REPORT

Island of Moen, Denmark, 12-15 September 2001

European Commission for the Control of Foot-and-Mouth Disease

Session of the Research Group of the Standing Technical Committee

AGA: EUFMD/RG/01

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

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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 on the Island of Moen, Denmark, from 12 to 15 September 2001.

Dr Per Have, Head of the Diagnostics Department of the Danish Veterinary Institute for Virus Research of Lindholm welcomed the Research Group members to the meeting. He informed them that this year is a special year in that the Institute is celebrating its 75th anniversary. It is therefore the perfect opportunity to host this important session of the Research Group taking into consideration the present FMD situation in Europe. This will no doubt put into focus this gathering. He wished all a very interesting few days in discussing different aspects of FMD. He regretted the fact that representatives from UBI were unable to attend due to the tragic situation in USA and that Nick Knowles from the Pirbright laboratory is also unable to attend.

The floor was then given to Dr Knud Borge Pedersen, Director of the Danish Veterinary Institute for Virus Research of Lindholm and the Danish Veterinary Laboratory who proceeded to welcome the Session to Denmark. He stressed how the outbreak in February in UK has reminded us that FMD is a very serious disease which can occur whenever and wherever, and new knowledge is still required, therefore this meeting is of particular importance.

He briefly described the founding of the Institute. The last outbreak of FMD in Denmark occurred in 1982/83 in 23 herds. At the beginning of the last century the disease was sporadic but in the 1920s a very large epidemic occurred. As a consequence, the Danish Government, under pressure of the Danish farm industry, decided to establish a research institute for FMD. It was intended to start by carrying out experiments and this led to the establishment of the Danish Veterinary Institute for Virus Research on the Island of Lindholm. This locality was selected in order to avoid the spread of infection. The island was subsequently purchased by the Danish State and thus the Institute became operational. The original intentions of the Institute were to establish only short-term experiments, but in 1933 there was an outbreak of Swine fever and this led to a broadening of the Institute and included other diseases. In the late 1930s the Institute also became involved in the development of vaccines.

He then briefly described the organization of the Danish Veterinary system which is part of the Ministry of Food, Agriculture and Fisheries in Copenhagen. There are two veterinary laboratories, the Danish Veterinary Laboratory and the Danish Veterinary Institute for Virus Research based in Aarhus and Lindholm. The intention is to merge these two Institutes into one unit thus becoming the Danish Veterinary Institute.

He wished the Session a useful and fruitful meeting and stressed the importance of the Research Group's contribution to this meeting taking into consideration the present situation in Europe. He took this opportunity to welcome everybody to visit the Institute during the duration of the meeting.

Dr Yves Leforban, Secretary of the European Commission for the Control of Foot-and-Mouth disease then took the floor. He welcomed all members of the Research Group on behalf of FAO, in particular the new members. The present Group was elected by the 34th Session of the Commission held in Rome in March 2001.

He thanked the Government of Denmark, in particular, Dr Pedersen, Director of the Institute and Dr Per Have for hosting this meeting near the institute of Lindholm. The venue is particularly appropriate for such an important meeting taking into consideration the new introduction of FMD in Europe which has had serious consequences.

He explained that at the same time, there is an important turnover in the staff of the EUFMD Secretariat. Ms. Joan Raftery retired immediately after the 34th Session after 24 years with the Commission. A replacement Administrative Assistant is being appointed by FAO. For the time being, Ms Egiziana Fragiotta is covering this interim period. He thanked John Ryan, who will be leaving in early October, for his valuable contribution to the activities of the Commission during the 3-year period of his assignment. He also thanked the Government of Ireland for funding his appointment.

He then proceeded to inform the meeting of his intentions to leave the post of Secretary during the year 2002. He emphasized that he will be leaving purely for personal reasons. He considered it important to inform the Group members at an early stage in order for them to have time to think about candidates for the position. The new post description has been submitted to the Executive Committee members and the vacancy will be issued within the next few months.

He took this opportunity to thank everybody for their cooperation and help which he highly appreciated. He enjoyed working with the Research Group members and always enjoyed the organization of the Research Group meetings. He stressed the fact that he will continue to carry out his duties as Secretary until his departure and will do his best to ensure a harmonious transition with the new Secretary. He wished the members a fruitful meeting and an enjoyable stay on the Island of Moen.

It was suggested that the election of the Chairman of the Research Group be done at this stage. It was unanimously agreed that Dr Kris De Clercq be re-elected to cover the next 2-year period.

Dr De Clercq accepted the task once again. However, he wished to point out that in future the procedure for election should be changed. He suggested that anybody wishing to present their own, or to propose another candidature, should contact the Secretariat before the meeting so that the nominations can be looked at in advance. He informed the meeting that he would do his best to carry out his duties as Chairman. He thanked the Danish Government, Dr Pedersen and Dr Have for hosting and arranging this meeting and congratulated them for the 75th year of the Institute. He also thanked the Secretariat of the Commission for the work carried out in preparation for the meeting. He thanked John Ryan for his contribution to the Commission and wished him success for his future. Particular gratitude was given to Dr Leforban as his contribution to putting the Commission back on the rails has been of utmost importance. He has done an excellent job and the new Secretary will have the important task of continuing the work that Dr Leforban has carried out so efficiently. He invited the members to either put forward their own candidature for the position or to suggest suitable candidates.

He then spent a few words on the Administrative Assistant of the Commission, Ms Joan Raftery. He informed the meeting that she has probably been with the Commission for longer than most of the members of the Research Group. She was very dedicated to the Commission and carried out her duties deligently and efficiently. He spoke to her a few days before the meeting and she requested that he convey her message to the Research Group on her behalf. She regrets not being present in Bulgaria and at this present meeting. This would have been her last meeting and would have been the perfect opportunity to say good-bye to all personally. She fell ill immediately after

the last meeting in Rome. She underwent major surgery and is presently under intensive chemiotherapy. She was not expecting this to occur, but is nonetheless quite serene and is doing her best to recover. Her message ended by conveying her warmest wishes to all.

Dr De Clercq took the opportunity to welcome both old and new members to the meeting of which approximately half are new members.

Prof. Reinhard Ahl of the Scientific Committee on Animal Health and Welfare of the European Commission in Brussels, conveyed the Committee's regards to the organizers and members of the Research Group. He reminded the group of the meeting in 1990 at this location which was chaired by the late Morton Eskildsen. He recalled the last meeting in Borovets, Bulgaria when at the time, FMD had been cleared from Albania, Bulgaria, Macedonia and the risks seemed to be well assessed and under control. However, this situation had changed dramatically this year and the ongoing threat of FMD in Europe had not yet been resolved. This meeting could be considered as a special one as the contributions would provide scientific advice and knowledge to the present disease situation.

The meeting was chaired by Dr Kris De Clercq (Belgium). Members of the Group present were: Drs. Aldo Dekker (the Netherlands); Franco De Simone (Italy); Chris Griot (Switzerland); Bernd Haas (Germany); Per Have (Denmark); François Moutou (France); Vilmos Pàlfi (Hungary); Sánchez-Vizcaíno (Spain); Ms Nilay Ünal (Turkey); Hagai Yadin (Israel); and, Soren Alexandersen who replaced Alex Donaldson as representative from the World Reference Laboratory. Observers attended from Turkey, WRL and the EC.

Adoption of the Agenda

The following Agenda was proposed for adoption.

Item 1	General information on the FMD situation in the world
Item 2	Reports on the outbreaks in Europe
Item 3	Reports on field and laboratory experiences during the crisis in Europe
Item 4	Special session on new kits by private companies and IAEA
Item 5	Serosurveillance
Item 6	Subclinical infection and carrier stages
Item 7	FMD diagnostics
Item 8	Pathogenicity
Item 9	Risk analysis and expert elicitation
Item 10	Vaccines and antigen banks: New type O in Turkey; Review of the list of strains to be included in the banks
Item 11	European Pharmacopoeia
Other items	Report of the Workshop on the Simulation Exercise held in Brno, 5-7 June 2001 Presentation of the new Reference Laboratory for Vesicular Diseases (CERVES) at ISZLE, Brescia, Italy Venues for the next Sessions of the Research Group The EC/EUFMD project FMD in small camelid

The Agenda was adopted as proposed.

Item 1 - General information on FMD situation in the World

John Ryan presented the FMD situation in Europe and over the world in 2001 (Appendix 1). He noted that the FMD situation world-wide has deteriorated significantly over the period 2000-2001 with different types of virus spreading beyond their traditional endemic areas.

Countries that had been free of the disease for long periods of time have had to cope with introductions of virus and the subsequent difficulties of disease eradication. Other countries that were considered to have improving situations with regard to FMD have experienced the reintroduction of the disease which abolished the advances made in recent years. The restrictions associated with the measures taken to control the disease have had severe societal and economic impacts.

Within serotype O, the PanAsian O strain has been particularly successful in spreading over long distances and affecting countries with a long history of freedom from FMD, such as Japan, Republic of Korea, Mongolia, South Africa, UK, Ireland, France and the Netherlands. This pandemic type O invaded the UK where the first cases were detected in February this year. From there the disease spread to France, the Netherlands and Ireland. Currently, all European countries with the exception of the UK have managed to eradicate the disease, either with (the Netherlands) or without (Ireland, France) emergency vaccination.

With regard to type A, massive outbreaks of disease caused by an A strain in South America have necessitated the return to mass prophylactic vaccination in Argentina and Uruguay. The complex situation with 3 distinct A strains circulating in the Middle East and Turkey continues to make control efforts difficult in this region.

Type Asia 1 continues to be endemic in South and South-East Asia, it is persisting in Turkey and Iran after its recent invasion and it continues to spread into the Caucasian countries.

Type SAT 2 has spread from its traditional endemic zone in Africa and has caused outbreaks in Saudi Arabia and Kuwait for the first time.

Nilay Ünal presented the situation of FMD in Turkey (Appendix 2). FMD continues to be endemic, with 3 serotypes (O, A, Asia 1) causing disease in Anatolia. The most prevalent serotype is type O, Asia 1 also continues to cause outbreaks, but type A was not as important as in other years, only two outbreaks were recorded and these belong to only one strain, A/Iran/96. Turkish Thrace experienced its first outbreak since 1996, with type O affecting a goat farm in Tekirdag province. The disease has been rapidly controlled by ring vaccination.

Soren Alexandersen presented a paper by Nick Knowles on the molecular epidemiology of recent type O isolates collected by the WRL from all over the world (Appendix 3). He highlighted that the interpretation of nucleotide sequence data and dendrograms should be undertaken with care due to differences in techniques and programmes employed and differences in the sections of the genome sequenced. In addition to the PanAsian type O virus, he reported that there are other genetically distinct O strains continuing to cause disease problems in South-East and East Asia, the Middle East, South America and Africa.

Aldo Dekker presented sequencing data on isolates from 24 of 26 FMD outbreaks in the Netherlands (Appendix 4). Only minor mutations were found. He concluded that the use of nucleotide sequencing remains limited in small outbreaks.

Conclusions

- Sequencing of the virus isolated in United Kingdom, France and the Netherlands indicated that the outbreaks were due to the same strain of virus.

- The FMD situation world-wide has deteriorated significantly over the period 2000-2001 with different serotypes and strains of virus spreading beyond their traditional endemic areas.

- No country is safe from the introduction or re-introduction of the disease and the risk of introduction to European countries remains significant.

- International trade in live animals (livestock, exotic pets, game species, zoo animals) and of animal products in most regions of the world is increasing. This remains the primary risk for the spread of FMD particularly because there is a general neglect of biosecurity issues and their hidden costs when driving trade liberalization measures forward.

- Improvements in roads, and in air and sea transportation increases the risk of disease spread.

- The deterioration of national veterinary services in many countries due to under-staffing, poor salaries and cut-backs in resources seriously undermines their ability to quickly uncover an exotic disease problem and respond appropriately.

- Despite the improved efforts of the Turkish authorities, the FMD situation in Turkey remains a threat to Europe.

Recommendations

- With regard to FMD, specific attention must be given to the biosecurity dangers of animal and animal product movements inherent in the structure of the animal production industry in Europe and world-wide.

- This requires that National Veterinary Services are funded and staffed to a level commensurate with their workload and responsibilities.

- All European countries should recognize the increased risk of FMD and take advantage of the lessons learned by the affected member countries to improve their contingency planning and prevention measures for FMD.

- The Commission should continue to support Turkey and the SAP Institute in their efforts to control FMD.

Item 2 - Reports on the outbreaks in Europe

François Moutou and Aldo Dekker reported on the outbreaks in France and in the Netherlands respectively, (Appendix 5 and Appendix 6) their countries and the representative from the World Reference Laboratory presented the work carried out by the Institute of Animal Health since the occurrence of the outbreak in the UK (Appendix 7). The representative from the EC also reported on the conclusions of the last meeting of the SVC on the UK situation held in Brussels on 10 September 2001 and circulated the report presented by UK. François Moutou provided additional

information on the findings of the FVO mission in which he participated as a national expert at the end of August.

Paul Barnett reported on the production of 500.000 bovine doses emergency aqueous aluminium hydroxide/saponine O1 Manisa FMD vaccine on the request of DEFRA. He described in detail the manufacture of the vaccine and the implications on the staff (Appendix 8).

Conclusions

- The FMD outbreak in UK demonstrated the important role of sheep.
- The recent epidemic had major economical consequences due to the ban of trade in all countries in Europe including free countries distant from the outbreaks.

Recommendations

- Based on the experience in the UK further research on FMD in sheep is encouraged.
- The possibility of zonification should be considered and proposals should be forwarded to OIE.
- Contingency plans should be prepared for at-risk situations or zones. EC legislation should include measures for pre-emptive culling.
- Methods for culling and disposal of carcasses should take into account the status of animals destroyed i.e infected farms, contact, vaccinated etc.
- Exchange of epidemiological and laboratory information between European countries and with international organizations including EUFMD should be encouraged. This can help laboratories in choosing the most appropriate methods for control and laboratory diagnosis (most appropriate cell and reagents for detecting a particular virus). The EUFMD should play a key role in this.
- The designation of a Community Reference Laboratory has been missing during the last outbreak in Europe. A Community FMD Reference Laboratory should be rapidly designated.
- A procedure to ensure availability of a large quantity of reagents in case of major outbreaks in Europe should be developed, possibly in cooperation with private companies. The creation of a reagent bank is a possibility.
- The situation where a major FMD outbreak occurs in the country of the laboratory designated as the Community Reference Laboratory should be foreseen.
- Methods and criteria for surveillance to regain FMD free status should be better specified in order to be included in the OIE International Animal Health Code.
- Implementation of the existing European legislations on identification of animals should be reinforced.
- There is a need for collaborative activities to be organized for virus antigen detection between FMD Reference Laboratories in Europe.

Item 3 – Reports on field and laboratory experiences during the crisis in Europe

José Sanchez-Vizacaìno, Franco de Simone, Per Have, Bernd Haas and Kris De Clercq reported on the experience of their countries during the recent FMD crisis in Europe. (Respectively Appendices 9, 10, 11, 12 and 13).

The reports from all countries showed that the responses to the outbreaks in the different countries were very similar:

- Clinical inspection on farms where FMD susceptible animals were imported from countries with an outbreak;
- Serological sampling of sheep, goats and deer imported from countries with an outbreak;
- In most cases culling of imported sheep, goats and deer.

Chris Griot summerized the issue of contact with the media (Appendix 14), which was also recognized by most of the participants. There were some differences in giving a press release when having a suspicion of FMD; in most countries a suspicion of FMD was not reported to the press. The major questions arising during the crisis were about zoonosis and vaccination. Discussion on the issue of information showed that information should be given by real experts otherwise any person who is a professor or a doctor will be asked for their comments.

Several countries used the 3ABC ELISA for screening of the samples collected from imported animals, or this ELISA was used next to the standard test. Franco De Simone and José Sánchez-Vizcaíno showed that the specificity of the 3ABC ELISA (± 99.7) was superior to the specificity of the tests for antibodies against structural proteins such as the LPBE.

France De Simone presented clear data showing that in case of doubtful results a second sampling is important to clarify whether it is a specific or non-specific reaction. (Appendix 10).

Bernd Haas reported that in Germany for mass-serosurveillance using the LPBE a cut-off of 1:90 was used as in Pirbright (Appendix 12). He stated that otherwise all holdings would have been found to contain seropositive animals. Therefore, some laboratories have replaced the LPBE by the SPCE as recommended at the previous RG meeting.

It was clear that the FAO cut-off serum selected three years ago is causing problems in several laboratories. Aldo Dekker presented VNT results from slaughterhouse sera, sera from recent outbreaks and sera from four outbreaks without clinical signs. Based on these data he concluded that the cut-off related to the FAO reference cut-off serum is too low (Appendix 15). He proposed that results of serum testing should be related to the different reference sera.

The participants reported on the FMD suspicions declared in their countries. Virological examination in all countries, with the exception of the UK, the Netherlands, Ireland and France did not reveal FMD infection. Most countries used RT-PCR next to standard virus isolation. Every laboratory using RT-PCR was confident that the use of RT-PCR could shorten the time needed for diagnosis. In laboratories handling strong positive samples cross-contamination was recognized as a problem which has to be taken care of.

Reinhard Ahl mentioned that in the experience of the FMD Diagnosis Laboratory in Tubingen, BHK 21 CT cell line is particularly sensitive to the isolation of all FMD virus strains. This cell line is available from this laboratory.

In several countries parapox viruses were identified by electron microscopy in samples submitted from sheep and cattle. In a few cases BVD and MCF seemed to have caused the clinical signs. But in most negative cases, no virological cause of the signs was identified.

Kris De Clercq showed the measures applied in Belgium which were very stringent (Appendix 13). He clearly showed that before a sampling scheme is implemented one should identify the purpose of the test. A distinction has to be made between surveys looking for the presence of virus at a certain prevalence or surveys for declaring freedom of infection.

The group discussed the issue of prohibition of movement of horses during an FMD outbreak and Alf Füssel explained that within EU different systems of certification are applied between countries.

Conclusions

- Media have had a major role in the recent epidemic and a better harmonization of the messages to be addressed to the public opinion at the European level should be encouraged. EUFMD should play a coordinating role in this respect.

- Responses to the recent epidemic among different countries was similar.

- Information to the public/farmers is very essential.

- Specificity of 3ABC ELISA is superior to the specificity of tests for antibodies against structural proteins.

- The FAO cut-off serum selected three years ago is causing problems in several laboratories.

- RT-PCR is a good addition to the standard virological techniques.

- Parapox, BVD and MCF were the only virological agents identified as differential diagnosis.

- The sampling scheme implemented should be based on the purpose of sampling.

Recommendations

- Coordination of responses at an international level is needed as exchange of information on frequently asked questions.

- FMD laboratories in Europe should ensure that they use the most sensitive cells for virus isolation of all FMD strains.

- Validated tests for the detection of non structural protein antibodies and especially the 3ABC ELISA has reached a high level of performance. There is a need for reference sera for these tests.

- The LPBE should be replaced by the SPCE for detecting antibodies against structural proteins.

- Standard references for RT-PCR are necessary.

- Results of the testing serum should be related to different reference sera.

- In case of doubtful serological results, second sampling of the same animal and other nearby animals is recommended.

- FMD experts, together with epidemiologists should prepare sampling schemes for different situations in cooperation with OIE.

- There is a need for harmonization of the rules of movement of horses and of certification in Europe in the case of an FMD outbreak.

Item 4 - Special session on new kits by private companies and IAEA

Christian Schelp, representing Dr Bommeli AG, Intervet Company, presented data on a 3ABC test kit developed on the basis of the Brescia/WRL tests (Appendix 16). In contrast to these tests, the Intervet test does not trap the 3ABC protein by monoclonal antibodies, but purified antigen is bound directly to the plate. The indirect test employs one conjugate for ruminants and another one for pigs. The data presented indicate a specificity of more than 99% for cattle, sheep and pigs. Seroconversion in infected animals was detected between 11 days p.i. in some animals and three weeks in others. Validation data were presented for cattle and sheep indicating a sensitivity similar to that of the original Brescia/WRL test and Intervet considers the tests to be ready for marketing. However, more sera from infected pigs will be needed to complete the validation for this species.

Promising first results obtained with a new ELISA for the differentiation of vaccinated and infected cattle based on synthetic peptides were presented by Joachim Grunmach, Bayer AG. The test is based on research conducted at the Federal Research Centre for Virus Diseases of Animals, Germany (Appendix 17).

Tim Doel, Merial presented a study on the induction of antibodies to NSPs after repeated application of 10 and 20 fold overdoses of Merial vaccines. The antibody response was tested in Ingrid Bergmanns laboratory by EITB. Only antibodies to the 3D NSP could be detected in significant quantity. So he concluded that no problems in respect to NSP serology would have to be feared with Merial vaccines applied according to normal vaccination procedures (Appendix 18).

As the UBI Company representative could not attend due to the tragedy in the US on 11 September, Kris De Clercq presented the data on two peptide based ELISAs he had received from UBI. One test detects antibody to structural protein VP1 (type O) and the second detects antibody to the 3B NSP of all serotypes. Data based on experiments preformed in several countries indicate that these tests are highly specific and have the potential to differentiate between FMD infection and vaccination in cattle, sheep and pigs. The test is already on the market, can be produced on a large scale and is very easy and convenient to use. Whether this test has the same sensitivity in cattle and sheep as the 3ABC ELISA needs to be further investigated (Appendix 19).

