LPBE detecting antibody to FMDV structural proteins (figures in bold for SPCE)
[3]ELISA detecting antibody to FMDV non-structural proteins ✓ = Test available but capacity not stated
[4] Monthly serodiagnostic capacity by addition of all available methods
[5]Monthly serodiagnostic capacity as a percentage of the total population of susceptible farmed animals (FAOSTAT)
*Country C scaled up to full testing capacity (after 10 weeks) is capable of testing 480,000 sera in regional laboratories using SPCE or NSP ELISA.

Table 2 Diagnostic capacity for detection of FMD antibody (maximum number of samples per month)

Sera per month	Number of Labs.
< 10,000	8
10-50,000	7
50-200,000	4
>200,000	1

Table 3 **Absolute serodiagnostic capacity per month (sum of testing capacity using all available methods)**

Sera/month as % Total susceptible animal population	Number of Labs.
<0.1%	9
>0.1% and ≤1.0%	8
>1%	3

Table 4 **Relative serodiagnostic capacity per month as a percentage of national populations of susceptible farmed animal species (cattle, small ruminants and pigs from FAOSTAT, 2001)**

	2000	2001	2002
Virus Neutralisation	13168	61933	12793
LPBE	44906	100719	59623
SPCE (WRL)	414	3079351	118461
Other SP ELISA[1]	6476	243977	14897
NSP ELISA[2]	1783	74260	18022
	66747	3560240	223796

[1]ELISA other than LPBE and SPCE (WRL) detecting antibody to FMDV structural proteins
[2]ELISA detecting antibody to FMDV non-structural proteins

Table 5 **Number of sera tested by different serological methods 2000-2002**

CODE	Trained Staff[1]	Vets.	Scientists	Technicians[2]	Other	Sera/day[3]	Sera per day per technician
B	6	-	-	-	-	4000	-
C	15	-	-	-	-	1200*	-
D	4	2	-	2	-	100-150	63
E	9	1	2	6	-	525	75
F	2	-	1	1	-	500	500
H	8	-	-	-	-	1750	-
J	4	2	1	1	-	200	200
K	3	-	-	-	-	80	-
M	19	-	-	9	-	2000	222
N	30	-	-	-	-	500	-
O	5	2	-	3	-	5500	1833
Q	7	-	-	-	-	5000	-
R	5	-	-	-	-	2000	-
S	5	1	-	4	-	400	100
Z	3	3	-	-	-	500	-
AA	17	4	-	9	4	500	56
AB	5	3	-	2	-	200	100
AC	2	-	-	-	-	500	-

[1]number of staff trained in serodiagnostic methods; [2]number of technicians (where specified);
[3]number of sera that can be tested per day for SP antibodies to FMDV with existing resources.
**resting serodiagnostic capacity of NRL (can be increased to >20,000 involving regional laboratories)*

Table 6 NRL staff numbers compared to serodiagnostic capacity

CODE	Available kits/reagents[1]			Sera/day[2]	Number of days testing[3]	*Source of 2^0 Reference Standards*
	LPBE	Other SP ELISA	NSP ELISA			
B	10000	400	200	4000	3	*WRL*
C*	-	-	2000	1200*	-	*in house*
D	-	-	-	100-150	-	*WRL*
E	-	100000	80000	525	190	*in house*
F	*20000$*	-	0	500	40	*in house*
H	4000	-	150	1750	3	*in house*
J	1500	-	-	200	8	*WRL*
K	1400	-	-	80	18	*WRL*
M	20000	-	500000	2000	10	*in house*
N	10000	-	500	500	20	*in house*
O	-	*120000*	-	5500	22	*in house*
Q	-	20000	60000	5000	4	*in house*
R	-	-	-	2000	-	*in house*
S	*18000$*	3000	1000	400	53	*other*
Z	1500	-	1000	500	3	*WRL*
AA	20000	10000	1000	500	60	*other*
AB	-	-	-	200	-	*in house*
AC	-	-	-	500	-	*WRL*
	106400	**253400**	**645850**			

[1]*number of sera that can be tested with available kits/reagents*
[2]*number of sera that can be tested per day for SP antibodies to FMDV*
[3]*number of days testing for SP antibody at full capacity with available kits/reagents*
**availability of kits/reagents not a limiting factor but capacity not stated; [$]depending on serotype*

Table 7 Available kits/reagents compared to serodiagnostic capacity

	Availability of trained staff	Supply of kits and reagents	Supply of control sera	Lack of equipment	Insufficient lab. space	Other factors
1st	**3**	**9**	**2**	**2**	**1**	**1**
2nd	**9**	**3**	**5**	**1**	**2**	**0**
3rd	**5**	**2**	**5**	**5**	**3**	**0**
1st/ 2nd	**12**	**12**	**7**	**3**	**3**	**1**

NB *column headings are possible limiting factors for increased serological testing (as per questionnaire) and the body of the table details the number of NRLs which prioritised each as first, second, third and either first or second most important.*

Table 8 Limiting factors for increased serological testing

	Arrangements for staff-training	Arrangements for supply of test kits/reagents	Standardisation and supply of control sera	Automation of testing	Adaptation and validation of diagnostic tests	Standardisation and QA/QC of test procedures	Guidelines for interpretation of serological tests
1st	4	4	5	2	2	3	0
2nd	3	8	3	2	2	1	2
3rd	0	0	2	5	5	4	1
1st / 2nd	7	12	8	4	4	4	2
Help needed	*2*	*6*	*12*	*7*	*6*	*6*	*2*
Could provide	*5*	*4*	*1*	*2*	*3*	*2*	*2*

NB *column headings are possible issues for consideration (as per questionnaire) and the body of the table details the number of NRLs which prioritised each as first, second, third and either first or second choices. The last two lines detail for each issue the number of NRLs where help is required or where help could be provided, respectively*

Table 9 Issues for consideration in a workshop on contingency planning for large-scale serosurveillance

Appendix 18

A European Diagnostic reagent bank - what should it contain?

Bernd Haas

Federal Research Centre for Virus Diseases of Animals

Introduction

While free trade in animals and animal products has led to improved productivity and lower prices, it has also increased the risks associated with animal diseases in at least two ways: animals and animal products can transmit diseases over greater distances in less time and the financial consequences of trade restrictions imposed in order to control outbreaks have become much more severe. Huge animal populations and the products derived from them suddenly become worthless since they cannot be traded in a commercially viable way, even if they are still perfectly fit for consumption. This applies in particular to FMD. In order to mitigate these consequences, new control strategies have been designed, which lead to an increased demand for laboratory investigations. The traditional main role of laboratories specialised on foreign animal diseases has been the rapid confirmation of new outbreaks. In future, tracing on and tracing back, proving freedom of disease and testing as basis for movement, slaughter and trade certificates will become more and more important and 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.

New regulations and lessons from the past

The 2001 FMD epidemic had shown that international and community rules and the ensuing practices had not taken sufficient account of the possibility offered by the use of emergency vaccination and subsequent tests to detect infected animals in a vaccinated population. With the new Council Directive on Community measures for the control of foot-and-mouth disease this will change. This Directive 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 (when the new directive was not yet in force), confirms that we have to be prepared to test hundreds, thousands or even millions of samples.

In the UK, despite the Institute of Animal Health is also World Reference Laboratory for FMD, and a staff of 60 people worked in 3 shifts, the diagnostic and logistic capacities did not allow for all suspect cases to be checked in the laboratory. At the peak of the epidemic, there were 50 new outbreaks a day and the authorities had to declare outbreaks on the basis of clinical signs. As a consequence, Pirbright is now establishing a high throughput system for real-time PCR. This method will also shorten the time between receiving a sample and giving a result. What is more important in the context of this paper is serological capacity: 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. In addition, at the time of the outbreaks, staff that had been hired for BSE testing could support the FMD teams. More than 50 people worked on the diagnosis of FMD. About 200 000 sera were tested (63161 sera in connection with suspect cases and screening, 61208 “pre-vaccination” tests, 10971 in connection with “pre-emptive slaughter”, and 45699 sera during “final screening”). These tests were performed using a mab-based competition-ELISA produced by ID-Lelystad. 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 not much 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 preformed by regional (state) diagnostic laboratories, while the national reference lab belongs to the Federal Research Centre. 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.

General requirements for tests kept in a FMD diagnostic reagents bank

The formal requirements are laid down in the EU regulation on FMD (ANNEX XIII). 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.

At least as long as there is no general agreement, in which situations type-independent NSP tests can replace type specific tests, tests for “anti-structural” antibodies for all seven serotypes must be available. Of course the reagent bank must contain an NSP tests for cattle. An NSP test for pigs (if a species specific extra test is needed) should be included as soon as sufficient validation data are available. The same applies to small ruminants, but it may turn out their immune reaction is too weak to permit the use of a NSP test. The European reagent bank should also contain standard sera for all serotypes and NSP serology in order to check and adjust the sensitivity of tests.