John Crowther reported on the experience of IAEA with NSP tests of various designs in many countries of the world (Appendix 20). He pointed out that available tests need more validation and better internal quality control and that also an external quality control system has to be introduced. Differences in the relative analytical sensitivity and diagnostic sensitivity and specificity of the available assays have been observed and must be taken into account when using the tests. Some indirect tests suffer from problems with certain species or individual sera giving background signals. A huge amount of data has been generated in different epidemiological situations and should be made freely available.

During the discussion Bernd Haas, Franco De Simone and John Crowther concluded that based on a comparison between the Brescia 3ABC ELISA and the UBI FMDV NS EIA, the sensitivity of the latter should be increased.

Conclusions

- Commercially produced complete test kits are now available. These tests have been validated extensively for cattle and also for sheep; less validation data have been generated for pigs. These tests will facilitate making use of the high throughput of regional and other laboratories normally not working with FMD. This allows to increase the testing capacity to the level required for whole herd testing of vaccinated populations. The tests are suitable for differentiation between vaccinated and infected animals on a herd basis, but will not reliably identify individual carrier animals in a vaccinated population.

- Modern purified vaccines normally will not induce antibodies to NSPs.

- Preliminary data indicate that most current tests may not be suitable for sera of wildlife species, new domestic species such as llamas, and certain breeds of buffaloes. Competition/inhibition assays may overcome this problem.

- There are differences in the relative analytical sensitivity and diagnostic sensitivity and specificity of the available assays. Further results on the performance of the existing NSP tests should be reported in the future.

- The experience of the IAEA revealed the importance of training and quality control for the application of NSP serology.

- The Brescia 3ABC ELISA has a higher sensitivity than the UBI FMDV NS EIA.

Recommendations

- Utilization of the existing tests for detection of NSP antibodies is encouraged for sero-surveillance in Europe.

- The use of these tests in vaccinated populations should be encouraged in order to reveal cases of FMD that had not been detected by clinical inspection, increase confidence in the effectiveness of eradication measures and gather experience with the tests in various epidemiological situations.

- A reference serum bank characterizing different epidemiological situation should be established, which contains sera of relevant species in sufficient quantities for reference and developmental purposes. Further validation studies of NSP tests should be performed for pigs.

- Competition/inhibition assays suitable for sera of all species should be developed to the stage of commercially produced complete kits.

- Validation data for NSP tests, especially from South America should be made freely available.

- Further studies correlating the antibody response to structural and non-structural proteins in sera and other types of samples with virus isolation and PCR data in carriers should be performed; these parameters should also be examined in pigs.

Item 5 - Serosurveillance

Nilay Ünal presented a paper on the results of the serological surveillance following the vaccination in Thrace region in 2000 (Appendix 21). Sera obtained from three different sets of animals were tested by a liquid phase blocking ELISA. Although a high level of immunity was observed at 28 days post vaccination, a rapid decrease was observed in the immunity levels after 60 days onwards especially against types O and Asia 1.

Nilay Ünal presented a second paper on the results of the 3ABC ELISA serosurvey conducted with the sera obtained from Thrace (Appendix 22). In this study a total of 2,639 sera were tested. The results showed that 1% of the sera were positive. She concluded that these positive sera might be as a result of false positives or an indication of a previous infection. There are indications that some of these animals might have been introduced into Thrace from Anatolia.

Yves Leforban informed the Group that OIE guide for surveillance of FMD is being established and he circulated the draft prepared by Dr Kitching for information and comments.

Conclusions

- Serological results should be interpreted in conjunction with virological and other laboratory findings and with epidemiological observations.

- The serosurveillance carried out in Thrace after the 2000 Autumn vaccination campaign has been very useful. The reason why the level of immunity after 60 days and onwards was decreased rapidly should be investigated further by Turkish authorities with the support of EUFMD and EC.

- The results of the 3ABC ELISA in Thrace are favourable. They demonstrated a 1% seroprevalence and based on this result there is a low probability of circulation of the virus in the region.

- The group supported the comments made by the Secretary to draft an OIE guide on surveillance. In particular because different sampling schemes are needed depending on the purpose of the screening and this must be stressed.

Recommendations

- Clear guides for FMD surveillance in Europe in different circumstances combining clinical and serological surveillance should be established in coordination with OIE.

- The guides should cover different epidemiological situations vis-a-vis FMD i.e. countries or zones which are currently FMD free and wish to be recognized officially free of FMDV infection by OIE or countries or zones which wish to regain a free status after having been infected.

- The use of NSP ELISA and particularly 3ABC should be encouraged. The tests based on 3 D may also be useful in certain circumstances.

- The continuation of the Serosurveillance in Turkish Thrace using 3ABC ELISA should be encouraged and supported by EUFMD or EC.

- The comments on the OIE guide on surveillance must be forwarded to OIE.

Item 6 – Subclinical infection and carrier stages

Kris De Clercq reported on the carrier workshop held in the Netherlands in June. The conclusions of the workshop were summarized (Appendix 23).

Aldo Dekker reported that FMD infection in sheep can easily be overlooked and therefore additional control measures in sheep, like serological screening during quarantine, seem necessary in areas where an FMD outbreak has occurred (Appendix 24).

Conclusions

- Sheep are frequently subclinically infected and FMD virus can persist in this species.

Recommendations

- If sheep are involved in an FMD outbreak, adequate serological screening must be performed.

Item 7 – FMD diagnostics

Four papers were presented on FMD diagnostic methods. The first, presented by Scott Reid, was an evaluation of automated RT-PCR systems to speed up FMD diagnosis (Appendix 25). Automation was carried out in a MagNA LC (Roche), followed by PCR amplification in a TaqMan 5700 thermal cycler. With the methods described, 64 samples from the UK 2001 outbreak could be tested in a normal working day with a higher sensitivity than ELISA. The results showed that a second passage in primary calf thyroid cell culture can be avoided and therefore much diagnostic time saved.

The second paper presented by José Sanchez-Vizcaìno on a new RT-PCR was based on detection of the 3D gene including a conserved *Ahd* 1 restriction site (Appendix 26). This allowed detection of all seven FMD virus serotypes as well as making a rapid digestion reaction following amplification. No cross reactions were detected with other *Picornaviruses.* The test described was evaluated on 48 different FMD virus isolates but included very few SAT serotypes.

Aldo Dekker presented a paper on the evaluation of a LightCycler based RT-PCR for the detection of FMD virus (Appendix 27). Twenty-six isolates from seven serotypes were tested. The evaluation showed that the RT-PCR is a sensitive technique. False positive reactions are mainly caused by cross-contamination with highly positive samples.

The fourth paper, presented also by Aldo Dekker, was a validation of a quick and simple monoclonal antibody-based ELISA for multi-species detection of antibodies in serum directed against type O FMD (Appendix 28). The ELISA had a specificity of 96%. The sensitivity was 98% relative to the virus neutralization test when testing cattle, pig and sheep sera collected from FMD-infected Dutch farms and scored 459 out of 484 virus neutralization test-positive experimentally derived sera correctly (95%).

Conclusions

- Automated RT-PCR systems with TaqMan amplification with no contamination has been evaluated. It allows 64 samples to be tested per working day. A second passage in cell culture can be avoided if RT-PCR were positive in the first passage.

- A LightCycler RT-PCR suitable for FMD diagnosis has been evaluated. It was up to 10 times more sensitive than virus isolation but contamination can still be a problem.

- The identification of the 3D gene by RT-PCR looks like a good diagnostic test for primary infections with any FMD virus serotype and the rapid digestion reaction can be used as an additional confirmation step.

- A monoclonal antibody based ELISA as a screening test for the multi-species detection of antibody to FMD virus serotype O was validated against field sera, slaughterhouse sera and sera from animal experiments in the Netherlands. The test had a lower sensitivity than the virus neutralization test.

Recommendations

- The automated RT-PCR systems need to be validated further on other serotypes and on probang samples.

- There is a need for more samples to be tested with a LightCycler RT-PCR and measures need to be taken to reduce contamination.

- The 3D gene RT-PCR must be evaluated with more FMD SAT serotypes.

Item 8 – Pathogenicity

Two papers were presented by Soren Alexandersen. The objective of the first study was to obtain data for FMD O viruses and a single type C isolate to enhance the capability of airborne virus simulation models (Appendix 29).

The collection of air samples near pigs infected with these strains has shown that the amount of virus (in $TCID_{50}$) emitted per pig per 24 hours was $10^{5.8}$ – $10^{7.6}$ for different FMD viruses (O1 and C Noville). Additionally, the results confirm that pigs compared to cattle and sheep are relatively resistant to infection by airborne FMDV.

The second paper dealt with aerosol excretion for the O UK 2001 in sheep and cattle (Appendix 30). An additional objective was to study the time course of virus load (infectivity and viral RNA) in nasal swabs, rectal swabs and in serum in order to assess transmission risks. Potential carrier status of sheep were examined at 28 days p.i. Infected sheep excreted around $10^{4.3}$ $TCID_{50}$ / 24 hours and airborne excretion picked on a single day vary easily after infection. Virus as well as viral RNA were detected in probang samples collected at 4 weeks after exposure.

Conclusions

- The findings indicate that the risk of airborne transmission from pigs will vary depending on the specific virus isolate and the species of the recipient animal.

- According to the presented data it is confirmed that the FMD infected pigs function as amplifier of the virus. However, risk of airborne transmission to pigs appears to be low.

- The second paper provides a basis for developing a more comprehensive picture of the various transmission risks from livestock especially sheep.

Recommendations

- The risk of aerosol transmission from pigs is variable but significantly high therefore infected pigs should be eliminated as soon as possible.

- The study of airborne transmission risk should be stimulated.

- More quantitative data are needed to allow detailed assessment of transmission risk under various conditions.

Item 9 - Risk analysis and expert elicitation

Chris Griot presented a paper (Appendix 31) on the risk of importing exotic animals into Switzerland, holding them in a USDA, APHIS approved transit quarantine for 30 days before continuing their transportation into the USA. A formal risk analysis defined as a process consisting of risk assessment, risk management and risk communication was implemented at the Swiss Federal Veterinary Office. The calculated risk of introducing a false negative animal (e.g. FMDV infected animal) was estimated to be 5×10^{-6} which is higher than the accepted probability of 10^{-6}.

John Ryan presented a follow-up report of the expert elicitation session on the risk of introduction of FMD into Europe which was held in Borovets, Bulgaria in 2000.

Conclusions

- It was concluded that exotic animals which are foreseen for transit quarantine should be handled the same way as for definitive import. International standards of laboratory testing should be considered when interpreting test results from the country of origin.

- Expert elicitation is a good tool to evaluate risks of introducing FMD into a country and should be expanded in the future.

Recommendations

- A formal risk assessment process should be considered when importing exotic animals.

- EUFMD should continue to pursue a risk assessment by performing a detailed study of trade flows in animals and animal products and movements of people and other goods and conduct an expert elicitation.

Item 10 - Vaccines and antigen banks

The representatives from Turkey presented two papers on the FMD viruses circulating in Turkey (Appendices 32a and 32b). Information on the genetic and antigenic characteristics of these viruses was provided. Antigenic characterization of recent type O viruses and showed that although some viruses gave low r values there is field evidence that these viruses can be covered by O Manisa vaccine. Sequencing of one type O isolate from 2000 showed that this virus was in the group of PanAsia. Analysis of type A viruses isolated in 2001 revealed that these viruses were related A/Aydin/98 (homologous to A Iran 96). No virus similar to A Iran 99 have been isolated recently in Turkey.

Nilay Ünal presented a talk on the activities regarding the improvement of the FMD vaccine's quality (Appendix 33). She gave brief information on the changes made at the Sap Institute and improvements achieved in the previous year.

A paper by Paul Barnett describing a novel formulation procedure able to extend the shelf-life of FMDV emergency vaccines was given (Appendix 34). The method involved preparing an oil vaccine with all ingredients into vials and storage of this formulation at ultra-low temperature until use. Experiments in guinea pigs indicated good long-term stability characteristics.

The Chairman asked the group to review the provisional recommendations from the World Reference Laboratory on FMD virus strains to be included in FMDV antigen banks in Europe (Appendix 35).

The information provided by Turkey on the adequate covering of O1 Manisa vaccine against the strains currently circulating in Turkey makes the need for inclusion of new type O in the bank less necessary. The group agreed that high potency vaccines against type O1 Manisa could be used. However, some members of the group were in favour of confirming this result through a challenge test.

Conclusions

- Improvements achieved at the Sap Institute were appreciated.
- The novel formulation procedure able to extend the shelf-life of FMDV emergency vaccines is a potentially useful method for storage of emergency vaccine for immediate use.

Recommendations

- Turkey should be encouraged to continue to characterize its strains and to send FMD samples to WRL to monitor the situation in the field.
- O1 Manisa and A/Aydin/98 seem to be suitable vaccine strains to be used in Turkey.
- Challenge test should be organized to assess the protection of O1 Manisa vaccine against recent isolates from Turkey.
- Utilization of vaccine with high payload antigen content is encouraged to give an adequate protection against new variants which may appear.
- The list of viral strains to be included in the banks as proposed by the World Reference Laboratory is endorsed by the group.

Item 11 - European Pharmacopoeia

Kris De Clercq reported on the meeting he attended with Group 15 V of the Eur.Phar. (Appendix 36). The proposed revised FMD monograph was discussed on 6 June. Several proposals made by EUFMD were taken into account. A new revision will be prepared by the Eur.Phar.

He also reported on a meeting with the CVMP/Immunologicals Working Party of EMEA. EMEA will organize a meeting on 19 September bringing together EMEA, EUFMD, OIE and EC.

The purpose will be to draft guidelines on safety, quality and efficacy of FMD vaccine production and on the introduction of new FMD strains.

Kris De Clercq explained that monographs cover vaccines for one species and there is no current monograph for pig breeding. Therefore, the question of vaccines to be used in pigs was briefly discussed by the group. Some members of the group were of the opinion that vaccines which passed the test in cattle can also be valid for pigs.

Recommendations

- The group recommended to continue the efforts in this field and to call on the EUFMD working group whenever necessary.

- To request the manufacturers to provide data obtained after vaccination of pigs.

Other items

John Ryan reported on the workshop on FMD simulation exercise held in Brno, Czech Republic from 5 to 7 June 2001 (Appendix 37).

Franco De Simone reported on the new Reference Laboratory for Vesicular Diseases at the IZSLE in Brescia, Italy (Appendix 38). The Chairman stressed the need of a laboratory for vesicular diseases in Italy and expressed the full support of the Research Group.

Yves Leforban informed the Group of the new EUFMD/EC project which is being signed. The project of US$1.5 million covering a period of 4 years intends to better define the activities supported by EC under the Trust Fund 911100. This includes support to the control programme in Turkey and in other countries where the situation is at risk for Europe and normative activities such as the organization of meetings and workshops (including the Research Group Sessions).

He also informed the Group of a request received from a small camelid breeder association in UK asking for advice to vaccinate their animals in order to avoid slaughter in the case of FMD. He had replied to this request by explaining that although scientific evidence exists that camelid are not very sensitive to FMD, the disease may be observed in camelid. This occurred recently in Mongolia and if special measures are applied to camelid they should also be applied to other species considered as low sensitive.

Nilay Ünal from Turkey confirmed the intentions of Turkey to host the next meeting of the Research Group in Izmir. The provisional dates are from 18 to 20 September 2002. Chris Griot confirmed the intentions of holding the Research Group meeting in Switzerland in 2003.

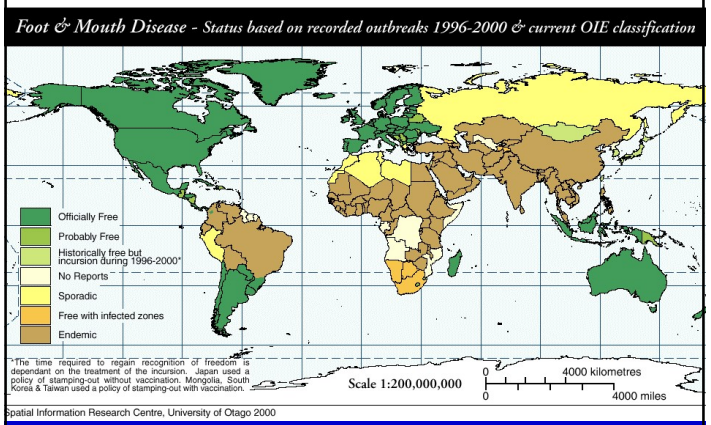
Adoption of the Report

The draft report of the meeting was discussed by the Session and accepted with some amendments.

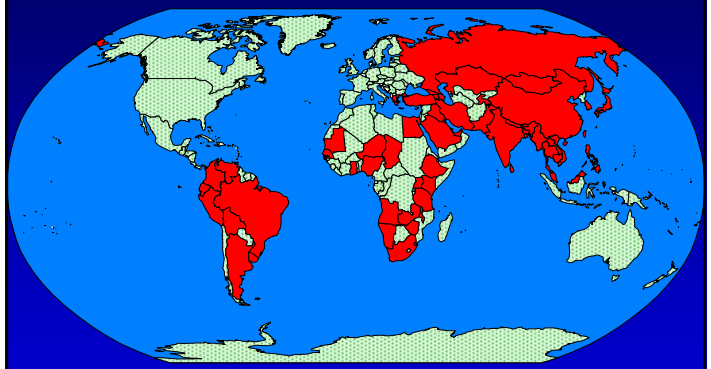
Closing remarks

The Chairman thanked the Government of Denmark on behalf of the Research Group for the kind hospitality offered. In particular, appreciation was expressed to Dr Pedersen and Dr Per Have of the Lindholm Veterinary Institute for all arrangements made for the smooth running of the meeting. Thanks were also extended to the members for their contributions.

FMD situation 2000-2001

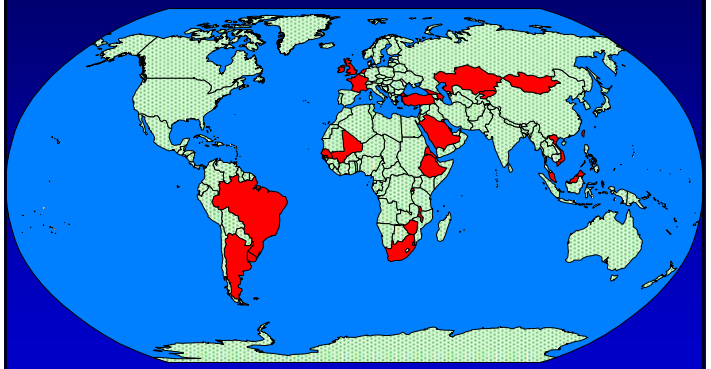


FMD outbreaks 2000



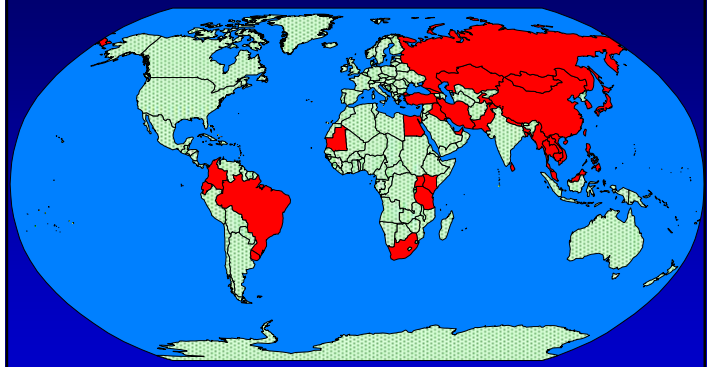
All serotypes as officially reported to OIE,WRL,FAO

FMD outbreaks 2001



All serotypes as officially reported to OIE,WRL,FAO

FMD Type O outbreaks 2000

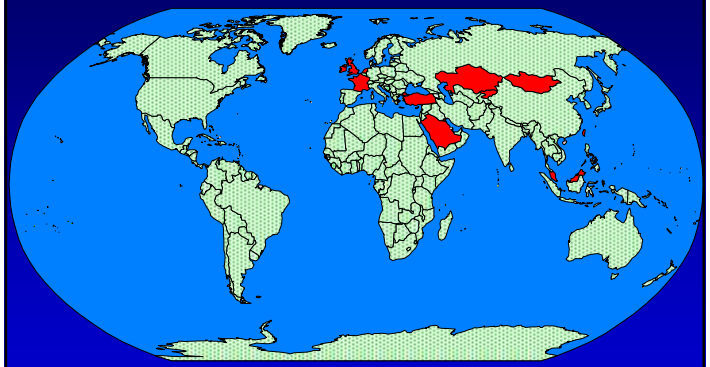


FMD Type O as officially reported to OIE,WRL,FAO

Evolution of PanAsian strain

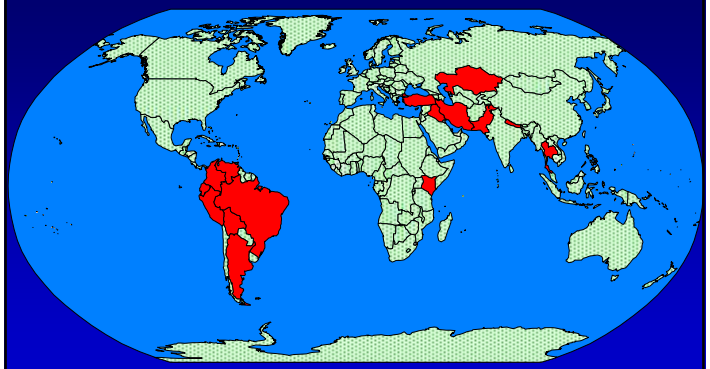
Prepared by Dr. P. Roeder, FAO from information provided by the World Reference Laboratory for FMD, IAH, Pirbright, UK

FMD Type O outbreaks 2001



FMD Type O as officially reported to OIE,WRL,FAO

FMD Type A outbreaks 2000

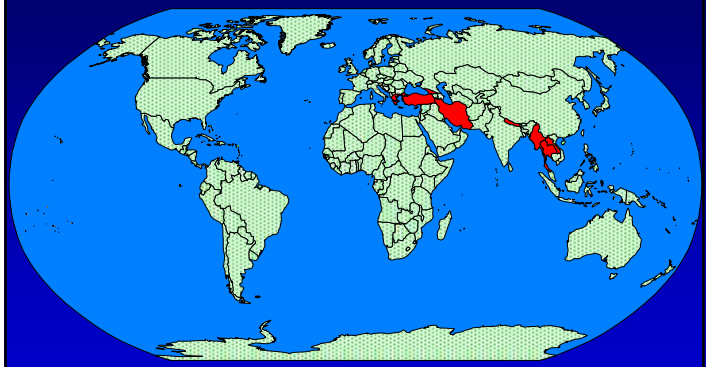


FMD Type A as officially reported to OIE,WRL,FAO

FMD Type A outbreaks 2001

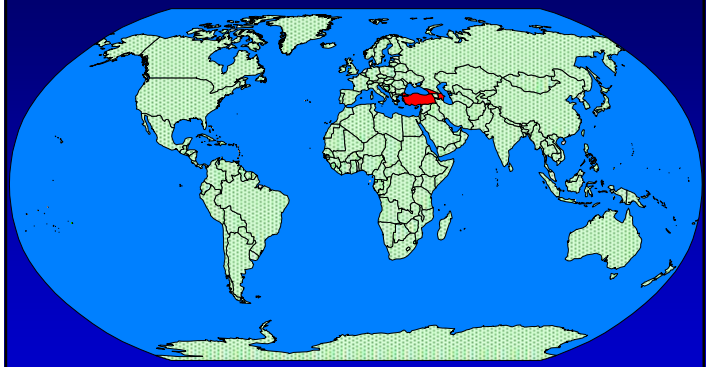
FMD Type A as officially reported to OIE,WRL,FAO

FMD Type Asia 1 outbreaks 2000



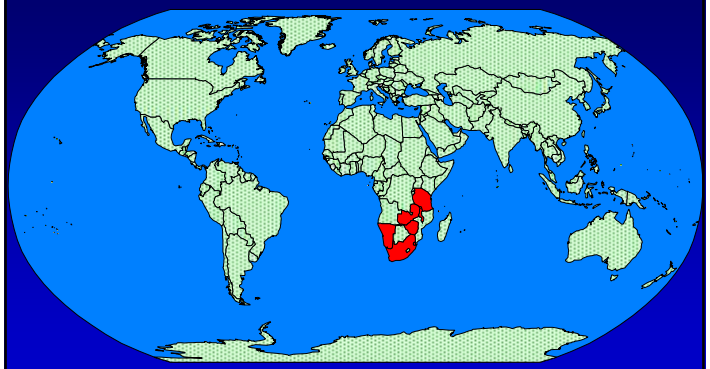
FMD Type Asia 1 as officially reported to OIE,WRL,FAO

FMD Type Asia 1 outbreaks 2001



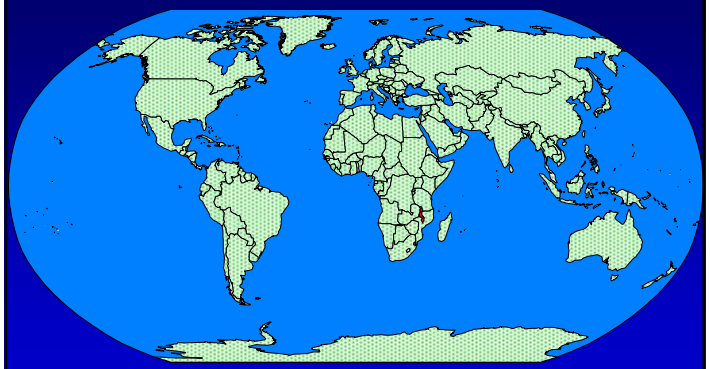
FMD Type Asia 1 as officially reported to OIE,WRL,FAO

FMD Type SAT1 outbreaks 2000

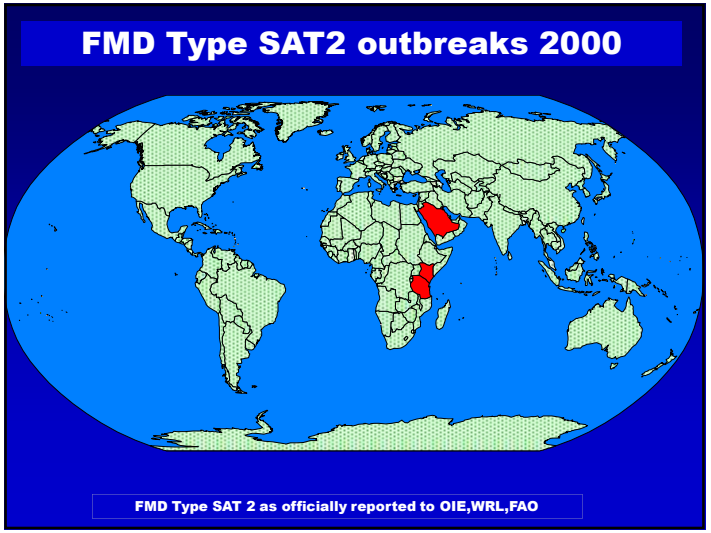


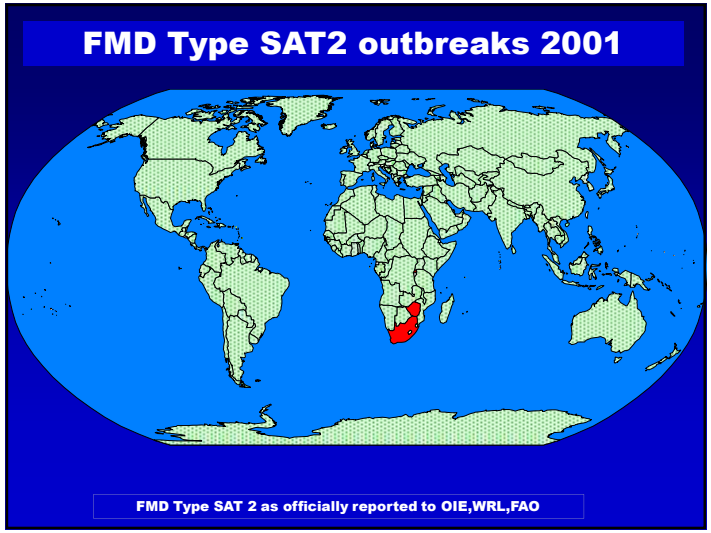
FMD Type SAT 1 as officially reported to OIE,WRL,FAO

FMD Type SAT1 outbreaks 2001



FMD Type SAT 1 as officially reported to OIE,WRL,FAO


FMD Type SAT2 outbreaks 2000
FMD Type SAT 2 as officially reported to OIE,WRL,FAO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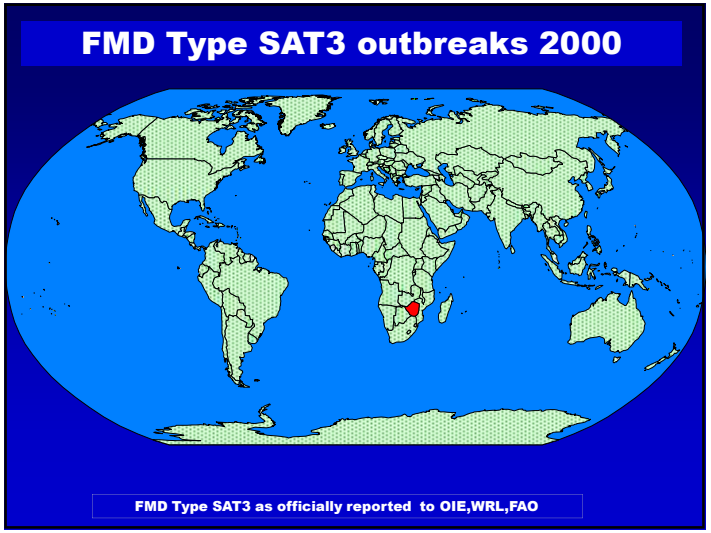
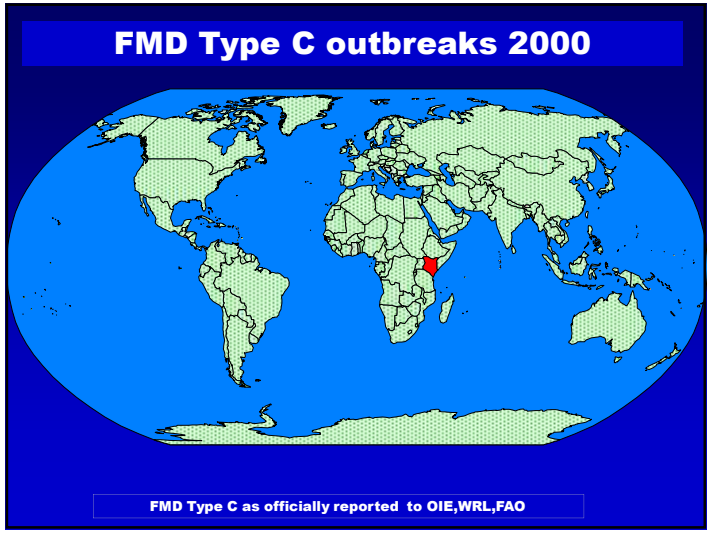
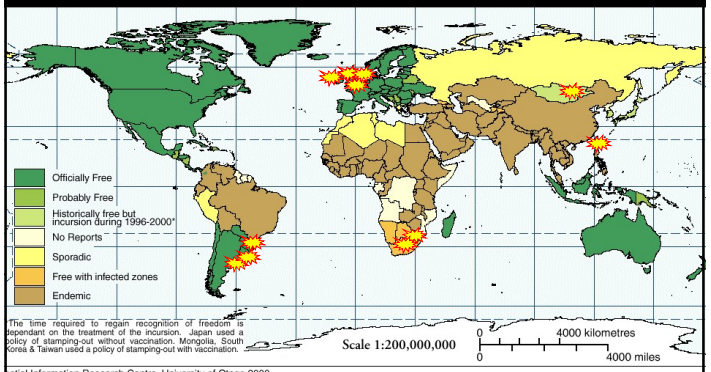
FMD Type SAT 2 as officially reported to OIE,WRL,FAO


FMD Type SAT3 outbreaks 2000
FMD Type SAT3 as officially reported to OIE,WRL,FAO


FMD Type C outbreaks 2000
FMD Type C as officially reported to OIE,WRL,FAO

Implications

Foot & Mouth Disease - Status based on recorded outbreaks 1996-2000 & current OIE classification



*The time required to regain recognition of freedom is dependant on the treatment of the incursion. Japan used a policy of stamping-out without vaccination. Mongolia, South Korea & Taiwan used a policy of stamping-out with vaccination.

Spatial Information Research Centre, University of Otago 2000

Appendix 2

THE SITUATION OF FMD IN TURKEY

Nilay Ünal
Sap Institute, P.O.Box 714, 06044, Ulus, Ankara, Turkey

Foot-and-mouth disease which is one of the most important diseases threatening livestock, remains endemic in Turkey.

Three FMD virus serotypes, O1 Manisa, A Aydin 98 (homologue A Iran 96) and Asia1 are circulating. In 2001, up to August, a total of 79 outbreaks were reported in Turkey. 46 out of them were O; 31 Asia-1 type and only 2 were A type (Table 1). These serotypes were identified at Sap Institute, by virus isolation, CFT, ELISA and RT-PCR.

One outbreak was reported in Malkara district, Tekirdag Province in the Thrace Region on 29th of June (OIE Bulletin, 6 July, Vol.14, No.27). 50 goats were affected in one herd. 6 goats which didn't show any clinical signs of FMD, had been introduced from neighbouring village a month ago. One goat was died in this herd.5 vesicular epithelium from suspected animals and 1 heart tissue post mortem sample were found O type FMDV positive by ELISA.

The team from Sap Institute went to infected village and took sera from the suspected animal. These sera were tested by LPB-ELISA and MAT-ELISA. The results of the samples were given in Table2.

Large and small ruminants in 12 villages around the outbreak were vaccinated and strict measures were taken. A serological survey was carried out 21 days after the vaccination by LPB-ELISA and 3ABC ELISA. According to the results of 3ABC ELISA, all sera were negative. The results of the serosurvey after the vaccination were given Table 3.

In 2001, 18 samples were sent to Pirbright IAH for strain identification, but no reply is being received yet. The list of the samples is given in Table 4. Some of them were the samples of last year.

Vaccination rate in the spring campaign in 2001 was about 60% both in Anatolia and Thrace Region. Because of some problems in vaccine production and modernization studies to improve the production conditions at Ankara, Sap Institute, sufficient quantity of FMD vaccine couldn't be produced at the beginning of the 2001.

In this period, due to the lack of the available vaccine, importation from other countries was intended, but non of the international producers put up to tender until the end of March, finally on April, Indian Biologicals Company accepted to provide 3 million doses of trivalent vaccine. As it was planned to vaccinate animals in the Thrace Region with the imported vaccine, the trivalent vaccine produced by Sap Institute as distributed starting from the eastern and south-eastern borders towards western part of Anatolia. So, the rate of the vaccination in Thrace Region couldn't reach to desired level.

Meanwhile, there is now sufficient vaccine at Sap Institute's stocks for the autumn campaign and it will start on October. In Turkish Thrace is going to be used a trivalent FMD vaccine

(O1 Manisa, A Aydin98 and Asia 1) which will be donated by the EU and a serosurvey will be carried out following this vaccination campaign.

Table 1: FMD outbreaks up to August 2001

Month	No.of FMD outbreaks in month identified	Virus types affected	Species	Susceptible	Cases	Death
January	8	1 A 4 O 3 Asia1	Bovine	4248	60	-
February	9	3 Asia 1 6 O	Bovine	2890	20	-
March	26	8 Asia1 1 A 17 O	Bovine Ovine	21322 3565	232 165	2 -
April	4	2 Asia 1 2 O	Bovine Ovine	2111 6500	86 150	15 62
May	11	6 Asia 1 5 O	Bovine Ovine	6500 1100	437 80	1
June	18	9 Asia 1 9 O	Bovine Ovine	8590 300	742 50	1 1
July	3	3 O	Bovine	2413	34	-
TOTAL	79	2A 46O 31Asia-1	Bovine Ovine	48074 11465	1611 445	19 63

Table 2: The results of samples from infected area in Thrace Region by ELISA

	Before the vaccination				Post vaccination		
Item (earlap)	**LPB-ELISA**			**MAT-ELISA**	**LPB-ELISA**		
	O	**A**	**Asia1**		**O**	**A**	**Asia1**
110	N	N	N	N	192	>256	128
137	N	N	N	N	>256	>256	192
115	N	N	N	N	128	256	192
118	N	N	N	N	96	>256	256
34451	128	>256	N	N	192	>256	192
207-99	96	192	N	N	192	256	256
103-98	N	N	N	N	128	>256	128
16975	N	N	N	N	128	256	256
166-99	128	45	N	+	192	256	256
111-98	96	128	N	+	128	256	256
215-99	128	192	N	+	>256	>256	192
152-96	192	N	N	N	192	256	256
284-95	192	N	N	+	>256	>256	256
229-94	N	N	N	N	128	>256	192
103-98	N	N	N	N	192	>256	256
44-96	N	N	N	N	128	192	192
245-97	N	N	N	N	192	>256	256
221-98	N	N	N	N	192	192	192
270-97	N	N	N	N	128	>256	>256
99-98	N	N	N	N	128	>256	192
1493	192	64	45	N	>256	>256	256
16959	64	N	N	N	128	256	192
1579	96	192	N	N	192	>256	>256
1377	N	N	N	N	128	>256	256
1541	N	N	N	N	192	192	256
244/97	N	N	N	N	128	256	256
18-93	N	N	N	N	96	>256	192

N: negative

Table3: The cumulative results of the post vaccination sera collected from the ring vaccination area in Thrace in 2001

	Large Rumimant (512)				**Small Ruminant (164)**			
FMD Types	**Positive**	**(%)**	**Negative**	**(%)**	**Positive**	**(%)**	**Negative**	**(%)**
O	360	70,5	152	29,5	109	66,4	55	33,6
A	394	77	118	23	113	68,2	51	31,8
ASIA1	379	74	133	26	103	62,8	61	37,2

Table 4: FMDV samples sent to Pirbright IAH

Province	**Serotype**
BOLU	O
KIRSEHİR	O
DÜZCE	O
AKSARAY	O
MALATYA	A
MANİSA	A
AMASYA	A
YOZGAT	A
ANKARA	Asia1
BALIKESİR	Asia1
DİYARBAKIR	Asia1
ERZİNCAN	Asia1
ERZURUM	O
ADIYAMAN	O
KARS	O
SAKARYA	O
KASTAMONU	O
NEVSEHİR	O

Appendix 3

MOLECULAR EPIDEMIOLOGY OF FOOT-AND-MOUTH DISEASE VIRUS: THE CURRENT SITUATION IN EUROPE AND THE MIDDLE EAST

N.J. Knowles, P.R. Davies and A.R. Samuel

Abstract

In February 2001 foot-and-mouth disease (FMD) appeared in Great Britain for the first time since 1981. Spread of the disease occurred to Northern Ireland, the Republic of Ireland (FMD-free since 1941), France (free since 1981) and the Netherlands (free since 1984). On the 20th February the World Reference Laboratory for FMD (WRLFMD) identified the causative virus as belonging to serotype O. Within 24 hours we had determined the complete sequence of the VP1 gene and had compared it to sequences on the WRLFMD database. This analysis clearly showed that the outbreak was due to the PanAsia strain, being closely related to viruses from recent outbreaks in Asia and South Africa (Knowles et al., 2001; Vet. Rec. 148: 258-259). Since then, VP1 sequences have been determined for over 25 UK virus isolates and also those from the outbreaks in the Irish Republic and France. Twenty three VP1 sequences of the Dutch outbreak viruses were also received from Dr. Aldo Dekker (ID-Lelystad). Comparison of all of these sequences showed that there was little genetic variation between all of the viruses examined. Routine molecular epidemiological surveillance of FMD type O viruses in the Middle East has revealed a new lineage present in the United Arab Emirates, Bahrain and Saudi Arabia in 2001 which is also present in India. This lineage appears to be most closely related to viruses from 1997 and more distantly related to the UK virus. Sequence analysis of FMD type O viruses from Turkey in 2000 has revealed the presence of two lineages one being closely related to the Iran/Iraq viruses from that year and the other being most closely related to PanAsia viruses from the Middle East in 1995/96. It would appear that individual viruses belonging to the PanAsia strain may have been evolving independently in different geographic regions and that the diversity at the tips of these lineages exceeds that previously stated for the variation seen within this virus strain.

Introduction

The spread of a pandemic foot-and-mouth disease (FMD) type O virus strain has recently been described (Knowles *et al*., 2000, 2001c). This strain, named PanAsia, has occurred throughout most of Asia from Turkey in the west to Japan in the east. It even spread into Europe in 1996 causing outbreaks in Bulgaria and Greece. The PanAsia strain has also managed to invade countries which have remained FMD-free for many years, e.g. Japan and South Korea.

The last significant outbreak of FMD to occur in the United Kingdom was in 1967-68 and was caused by a type O virus. Since then only two small outbreaks have occurred. The first was on the Channel Island of Jersey in 1974 and was caused by FMD virus type C. The second was due to FMD virus type O and occurred in 1981 both on Jersey and the Isle of Wight, just off the south coast of England.

On the 20th February 2001 FMDV type O was identified in samples of pig epithelium from an abattoir in Brentwood, Essex. Subsequently, the probable source of infection was traced to a swill-feeding pig farm at Heddon-on-the-Wall in Northumberland. It was suspected that infection had been present on that farm for a number of weeks and that spread had occurred, possibly by the windborne route to a nearby farm which kept sheep. Once within the sheep population, spread was able to occur, mainly by contact, due to the many uncontrolled movements that occur in the UK. Disease has now been confirmed on 2013 premises (Table 1; Fig. 1) with the resultant destruction of some 3,854,000 animals. These were comprised of 594,000 cattle, 3,104,000 sheep, 139,000 pigs, 2,000 goats, 1,000 deer and 14,000 other animals.