The numbers should be sufficient to cover the time until newly produced kits are available, which may take at least one to two months. Considering the maximum throughput of the veterinary laboratory system of a major country, tests for at least one million samples would be required in order to avoid a situation in which kits become the “bottleneck” of disease control.

The minimum time for delivery probably has a huge influence on costs. Here, an expert group should discuss with kit producers reasonable time limits. Components for the SPCE could reach the customers within 24 – 48 hours, if the users coat the plates themselves. Complete kits with coated plates probably could be shipped within 2 to 4 weeks. This would be acceptable for NSP serology for the purpose of identifying infected animals in a vaccinated population.

To ensure quality and compliance with QM rules, check stability during storage and prepare for rapid finishing, packaging and delivery to the customers exceed the capabilities of most national laboratories. Therefore, these tasks should be performed by a diagnostic kit producer, even if some components of the kits may come from national laboratories or, in case of the antigen, a vaccine producer.

Of course 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

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.

Ways to reduce costs

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: Produce antigen, antibodies and other reagents, produce and test a pilot batch, and then store the reagents. In order to ensure the availability of suitable ELISA plates in sufficient numbers, a contract with an ELISA plate producer would be necessary. He would have to considerably increase the stock of plates, in order to be able to meet the sudden increase in demand. However, plates could be regularly turned over and sold, so no cost for discarding plates would have to be expected. A standby contract with a company that would rapidly coat the plates would also be necessary, unless the kit producers do this step themselves or it is decided to do that in the customers' laboratories - the later approach possibly compromising performance and stability of the test.

In "peace times" there would only be costs for the production of reagents, the storage of components and reservation of coating, testing and packaging capacity, but no test kits would have to be discarded. A limited number of test kits produced as "ready reserve" should be used for training and ring tests before they expire. In a crisis, customers would have to pay for preparing complete kits and replacing them after they had been used or expire. However, during an epidemic the money would be available.

Since NSPs are type-independent, theoretically, one could use one test for anti non-structural protein (NSP) antibodies to cover all serotypes. Since NSPs are generally less immunogenic than structural proteins, seroconversion to NSP in non-vaccinated animals may be slightly delayed compared to conventional, type specific tests. Nevertheless, using a NSP test for cattle and pigs may be acceptable. In respect to small ruminants, this appears doubtful. However, no NSP test has been fully validated and accepted for this purpose.

A representative of a test kit producer recently suggested a possible solution for cases, when there is a limited regular market for a kit "in peace times", but the rapid production of sufficient quantities for emergencies in Europe would be difficult. Here, veterinary authorities should buy a certain number of kits, which are stored by the producer and replaced e.g. 3 times a year. The batch which has to be replaced and which still is fully usable for a reasonable period of time, will be sold on the regular market. This avoids the expensive discarding of expired test kits. Some kits could also be used for the regular training at regional labs before they expire.

For international reagent banks, labels and manuals should be in English, and additional national manuals could be prepared with support of the national laboratories.

Comments on specific tests

Assays for antibody to structural proteins

Cedi test FMDV type O
Cedi Diagnostics B.V. offers a competitive, mab-based ELISA for antibodies against FMD type O, which is very easy to use. This test was already used in 2001, but it has to be shown whether the test can match the sensitivity of the SPCE or LPBE while maintaining a satisfactory specificity. Some laboratory prototypes of tests for serotypes A, Asia and C have been produced, but have not reached the same stage yet.

Solid-Phase-Competition-ELISA (SPCE)
The solid-phase-competition-ELISA (SPCE) was developed at the WRL, Pirbright as a replacement for the liquid-phase-blocking-ELISA (LPBE), currently the prescribed test, because the LPBE is not fully satisfactory in respect to specificity and robustness.
The SPCE basically employs the same main reagents (rabbit and guinea pig antibodies) as the LPBE. In the original publication a highly purified antigen was used, but inactivated supernatant (like in the LPBE) or vaccine antigen concentrate can also be used. For serological screening in the UK in 2001, O Manisa vaccine antigen concentrate was supplied by Merial. Supply of antigen concentrate of strains not regularly produced would be problematic, or, if produced specifically for diagnostic purposes, very expensive. Plates were coated by the regional laboratories. Rabbit and guinea pig sera were provided by the IAH, which also retested doubtful sera by VNT.

Assays for antibody to non-structural proteins

3ABC-ELISA from Intervet/Bommeli (Chekit FMD-3ABC bo-ov)
This test is partially based on the 3ABC ELISA developed at the Italian national reference laboratory in Brescia. Prototype kits have been validated at various laboratories, including the BFAV and several German regional laboratories. The test is robust and easy to use. It recognizes about 80% of vaccinated and subsequently challenged cattle.

3ABC-ELISA from Panaftosa, Brazil
This Elisa is used successfully in South America. Doubtful reactions are checked by EITB. Preliminary results indicate that for usage in Europe modifications would be necessary to increase specificity.

Competitive ELISA from Cedi Diagnostics
This Elisa is based on a laboratory prototype developed at the Danish national FMD laboratory in Lindholm. In experiments with vaccinated and subsequently challenged cattle performed in Tübingen and Lindholm, this test had the best performance of all tests compared. First prototypes for the commercial version are expected in late 2003.

Peptid-ELISA from UBI (UBI FMDV NS EIA)

This test was developed by UBI, USA, and showed good results with pigs. However, as for any other NSP test, there are few data on vaccinated and subsequently challenged pigs. The original version of the test did not fully match the sensitivity of the best European test in respect to vaccinated and subsequently challenged cattle. Later versions performed better, but should be further validated regarding specificity with European sera.

Peptide-ELISA from Armin Saalmüller (BFAV, Tübingen)
A laboratory prototype based on linear B-cell epitopes, recently published in Journal of Virology showed very promising results.

Enzyme-linked immunotransfer blot assay (EITB) from Panaftosa, Brazil
This Western Blot for the confirmation of doubtful ELISA-results is used successfully in South America. However, extensive validation for European conditions would be necessary, interpretation is difficult and subjective and the availability is limited.

Appendix 19

Estimating the size of FMD outbreaks

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1. Introduction

It was clear in 2001 that the FMD epidemic that developed in the UK and spread to France, Ireland and the Netherlands was not expected. Not that way, not in these countries, not that size. It was also a surprise as models had been developed, using sophisticated mathematical programmes and software explaining that control should be achieved more rapidly. Even more, training sessions had been organised over Europe since 1992 and the vaccination ban, where none of the studied scenario ever proposed such a situation. This means that we still have to improve our prediction tools, which may not be so easy.

This presentation will try to put together some of the information needed to face any FMD outbreak, and discuss some of the ways to use them.

2. A need for more information and data

Do we know today, in 2003, everything we need to understand the 2001 situation? Do we really know how the virus entered UK, France, Ireland and the Netherlands and how it spread? If data are still lacking, we may never be able to answer these major questions. For instance, most of the archives of 1981 Brittany outbreaks (previous outbreaks in France, 20 years ago) have been destroyed, as we realised recently when we looked for them. The real information may be missing for good. This is especially true if we are facing illegal movements of animals or of animal products. At the same time, reconstructing piece by piece a past outbreak is certainly useful, but we have to admit that it may be useless to anticipate the next outbreak only from it. As FMD epidemics are now very rare, each of them may be following its own route, different from any other. Parameters will be different each time. The idea linked to use an "average" outbreak, built from past episodes and experience, to estimate what may happen next time could be misleading. So, what are the possibilities left to anticipate and to predict? This also means that models need time to be run: new field data must be available to make any estimation of the parameters, as the parameters of a previous outbreak will not fit anymore in this new peculiar situation.

Surveys opinions have been used, as the CVOs opinions survey presented here at this session. We also remember the "experts" opinions survey of 2000, presented at the Bulgaria EUFMD research group meeting. The least probable countries at risk were British Islands, and the more probable origin of the virus was Near-East countries. Five months later the reality was quite different.

In any case, if we wish to predict, we do need information, data. These we can list. A better knowledge of FMD situations, outbreaks, around the world and of the virus strains circulating

is essential. Can we improve this information world wide? There are still too many missing data for this first question. It would be useful for many countries, not only for ours.
Then the importance of animals and animal products trade is clearly a major topic. Trade routes, their trends and evolution should be monitored. The improvement of roads, the development of the world market, all are increasing the volume of goods circulating between countries and the rapidity of their circulation. This is clearly in contradiction with better sanitary measures and controls.