On the 12th March 2001 FMD was detected in France in six cattle at Baroche Gondoin, Mayenne. The animals became infected after having been in close proximity to sheep imported from the United Kingdom. The imported sheep, kept in a holding 500 metres from the affected establishment, were slaughtered and then destroyed (as were in-contact animals) on 27th February 2001. They had originated from British outbreak FMO/2001/11 (Llangaron, Herefordshire) where disease had been confirmed on the 26th February 2001. A second outbreak was detected in France at Mitry-Mory, Seine et Marne on the 23rd March 2001.

A series of outbreaks occurred in Northern Ireland starting on the 28th February 2001 with a farm at Meigh, South Armagh. The second outbreak was detected over six weeks later on the 13th April 2001 at Ardboe, nr. Cookstown, County Tyrone and the third on the 15th April 2001 at Cushendall, County Antrim. The fourth outbreak occurred near the second at Ardboe, but about nine days later on the 22nd April 2001.

Infected animals were found in the Republic of Ireland on the 22nd March 2001 at Broughattin, Proleek, County Louth, just a few miles from the first outbreak in Northern Ireland.

Between the 21st March and the 22nd April 2001, 26 infected premises were detected in the Netherlands (see A. Bouma, P. Eble, E. v. Rooij, A. Bianchi, A. Dekker, this meeting). An infected farm in Oene (Dutch outbreak number 3) housed 74 veal calves from Ireland. These calves had been part of a larger shipment of Irish calves which had been laid up at a holding point in Baroche Gondoin, department of Mayenne, from 4 pm on 23rd February to 4 am on 24th February. It is therefore thought that the calves may have become infected during those 12 hours through contact with infected British sheep and then transmitted disease to the Netherlands.

The VP1 genes of viruses isolates from a number of the British, Irish and French cases were sequenced and compared with sequences of the Dutch virus isolates determined at ID-Lelystad and with FMD type O virus isolates from various other countries. In addition recent FMD type O viruses isolated from samples received from various countries in the Middle East were also sequenced and compared.

Materials and Methods

Viruses. All the virus isolates were obtained from the WRLFMD strain collection either as 10% epithelial suspensions or as cell culture passaged material. Details of the viruses studied are

shown in Table 1. RNA was extracted directly from these samples using RNeasy spin-columns (Qiagen) as per the manufacturer=s instructions.

Oligonucleotide primers. Oligonucleotide primers with a Cy5 amidite fluorescent dye for use with the ALFexpressJ automated sequencer were purchased from a commercial source (Amersham Pharmacia Biotech, Sweden). Unlabelled primers used for PCR were purchased from Cruachem (UK). The sequences of the primers have been described previously (Knowles and Samuel, 1995).

RT-PCR. Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the primer set ARS4/NK61 (1301 bp) essentially as described by Knowles and Samuel (1995).

Cycle sequencing. fmolJ DNA sequencing kits (Promega, UK) which use the cycle sequencing method described by Murray (1989) were used according to the manufacturer=s protocol with the following amendments: approximately 80 fmoles of cDNA template was used in the reactions and 1.5 pmoles of Cy5 amidite-labelled primer. The reactions were heated to 95°C for 2 minutes and subjected to 30 cycles of the following programme on a thermal heating block (Omnigene, Hybaid UK): 95°C for 30 seconds, 42°C for 30 seconds and 70°C for 1 minute. The reactions were terminated by adding 4µl of Cy5 sequencing stop solution (Amersham Pharmacia Biotech, Sweden) and cooled to 4°C. The reactions were heated to 95°C for 3 min prior to loading on an ALFexpressJ DNA Sequencer (Amersham Pharmacia Biotech, Sweden). The software, ALFwin Sequence Analyser v2.10.06 (Amersham Pharmacia Biotech, Sweden), was used to process the data which was then exported as an ASCII text file, aligned manually and analysed using the EpiSeq v2.0 suite of computer programs (N.J. Knowles, unpublished).

Phylogenetic analyses. Nucleotide sequences were analysed on an IBM compatible personal computer using programs written by one of the authors (NJK). All pairwise comparisons were performed by giving each base substitution equal statistical weight (ambiguities were ignored). A binary tree was constructed according to sequence relatedness across the complete VP1 gene (639 nucleotides) using the Neighbor-joining algorithm (Saitou and Nei, 1987) as implemented in the computer program NEIGHBOR (part of the PHYLIP 3.5c phylogeny package; Felsenstein, 1993). The subsequent unrooted tree was plotted using TreeView v1.6 (Page, 1996).

Results and Discussion

The FMD situation in the Middle East

Four virus isolates from Turkey in 2000 were sequenced and compared. They fell into two groups, i) O/TUR/5/2000 and O/TUR/8/2000 which were closely related to viruses resulting from the most recent spread of the PanAsia strain (Fig. 2); and ii) O/TUR/2/2000 and O/TUR/7/2000 which were closely related to the PanAsia viruses present in Turkey in 1996 (based on partial VP1 sequences; data not shown).

Phylogenetic analysis of viruses isolated during 2001 from samples received from Bahrain, Saudi Arabia and the United Arab Emirates (UAE) revealed that a new lineage was present in the Middle East (Fig. 2). Sequences derived from viruses isolated from three different Indian states were submitted to the WRLFMD sequence database by R. Venkataramanan (IVRI-Mukteswar).

These proved to be very closely related to this new lineage. The next most closely related virus sequences were those derived from Bahrain, Kuwait and the UAE in 1997 (Fig. 2). These 1997 isolates had previously been classified as members of the PanAsia strain (Knowles et al., 2000, 2001c), however, it is not clear if the newer (2001) viruses should also be included as members of this strain. Clearly the evolution and co-existence of multiple genetic lineages (Samuel et al., 1997) is a very complex issue and needs further study.

Previously, a maximum level of 5% nucleotide difference was used to group viruses within the PanAsia strain (Knowles et al., 2000, 2001c). As different lineages evolve newer isolates may be related to previous ones by less that 5%, however, isolates at the tips of the lineages become more and more distantly related. Figure 3a shows a hypothetical case where a progenitor virus isolate AA@ gives rise to two descendants AB@ and AC@, each differing by 5% to AA@. Further evolution gives rise to AD@ and AE@ from the AB@ isolate and AF@ and AG@ from the AC@ isolate. The resulting relationship are shown in Table 3.

In the past we have used UPGMA (**U**nweighted **P**air **G**roup **M**ethod with **A**rithmetic mean) trees to reconstruct phylogenetic trees, however, recently we have started to use the Neighbor-joining method. The latter method is better at reconstructing these trees, particularly when all the viruses being examined are not contemporaneous. Using the Neighbor-joining method the hypothetical evolutionary case mentioned above (Fig. 3a) is accurately reconstructed (Fig. 3b). However, using the UPGMA method (which assumes an evolutionary clock and that all viruses are contemporaneous) a number of mistakes are made (Fig. 3c). The UPGMA method can successfully reconstruct the tree topology if only contemporary virus isolates are used (Fig. 3d).

The FMD situation in Europe

Phylogenetic analysis of 18 FMD type O viruses from the UK epizootic in 2001 showed them to be very closely related (Fig. 2). Similarly isolates from the Republic of Ireland, France and the Netherlands also closely related to each other and to the UK viruses (Fig. 2). Further analyses are currently in progress, particularly to compare in more detail the Hereford (UK), French and Dutch viruses which are thought to be directly linked. The suspected routes of spread of these European outbreaks are shown in Figure 4.

The FMD situation in South America

In the past few years FMD type O has occurred in a number of South American countries, i.e. Bolivia (1998), Brazil (Matto Grosso do Sul, 1998), Brazil (State of Rio Grande do Sul, August-September 2000), Uruguay (Department of Artigas, October 2000) and Colombia (Department of Antioquia, August-September 2000) . Examination by RT-PCR and nucleotide sequencing of theVP1 gene of viruses isolated during these outbreaks has demonstrated that none were closely related to the PanAsia strain (O/URU/1/2000 shown in Fig. 2, otherwise data not shown).

Currently further FMD type O virus isolates from the UK outbreaks are being examined to elucidate the extent of diversity in the course of the epizootic. In addition the complete genome sequence of one UK isolate is being determined in order to compare it with other PanAsia viruses. Newer virus isolates from the Middle East and other parts of the world are also being studied to establish which strains are present.

Acknowledgments

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Table 1. Number of infected premises in the UK 2001 FMD epizootic (n=2013*).

County/region	No.	County/region	No.
Anglesey	13	Monmouthshire	26
Berkshire	2	Northants	1
Borders	11	Northumberland	77
Cheshire	16	North Yorkshire	134
Co Durham	93	Oxfordshire	2
Cornwall	4	Powys	69
Cumbria	886	Shropshire	11
Derbyshire	8	Somerset	8
Devon	173	Staffordshire	48
Dumfries & Galloway	176	Teesside	5
Essex	11	Tyne & Wear	6
Glamorgan	5	Warwickshire	2
Gloucestershire	76	West Yorkshire	6
Herefordshire	43	Wiltshire	7
Kent	5	Worcestershire	26
Lancashire	53	Northern Ireland	4
Leicestershire	6		

* as of 9th September 2001

Table 2. Details of the foot-and-mouth disease type O viruses studied.

Virus designation	Geographic origin	Date of collection	Species	Accession number	Reference
O/ALG/1/99	Souidania, Governorate of Greater Algiers, Algeria	02/1999	bovine	AJ303467	Samuel et al., 1999
O/BAR/2/97	Bahrain	1997	nk	AJ318824	Knowles et al., 2001c
O/BAR/8/98	Bahrain	1998	bovine	AJ318825	Knowles et al., 2001c
O/BAR/6/99	Bahrain	1999	bovine	-	This work
O/BAR/1/2001	Bahrain	03/2001	bovine	-	This work
O/CAM/6/99	Kâmpóng Thum, Cambodia	28/01/1999	bovine	AJ318827	Knowles et al., 2001c
O/CAM/2/2000	Angkor Chum, Siem Reap, Cambodia	27/01/2000	bovine	AJ318828	Knowles et al., 2001c
O/CHA/1/99*	Tibet, P.R. China	05/1999	bovine	AJ318830	Knowles et al., 2001c
O/CHA/2/99*	Tibet, P.R. China	05/1999	bovine	AJ318831	Knowles et al., 2001c
O/CHA/3/99*	Tibet, P.R. China	05/1999	bovine	AJ318832	Knowles et al., 2001c
O/CHA/4/99*	Hainan, P.R. China	05/1999	bovine	AJ318833	Knowles et al., 2001c
O/FRA/1/2001	Baroche Gondoin, Dept. of Mayenne, France	12/03/2001	bovine	-	This work
O_1/Kaufbeuren/FRG/66	Kaufbeuren, West Germany	1966	bovine	X00871	Forss et al., 1984
O/GHA/5/93	Kintampo, Ghana	06/01/1993	bovine	AJ303488	Samuel and Knowles, 2001
O/HKN/1/99	Mong Tseng Tsuen, Yuen Long, Hong Kong	05/01/1998	porcine	AJ294925	Knowles et al., 2001a
O/HKN/4/2001	Hong Kong	04/2001	nk	-	This work
O/IND/83/2001H	Karnataka, India	2001	nk	-	R. Venkataramanan, pc 2001
O/IND/96/2001H	Punjab, India	2001	nk	-	R. Venkataramanan, pc 2001
O/IND/116/2001H	Haryana, India	2001	nk	-	R. Venkataramanan, pc 2001
O/IRN/9/99	Iran	1999	nk	AJ318838	Knowles et al., 2001c
O/IRN/16/2000	Alostan, Sardasht, West Azerbaijan, Iran	28/06/2000	ovine	AJ318840	Knowles et al., 2001c
O/IRQ/30/2000	Iraq	09/04/2000	bovine	AJ303499	Samuel and Knowles, 2001
O/IRL/134/2001	Broughattin, Proleek, County Louth, Republic of Ireland	22/03/2001	ovine	-	This work
O/KEN/83/79	Mweiga, Nyeri Dist., Central Prov., Kenya	1979	bovine	AJ303511	Samuel and Knowles, 2001
O/KUW/3/97	Kuwait	1997	bovine	-	This work
O/KUW/1/98	Warfra (Al Warfah), Kuwait	02/05/1998	bovine	-	This work
O/JPN/A/2000	Miyazaki, Japan	07/04/2000	bovine	AB050978	Knowles et al., 2001c
O/LAO/2/2000	Laos	26/01/2000	nk	AJ318844	Knowles et al., 2001c
O/MAY/2/2000	Kilang Papan, Batu Arang, Selangor, Malaysia	02/02/2000	bovine	AJ318846	Knowles et al., 2001c
O/MAU/19/2000	Nieleba, Guidimakha, S.E. Mauritania	01/10/2000	nk	-	This work
O/MOG/2000	Ulaanbadrakh, Dornogovi, Mongolia	04/2000	nk	AJ318847	Knowles et al., 2001c
O/NEP/12/2000	Kathmandu, Nepal	14/06/2001	bovine	-	This work
O/NET/1/2001I	Olst, Netherlands	21/03/2001	nk	-	A. Dekker, pc, 2001
O/NET/3/2001I	Oene, Netherlands	22/03/2001	nk	-	A. Dekker, pc, 2001
O/NET/5/2001I	Oene, Netherlands	25/03/2001	bovine	-	A. Dekker, pc, 2001
O/NET/11/2001I	Oene, Netherlands	29/03/2001	nk	-	A. Dekker, pc, 2001
O/PHI/7/96	Mahabang Parang, Angono, Philippines	1996	porcine	AJ294926	Knowles et al., 2001a
O/QTR/3/99	Al-Wabra, Wildlife Preservation, Dohar, Qatar	27/02/1999	Dorcas gazelle	-	This work
O/1734/RUS/2000	Elitnoye, Ussuriysk, Primorskiy, Russian Federation	04/2000	porcine	AJ318850	Knowles et al., 2001c
O/SAR/1/2000	Camperdown, Kwazulu-Natal, South Africa	09/2000	porcine	AJ318860	Knowles et al., 2001c
O/SAU/2/97	Riyadh, Saudi Arabia	05/1997	bovine	AJ318851	Knowles et al., 2001c
O/SAU/38/98	Al-Kharj, Saudi Arabia	1998	bovine	AJ318852	Knowles et al., 2001c
O/SAU/2/99	Saudi Arabia	1999	nk	-	This work
O/SAU/11/2001	Saudi Arabia	04/2001	bovine	-	This work
O/SKR/1/2000	Papyung, P=aju City, Kyunggi, South Korea	26/03/2000	bovine	AJ318854	Knowles et al., 2001c
O/TAW/81/97	I-lan, Taiwan POC	17/04/1997	porcine	AJ296321	Samuel and Knowles, 2001
O/TAW/2/99	Kinmen, Taiwan POC	06/1999	bovine	AJ294927	Knowles et al., 2001a
O/TAI/4/99	Mae Hong Son, Thailand	01/03/1999	bovine	AJ303536	Samuel and Knowles, 2001

O/TAI/1/2000	Nong Khai, Thailand	01/01/2000	buffalo	-	This work
O/TAI/2/2000	Songkhla, Thailand	18/01/2000	bovine	-	This work
O_1/Manisa/TUR/69	Manisa, Turkey	01/04/1969	bovine	AJ251477	Aktas and Samuel, 2000
O/TUR/2/2000	Balikesir, Merkez, Turkey	2000	bovine	-	This work
O/TUR/5/2000	Nigde, Turkey	2000	bovine	-	This work
O/TUR/7/2000	Diyarbakir, Turkey	05/05/2000	bovine	-	This work
O/TUR/8/2000	Konya, Turkey	2000	bovine	-	This work
O/UAE/7/97	Al Ain, United Arab Emirates	05/1997	bovine	AJ318856	Knowles et al., 2001c
O/UAE/4/99	United Arab Emirates	1999	antelope	-	This work
O/UAE/1/2000	Al Rawabi, Dubai, United Arab Emirates	06/05/2000	bovine	-	This work
O/UAE/2/2000	Al Rawabi, Dubai, United Arab Emirates	06/05/2000	bovine	-	This work
O/UAE/3/2000	Al Rawabi, Dubai, United Arab Emirates	06/05/2000	bovine	-	This work
O/UAE/6/2001	Al Hayk'l Slaughter House, 75 km from Bubai, United Arab Emirates	03/2001	bovine	-	This work
O/UKG/12/2001	Essex, England, UK (FMO/2001/01)	20/02/2001	porcine		Knowles et al., 2001b
O/UKG/123/2001	Essex, England, UK (FMO/2001/03)	22/02/2001	bovine	-	This work
O/UKG/128/2001	Northumberland, England, UK (FMO/2001/04) (index case)	23/02/2001	porcine	-	This work
O/UKG/130/2001	Essex, England, UK (FMO/2001/05)	23/02/2001	porcine	-	This work
O/UKG/150/2001	Northumberland, England, UK (FMO/2001/06)	23/02/2001	bovine	-	This work
O/UKG/174/2001	Devon, England, UK (FMO/2001/07)	24/02/2001	bovine	-	This work
O/UKG/195/2001	Wiltshire, England, UK (FMO/2001/08)	26/02/2001	ovine	-	This work
O/UKG/198/2001	Devon, England, UK (FMO/2001/09)	26/02/2001	bovine	-	This work
O/UKG/438/2001	Co. Armagh, Northern Ireland, UK (FMO/2001/1700)	28/02/2001	ovine	-	This work
O/UKG/478/2001	Dumfries & Galloway, Scotland,, UK (FMO/2001/28)	01/03/2001	ovine	-	This work
O/UKG/3730/2001	Dumfries & Galloway, Scotland, UK (FMO/2001/806)	30/03/2001	nk	-	This work
O/UKG/3802/2001	Devon, England, UK (FMO/2001/801)	30/03/2001	bovine	-	This work
O/UKG/4021/2001	Cumbria, England, UK (FMO/2001/911)	01/04/2001	ovine	-	This work
O/UKG/4027/2001	North Yorkshire, England, UK (FMO/2001/766)	29/03/2001	ovine	-	This work
O/UKG/4553/2001	Borders, Scotland, UK (FMO/2001/1101)	07/04/2001	ovine	-	This work
O/UKG/5060/2001	Co. Tyrone, Northern Ireland, UK (FMO/2001/1701)	13/04/2001	bovine	-	This work
O/UKG/5565/2001	Co. Antrim, Northern Ireland, UK (FMO/2001/1702)	15/04/2001	bovine	-	This work
O/UKG/9359/2001	North Yorkshire, England, UK (FMO/2001/1602)	16/05/2001	bovine	-	This work
O/UGA/5/96	Mbarara, Uganda	17/01/1996	bovine	AJ296327	Samuel and Knowles, 2001
O/URU/1/2000	Artigas, Uruguay	25/10/2000	bovine	-	This work
O/VIT/2/97	Vietnam	1997	bovine	AJ294929	Knowles et al., 2001a
O/VIT/3/97	Vietnam	1997	porcine	AJ294930	Knowles et al., 2001a

*, Lanzhou Veterinary Research Institute reference number
H, Indian Veterinary Research Institute-Mukteswar reference number
I, ID-Lelystad reference number

Table 3. Percentage nucleotide relationships between a hypothetical progenitor virus isolate AA@ and its descendants.

	A	B	C	D	E	F	G
A	0.00						
B	5.00	0.00					
C	5.00	10.00	0.00				
D	10.00	5.00	15.00	0.00			
E	10.00	5.00	15.00	10.00	0.00		
F	10.00	15.00	5.00	20.00	20.00	0.00	
G	10.00	15.00	5.00	20.00	20.00	10.00	0.00

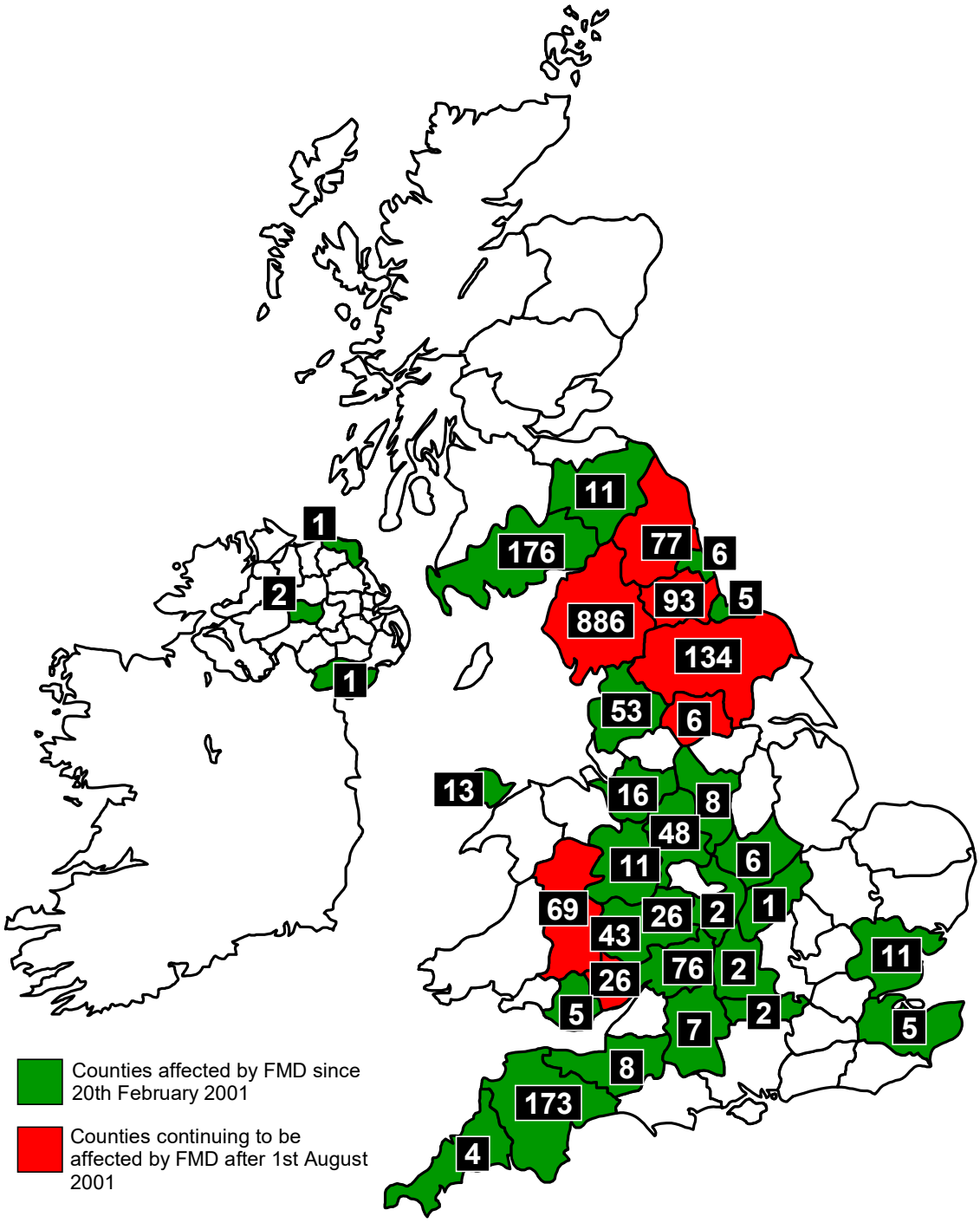


Fig. 1. Number of infected premises by county in the 2001 UK outbreak of FMD. A total of 2013 were recorded as of 9th September 2001.

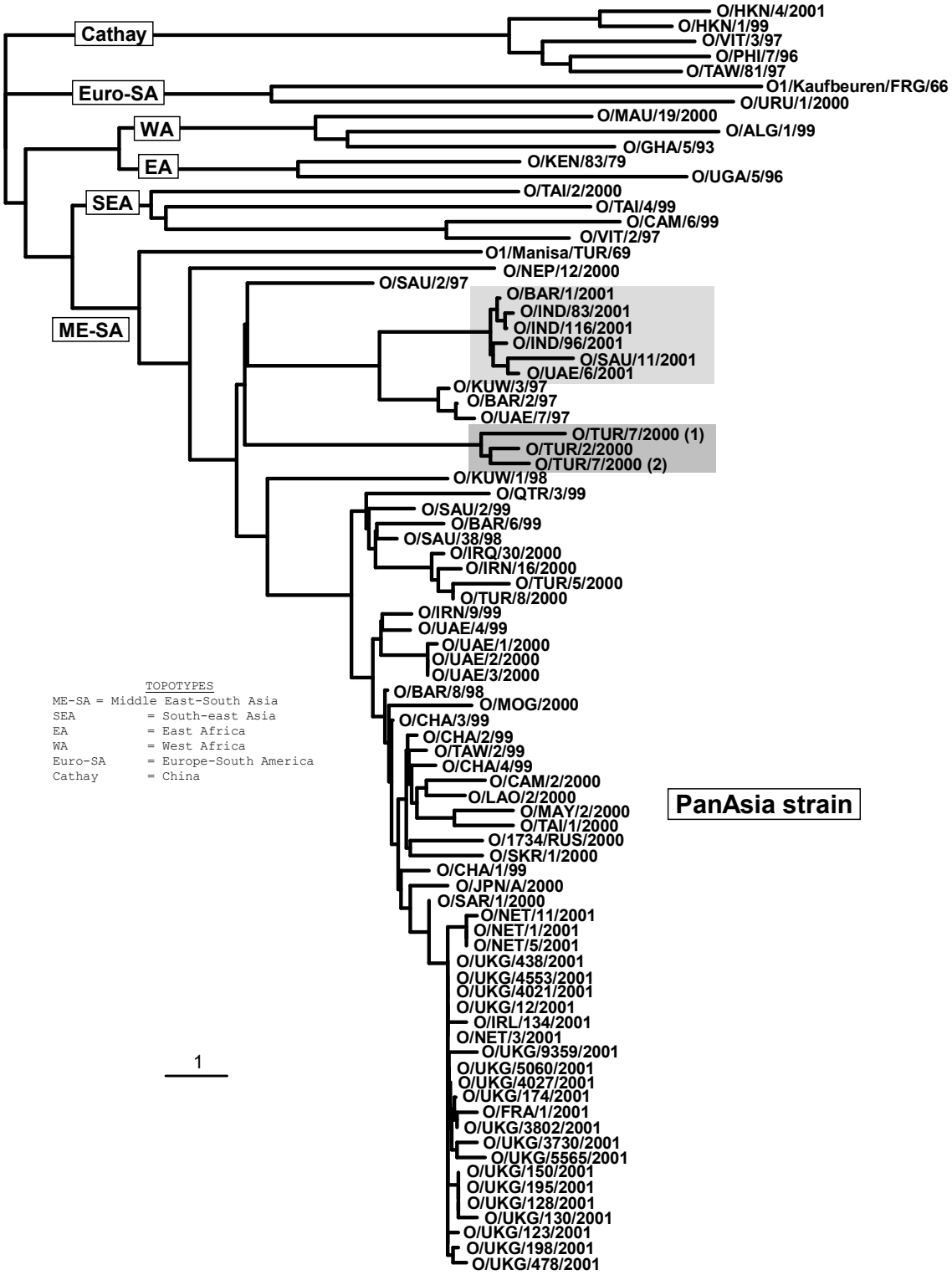


Fig. 2. Genetic comparison of the complete VP1 genes of recently isolated foot-and-mouth disease viruses.

Appendix 4

Sequence data of the foot-and-mouth disease outbreaks in the Netherlands; how do they correspond with the results from tracing

A. Dekker, C. Boonstra-Leendertse, J. Boonstra

During an outbreak of a notifiable disease tracing of contacts of infected farms is always difficult. In the Netherlands the compensation paid to the farmers are cut, if regulations with regard to identification and registration or hygiene have not been followed. Therefore, farmers are often reserved to reveal information. In the 2001 foot-and-mouth disease outbreak on 7 farms possible contacts came to light. In 17 cases, there was an infected farm nearby but the real contact could not be traced. To study whether sequencing the virus isolates could help to reveal the possible contacts, all outbreak viruses were sequenced and compared to a known UK outbreak strain.

Standard methodologies for RNA isolation, RT-PCR and sequencing, using primers advised by the world reference institute, were used. In all cases, RNA was isolated from the original vesicular material, without a cell passage.

The VP1 sequence of the index case (NET 3/2001) was identical to several UK strains. Within the outbreak strains only small differences were found. Two nucleotides in VP1 and one in the beginning of 2A changed very early in the epidemic. Nine isolates had these changes. The other 13 isolates had one additional nucleotide change in VP1, which in 11 isolates resulted in an amino acid substitution (Figure 1).

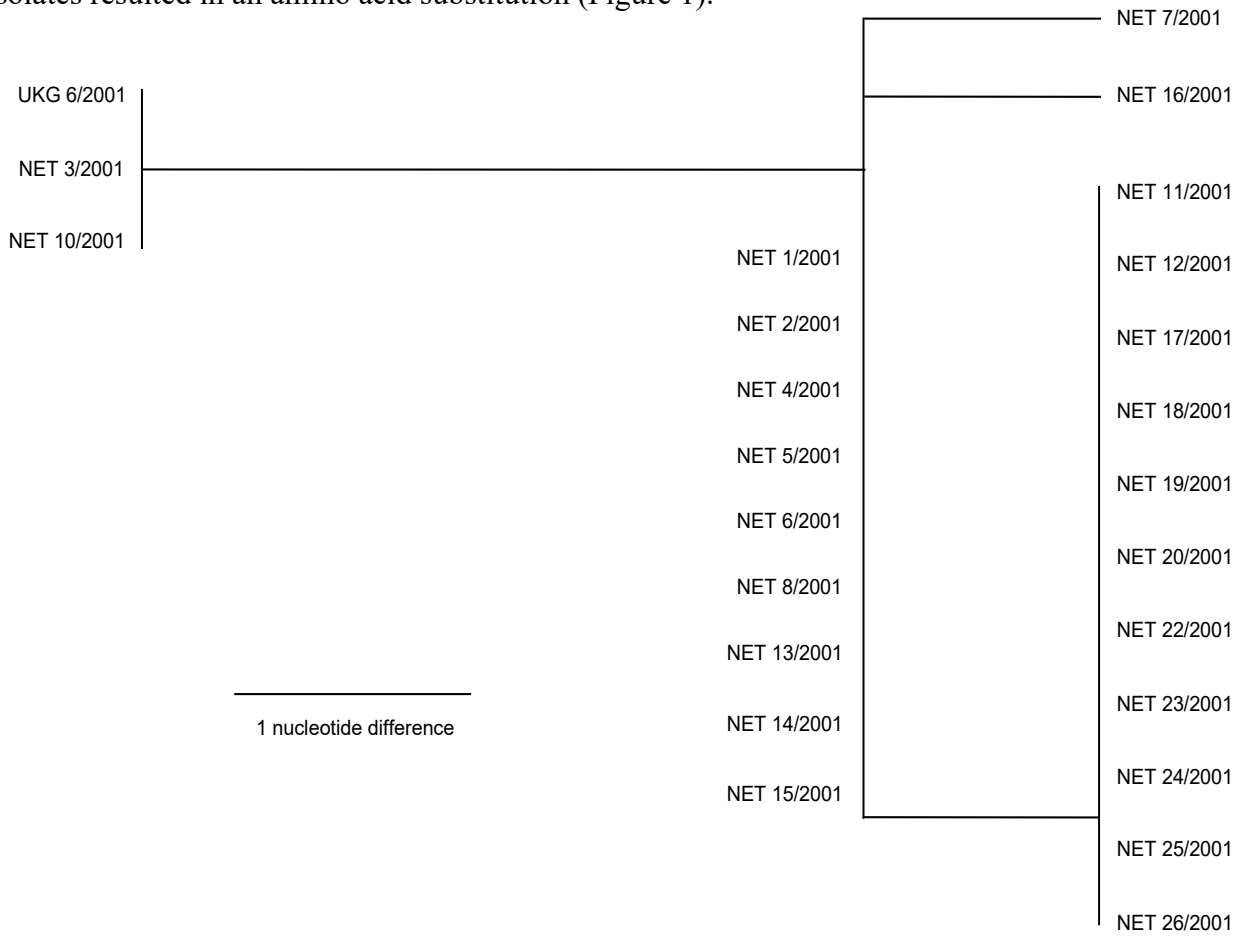


Figure 1: Neighbour-joining tree of all Dutch outbreak isolates (based on VP1 and first 63 nucleotides of 2A)

In this figure, NET 9/2001 is missing, because this farm was diagnosed based on serology and the epidemiological link, transport of infected goats, and strain NET 21/2001 has not been sequenced yet.
Geographically strains with equal sequence were often found in the same area (Figure 2). Some contacts identified during the inquiry did not seem logical when looking at the sequences of the isolates and the serological results found on the farm.

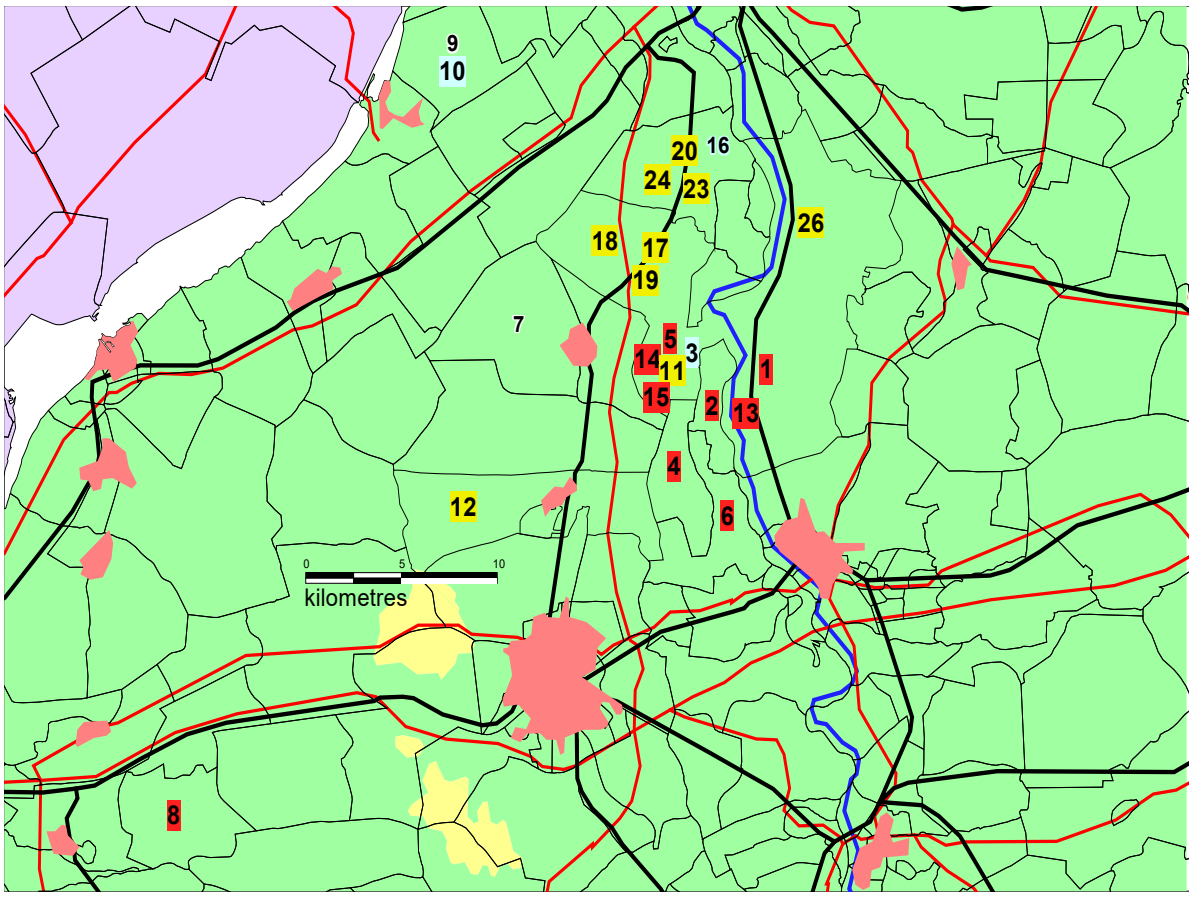


Figure 2: Geographical distribution (approximation based on postal code areas) of the infected farms in the centre of the Netherlands.

The ability to trace contacts is essential for disease control. All people having contact with farm animals should register all contacts during an outbreak of a notifiable disease, to enable epidemiological tracing. The fact that the geographical distribution of sequences was not randomly suggests that spread of virus within a small area often occur. This Implicates that hygiene by farmers, and by disease control personnel has to be very strict. If control measures cannot be performed without these very strict hygienic measures, other control measures like vaccination should be pursued.

This study shows that sequence data can help to understand the epidemiology of FMD. But due to the fact, that only minor mutations were found in the part that was sequenced, the use of nucleotide sequencing remains limited in small outbreaks like this one.

Appendix 5

REPORT ON THE OUTBREAK IN FRANCE

François Moutou

* 20 February 2001 Notification of FMD in the UK

* 1-21 February 2001 31476 sheep imported from the UK

20 February – 2 March 1254 animals from Ireland
20 February – 5 March 15787 animals from the Netherlands

* 59 968 animals slaughtered in 117 farms ; 43% imported, 53% in-contact.

* 5404 blood samples

5398 negative from 189 farms
28 positive from 6 farms

* First outbreak on 13 March in Mayenne
* Second outbreak on 23 March in Seine-et-Marne.

Serology against FMD in 2001

* February – June 17932 blood samples
893 lots
68 departments

* Imported – contact sheep

9524 blood samples
590 lots
61 departments

* "Native" sheep (May – June)

Resident and "nomadic" (Alps and Pyrénées)

8408 blood samples
303 lots
18 departments

All negative

- 30 blood samples / flock.

Appendix 6

THE EPIDEMIC OF FOOT-AND-MOUTH DISEASE IN THE NETHERLANDS IN 2001: LABORATORY EXAMINATIONS

A. Bouma, P. Eble, E. v. Rooij, A. Bianchi, A. Dekker

After the outbreak of foot-and-mouth disease (FMD) in the United Kingdom, an outbreak of FMD occurred in the Netherlands. The first farm infected (NET 3/2001) was a mixed, veal calf / goat farm in the central part of the Netherlands. The most likely route of infection was the import of Irish veal calves via an FMD contaminated staging point in France, which was located near the first outbreak of FMD in France. Because the virus grew poorly in the secondary pig kidney cells used for virus isolation the diagnosis took several days. Before the first farm was confirmed, in the laboratory, already two other clinical cases were detected (NET 1/2001 and NET/2/2001). Despite the control measures, more outbreaks of FMD occurred within the area around Oene. In total 26 outbreaks occurred (Table 1).

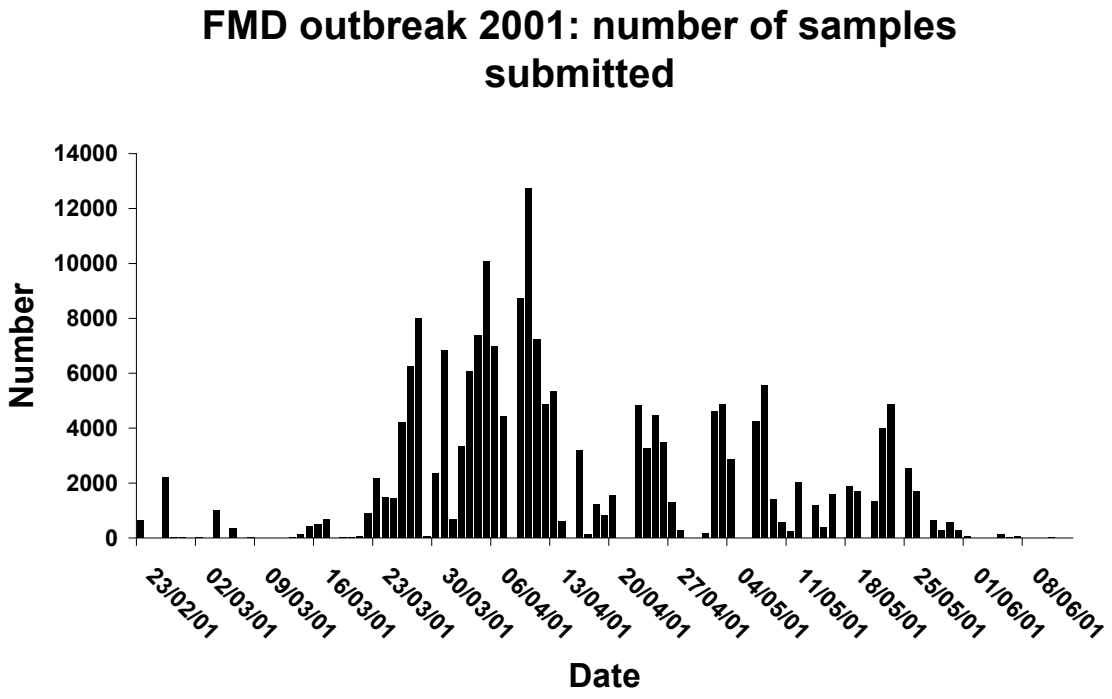
Table 1: Outbreaks of FMD in the Netherlands (2001)

Number	Date Diagnosis	Date Culling	Location	Number of animals present				Number of samples taken before culling	
				Cattle	Sheep	Pigs	Goat	Total	Positive
1	21/03/01	21/03/01	Olst	59	74	0	0	4	1
2	21/03/01	22/03/01	Welsum	121	0	0	0	122	0
3	22/03/01	17/03/01	Oene	74	0	0	545	184	94
4	24/03/01	24/03/01	Nijbroek	1197	16	0	3	113	1
5	25/03/01	26/03/01	Oene	164	0	0	0	163	0
6	27/03/01	28/03/01	Terwolde	0	0	0	624		
7	27/03/01	28/03/01	Tongeren	69	86	0	0	64	1
8	28/03/01	27/03/01	Kootwijkerbroek	457	3	0	1	67	0
9	28/03/01	21/03/01	Oosterwolde	327	36	0	50	129	47
10	29/03/01	29/03/01	Oosterwolde	113	0	0	0	57	0
11	29/03/01	30/03/01	Oene	179	0	0	43	223	2
12	01/04/01	02/04/01	Vaassen	78	19	0	0	94	0
13	03/04/01	03/04/01	Olst	46	0	0	0	12	2
14	03/04/01	03/04/01	Oene	31	0	0	0	1	0
15	03/04/01	03/04/01	Oene	54	0	0	0	51	0
16	07/04/01	06/04/01	Wapenveld	23	0	0	0	23	0
17	07/04/01	07/04/01	Heerde	91	0	0	0	71	0
18	07/04/01	08/04/01	Heerde	334	0	0	0	180	0
19	09/04/01	08/04/01	Heerde	47	1	0	0	43	0
20	09/04/01	09/04/01	Wapenveld	11	22	0	0	15	0
21	10/04/01	09/04/01	Heerde	47	1	0	1	93	0
22	11/04/01	11/04/01	Ee	103	0	0	0	8	0
23	11/04/01	12/04/01	Wapenveld	54	0	0	0	55	2
24	11/04/01	12/04/01	Wapenveld	89	0	0	0	86	0
25	11/04/01	12/04/01	Anjum	133	0	0	2	33	1
26	22/04/01	22/04/01	Wijhe	76	0	0	0	58	1

In 23 of the cases antigen was detected by ELISA, in two cases the virus had to be amplified by passage in cell culture. In one case (NET 9/2001) the diagnosis was based on positive serology in combination with epidemiological evidence of transport of infected goats.