If we just take the example of our main airports, it proves to be difficult to check every hand luggage to look for meat product for instance. When custom officers decide to control all the passengers of a specific flight, the volume of seized food products is impressive, and this is not really illegal trade. Who is in charge of these controls, who is looking for these products? Veterinary services may not be the only concerned. Can we improve this monitoring (quantities, origins, quality) and these controls at the European level? Any action decreasing the risk of introducing the virus can be seen as decreasing the probability of a new outbreak. Information of passengers may be one of the solutions, but certainly not the only one.

At this stage, it can be recalled than we are facing three different risk categories: the risk of introduction of the virus, the risk of occurrence of a first (primary) outbreak if the introduced virus reaches a sensitive animal, and the risk to turn this first outbreak into an epidemic. Each step is linked to its own monitoring and surveillance systems. We have just seen the situation of the introduction risk, as the virus is no more present in our countries, except in laboratories confinement. We can just add that the causes of introducing the virus may be trade, neighbourhood, accidental or criminal. The probability to find rapidly a first outbreak is related to a good information and awareness within the field actors, like farmers, veterinarian practitioners and also animal traders, may be less informed than the first two. Diagnostic capacities are essential to recognise rapidly any clinical suspicion and a contingency plan must be ready, and known.

Other useful information is linked to what is related to the index outbreak.
International trade is certainly important to monitor, but we realised in 2001, that intra-national and intra-European live animal trade was also of major importance. Here we could also improve our knowledge.

Ageing the lesions of the infected animals can give information of the time when the virus arrived. The species concerned (pig versus ruminants) give a different probability of spreading the disease, and the density of farms and kind of productions in the surrounding can also change the picture. A possibility could be to give different ranks to the risk following the estimated age of the oldest lesion, the species concerned, the place where the suspicion is discovered and the density of animal farming in the area (Huirne & Windhorst 2003). Finding a suspect animal in a farm is different from finding it on a market or in a slaughterhouse. The fact that the area of concern is heavily exporting livestock or not, is another important item.
If a first blister is identified on the tongue of a cow in a dairy farm in a low density farming area, the index case may really be the primary outbreak. Any predictive modelling can start with this hypothesis. If the first clinical suspicion is already some days old and coming from a pig on a market or in a slaughterhouse, this index case may not be the primary case and another attitude should be more adapted. A predictive modelling could then start with more than one outbreak.

Another very important parameter is time, as incertitude will decrease with time. It may be adapted, following the situation, to wait some days before taking decisions like emergency vaccination or to implement a broad preventive slaughtering (Mahul & Durand 2000, Durand *et al*. 2002, Wilpshaar *et al*. 2002). The earlier the modelling and the prediction are started, the higher is the risk of a misleading conclusion. The later, the less useful it becomes. This delay is necessary to estimate the parameters of the models.

Worse and less worse scenarios can be proposed as soon as the suspicion is known, fuelled with new data as soon as they are known, but always with an attitude of uncertainty.

3 – A good use of data

Behind modelling and prediction, there is also communication of the results. At the last EUFMD general session of April 2003 in Rome, a presentation of a risk analysis linked to the probability of a new FMD outbreak occurring in the UK in connection with illegal introduction of meat from the Middle-East was presented. From the scenario followed and the hypothesis used, the probability was estimated to be of one outbreak in 130 years with a confident interval limits between one outbreak every 41 year and one every 1,110 year, at a confidence level of 90%. Here, the main issue is to explain to CVOs and to decision makers how to deal with such a result. If this fails, all research funds for FMD could be reoriented towards other diseases as even 41 year is beyond any government duration.

This means that modelling cannot be seen just as a way to face lack of data. It is certainly something else, as presented last year (Moutou & Durand 2002). One of the best ways to use models is for training purposes and for comparison of different scenarios, once hypothesis have been accepted. Even if some of the assumptions prove later not to be true, the comparisons of different scenarios may be useful anyway.

4. Improving data quality

The question of increasing the knowledge on FMD and FMDV strains around the world has already been addressed, as well as the point concerning the control of what is entering in every country. In any country, other steps can also be improved, all of them being able to decrease the consequences of a virus introduction. However, we have to admit that contingency plans have only a little impact on the probability of introducing the virus, as this is mainly linked to commercial rules.

Diagnostic capacity is a sensitive item. If the clinical suspicion must be rapidly recognised, the virological and serological diagnostic capacities must be able to react rapidly and securely. Usually, a single national laboratory should be enough to face all samples sent for virological analysis and diagnosis. One of the new questions is linked to serology, as preventive vaccination could be used during next outbreaks. What is the capacity expected for any country? Should we propose a ratio linking national herd figures and the number of serological tests that could be performed by week? Is it better to have a single centralised laboratory or different local facilities? As the recovery of a FMD free status without vaccination will depend on serological tests, this question is important.

The discussion is going on in France for instance, with a first draft oriented toward the possibility of having 5 or 6 local laboratories dedicated to FMD serology. However, this is not yet decided and new questions arise. What should be the capacity in term of serological kits? Who shall be in charge of a reagents bank? Shall we foresee 10,000 or 80,000 serological

tests? How can we organise the training of the people of these laboratories? Are the biosecurity questions well coped? What do we know about good laboratory practices and quality control in this context? The questions are clearly not so simple.

5. Discussion

Recently for a new master in public health passed at Paris VI University, a FMD outbreak modelling was realised (Le Menac'h 2003). Following the hypothesis of 20 primary outbreaks spread at random over the whole territory, but only connected with ruminants farms, the model used here computed an average (from 200 different tries) epidemic of 9,551 infected premises lasting 348 days. We may expect that the software is a little too pessimistic.

Predicting or estimating the location and the size of next FMD epidemic is a real challenge and we may not have all the tools to achieve this properly. However, it is clear that we do know where to improve epidemiological surveillance so that the surprise, once it happens, should not be too big.

There is to improve our knowledge on FMD situations around the world, and specially the evolution of FMDV strains. Better information on animals and animal products trade routes is also essential. This should be done at an international level and at an intra-national level, as we learnt from 2001 epidemics. At the end, we need to get sure that all our farming (farmers, animal traders) and sanitary actors (veterinary practitioners, veterinary officers, customs officers) are aware of these challenges. The diagnostic capacities should also be adapted.
Then, estimating next epidemic size could be easier, what could be the tool or the model used, and even if we wish no new epidemic at all.

6. References

Durand B., Moutou F., Mahul O. (2002) - Apport de la modélisation pour l'aide à la décision lors d'épizootie de fièvre aphteuse. *Epidémiol. Santé Anim.*, **42** : 43-56.

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Le Menac'h A. (2003) Modélisation de la diffusion spatio-temporelle des épizooties de fièvre aphteuse en France, DEA de Biomathématiques, INSERM U144, Université Paris VI, 19p.

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Wilpshaar H., Meuwissen M.P.M, Tomassen F.H.M., Mourits M.C.M., van Asseldonk M.A.P.M., Huirne R.B.M. (2002) Economic decision making to prevent the spread of infectious animal diseases. 20th Conference of the O.I.E. Regional Commission for Europe, Finland,16p.

Appendix 20

Difficulties in sending FMDV specimens to WRL (Draft paper)

Vilmos Pálfi

Report of the thirty-Fifth Session of the EC for the Control of FMD

Item 3. Discussion

The number of samples from which no virus could be recovered at the WRL probably reflected in part of the quantity of the material collected and inadequate conditions of transport.

Recommendations 14 and Note for 8.2 :

The Session called for an investigation by the EUFMD, OIE and WRL of the current difficulties of the international transport of FMD samples by air in order to arrive at solutions for these problems

Important Web Sites on Shipping Matters

1. http://www.who.int/emc/pfds/emc97_3.pef
WHO /EMC/97.3
Guidelines for the Safe Transport of Infectious Substances and Diagnostic Specimens

2. http://www.saftpak.com
2003 CD – Comprehensive Guide to Shipping Infectious Substances and Diagnostic Specimens

3. http://www.iata.org/cargo/db and http://www.aita.org/dangerousgoods/index
Dangerous Goods by Air 2003 Revised 44th edition

4. http://www.absa.org/resbspubs.html
American Biological Safety Association
Infectious Substances Shipping Guidelines, published annually only in English
Link for IATA online store:
http://www.iataonline.com/shop/product.asp?sku=9052%D03

5. http://hazmat.dot.gov/icao.htm
ICAO Technical Instructions
Link to publications containing international requirements

Important publications on shipping matters

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. Requirement for packaging and despatch of biological materials to the IAH,Pirbright Laboratory,UK from overseas. IAH, Pirbright Laboratory, 5th November 2001
3. International transfer and laboratory containment of animal pathogens
OIE Int.Anim. Health Code, 2000
4. DSMZ, Shipping of Infectious, Non-Infectious and Genetically Modified Biological Materials. International Regulations
5. IATA: Infectious Substances Shipping Guidelines 2003, 44th edition
6. ICAO: Technical Instructions for the Safe Transport of Dangerous Substances by Air 2003/2004
7. ABSA: Infectious Substances Shipping Guidelines
8. SAFTPAK, 2003 CD- Comprehensive Guide to Shipping Infectious Substances and Diagnostic Specimens
9. WHO, Geneva 1997: Guidelines for the safe Transport of Infectious Substances and Diagnostic Specimens

Terrestrial Animal Health Code 2003
Article 1.4.6.4. Classification of animal pathogens

d., Group 4 animal pathogens
Disease producing organisms which are either exotic or enzootic but subject to official control and which have a high risk of spread from the laboratory

Geographical spread if released from the laboratory is widespread

Direct animal to animal transmission occurs very easily
The statutory control of animals movements over a wide area is necessary

The disease is of extremely severe economic and/or clinical significance

FMDV belongs to Group 4 Animal Pathogens

Transport Planning

It is the responsibility of the sender to ensure the correct designation, packaging, labelling 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.