Most infections appeared in the proximity of other outbreaks, only in 7 out of the 26 cases a possible contact could be traced. In two cases transport of infected animals, in one case contact via the milk tanker and in four instances persons who had been on both farms. The first control strategy was pre-emptive culling in a 1 km zone. Because the number of farms identified for pre-emptive culling became higher than the capacity for pre-emptive culling, available on short notice, the Ministry of Agriculture decided to implement a emergency vaccination strategy for all biungulates in a large area around Oene, the "Noord Veluwe". All susceptible animals (± 200.000) on approximately 1,120 farms in this area were vaccinated.

All vaccinated herds were checked for infection by clinical examination and serology. This resulted in a very large number of samples submitted in this period, starting 28 March 2001 (see Table 2 and figure below).



All serum samples were screened in a single dilution in the neutralisation test or in a newly developed monoclonal-based screening ELISA. This enabled us to test over 50.000 samples per week. The definite result was based on the titre in the neutralisation test. Based on the sera taken from the vaccinated animals three additional farms were identified. On one cattle farm clinical signs were evident at the time of pre-emptive culling, but no samples were submitted because the animals were killed anyway. The two other farms were goat and sheep farms were clinical symptoms were not seen. Based on OIE and EU regulations and for economic reasons alone, all animals vaccinated were pre-emptively culled.

In the cases that animals were not vaccinated, positive serological results were always followed by the collection of additional samples. Except for the three farms mentioned above, all other positive results were considered as false positive. During final screening all farms in a 10 km zone around the vaccination area were examined for clinical signs of disease and in a sample of these farms 45,699 sera were collected (Table 2).

Table 2: Serological results during the outbreak (without the results obtained at the outbreak farms)

Reason serological investigation	Number of sera		Percentage
	tested	Positive	
Suspicion or screening	63161	95	0.15%
Pre-vaccination	61208	72	0.12%
Pre-emptive slaughter	10971	14	0.13%
Final screening	45699	6	0.01%
Total	181039	187	0.10%

During this final screening 6 animals on 6 farms were found positive by ELISA and the VNT. In three cases no serological positive samples were found at re-sampling. In two cases the same animal was positive on re-sampling, so this was a singleton reactor. The seropositive animals were culled. In one case the cow was born in 1989 and had been vaccinated so no further investigation followed.

In total, 26 outbreaks occurred, the last outbreak on 22 April 2001. The country was declared FMD-free in August 2001.

Appendix 7

PIRBRIGHT'S ROLE IN THE UK 2001 FMD EPIDEMIC AND ITS RESPONSE TO THE EMERGENCY

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SUMMARY

An initial diagnosis of type O FMD in pigs at an abattoir near Brentwood, Essex was made on Tuesday 20 February by the Institute for Animal Health (IAH) Pirbright. Soon afterwards AID visited the premises and investigated the disease situation. In the meantime the laboratory diagnosis was repeated and confirmed. The CVO was given a report by telephone from the abattoir that evening. He decided to declare an outbreak which was done officially the following morning. The first sequencing results, available after 36 hours, showed that the UK virus was a member of the PanAsia group of strains within type O.

After a lull of 3 days, a series of outbreaks occurred, first in Northumberland, in the northeast and then in Devon, in the southwest. Experts from Pirbright (SA and RPK) visited premises in those regions and later a premises in Essex, about 30 km from the abattoir where the disease was first diagnosed. The objectives were to identify the periods when infection was probably introduced and in the case of the pig premises to analyse the risk of airborne spread both locally and over distance, including to the continent. This was done by ageing lesions, by laboratory investigations and by the use of models to generate simulated plumes of airborne FMD virus. The modelling was done in collaboration with meteorologists in Denmark and the UK.

The finding of lesions around 12 days of age in pigs at the swill fed premises in Northumberland suggested that infection was probably present from the beginning of February. This was the earliest indication of infection and so it was concluded that this holding was probably the primary outbreak. It was concluded from further investigations that pigs from this premises probably spread infection to the abattoir in Essex. The movement of sheep from a farm at Ponteland, previously infected by airborne virus from the primary outbreak, was the probable mechanism of spread to Devon. The second outbreak investigated in Essex, a pig farm near Canewdon, was probably infected by contact with the abattoir near Brentwood.

As soon as the first laboratory diagnosis was made Pirbright started to increase its capacity to handle an increased number of samples. Personnel were deployed from other departments on site and from our sister laboratory at Compton. Volunteers soon arrived from other laboratories in England, Scotland, Ireland, Australia and New Zealand. Previous employees obtained leave of absence from their jobs and offered their services. In a short time the number of people available for diagnostic work (including database operation and reporting) increased from 20 to over 60. Veterinarians from the UK and

overseas, including Ireland, Australia and Italy (FAO and EUFMD) were recruited to man the interfaces between the laboratory and MAFF, HQ and the laboratory and the field, to provide expert advice to MAFF and deal with the enormous number of inquiries from the media, the public and politicians.

As the epidemic took off the diagnostic workload increased dramatically and so duty rosters were established to enable the work to continue around-the-clock and through weekends. The ELISA for antigen detection, tissue culture for virus isolation and the LPBE were used for virological and serological investigations, respectively. Sheep were the species predominantly involved and so a large number of blood samples were tested for both virus and antibody. A small number sheeep probang samples were tested by virus isolation and PCR. Todate (03/08/01) over 14,000 diagnostic samples have been processed. During these activities a validation exercise was begun to compare an automated, real time RT-PCR with conventional ELISA/virus isolation procedures.

During the second month of the epidemic a major serological surveillance was initiated aimed mainly at freeing up the southern and eastern regions of the country. The demand on the laboratory increased and additional personnel were recruited, mainly from the Veterinary Laboratories Agency, Weybridge. The number of tests performed per week steadily increased and then leveled off at between 50 and 60,000 per week. By the fifth month of the epidemic Pirbright had tested more than 500,000 samples. This included sera from the Republic of Ireland and Northern Ireland. The screening test mainly used was the new solid phase competitive ELISA with verification of doubtful results by virus neutralisation. At the time of writing a robotic system with a specified capability of processing 100,000 sera per week was being installed and commissioned.

The possibility of vaccination has been continuously debated during the epidemic. An early action was to antigenically characterise the UK virus to determine its relationship with antigens stored in the International Vaccine Bank. A close relationship ($r = 1.0$) was found with the O Manisa strain. In March the International Vaccine Bank formulated 500,000 doses of vaccine which was held in readiness but not used.

The epidemic has been a stimulus to complete research projects in progress and to initiate new work, including: (a) studies to determine the quantities of airborne virus excreted by pigs and sheep infected with the UK strain of virus to update models for predicting airborne spread; (b) investigations to examine the virulence of the UK strain in different species and also its transmissibility; (c) the application of real time RT-PCR to investigate the infectiousness of the UK strain in sheep; (d) studies of the serial passage of FMD virus in groups of sheep; and (d) the sequencing of the VP1 gene of 27 isolates of the UK strain.

Appendix 8

REPORT ON THE PRODUCTION OF EMERGENCY 01 MANISA FOOT-AND-MOUTH DISEASE VACCINE FOR THE UNITED KINGDOM BY THE INTERNATIONAL VACCINE BANK (IVB), 22nd MARCH – 4th APRIL 2001

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Summary

On the 20th February 2001 the United Kingdom confirmed its first case of foot-and-mouth disease (FMD) for 20 years and during the initial stages, the International Vaccine Bank (IVB), Pirbright, prepared itself for the possibility of producing emergency FMD vaccine. Serological evaluation and sequencing data undertaken at Pirbright had indicated that the most suitable vaccine strain against this particular field isolate would be O_1 Manisa. On the 22nd March, the Department of Environment, Food and Rural Affairs (DEFRA), formerly the Ministry of Agriculture, Fisheries and Food (MAFF) requested the formulation of 500,000 bovine doses of aqueous aluminium hydroxide/saponin O_1 Manisa vaccine. This was the first time in the Banks' history that it had been summoned to produce vaccine in 'anger'. This report details the subsequent manufacture of vaccine following request.

Manufacture of emergency FMD vaccine

The 500,000 bovine doses of O_1 Manisa vaccine were formulated over four separate batch runs. The first three runs consisting of 150,000 cattle doses, and a final run of 50,000 bovine doses. Because of the volumes required, the 500 litre vessel OV1 vessel was used for the blending of each of the 150,000 dose batches (equivalent to 450 litres), whilst the final 50,000 dose run (equivalent to 150 litres) was blended in the 300 litre vessel LH1. Each individual run took three days to complete from preparation and sterilisation to filling and capping. In accordance with Good Manufacturing Practice (GMP), records were made of each manufacturing stage, which were countersigned by the appropriate member of staff and any problems entailed were also noted.

Some 26 staff members were either directly or indirectly involved in assisting in the production of the vaccine. This included 6 bottling personnel, 5 personnel for dispatch (1 transporting vaccine to hatch, 2 adding documents, freezer packs and checking, 2 strapping boxes and 3 personnel packing). Bottling rate of the vaccine was approximately 280 units per hour and a filling run into nominal 300 ml polypropylene bottles of 150,000 bovine doses took approximately 6 - 6.5 hours. Some 494,657 bovine doses were finally dispensed, which were hand labelled, packed appropriately in 20 unit amounts and stored in the 1VB's + 4ºC cold room. The only dispatched vaccine, Batch 1/01, which was transported to Penrith in Cumbria, also included cool packs (1 per box), an aqueous vaccine package insert/data sheet and a disclosure sheet notifying the user of the number of doses per vaccine bottle. These sheets were similarly produced for the other 3 batches awaiting dispatch. The number of doses per bottle tended to vary slightly from run to run.

The minimum of at least 30 retention samples were kept from each batch for subsequent analyses or sterility testing. In addition, during the transfer of components and blending of each batch, in-line samples were also taken for sterility checks. The vaccine, Batch 1/01, which was dispatched to

Cumbria was subsequently returned to Pirbright and all four batches of vaccine are still currently stored in the IVB's +4°C fridge.

Quality control

Safety, according to current European Pharmacopoeia guidelines, and potency of the emergency vaccine by serology, were undertaken in-house. Sterility of the final product was carried out independently by a third party to full European Pharmacopoiea compliance. Two production batches were used for the various tests. Batch 1/01, which was dispatched to Penrith in Cumbria, underwent sterility and safety testing. Batch 2/01 was used for potency analysis in 8 cattle. In addition, Batch 4/01 has been used to monitor the stability of the final product at +4°C.

Results

a) Safety test on emergency 0_1 Manisa vaccine Batch 1/01- Carried out in accordance to the European Pharmacopoeia safety test for veterinary vaccines. Briefly, two cattle were inoculated with 2 x bovine dose (6 ml) of aqueous AI $(OH)_3$/saponin vaccine, Batch 1/01, subcutaneously. Body temperatures were recorded and the animals were monitored daily for well being and local reactions.

Table 1 Results of safety test on emergency 0_1 Manisa vaccine Batch 1/01

Animal	5/4/01	6/4/01	7/4/01	8/4/01	9/4/01	10/4/01	11/4/01	12/4/01	13/4/01	4/4/01
UJ70	38.6°C	39.0°C	39.1°C	38.8°C	38.9°C	38.5°C	38.4°C	38.5°C	38.7°C	38.6°C
UJ71	38.2°C	39.0°C	38.2°C	38.5°C	38.7°C	3 8.4°C	38.2°C	38.2°C	38.4°C	38.4°C

No adverse reactions were observed following vaccination and animals remained healthy and body temperatures remained normal during period of monitoring.

b) Cattle potency test of emergency 0_1 Manisa vaccine Batch 2/01 - Using the same vaccine formulation, this antigen was originally potency tested for acceptance into the IVB in 1991 and was found to have a PD_{50} value 112. Batch 2/01 was therefore tested in accordance to a mini IVB cattle potency test which is routinely undertaken every fifth anniversary following acceptance. Briefly, eight cattle were subcutaneously vaccinated with a 1/10 cattle dose of antigen as a 3 ml aqueous AI $(OH)_3$/saponin vaccine. At 21 days post-vaccination animals were bled for serology and a PD_{50} value estimated from the neutralising antibody titres at 21 days by computer model analysis using logistic regression.

Table 2 Cattle potency test on emergency 0_1 Manisa vaccine Batch 2/01

Animal Number	Antibody titres (Log SN50 @100 TCID50) at 21 days p.v.	Probit - % Probability of Protection
UJ91	1.505	40.7%
UJ92	1.95*	>90%
UJ93	1.95	>90%
UJ94	1.95	>90%
UJ95	1.806	79.7%
UJ96	1.95	>90%
UJ97	1.95	>90%
UJ98	1.95	>90%

Expected protection for 0_1 Manisa Batch 2/01 = 6.606/8 (>10 PD_{50}) Variance = 0.942 t - statistic - 2.6849606

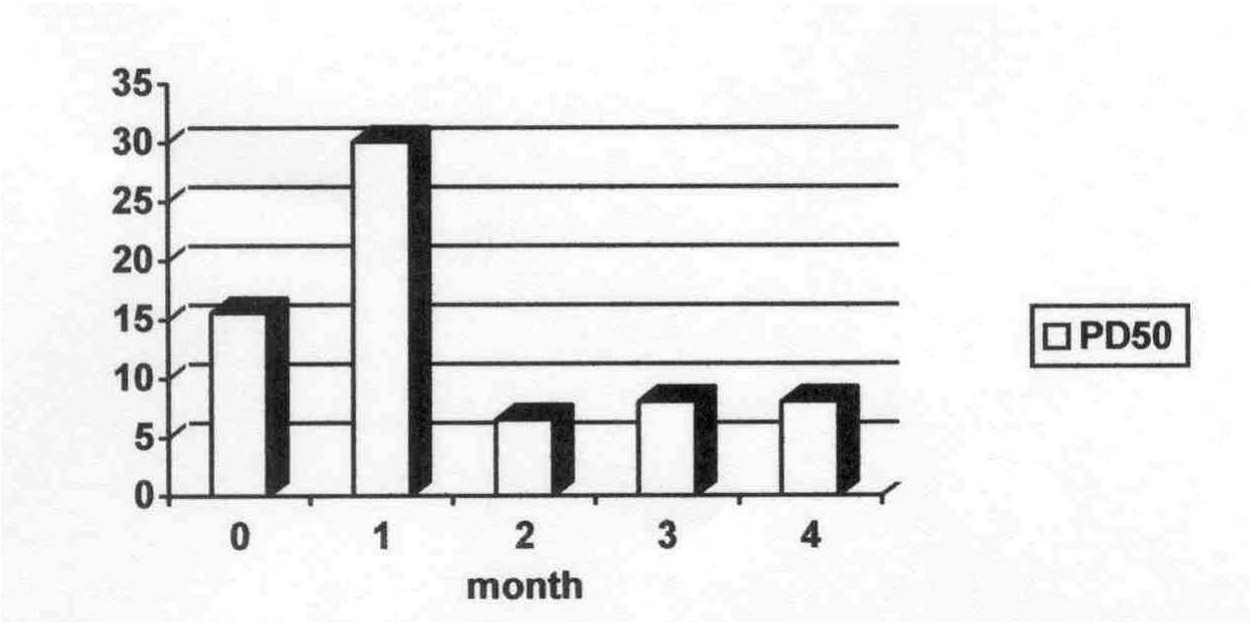
chance that PD_{50} > than dilution used (10) = 98.61%

* - all the 1.95 titres were >1.95 and therefore the calculation is lower that the probable value, however, the potency was in excess of its requirement.

c) **Sterility test on emergency 0_1 Manisa vaccine Batch 1/01** - The testing regime which was done in accordance to the current European Pharmacopoeia and undertaken independently found Batch 1/01 to be sterile. In addition, all line samples taken during the different stages of manufacture showed no evidence of contamination.

d) **Guinea pig potency/stability test on emergency 0_1 Manisa vaccine Batch 4/01** - The testing regime followed that previously described (1) except that the animals receiving a specific dilution of vaccine were always in groups of five and the vaccines were only diluted threefold up to 1/27. Testing was repeated monthly over a 4 month period. PD_{50} values were calculated by the method of Karber (2).

Figure 1 Guinea pig potency/stability values of Batch 4/01 stored over 4 months at +4ºC



Discussion

The emergency foot-and-mouth disease vaccine requested on the 22nd March 2001 by the Department of Environment, Food and Rural Affairs (DEFRA), formerley the Ministry of Agriculture, Fisheries and Food (MAFF), and produced by the IVB at Pirbright, was shown to be safe, sterile and of the required potency with a PD_{50} value in excess of 10. A dossier of all the relevant quality control testing of 0_1 Manisa antigen was compiled for scrutiny by the Veterinary Medicines Directorate.

References

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Appendix 9

THE SPANISH DIAGNOSIS EXPERIENCE DURING THE 2001 FMD EUROPEAN CRISIS

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Since an outbreak of FMD was declared in United Kingdom on the 20th February 2001, until June of this year, about 28.000 samples were collected in Spain and tested in our laboratory CISA at Valdeolmos for monitoring the situation of possible animals infected of FMD virus in the country. The distribution of these sera by animal species was as follows: pig sera 15.000, bovine 8.862 and ovine 4000. The diagnostic strategy consisted of the inspection of samples by PCR (using "universal" primers selected in our lab) and 3ABC-ELISA (indirect test developed and validated previously in Valdeolmos), LPB Elisa and seroneutralization.

None of the analysed samples by PCR were positives. The clinical lesions suspects of FMD and submitted to Valdeolmos were mainly collected from sheep and all of them were negatives to FMD virus and a few cases were positives to ecthyma virus.

Serum were studied by 3ABC protein, Liquid Phase Blocking ELISA using the reactive supplied by WRL from Pirbright and Seroneutralization test using BHK21 cell cultures. None of the sera analysed by Seroneturalization test was positive. False positives were found in a higher percentage analysing sheep sera: 0,67% using LPBE and 0,2% using 3ABC-ELISA. The percentages of false positives in bovine sera were 0,51% using LPBE but only 0,07% when the 3ABC-ELISA was used. Among the pig sera the percentage of false positive were very low; 0,06% and 0,02% using LPBE or 3ABC test respectively.

Concerning the number of sera found doubtful (close or equivalent to cut-off value), using the 3ABC-ELISA those dates were 0,3% (sheep), 0,1% (pigs) and 0% (cattle). However, using LPBE these percentages were slightly higher: 2,1% (sheep), 0,3% (pigs) and 1,2 % (cattle).

Summarizing these results suggest that the 3ABC test used in CISA can be a useful tool in the diagnosis and serosurveillance of FMD since this test is easy to perform, rapid and specific, being the percentage of false positive as well as the number of doubtful sera that required further diagnostic confirmation very low.

Appendix 10

SURVEILLANCE OF FMD IN ITALY DURING THE YEAR 2001

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This report concerns results of the FMD surveillance performed during the year 2001 at the CERVES (Italian Reference Centre for Vesicular Diseases), Brescia, Italy.
The period taken in consideration lasts from 21 February till 18 May, when restrictive regulations on animal trade were enforced by the E.U.
Surveillance was extended also to consignments of FMD susceptible animals imported in Italy since 1st February 2001.

Materials and Methods

Materials and Tests used at the CERVES are as follows:

Serology

FMD viruses in use were the O Manisa and O Switzerland subtypes. The screening ELISA employed inactivated antigens.
Screening ELISA The test was a monoclonal antibody-based LPBE. The cut-off is the titre corresponding to the Weak Positive Reference Serum.
SN The test was performed according the "Manual of Standards for diagnostic Tests and Vaccines", OIE, 2000.
3ABC ELISA The test was a trapping-indirect ELISA for the detection of antibodies to the NS polypeptide 3 ABC of the FMD Virus. Results were evaluated as T/P (Test Serum/Positive Serum) Ratio.