Table 1. ***Guidance on the laboratory requirements for the different containment groups***
Source : Terrestrial Animal Health Code 2003. Chapter 1.4.6.

http://www.oie.int/eng/normes/Mcode/A_00023.htm

Requirements of the laboratory:

A., Laboratory siting and structure
Points 1-10
B., Laboratory facilities
Points 11 - 18
C., Laboratory discipline
Points 19 – 25
D., Handling of specimens
Points 26 - 29

	CONTAINMENT GROUP		
REQUIREMENTS OF THE LABORATORY	**2**	**3**	**4**
A) Laboratory siting and structure			
1. Not next to known fire hazard	Yes	Yes	Yes
2. Workplace separated from other activities	Yes	Yes	Yes
3. Personnel access limited	Yes	Yes	Yes
4. Protected against entry/exit of rodents and insects	Yes	Yes	Yes
5. Liquid effluent must be sterilised		Yes and monitored	Yes and monitored
6. Isolated by airlock. Continuous internal airflow		Yes	Yes
7. Input and extract air to be filtered using HEPA or equivalent		Single on extract	Single for input, double for extract
8. Mechanical air supply system with fail–safe system		Yes	Yes
9. Laboratory sealable to permit fumigation		Yes	Yes

10. Incinerator for disposal of carcasses and waste	Available	Yes	Yes on site
B) Laboratory facilities			
11. Class ½/3 exhaust protective cabinet available	Yes	Yes	Yes
12. Direct access to autoclave	Yes	Yes with double doors	Yes with double doors
13. Specified pathogens stored in laboratory	Yes	Yes	Yes
14. Double ended dunk tank required		Preferable	Yes
15. Protective clothing not worn outside laboratory	Yes	Yes	Yes
16. Showering required before exiting laboratory			Yes
17. Safety Officer responsible for containment	Yes	Yes	Yes
18. Staff receive special training in the requirements needed	Yes	Yes	Yes
C) Laboratory discipline			
19. Warning notices for containment area	Yes	Yes	Yes
20. Laboratory must be lockable	Yes	Yes	Yes
21. Authorised entry of personnel	Yes	Yes	Yes
22. On entering all clothing removed and clean clothes put on		Yes	Yes
23. On exiting all laboratory clothes removed, individual must wash and transfer to clean side		Yes	

24. Individual must shower prior to transfer to clean side			Yes
25. All accidents reported	Yes	Yes	Yes
D) Handling of specimens			
26. Packaging requirements to be advised prior to submission	Yes	Yes	Yes
27. Incoming packages opened by trained staff	Yes	Yes	Yes
28. Movement of pathogens from an approved laboratory to another requires a licence	Yes	Yes	Yes
29. Standard Operating Procedures covering all areas must be available	Yes	Yes	Yes

Packaging requirements for transport of infectious substances

The packaging requirements are determined by the UN and are contained in IATA regulations in the form of Packaging Instructions 602 .The system consists of three layers (Primary and secondary receptacle and outer shipping package).

Detailed packaging instructions for sending biological materials to IAH Pirbright were published in November 2001 (see List of Important publications), Web site: http://www.iah.bbsrc.ac.uk/indexhtml -NOT FOUND

Additional information:
Maximum Net Quantity per Package (IATA Regulation)

Substance	Maximum Net Quantity per Package	
	Passenger Aircraft	Cargo Aircraft
Infectious substance, liquid	50 ml	4 l
Infectious substance, solid	50 g	4 kg

"Difficulties" in sending FMDV samples to WRL:

1., People shipping infectious substances require to be trained on the current IATA/ICAO rules/regulations which should be updated frequently to minimize the risk associated with the transportation of infectious materials.

2., Selection and collection of specimens submitted to WRL for investigation of vesicular virus infections demands specific knowledge. During transport specimens must be kept under optimal conditions ensuring satisfactory results of investigations.

Source of difficulties:

Relevant information for collection and shipment of specimens (infectious substances) currently are available separately.

Solution of "difficulties":

Manual should be compiled all the necessary information and instructions for collection and shipment of infectious substances to WRL for investigation of vesicular viruses.
This Manual should collect all general (IATA/ICAO) and specific information (WRL) ensuring the safe transport and satisfactory results of investigations.

This Manual should appear on FMD website of WRL, Pirbright.

Appendix 21

An assessment of guidelines for treatment of meat from a FMD vaccination zone

Per Have, Danish Veterinary Institute, Lindholm, Kalvehave

According to the OIE animal health code a waiting period following the last outbreak of FMD is necessary before a country or zone can regain its status as free from FMD with or without vaccination. The waiting period is primarily meant to allow for disclosure of residual infectivity, if any, by exposure of susceptible species at risk and development of clinical signs or signs of infection, according to the 2002 revision of the OIE FMD animal health code.

Animal products prepared during the waiting period may constitute a risk of transmitting FMD, depending on species, vaccination status and product type, among others. The risk may be linked either to the presence of carriers or recently infected naive animals at risk. Emergency protective vaccination clearly will reduce the risk of encountering recently infected animals, whereas the risk posed by carriers established prior to vaccination would not be significantly altered by vaccination.

Fresh meat derived from carrier animals (cattle and sheep) does not contain infectious virus (Sutmoller, 2001) whereas fresh meat from recently infected (clinically or subclinically) animals may contain significant amounts of virus. In ruminants, maturation to pH<6, deboning and removal of visible lymphatic tissue greatly reduces any viral infectivity and is often used as a risk reducing step when importing beef from countries that are not free from FMD without vaccination. In contrast, maturation to pH<6 is not a reliable feature of pork, hence thermal treatment is normally applied as a risk reducing step rather than maturation and deboning.

The risk presented by fresh meat is tightly associated with the occurrence of recently infected animals at the time of slaughter. The occurrence of such animals is dependent on availability of susceptible animals at risk, i.e. before vaccination or after vaccination until the onset of immunity in vaccinated species and obviously also in species left unvaccinated. It is worth emphasizing here, that the value of a waiting period to demonstrate (clinically or by NS-serology) absence of viral activity also depends on availability of susceptible animals at risk. Conversely, since vaccination eliminates animals from population at risk (also in terms of NS-serology) it follows that monitoring for viral activity and risk managment for fresh meat should both focus on non-vaccinated animals if possible.