Virology

Materials submitted to virological tests were the homogenates of epithelial tissues (from tongue, gum and lips), tonsils, bone marrow and scabs collected in the field or at slaughterhouses.
Tissue Culture IBRS-2 and BHK are the cell lines currently in use. The sample was scored as negative after three blind TC passages without showing CPE.
Antigen detection The test currently in use is a sandwich ELISA performed with a combination of rabbit immune sera and MAbs specific for O, A, C and Asia types.
RT-PCR The test was performed using two sources of primers, namely:
- F17 and F21 (21-40 and 210-228 of the 3D gene), (Rodriguez A. , Virology 1992)
- LD2 and LR2 (209-226 and 403-420 of the 3D gene), (Lomakina N., unpublished, 1998)

TEST "in vivo" Suckling mice (3-5 days old) were inoculated "in peritoneum" and kept under observation for 5 days.

Results and Discussion

Serological Examinations

A final 43 166 serological tests have been performed during the emergency period.
Sera have been collected from 29 721 animals among which cattle (14 515), sheep and goats (11 775) and swine (3 421), officially submitted for examination.
The origin of consignments was from E.U countries as well as from Italy. Species involved were bovine, ovine and swine. Very few samples originated from other countries (East Europe, Uganda) or species (elephant, camelids).

All sera received during the emergency period have been submitted to the screening ELISA (1st sampling).
Positive and/or doubtful reactors were sampled again (2nd sampling). Sampling was usually extended to further animals in contact with reactors and submitted again to the screening ELISA.
All doubtful and/or positive sera in the screening ELISA were examined by the 3 ABC test.
In order to improve the knowledge of the performances of the 3ABC test, besides the examination of sera identified as reactors by the screening ELISA, also all the sera received from 18th February to end of March (n = 10 487 sera) have been submitted to this test.
In **Table 1** results of 1st and 2nd samplings are reported
Titres of reactors of the 1st sampling resulted unchanged or decreased at the 2nd sampling and never an increase of titres has been observed.
All reactors identified during the two sampling phases with the screening ELISA (n =782) resulted negative with the 3 ABC ELISA . On the contrary, within sera negative in the screening ELISA (n =10 487), 26 reacted in the 3 ABC test. Among them 5 scored clearly positive whilst 21 resulted borderline. Twenty-five out of 26 sera originated from cattle (n = 7468), one (borderline) from a sheep (n =1976) and none from pig (n = 1049). The follow up of these herds demonstrated that any peculiar FMD sign has not been observed onwards so it is reasonable to consider that positive titres detected in 782 sera with the screening ELISA and in 26 sera with the 3 ABC ELISA were not specific.

Finally 586 out of 782 reactors in the screening ELISA were submitted also to SN.
Thirty-six among these reactors were able to neutralise to some extent the infectivity of O Manisa strain (6.2%).
The distribution in classes of titres of 586 sera positive in the screening ELISA and the number of SN reactors found in each class is shown in **Table 2**.
The summary of serological tests performed during the emergency period is reported in **Table 3**.

Virological Examinations

Table 4 reports the results of virological tests performed to clear suspicions due to observation of clinical signs or after positive serological results or because herds in contact with the above mentioned ones.
Often the occurrence of vesicles was claimed but never confirmed at receiving of suspect samples.
Suspicions for positive serology confirmed at re-sampling was usually followed by slaughter of the reactor animals and collection of tonsils for virological studies.
All (n = 26) of such cases has been examined in tissue culture resulting negative: 13 out of them were negative also in RT-PCR tests.
Tonsils from one cattle (origin Poland) with questionable serology showed CPE in both cell lines currently used. A positive reaction was obtained in an RT-PCR test using the primers described by Rodriguez but not with the ones described by Lomakina. The CPE was unaffected by treatment at pH 5 so it is unlikely to be an FMD virus. TC fluids resulted negative to all other virological tests.
Strong alarm (serological findings and clinical signs) was connected to 3 consignments from different places of France: alarm became panic when the first outbreak was declared in France. A "parapox" virus was immediately seen by Electron Microscopy in pathological samples from two out of three consignments, whilst all tests for FMD virus resulted negative.
Electron microscopy gave again a powerful help on two other similar occasions due to "Ectima" in sheep and "Papular Stomatitis" in cattle.
In one clinical suspicion due to the presence of vesicles on cattle tongue a BVD virus was demonstrated.
All other samples from clinical or serological suspicions resulted negative to virological tests.

TABLE 1 : Serological results with the MAbs-based LPB-ELISA and the 3ABC-ELISA

ORIGIN	SPECIES (*)	LPB-ELISA				3ABC ELISA			
		1st sampling		2nd sampling		1st + 2nd sampling POS LPB-ELISA		1st + 2nd sampling NEG LPB-ELISA	
		NEG	POS (%)	NEG	POS (%)	NEG (%)	POS	NEG	POS (%)
ITALY	Cattle	68	9 (13,2)	0	0	9	0	nd	
	G and S	9742	112 (1,1)	519	28 (5,4)	140	0		
	Swine	102	0	0	0	0	0		
TOTAL		9912	121 (1,2)	519	28 (5,4)	149	0		
E.U.	Cattle	12532	345 (2,7)	1915	256 (13,3)	601	0		
	G and S	664	21 (3,1)	850	9 (1,0)	30	0		
	Swine	3319	2 (0,06)	0	0	2	0		
TOTAL		16991	368 (2,1)	2765	265 (9,6)	633 (100)	0		
TOTAL	Cattle	12600	354 (2,8)	1915	256 (13,3)	610	0	7471	25 (0,33)
	G and S	10406	133 (1,2)	1369	37 (2,7)	170	0	1967	1 (0,005)
	Swine	3421	2 (0,05)	0	0	2	0	1049	0
TOTAL		26437	489 (1,8)	3284	293 (8,9)	782 (100)	0	10487	26 (0,25)

Legenda: (*) G and S = Goat and Sheep

TABLE 2 : Distribution in classes of titres of 585 sera positive in the LPB-ELISA. In brackets: number of sera resulted positive to Serum Neutralisation.

>20-50	> 51-100	> 100-200	> 201
300 (22)	223 (13)	43 (2)	20 (1)

TABLE 3 : Summary of serological tests performed during the FMD emergency period (February-May 2001)

LPB-ELISA	30.503
3ABC-ELISA	12.077
Serum neutralisation	586
TOTAL	43.166

TABLE 4 : Tests on animals with clinical and/or epidemiological and/or serological suspicions of FMD.

HOLDING PROVINCE	NUMBER SPECIES	ORIGIN (clinical suspicions)	SEROLOGICAL TESTS ELISA FMDV O Man		VIROLOGICAL TESTS						
			POS/ N°samples	RANGE	SAMPLE	Tissue colture		PCR		ME	other tests
						IBRS 2	BHK 21	primers A	primers B		
DL Pescara	sheep	France (c.s.)	9 / 9	20-70	tonsils	neg				nd	
DL Pescara	262 sheep	France (c.s.)	8 / 56	20>270	tongue vesicles	neg				para pox	nd
DL Pescara	369 sheep	France (c.s.)	1 / 360	60	tongue vesicles	neg				para pox	nd
ZM Varese	49 goats	France (c.s.)	0 / 49	nd	lips epith.	neg				para pox	nd
PR Modena	cattles	Italy (c.s.)	0 / 20	nd	foot and mouth ep.	neg				para pox	neg
AO Piacenza	cattles	Italy (c.s.)	0 / 1	nd	gum epith.	neg				neg	
TFG Pisa	332 sheep	France (c.s.)	0 / 12	nd	tonsils	neg				nd	
TFG Pisa	323 sheep	France (c.s.)	0 / 1	nd	bone m. foot ep.	neg				nd	
LM Novara	cattles	Italy (c.s.)	0 / 2	nd	tongue vesicles	neg				nd	BVD
TL Ravenna	pigs	Italy (c.s.)	0 / 20	nd	vesicles	neg		nd		nd	neg
BP Como	cattles	Italy (c.s.)	nd		gum epith.	neg		nd		nd	
Bioparco Roma	elephant	Italy (c.s.)			vesicle	neg		nd		neg	
SF Pordenone	pigs	Italy (c.s.)			vesicle	neg		nd		nd	
MD Ravenna	pigs	Italy (c.s.)			scabs	neg		nd		nd	
N° 27 holdings	cattles, sheep, goats	Ita, Fra, Ger, Pol (°)	positive serology		tonsils	neg		13 neg (*)		nd	
	cattles	Poland	1 / 31	40	tonsils	pos		pos	neg	nd	

Legenda:
Primers A – Rodriguez et al., Virology 1992; Primers B – Lomakina, unpublished, 1998.
(°) Ita = Italy, Fra = France, Ger = Germany, Pol = Poland
(*) Number of examined holdings

Appendix 11

PREVENTIVE MEASURES AND DISEASE VIGILANCE IN DENMARK FOLLOWING THE CURRENT OUTBREAK OF FMD IN THE UK

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Preventive measures

Following the declaration of the first outbreak of FMD in the UK on February 20 2001, a cascade of events were immediately initiated in Denmark. Many questions were raised concerning the then unknown risk of introducing the disease: what was the source in UK and might that also present a risk to Danish animals, had potentially infected animals or animal product been imported before import bans became effective, did international transportation of goods and commodities represent a risk like movement of humans might also do, and, since the first outbreak was encountered in pigs, was there a risk of airborne transmission? Rapid answers to all of these questions, and many more, were urgently needed in order to implement actions and measures aimed at minimizing the risk of introducing FMD. Should the disease be introduced in spite of these measures, it was important to ensure utmost vigilance (early detection) and minimizing spread of the disease between herds.

It was strongly advised not to import any live cloven-hoofed animals from any country until the situation was clarified. Fresh meat on the bone and other products (food and non-food) imported from the UK were traced and dealt with. Rules concerning catering and kitchen waste were firmly implemented and controlled and a total ban of import of animal products by tourists was imposed.

In order to minimise contact between animals and herds markets and shows were prohibited and restrictions were put on collection and transport of animals, including reinforced measures for cleaning and disinfection of transport vehicles. All other contacts between herds (transports, persons etc.) were to be minimized. Such recommendations were put forward in close collaboration with and supported by representatives of the farming community.

Laboratory investigations

During the months after the initial outbreak on February 20 in the UK, 10 suspect cases of FMD have been investigated in the laboratory. Two of these were from Norway and Finland, respectively. All cases were from cattle except for one suspect pig found dead in a forest. A further 35 herds were put under restriction due to contact with these suspect herds.

Epithelial samples from suspect cases were examined by antigen ELISA (Have et al. 1984. Acta Vet. Scand. 25(2), 280-296), virus isolation in tube cultures of primary or secondary bovine thyroid, kidney and porcine kidney cells and PCR. Tube cultures were frozen and thawed after 48h and submitted to a second blind passage.

The primers chosen for the PCR were 1F and 1R, located in the 5' untranslated region and designed to detect all seven serotypes of FMDV (Reid et al. 2000. J. Virol. Meth. 89; 167-176). The expected size of PCR amplification product was of 328 base pairs. Viral RNA was extracted by QIAamp Viral RNA kit (QIAGEN). For cDNA synthesis RETROscript kit (Ambion) was used and primers for the cDNA synthesis were random decamers provided in the kit. PCR was performed with reagents from Applied Biosystems. The PCR was performed with the following cycling conditions: step 1: 95°C, 10min; step 2: 95°C for 10 sec, 56°C for 10 sec, 72°C for 15 sec for 40 cycles; step 3:

72°C for 10 min. The PCR was run in an ABI PRISM 7700 Sequence Detection System (Applied Biosystems) in which the PCR product after amplification was analysed by performing a melting curve showing the specific melting temperature of about 89°C. Furthermore, the PCR product was analysed in a 1% agarose gel.

All tests for FMDV proved to be negative.

No registered imports of susceptible live animals into Denmark had taken place during 2001. However, due to the inconsistent reporting of sheep movements within the Community, it was decided to carry out a screening of sheep sera, collected at slaughterhouses, for antibodies to FMDV. Two thousand sera were examined in a solid phase blocking ELISA (Have & Holm Jensen 1983. Session of the research group of the standing technical committee of the European commission for the control of foot-and-mouth disease, Lelystad, Netherlands, p.44-51) using O1 Manisa reagents. Also 941 serum samples from small ruminants were received from Portugal as part of a serosurveillance in these species. None of the sera contained antibodies against FMDV type O.

It was felt that the current protocol for virus isolation (duration 96 h, Manual of diagnostics, OIE 2000) put an unacceptably long period of restriction on suspect farms and associated logistic ressources and further allowed trading partners to react categorically, thus adversely affecting international trade. It should be considered if and when combinations of virus isolation, ELISA and PCR may shorten the time required to firmly establish a negative diagnosis.

Appendix 12

PREVENTIVE MEASURES AND LABORATORY EXAMINATIONS IN GERMANY FOLLOWING THE CURRENT OUTBREAKS OF FMD IN THE EU

Bernd Haas, Federal Research Centre for Virus Diseases of Animals

Measures to prevent the spread of FMD:
In Germany about 7000 animals were destroyed because of the FMD cases in neighboring countries. In North-Rhine-Westphalia, 2153 sheep were destroyed because they either came from the UK or had contact with sheep from the UK and the FMD cases in the Netherlands led to the destruction of 4419 pigs and 231 cattle. Markets were prohibited and the collection and transportation of animals were restricted. Transportation of animals required a license, which was given only under certain conditions. In principle, transportation of animals was only possible directly to an abattoir or another holding, avoiding contact with animals from other herds. Animals had to stay in their holding for certain periods of time before they could be moved. Vehicles had to be cleaned an disinfected before and after every transport. When the disease situation improved, the restrictions were reduced step by step.

Laboratory examinations
Reasons to perform serology:
1493 samples because of connections with the UK
7039 samples because of connections with the Netherlands
131 samples because of connections with France
2126 samples because of other reasons, mainly clinical signs in the same or contact holdings
10789 total

Species:
8438 samples form pigs, 1472 samples form sheep and goats, 879 bovine samples

Methods:
LPBE = Liquid-phase blocking ELISA, Hamblin et al. (1986)
A cut off of 1:90 was used because otherwise more or less all holdings would have been found to contain seropositive animals. This was done in agreement with the recommendations form Dr. P. Kitching.
SPCE = Solid Phase Competition ELISA, Mackay et al. (2001), cut off titre was 1:5

Results**:**
No specific antibodies to FMDV were found with the exception of one holding with old cows that had been vaccinated before 1991. Unspecific reactions led to immediate resampling, in one case a VNT was performed with negative results.

Clinical signs that led to laboratory examinations:
Suspect cases with clinical signs were examined by virology (plaque tests with BHK21-CT cells), RT-nPCR and serology with negative results. Clinical suspicions led to the testing of samples from 63 holdings until 26 April, which means there was about one suspect case per day for about 2 months. Usually there are 2 – 8 suspect cases per year. This year, in 57 holdings clinical signs, usually mouth or foot lesions or lameness, were reported. Virological samples were taken also from some possible contact holdings without specific signs. The species affected were bovines (19 cases), sheep (19 cases), pigs (16 cases), goats (2 cases) and roe (1 case).

However, actual vesicles were rarely seen, but mostly just "erosions" or "lameness" . In four cases bovines showed strong salivation for no obvious reason. In two cases strong salivation in sheep was reported. According to the information the FMD laboratory received, in at least 7 cases parapoxvirus ovis was the most probable reason for the observed signs in small ruminants. MD was diagnosed in one case in cattle and a combination of stomatitis papulosa and a respiratory infection in another case. Often no reason for the clinical signs were established with certainty, although usually there were indications of mechanical injuries or bacterial infections. In two cases, carcasses of domestic pigs with foot lesions were found near roads.

Appendix 13

THE FMD CRISIS 2001: FIELD MEASURES, LABORATORY TESTS AND PROCEDURE FOR MASS SCREENING IN BELGIUM

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1. Field measures taken in Belgium during the FMD crisis 2001

After a FMD outbreak in the UK all import of cloven-hoofed animals and animal products was prohibited. The same was applied for France and the Netherlands. The import of hay, animal food, slurry etc was also stopped. Also the import of horses was forbidden because of the danger of the transport vehicle not being used only for horses. All transport vehicles from those countries had to be cleaned and disinfected. Gathering of all kind of animals at markets, shows, competitions, etc was forbidden. Access to farms was limited and special hygienic measures had to be applied. French and Dutch owners of farms in Belgium were not allowed to visit their farms anymore. Zoos and farms for school children were closed. The limited swill feeding after heat treatment that was still allowed was immediately forbidden. A buffer zone between Belgium and France or Belgium and the Netherlands was established for a limited period of time after the FMD outbreaks in these countries.

All animals present on a farm that imported animals from a country where FMD was confirmed were killed on the spot and brought to a rendering plant. Blood and saliva samples were taken (see below). About 8800 sheep were imported from the UK.

A one-month screening for clinical signs every 4 days was done on all farms that imported animals and on the farms in the 10 km zone around the farm. Blood samples from sheep and goats were taken every 8 days. All animal movements from and on the farm were prohibited: a complete and immediate stand still.

After the screening transport of cattle and pigs to the slaughterhouse was allowed. Ten days later a one to one transport for cattle and pigs was allowed. The same was allowed for sheep and goats another 10 days later but only after a serological examination of the farm. This was done because it was not absolutely sure that all imported sheep were found and FMDV could still persist sub clinically in these herds.

End of April: transport of cloven-hoofed animals to several farms was allowed again.
Half of May: markets for non-cloven hoofed animals were re-established. Access to farms was allowed again but hygienic measures were continued.
End of May: blood sampling of sheep before transport was lifted. The gathering of cloven-hoofed animals except sheep and goats was allowed again. The import limitation of horses from the UK and cattle for slaughter from the Netherlands was lifted.
Half of June: all measures except for the UK were lifted.
Beginning of September: border control was reinforced for the UK.

2. Laboratory tests

All samples form suspicions and screenings for virological examination were checked by Ag-ELISA and virus isolation on FLK cells (sheep, goat or cattle samples) or SK-6 cells (pig samples). All serum samples were analysed with the SPCE (solid phase competition ELISA). Positive samples were checked by VNT.

Between the end of February and end of June 50 suspicions were recorded: 26 sheep, 16 cattle, 5 pigs, 3 goats. In total 700 samples from these suspicions were analysed: skin and mouth lesions, saliva, sera, blood, tonsils, spleen. Two suspicions were very serious. One was on a pig farm where 3 pigs with fever and snout lesions were found. This was probably due to a caustic agent (exaggerating disinfection?). The second was on a cattle farm where 2 salivating animals were found, one with fever. When the veterinary inspector arrived already eight animals had fever and 3 were salivating. This turned out to be maligne catharal fever in one animal that died soon. Salivation and fever in other animals was probably due to chasing the animals for inspection.

For the screening 8656 sera were analysed at a 1:10 dilution by SPCE: 6808 sheep, 1060 goats, 512 cattle, 274 swine, 1 deer, and 1 human. Some difficulties were encountered with sheep and goats giving sometimes inhibition percentages near the cut off of 30%. The presence of FMDV was checked in saliva from sheep and goats: 2503 Ag-Elisa's and 2759 virus isolations with minimum one blind passage.

All samples were negative. Belgium had no FMD.

3. Procedure for mass-screening

3.1. Mass-screening in periods of increased vigilance

Mass-screening to identify FMDV infected herds is based on the detection of clinical signs in animals on the spot. The procedure is put in place when the presence of FMDV is confirmed within Belgium, in the neighbouring countries or with a trade partner. This procedure demands a complete standstill on all herds involved in the screening.

All cattle and pigs on the farms involved are checked for clinical signs every fourth day. When clinical signs indicate the presence of FMDV the 'Procedure for Suspicion' (see Emergency plan) is initiated. Samples are taken for a virological examination. When clinical signs are absent the procedure for checking all animals every fourth day is maintained for 3 weeks.

All sheep and goats on the farms involved are put in quarantine and checked for clinical signs every fourth day. When clinical signs indicate the presence of FMDV the 'Procedure for Suspicion' (see Emergency plan) is initiated. Samples are taken for a virological examination. When no clinical signs are found samples are taken at day 0 and then every eighth day. A maximum of 60 at random selected animals is blood sampled for serological investigation and saliva samples are taken from the mouth for virological tests. This is continued until 30 days after the start of the procedure.

3.2. Mass-screening during culling

To determine the infection rate within a farm in case of preventive culling, a clinical examination is done first. When clinical signs indicate the presence of FMDV the 'Procedure for Suspicion' is initiated. Samples are taken for a virological examination.
When clinical signs are absent in cattle or pigs, no samples are taken. The FMDV prevalence in this herd will be so low that all animals should be sampled and only very sensitive virus detection tests could demonstrate the presence of FMDV (virus isolation, PCR).
If clinical signs are absent in sheep or goats, saliva samples are taken from maximum 60 at random selected animals on the farm for a virological examination. The number of blood samples to be taken in at random selected sheep and goats for serological investigation is given in table 1.

If a FMD outbreak in a herd is confirmed samples can be taken to determine the herd prevalence. The number of blood samples to be taken in at random selected animals for serological investigation is given in table 1. In the absence of clinical signs determination of FMDV prevalence during a FMD crisis is only done for sheep and goat flocks. For cattle and pigs, saliva samples for virological examination can be taken from maximum 60 at random selected animals and stored, to determine the infection status of the animals in a retrospective way.