Heat treatment

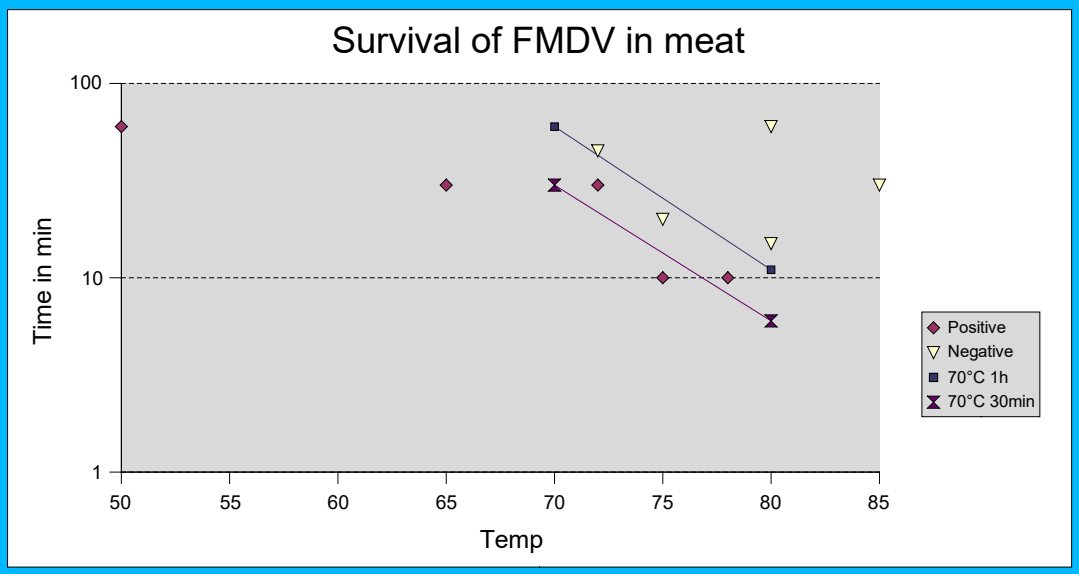
Thermal inactivation is widely used to reduce or eliminate microorganisms (bacteria, viruses) in animal products. The effect of heating is always determined by a combination of time and temperature. For practical purposes, the kinetics of inactivation is often assumed to be first-order but this is not always the case. Quite often the survivor curve shows a biphasic shape which may be approximated by describing a linear decay of two discrete populations, i.e. a sensitive and a more resistant fraction within a given population of organisms. The degree of biphasic behaviour may be temprature dependent.

The decimal reduction time D_T at a given temperature T denotes the time needed to reduce the viable population by 90%. A semi-log plot of D-values against temperature often yields a linear relationship, from which a z-value may be calculated. The z-value is defined as the number of

degrees temperature required to change D by one log unit, that is the change in temperature needed to raise or lower the death rate by a factor ten. The z-value is normally considered to be constant for a given strain of microorganism in a given product.

In practise, heat treatment includes a heating phase and cooling phase. 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 ($\sum 10^{(T-Tr/z)} * \Delta t$ where T is the temperature during the time interval Δt and T_r is the reference temperature e.g. 70°C).

The heat resistance of FMDV in meat products has been investigated in a number of studies. Thus Blackwell et al. (1988) found that FMDV in infected lymph node survived a thermal processing time of 1.45 hr reaching a final core temperature of 71.2°C. However, the period above 70°C was appreciably less than 30 min. FMDV did not survive 79.4°C reached after 2 hr. Garcia-Vidal et al. (1988) found that processing at 75°C for 20 min or 80°C for 15 min eliminated FMDV. In a more recent study (Masana et al., 1995) it was found that FMDV survived processing at 75°C for 2 hr but not for 4 or 5.75 hr. Although these studies used an overall similar approach, minor technical differences in conjunction with absence of kinetic data (rate of inactivation) makes it difficult to make a proper comparison, especially because estimates of D and z values have not been made. Below are the results of Garcia-Vidal et al. plotted into positive or negative relative to lines corresponding to treatment of 70°C for 1 h or 30 min, respectively, and assuming a z-value of ≅ 14, which has been found to be a reasonable approximation for a number of viruses (unpublished data).



Control measures in the new EU directive

If protective vaccination is applied, a surveillance area around the vaccination zone must be defined, where intensified surveillance can take place (Article 52-2). However, there is no indication how such a surveillance should be performed.

The waiting period until regaining freedom from FMD when applying protective vaccination is divided into 3 phases according to overall perceived risk:

- *Article 54: Measures applicable in the vaccination zone during the period from the beginning of emergency vaccination until at least 30 days have elapsed following the completion of such vaccination (Phase 1)*
- *Article 55: Measures applicable in the vaccination zone during the period (not earlier than 30 days) from (the date of completion of) emergency vaccination until the survey and the*

classification of holdings are completed (Phase 2)

- *Article 58: Measures applicable in the vaccination zone after the completion of the survey and the classification of holdings until the foot-and-mouth disease and infection free status is recovered (Phase 3)*

The following treatments are prescribed for meat and milk, respectively, for each of the 3 phases.

		Cattle	***Sheep***	***Milk***	***Pork***
Phase I	+vacc	heat treatment[1]	heat treatment[1]	UHT or HTST	heat treatment[1]
	-vacc	n.a.	not allowed	n.a.	not allowed
Phase II	+vacc	deboning	deboning	UHT or HTST	heat treatment[1] or fresh meat[2]
	-vacc	n.a.	? (e.g. sheep)	n.a.	?
Phase III	+vacc	deboning	deboning	UHT or HTST	heat treatment[1] or fresh meat[2]
	-vacc	none	none	n.a.	none

[1]A number of options are available, as specified in Council Directive 2002/99/EC of 16 December 2002 laying down the animal health rules governing the production, processing, distribution and introduction of products of animal origin for human consumption

[2] can only be placed on national market until 3 months after last outbreak; later rules to be decided by committee procedure

In phase I and II only meat from vaccinated animals can be marketed, reflecting a relative lower risk of vaccinated animals to non-vaccinated animals. In phase II the requirement of heat treatment of beef is replaced by maturation and deboning, reflecting a less stringent effect of deboning compared to heat treatment. In phase III the requirement for treatment of meat from vaccinated animals is maintained, whereas meat from non-vaccinated animals can be marketed without treatment. The latter is a dramatic change from phase II and can hardly be justified by relative risk assessment of meat from vaccinated vs. non-vaccinated animals. Likewise, the particular restriction on marketing of pork to the national market from vaccinated animals is not in line with the fact that, should any viral activity remain, it would mainly constitute a risk to non-vaccinated animal (pigs not being carriers).

The heat treatments referred to in Council Directive 2002/99/EC prescribe the following options**:**

- *(a) Heat treatment in a hermetically sealed container with an F_0 value of 3,00 or more*
- *(b) Heat treatment at a minimum temperature of 70 °C, which must be reached throughout the meat*
- *(c) Heat treatment at a minimum temperature of 80 °C, which must be reached throughout the meat*
- *(d) Heat treatment in a hermetically sealed container to at least 60 °C for a minimum of 4 hours, during which time the core temperature must be at least 70 °C for 30 minutes*
- *(i) Heat treatment ensuring a core temperature of at least 65 °C is reached for the time necessary to achieve a pasteurisation value (pv) equal to or more than* 40.

Discussion

The lethal effect of option b and c will depend on the length of heating and cooling period and it is therefore not possible to make a direct comparison and assessment of each option. If, as mentioned above, a lethal effect corresponding to 70°C for 1 hour is used as a guideline, option d can be shown (by lethal rate integration) to correspond to 70°C for approx. 70 min. Option a corresponds to 121°C for 3 min and exceeds by far 70°C for 1 hour.

In conclusion, the issue of safeguarding meat from FMDV needs particular consideration of what must be treated, i.e. an assessment of the relative risk of occurrence and quantity of FMDV in meat from vaccinated and non-vaccinated animals, respectively as well as how such meat should be treated. The current requirements for the heat treatment of meat from FMDV vaccinated animals, although based on empirical data, can be considered to provide a high degree of safety when applied to low-level contaminated products such as meat from vaccinated animals.

Until specific estimates of death rates and heat stability of FMDV become available it will difficult to set up more specific guidelines in terms of quantitative risk reduction of FMDV in meat. Such guidelines should also take into account the relative risk of occurrence and quantity of FMDV in meat from vaccinated and non-vaccinated animals, respectively.

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Appendix 22

Treatment of meat and milk from vaccinated herds/animals which test negative by NSP test; a risk analysis

A. Dekker

At the 2002 session of the research group Dr. Donaldson noted that in 2001 there was disparity between the export restrictions faced by the FMD affected countries in the EU compared to those in South America. Dr. Donaldson had reviewed "The minimum requirements for importation into Europe of live animals, fresh meat and offal of the bovine species". Dr. Donaldson rightly suggest that EU and OIE regulations for trade should be identical and therefore if special rules are considered necessary for the trade of de-boned beef or other meat products, these should be recommended to the OIE for their consideration. Dr. Have had commented on the survival of FMD after heat treatment and rightly states that kinetic data (rate of inactivation) in almost all papers on FMD survival in meat is missing (McKercher et al. 1980, Blackwell 1984, Mebus et al. 1997). Producing FMD inactivation curves of meat or meat products is difficult. Determining the inactivation rate of FMD in milk is possible (de Leeuw et al. 1980), but most others only looked at the result at the endpoint (Hyde et al. 1975, Cunliffe et al. 1977, Cunliffe et al. 1978, Cunliffe et al. 1979, Walker et al. 1984). Dr. Have presented information that the rate of inactivation of viruses in meat increases approximately 5 times if the temperature is increased with 10 degrees Celsius. This is a higher increase in reaction rate than normally observed for biological reactions. Others have shown that the inactivation rate of FMD depends on the substances added to the virus suspension and virus can be stabilised by drying (Ferris et al. 1990, Dekker 1998). As Dr. Have stated there is certainly need for more data of FMD inactivation by heat in meat and milk of infected animals. The question remains at which reduction of FMD virus titre one should aim.

The Sanitary and Phytosanitary (SPS) agreement of the World Trade Organisation states that the risk should be reduced to an acceptable level of risk, **no** unnecessary trade barriers should be realised. Even more important the SPS agreement mentions the "equivalence principle", which means that different means of risk reduction can be exchanged. This last principle is very important, because would titres of 10^6 be expected in vaccinated animals? If not, do we need 6 log reduction?

Different parts of this issue will be discussed during the meeting.

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Appendix 23

Assessing outbreak-specific control strategies against FMD – A simulation approach

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Worldwide, the productive livestock industry depends on the health status of its animals. One of the greatest economic threats to this branch of production are highly contagious diseases – for example Foot and Mouth Disease (FMD). During the last outbreak in the UK in 2001, cases were reported on over 2000 farms, approximately 6 million animals had to be slaughtered, and the cost of the outbreak was estimated as 8 billion Euros[i].

In late March 2001, the British Government decided to implement the following control strategies: slaughter of animals on infected farms within 24 hours, and within 48 hours on contiguous farms. In one part of the country (notably Cumbria, and the adjacent county of Dumfries & Galloway in Scotland), compulsory slaughter of all sheep within a 3-km radius of infected holdings in defined areas was undertaken because of concerns that extensive disease in sheep might pose a threat to dairy and beef cattle in the area.[ii] Although discussed early on, emergency vaccination was not performed for various reasons.

As a consequence of the UK epidemic alternative contingency plans should be considered and evaluated during "peace time". The Swiss Veterinary Office in collaboration with the IVI invited several Swiss organisations and stakeholders to discuss the use of prophylactic vaccination and the possible reaction of consumers towards the marketing of meat and meat products originating from vaccinated animals. One of the main conclusions from this "round table discussion" was that an emergency protective vaccination strategy, in addition to stamping out of FMD infected herds, was preferred by all organisations.[iii]

Based on the conclusions of this meeting it is obvious that alternative control strategies to the mass slaughter of animals have to be considered. So far, in Switzerland there is no defined strategy for the implementation of vaccination and other strategies alternative to stamping out. Therefore, different outbreak scenarios are needed to evaluate the impact of strategies and to assess their benefit. To approach this task, we are using a computer model to simulate the spread of FMD in different outbreak scenarios and to quantify the effects of different control strategies. On the basis of the output of the different scenarios recommendations in respect of control measures can be derived and eventually implemented in case of emergency. The simulation model we are using is called InterSpread Plus (ISP) and was developed by Massey University in Palmerston North, New Zealand. A pre-cursor of ISP was used in the assessment of vaccination in the UK outbreak.

ISP is a stochastic, spatio-temporal computer simulation model for infectious diseases in farm animals. The program creates a virtual population based on data provided (i.e. number and geographical locations of the farms, species and their respective numbers on each farm). The initial infection is randomly assigned to one or more of these farms and the model then calculates the spread of disease on a daily basis taking account of the following spread mechanisms:

- spread through movement (animals, persons and vehicles)
- airborne spread
- local spread
- dairy tanker routes

Additionally, these mechanisms are influenced by several other pre-defined parameters.

ISP requires a variety of input data from many different sources. This information had to be collected prior to running the simulations. Part of this information could be extracted from existing animal databases such as the Swiss agrarian-political information system (AGIS) and the animal movement database (TVD). Additional data was gathered by an extensive screening of scientific publications. In the case where no written information was available, we relied on expert opinion.

Final results of the simulation are not yet available. First, a baseline scenario was defined reflecting the eradication strategy lined out in the current Swiss legislation. This contingency plan included the following measures:

- depopulation of infected herds
- movement ban of all farmed livestock throughout Switzerland during at least a 7 day period
- the establishment of protection and surveillance zones, with a radius of 3 km and 10 km respectively, around infected premises. All farms within these zones will be put under surveillance and movement restrictions will apply
- forward and backward tracing of dangerous contacts and surveillance of these contact farms

The efficiency of the different control strategies can be evaluated during the course of the modelled epidemic. It is possible to specifically apply the strategies spatially, as well as temporally, differentiated. The extent of an outbreak occurring either in a sparsely or a densely populated area will be compared. Further, we'd like to examine the effect of the species infected on the index farm – cattle vs. swine vs. sheep – on the course of the epidemic. Based on these scenarios other control strategies such as pre-emptive culling of dangerous contact farms, the pre-emptive culling of premises lying within a defined radius to an infected herd, and different vaccination strategies (mass prophylactic vaccination vs. ring vaccination) will be evaluated either as a single measure or in combination to other strategies. From the final output of these simulations we will provide the authorities with the necessary base information on how to best deal with a specific outbreak and to minimize financial as well as animal losses.

This presentation will be a synopsis of our preliminary results and an outlook on future work.

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Appendix 24

Further validation of the solid-phase competitive ELISA for FMDV types A, C & Asia 1

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Institute for Animal Health, Pirbright, UK

Specificity

160 UK negative sheep, 159 UK negative cattle and 156 UK negative pigs were tested by the type A. C and Asia 1 SPCE.

FMDV Type A						
	Sheep (n=160)		Cattle (n=159)		Pigs (n=156)	
Cut-off	Mean (PI)	Specificity	Mean (PI)	Specificity	Mean (PI)	Specificity
40%	14.0	99.8%	16	99.4%	9.9	100%
50%	14.6	100%	16.2	100%	9.9	100%
60%	14.6	100%	16.2	100%	9.9	100%

FMDV Type C						
	Sheep (n=160)		Cattle (n=159)		Pigs (n=156)	
Cut-off	Mean (PI)	Specificity	Mean (PI)	Specificity	Mean (PI)	Specificity
40%	6.1	99.4%	17.5	95.6%	4.5	100%
50%	6.3	100%	18.6	100%	4.5	100%
60%	6.3	100%	18.6	100%	4.5	100%

FMDV Type Asia 1						
	Sheep (n=120)		Cattle (n=159)		Pigs (n=156)	
Cut-off	Mean (PI)	Specificity	Mean (PI)	Specificity	Mean (PI)	Specificity
40%	1.0	98.1%	2.4	98.1%	0.36	100%
50%	1.3	98.5%	2.7	98.7%	0.36	100%
60%	2.2	99.2%	3.0	99.4%	0.36	100%

Conclusions:

- Using a cut-off of either 50% or 60% resulted in 100% test specificity for all species against types A and C.
- Using a cut-off of 40% for types A and C, the specificity was greater than 99%, with the exception of type C cattle which gave the lowest specificity of 95.6%.
- Using a cut-off of 40%, 50% or 60% for Asia 1 with pig sera gave 100% specificity. Using any of the cut-off values for Asia 1 sheep and cattle sera resulted in a specificity of greater than 98.1%.

- The SPCE for types A, C, and Asia 1 show a similar specificity to that described for type O. These assays should be suitable for use in sero-surveillance.

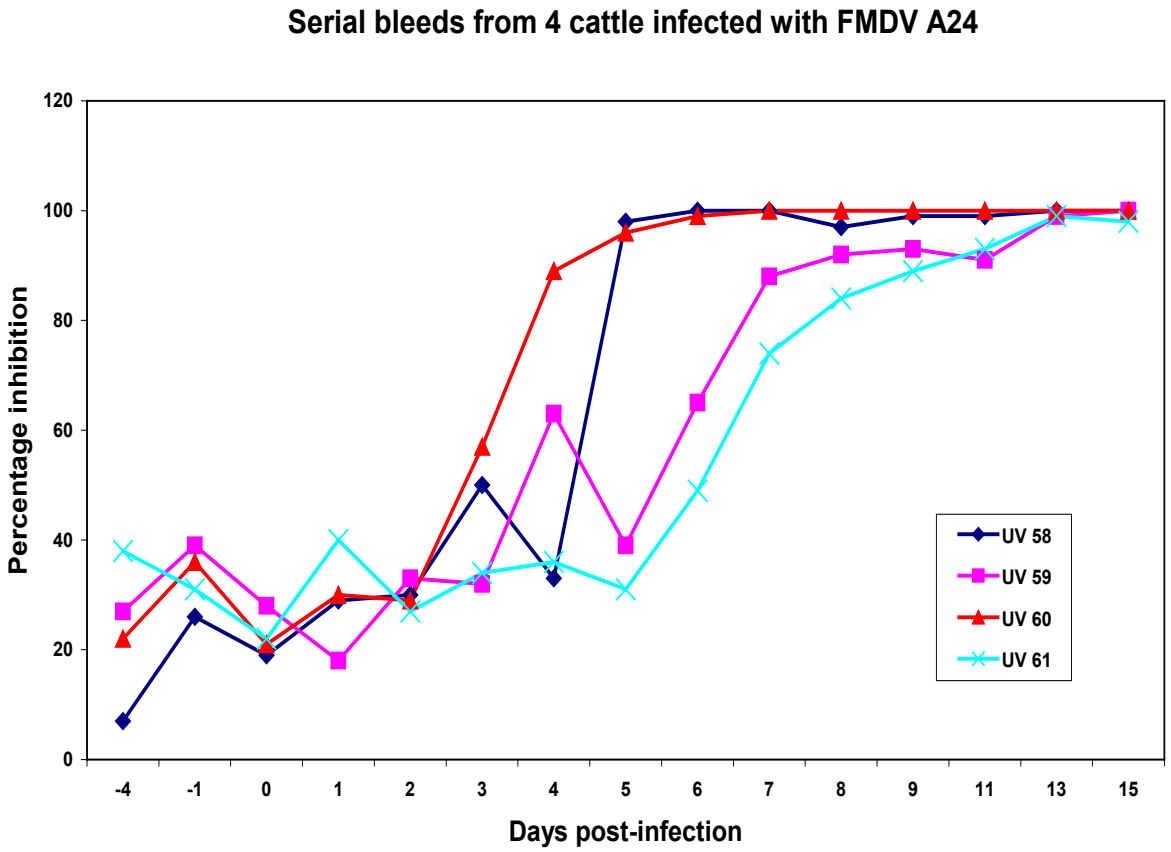
Note: Many of the false positive sera were far beyond the normal distribution of negative values and correlated with poor quality samples with either bacterial or fungal contamination.

Sensitivity

Where possible serial bleeds from animals infected with types A, C and Asia 1 have been used. In their absence serial bleeds from animals vaccinated and subsequently challenged with live virus were used.

Type A

a) Sera (69) collected daily from 4 cattle infected with FMDV A24 were examined by SPCE. All cattle were sero-positive (>50%) between 4-7 days post infection (dpi). All gave PI values >90% by 11 dpi.



b) Sera (48) collected at 7 day intervals from 6 sheep vaccinated with FMDV A22 vaccine all gave a good response to the vaccine as determined by VNT. Four were SPCE positive (>50%) by 7dpv which correlated with the VNT results. The remaining 2 sheep were sero-positive by 14dpv.

c) Sera (52) collected at 7 day intervals from cattle vaccinated and subsequently challenged with FMDV A22 all gave a good response to vaccine as determined by VNT. All eight cattle were SPCE positive by 15 dpv with PI values between 66-88%. This agreed with the VNT results except for 3 animals which were VNT positive at 7dpv. All animals gave SPCE values greater than 91% by 7 days post challenge.

d) Sera (40) collected at 7 day intervals from 8 cattle vaccinated and subsequently challenged with FMDV A24 gave a poor response to vaccine (VNT data not available) and all gave SPCE values of 77-99% by 7dpc.

e) Sera (41) collected at 7 day intervals from 10 cattle vaccinated and subsequently challenged with FMDV A22 gave a good response to vaccine (VNT data not available) and all gave values between 65-99% by 15dpv (one animal 79% at 7dpv). All gave SPCE values >91 at 7 dpi.

Type C

a) Sera (52) collected at roughly 7 day intervals from 13 sheep vaccinated with FMDV C Oberbayern. All animals were SPCE positive (>50% PI) between 13-19dpv (not all animals sampled on 13dpv). These results were in agreement with the VNT results in 10/13 animals. The VNT detected three animals as being positive at the earlier sample than the SPCE.

b) Sera (32) collected at 7 day intervals from 8 cattle vaccinated with FMDV C Oberbayern. All eight cattle gave SPCE values greater than 60%PI at 14dpv.

Asia 1

a) Sera (53) collected at 7 day intervals from cattle vaccinated and challenged with Asia 1 Shamir. Vaccine response appeared to be poor (VNT data not available) with only 3/11 cattle giving SPCE values >50%. At7dpc 8/11 cattle gave SPCE values greater than 95% with 3/11 giving values between 50-60%.

Conclusions

- Where post-infection sera were available the sensitivity of the A, C and Asia 1 tests appeared similar to that shown by the type O SPCE. i.e. 100% chance of detecting infected animals by 8 days post-infection.

- Antisera from vaccinated animals showed a good correlation with VNT and in most cases the humoral antibody response to vaccination was detected by 14 days post-vaccination. Very high PI values (>90%) were obtained at 7 days post-challenge.

- Urgently need more serial bleeds from infected animals.

Subtype specificity and test cross-reactivity

A panel of antisera raised against different subtypes of FMDV were examined to establish the range of detection of the type O, A, C and Asia 1 solid-phase competitive ELISAs. This also gave an indication of cross-reactions between types. The antisera were mainly from vaccine experiments, some of which were polyvalent. All antisera gave VNT titres equal to or greater than 1/45.

KEY: Red denotes >70% PI **Yellow denotes 51-70% PI**

TYPE O ANTISERA	SPCE Type O PI%	SPCE Type A PI%	SPCE Type C PI%	SPCE Type As1 PI%
O Manisa (5879)	104	23	23	3
O Manisa (ALSA) (ID-DLO)	99	26	17	5
O Manisa Lelystad (1733/-99)	94	40	28	0
O Manisa Lelystad (1734)	89	16	33	1
O Manisa (1458) pooled? diluted	102	15	35	9
O 3039	101	32	23	0
O 4174 (1621)	87	15	50	0
O Phil-95 14/10/93	95	26	40	0
O Phil-95 15/9/99	92	31	29	0
O IND 53/39 (1382)	91	23	35	0
O IND 53/79 (1329)	100	70	12	6
O IND 53/79 (1257+)	92	27	14	7
O BFS (1393)	90	4	1	0
O BFS (1860)	99	43	5	23
O LAUS BUS	89	22	21	0
O ISR 2/85 (1410)	100	44	17	16
O ISR 2/85 (1439)	90	74	9	0
O ISR 2/88 (1462)	95	33	7	0
O ISR 2/88 (Dalton) (1462)	97	21	3	10
O TAIWAN-98-Lelystad (1493-? Porcine)	65	8	0	0
O K77/78	61	42	34	0
O Campos (1364)	94	18	8	0
O Campos	98	25	19	8
O HK 6/83 o;14/12/88	75	9	4	4
O HK 6/83 o;22/12/88	68	10	6	0

O HK 6/83 RJO-6.1-89	97	46	19	3
O THAI/189 (210)	89	16	27	11
O BKK 60	84	15	15	8
O THAI 189/87 (#83)	99	95	73	91
O CHINA (?Porcine)	71	9	12	1
O MUKTESWAR (AEFHIJ pool)	95	84	79	90

TYPE A ANTISERA	SPCE Type O PI%	SPCE Type A PI%	SPCE Type C PI%	SPCE Type As1 PI%
A22 Iraq (1481)	0	76	16	0
A SAU 23/86 (1569)	36	45	25	0
A IND 17/82	75	90	64	23
A KEN 35/80	36	61	37	13
A THAI (482)	96	97	80	82
A POR 1/83 (1260)	0	28	18	6
A IND 7/82 (16399)	31	82	21	0
A IND 7/82 (16231)	66	92	24	8
A22 IND 7/82	68	93	28	4
A IND (16293)	67	92	30	13
A IND 7/82 (16387)	64	91	26	0
A IND 7/82 (16228)	67	92	30	0
A IND 7/82 (16234)	72	93	37	0
A IND 7/82 (16204)	67	93	43	0
A IND 7/82 (16275)	65	92	42	0
A IND 17/82	68	90	55	28
A SAU 41/91	28	62	11	0
A SAU 23/86 (1430)	82	74	73	38
A24 20/87 (pooled)	32	53	26	20
A24 CRUZ 48-54	35	55	30	10
A24 CRUZ (B2 pool)	58	61	52	45
A2 CRUZ	52	56	50	39
A22 IRAQ 24/64 (Wellcome)	19	71	32	0
A22 IRAQ (1401)	0	77	41	0
A22 Mahmatli	39	81	32	0
A SAUDI 41/91 SI 96	0	55	25	0

TYPE C ANTISERA	SPCE Type O PI%	SPCE Type A PI%	SPCE Type C PI%	SPCE Type As1 PI%
C INDIA 51/79 (1001)	75	54	94	67
C3 INDIA (mars) (1361)	15	32	98	46
C3 INDAIAL	25	14	67	21
C PANDO URUGUAY (pc49-52) (1277)	44	32	93	25
C NOV	40	28	81	35
C INDIA 51/79 (1001)	63	55	94	70
C NOV (KA 52)	22	38	87	38
C OBER (from IVB)	5	38	71	3
C1 Detmold (19)	37	47	86	0
C SPAIN (99) 22/12/88	18	22	63	3
C SPAIN (99) 14/12/88	22	10	68	5
C SPAIN (99)	16	11	67	0
C SPAIN (98)	30	15	60	0
C SPAIN (97)	13	13	45	0
C SPAIN (96)	49	13	78	0
C SPAIN (95)	30	24	64	0
C SPAIN (94)	48	17	69	0

TYPE ASIA 1 ANTISERA	SPCE Type O PI%	SPCE Type A PI%	SPCE Type C PI%	SPCE Type As1 PI%
ASIA INDIA 8/79 (RA29)	0	13	13	57
ASIA CAMB (C-SIII)	53	18	34	69
ASIA (HOECHST)	75	66	69	95
ASIA TIA 1/85 (499)	59	61	59	91
ASIA TAI (trivalent Asia/O/A)	98	80	58	99
ASIA ISR 3/89	25	8	14	88
ASIA TUR - 73 (1234)	25	28	8	60
ASIA SHAMIR (1536)	95	98	87	82

PHASE XVII SERA

Antisera	SPCE Type O			SPCE Type A			SPCE Type Asia 1		
O	96	94	97	26	19	23	18	5	9
A	64?	45?	52?	91?	89?	86?	95?	96?	97?
Asia 1	21	8	35	65	60	59	97	95	97

Conclusions

- Type O SPCE capable of detecting all serotypes. Little cross-type reactivity (Thailand and O Mukteswar appear to be from polyvalent vaccine).

- Type A SPCE has greater cross-type reactivity. Apparent lower sensitivity for antibodies to A24, not borne out by serial bleeds from A24 infected animals (see Sensitivity section and Phase sera). The A Thai antiserum is possibly from a polyvalent vaccine.

- Some cross-type reactivity with type C assay. Poor sensitivity with C Spain 1988 antisera (low titre VNT).

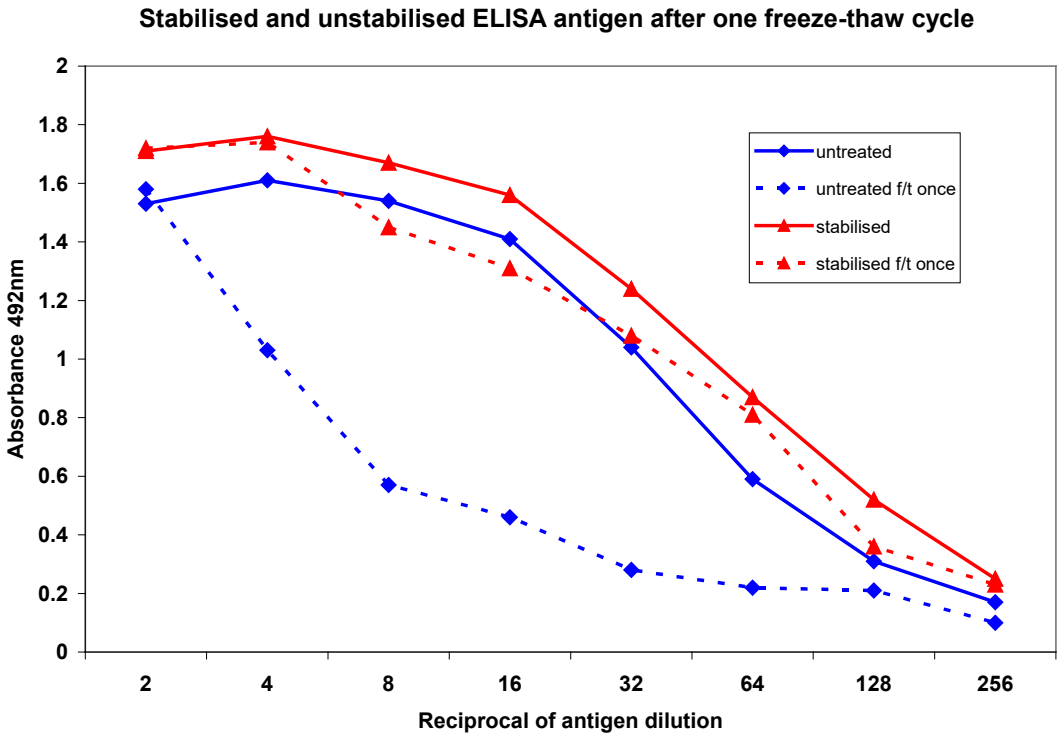
- Asia 1 assay very cross-type reactive, particularly Asia 1 Shamir antisera.

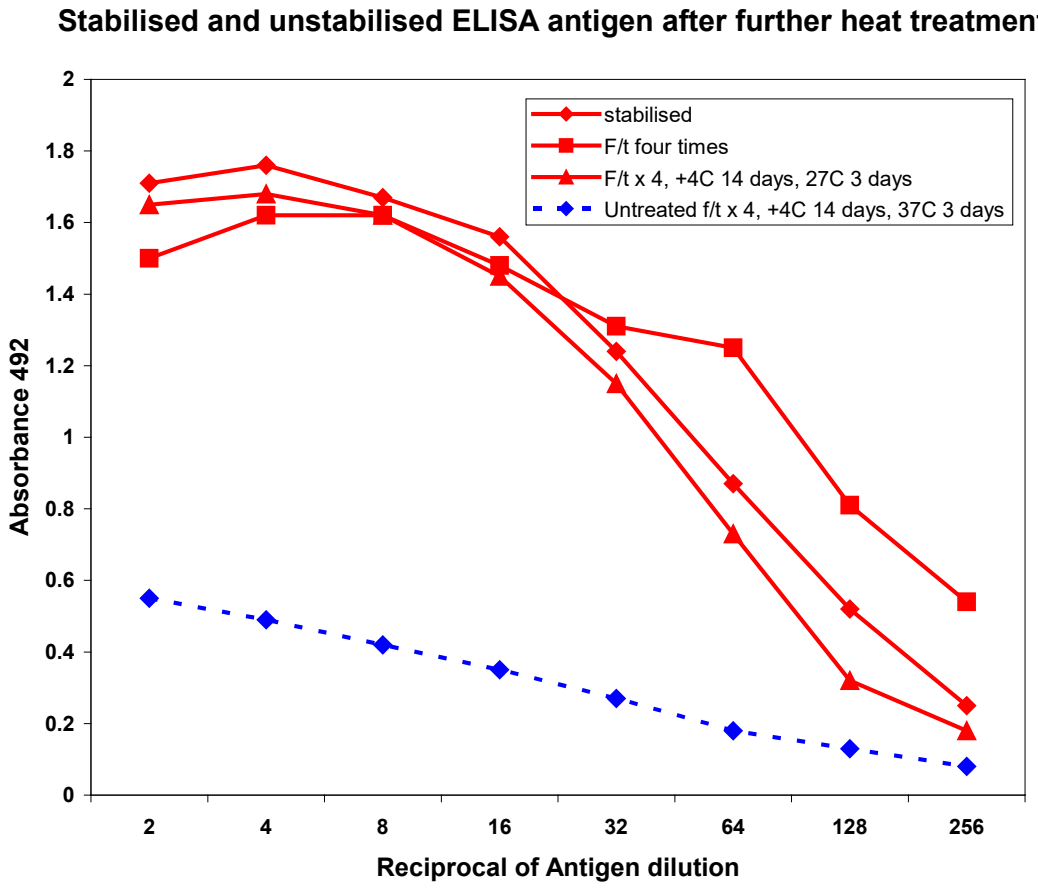
- The O, A, C and Asia 1 SPCE ELISA are suitable for use in serological surveillance without the need for additional combinations of reagents to cover different serotypes.

Stabilisation of the SPCE antigen

One of the problems in introducing the SPCE has been the stability of the FMDV antigen used in the test. The antigen is concentrated semi-purified vaccine antigen which needs to be kept at -70^{o}C. Repeated freeze-thawing the antigen, exposure to room temperature or storage at -20^{o}C in glycerin all result in significant loss of activity.

We hope to have overcome this problem by using a stabilization process.


Stabilised and unstabilised ELISA antigen after one freeze-thaw cycle
Absorbance 492nm
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
2
4
8
16
32
64
128
256
Reciprocal of antigen dilution
untreated
untreated f/t once
stabilised
stabilised f/t once



Conclusions

- Stabilisation of the antigen appears to be successful and will allow incorporation of the antigen into a kit.

- Stabilised antigen to be sent out on trial to a selection of Laboratories for further evaluation.

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