The procedure mentioned is only applied in non- vaccinated herds. Mass-screening in a vaccinated herd without clinical signs is only valuable if all animals are sampled and virus detection is done with very sensitive tests.

Table1: number of samples to be taken (n) depending on the number of animals present (N)

N animals per herd	n samples per herd	N animals per herd	n samples per herd	N animals per herd	n samples per herd	N animals per herd	n samples per herd
10	10	110	60	250	73	1000	89
20	20	120	60	300	76	1200	90
30	30	130	60	350	78	1400	91
40	40	140	60	400	80	1600	92
50	50	150	60	450	82	1800	92
60	60	160	62	500	83	2000	94
70	60	170	63	600	85	3000	95
80	60	180	64	700	86	5000	96
90	60	190	65	800	87	>5000	96
100	60	200	70	900	88		

3.3. Mass-screening for declaring a region free of FMDV infection

For the purpose of declaring a region where no emergency vaccination is applied free of FMDV infection blood samples for serological investigation are taken from a maximum of 11 at random selected animals from all herds in the region 30 days after culling the last infected herd. When all samples are negative the region can be declared free of FMDV infection with 95% accuracy.
If the number of blood samples taken per herd is increased then the number of herds to be sampled can be decreased. If 20 samples per herd are taken in a region of 10.000 herds then

only 5988 herds have to be checked. The latter implicates that the number of herds to be visited have to be calculated for each region and depends on the number of samples taken per herd.

In a region where emergency vaccination is applied all animals are clinically examined twice with an interval of two weeks. All animals are blood sampled for the detection of antibodies against non-structural proteins. False negatives are still possible as the sensitivity of the NSP-ELISA is below 100% and the FMDV prevalence will be low.

THE 2001 FOOT-AND-MOUTH DISEASE OUTBREAK IN THE EU: AN UNFORESEEN INTEREST FOR THE DISEASE IN SWITZERLAND

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Foot-and-mouth disease (FMD) continues to be a major threat to the livestock population throughout the world. The last major outbreaks in Switzerland were recorded in 1965/66, in which more than 1000 farms where affected by FMD virus serotype O. As with other EU countries, Switzerland employed as FMD vaccination policy until 1990. During this period, approximately 30% of the susceptible livestock population (predominately dairy cattle) was annually vaccinated using a trivalent vaccine. Since 1990, Switzerland no longer applies such a vaccination policy. Instead, an emergency vaccine bank was created, and maintained with 300,000 doses of the 4 serotypes O, A, C and Asia1.

In an era of global movement of animals and animal products, any disease can easily be introduced into a country. Several different measures for the control of such movement, at the level of the government as well as the cantonal veterinary offices, are in place. The national reference laboratory provides the diagnostic service, expertise and continuing education on the subject of FMD (and other list A diseases), and in addition, systems for continuous animal health monitoring and surveillance are in place. Because recognition of the first case (index case) is extremely important, the disease awareness level for FMD among district veterinarians has to be maintained as high as possible. For this purpose, mandatory courses are held each year, in which clinical recognition, submission of samples to the National Reference Laboratory (IVI), diagnostic aspects, and the correct handling of a suspect field case are presented and discussed. Furthermore, if FMD should be introduced into Switzerland, emergency vaccination of the livestock population at risk would be possible within 4 days.

After the first report of the current FMD outbreak in the UK on February 21 (BBC News), the Swiss Federal Veterinary Office and the National Reference Laboratory experienced a massive interest in the different aspects the disease. Selected areas of this public interest, in particular those where in the National Reference Laboratory was involved, will be presented. This includes an in-depth analysis of (i) media activity, (ii) public perception of the disease, and (iii) handling of suspect FMD field cases and their subsequent submission to the IVI. Furthermore, the cantonal veterinarians were interviewed during May/June 2001 on how the FMD "crisis" was handled by the Swiss Federal Veterinary Office and the National Reference Laboratory IVI. The results of this questionnaire will be presented.

Taken together, it was observed that the FMD outbreak received a high level of attention by the public in Switzerland. Therefore, it can be speculated that disease awareness among personal involved with livestock should be at a high level, at least at the time of writing of this abstract. However, it is uncertain as to how long this level of disease awareness can be maintained.

Nevertheless, the lessons learnt after these outbreaks should have a long lasting beneficial effect on animal disease control, not only in Switzerland, but also in the EU member countries. Animal production practices which favor the spread of any disease should be re-thought. It would be wrong if after the last case in the UK we returned to "business as usual".

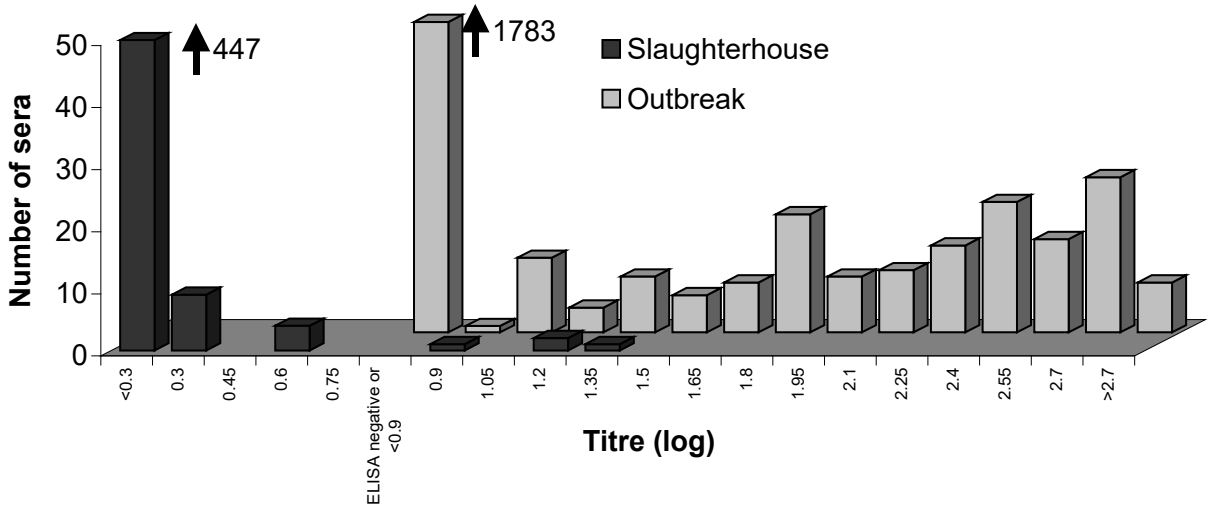
Validation of the FAO type O reference sera using sera collected on outbreak farms

A. Dekker, F. van Hemert-Kluitenberg, K. Miedema, G. Chénard

Correct classification of serological positive and negative animals is very important, especially in a notifiable disease like foot-and-mouth disease. For this reason, the FAO commissioned the World Reference Laboratory for FMD to produce standard reference sera. The cut-off used in each serological test should be on the level of the cut-off reference serum. In fact in each test, the cut-off serum, or a serum related to this serum, should be included. Previous work, however, showed that a small population of non-infected animals has neutralisation titres above the titre of the cut-off serum. Therefore, a slightly higher cut-off was proposed. A higher cut-off serum would give rise to more false negative results, but it was not clear how many positive sera would be negative. During the 2001 foot-and-mouth disease outbreak in the Netherlands, sera were collected on all outbreak farms. All sera were tested in the virus neutralisation test, starting with a 1/8 final dilution (0.9 ^{10}log). Sera with a titre of 1/11 (1.05 ^{10}log) or lower were considered negative which is the same cut-off used by the World Reference Laboratory as stated in the OIE manual. The FAO cut-off serum, however, was consistently negative in our test with a titre between 1/3 (0.45 ^{10}log) and 1/6 (0.75 ^{10}log). To study whether the cut-off used is correct we compared the distribution of titres found on the outbreak farms to the distribution of titres found in non-infected slaughter cows collected in spring 2000.

Figure 1 shows the distribution of neutralisation titres found in both sets of sera. The distribution of slaughterhouse sera clearly shows that for a good specificity of the test the cut-off defined by the FAO cut-off reference serum (titre 0.6 tot 0.75) is too low. Approximately 2% false positive results would be encountered, which is too high when you are dealing with an outbreak.

Figure 1: Comparison of all outbreak sera to slaughterhouse sera



Neutralisationtest titres FMDV type O Manisa

The distribution of sera with a titre above the starting dilution (0.9 ^{10}log) from outbreak farms shows a tailing off to the left side. Resulting in quite a number of sera with a titre just below or above the cut-off (1.2 ^{10}log) used in the outbreak, indicating that in an outbreak many animals can be encountered with a low virus neutralisation titre. The most important question is; would this be a problem to detect seropositive herds? Probably not, because most of the positive sera used in this study were from farms where clinical signs were seen and sera were collected from animals that just sero-converted. There was one outbreak farm (NET 9/2001), however, where clinical signs were not detected (Figure 2).

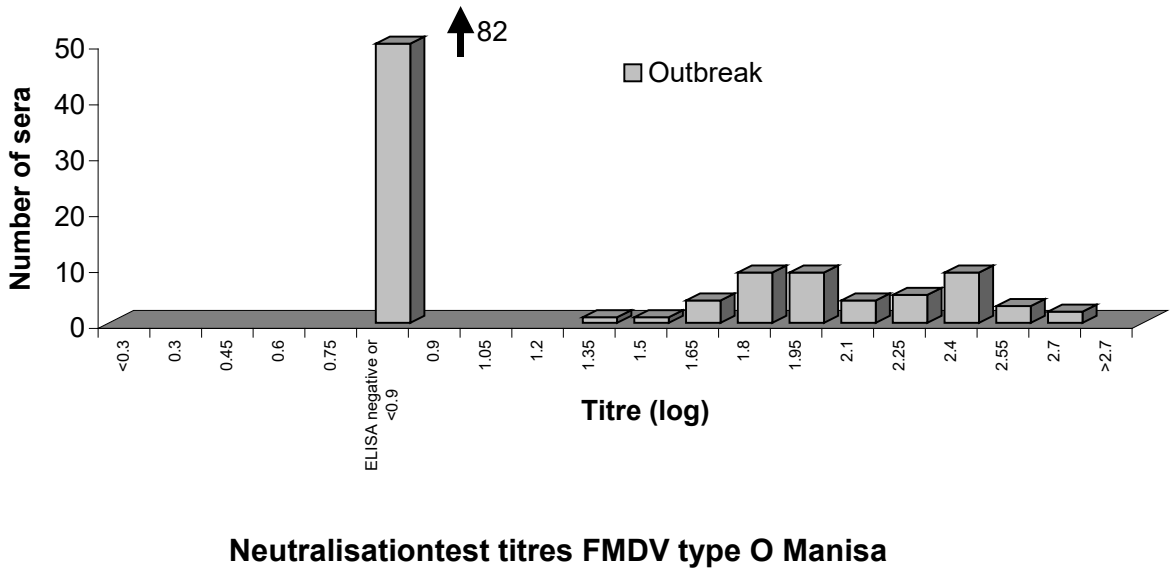


Figure 2: Titres found on outbreak NET 9/2001

On this farm, there is a clear-cut difference between negative and positive results. Indicating that the cut-off used (≥1.2 ^{10}log) was sufficient for detection of old infections without producing too much false positive results. During the screening after vaccination, three farms were found with positive serology, which were not declared an outbreak. The distribution of positive sera on these farms was comparable to the distribution on outbreak NET 9/2001.
Testing sera for antibodies against a disease is not a technique to detect an early infection. The fact that outbreaks with recently infected animals have low antibody titres does not mean the cut-off in the serological test has to be low. Based on the observations on the four farms without clinical signs it can be concluded that a cut-off level higher than the cut-off defined by the FAO reference serum does not affect the sensitivity of the neutralisation test on these farms. All other farms were detected by detection of virus or antigen, and serology was not needed for the laboratory diagnosis. The increase of the cut-off surely improves the specificity.

Analysis of the sera collected in the UK will further help to define a sound cut-off level for FMDV serology.

Bommeli DIAGNOSTICS

CHEKIT®-FMD-3ABC

Bommeli DIAGNOSTICS

CHEKIT®-FMD-3ABC

3ABC ELISA PROJECT

- Collaboration between:
  - Institute for Animal Health (IAH), Pirbright, United Kingdom
  - Istituto Zooprofilatico Sperimentale della Lombardia e dell'Emilia-Romagna (IZP), Brescia, Italy
  - Bommeli Diagnostics, Bern-Liebefeld, Switzerland

Bommeli DIAGNOSTICS

CHEKIT®-FMD-3ABC

ORIGINAL IAH/IZP 3ABC ELISA (trapping indirect ELISA)

Strengths:

- Validated and established test
- Differentiation between vaccinated and infected animals

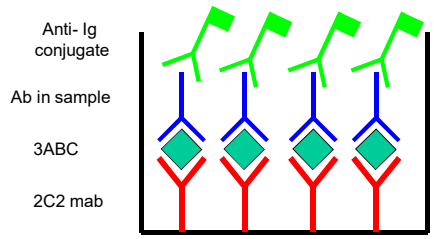
Weaknesses:

- Not ready for use --> standardization?
- Control wells without 3ABC (biphasic test)
- Not available for large scale testing

Bommeli DIAGNOSTICS

CHEKIT®-FMD-3ABC

ORIGINAL IAH/IZP 3ABC ELISA (trapping indirect ELISA)



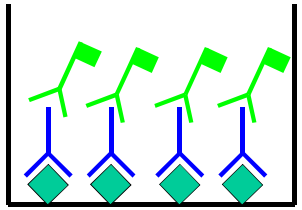
CHEKIT-FMD-3ABC

CHEKIT-FMD-3ABC

Anti-Ig conjugate

Ab in sample

3ABC



CHEKIT-FMD-3ABC

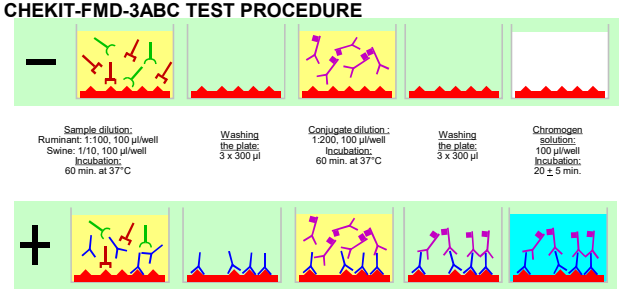
CHEKIT-FMD-3ABC

Strengths:

- Test based on validated and established system
- Ready for use reagents
- Monophasic assay
- Established large scale production
- Commitment from IAH & IZP for 3ABC system
- One test for all major species (cattle, sheep and swine)

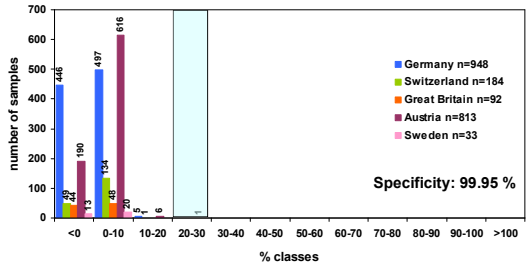
CHEKIT-FMD-3ABC

CHEKIT-FMD-3ABC TEST PROCEDURE

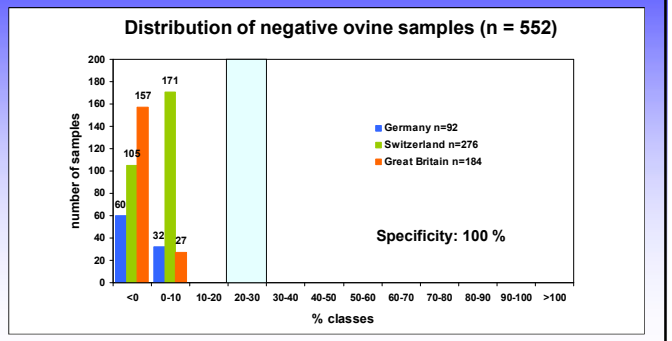


CHEKIT-FMD-3ABC

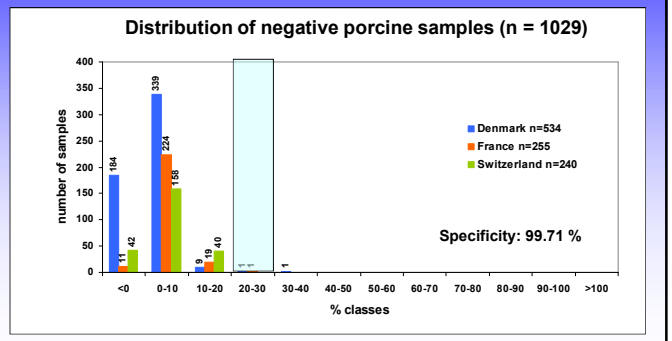
Distribution of negative bovine samples (n = 2070)



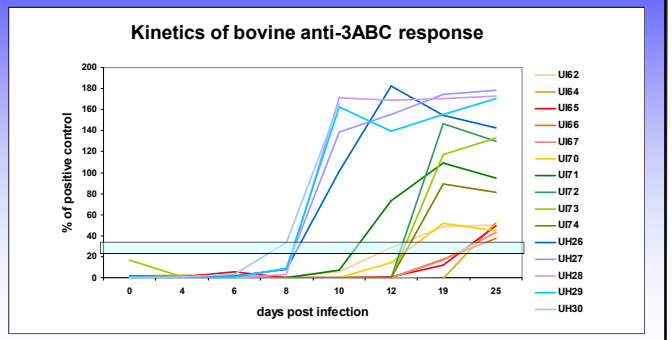
Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC



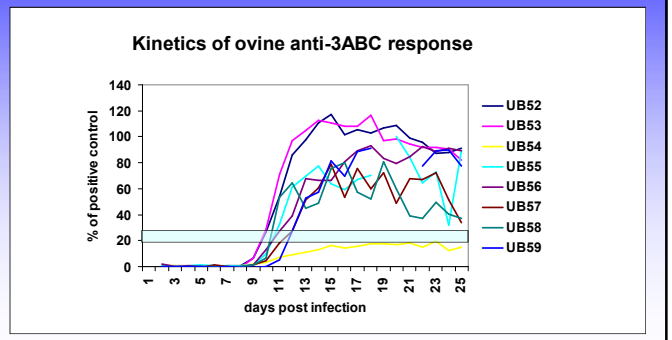
Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC



Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC



Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC



Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC

Comparison of 3ABC-ELISAs

bovine sera

designation	Sera Status	Sera Serotype	3ABC-703 OD	3ABC-703 %	Pirbright ratio
NC			0.089	0	
PC			1.283	100	
154	Reference sera		3.281	267	
O9	Brescia		2.145	172	
D2			0.611	44	"cut off"
N1	Reference sera		0.116	2	
P1	Pirbright		1.873	149	
P2			0.408	27	"cut off"
100	PV	A	0.097	1	0.07
106	PV	A	0.122	3	0.00
108	PV	C	0.087	0	0.03
97	PV	O	0.105	1	0.08
98	PV	O	0.121	3	0.06
99	PV	O	0.090	0	0.08
42	PI	A	4.000	327	1.25
47	PI	A	2.341	189	1.09
40	PI	O	2.298	185	1.15
25	PI	SAT-1	1.502	118	1.15
76	PI	SAT-2	1.004	77	0.47
108	NI		0.094	0	
109	NI		0.092	0	
110	NI		0.083	0	
111	NI		0.083	-1	
114	NI		0.091	0	
116	NI		0.099	1	

ovine sera

dpi	80 OD	80 %	Pirbright
1	0.104	0	0.06
3	0.165	4	0.05
4	0.070	-2	0.08
5	0.129	1	0.08
6	0.130	1	0.08
7	0.111	0	0.03
8	0.123	1	0.06
9	0.108	0	0.05
10	0.089	-1	0.06
11	0.127	1	0.05
12	0.094	-1	0.06
14	0.399	19	0.15
16	1.229	71	0.54
19	1.996	120	0.83
22	2.074	125	0.72
26	1.787	107	

Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC

Screening of sera from vaccinated sheep

pv (weeks)	3ABC (%) ⌀	n = 5 STD	A22 Iraq VNT	O1 Manisa VNT	Asia1 Shamir VNT
0	-2.3	0.53	<0.30	<0.30	<0.30
1	-2.5	0.72	<0.30	0.75	0.99
2	-2.2	0.41	0.63	1.29	1.98
3	-2.5	0.28	0.57	1.53	1.62

Vaccine strains:
A22 Iraq
O1 Manisa
O1 Marocco
Asia-1 Shamir

Screening of sera from vaccinated cattle

	pv (weeks)	Group 1 3ABC (%) ⌀	Group 1 n = 2 STD	Group 1 O1 Manisa VNT	Group 2 3ABC (%) ⌀	Group 2 n = 2 STD	Group 2 O1 Manisa VNT	Group 3 3ABC (%) ⌀	Group 3 n = 2 STD	Group 3 O1 Manisa VNT
	0	-0.7	2.65	<0.30	-3.1	0.16	<0.30	-2.7	0.60	<0.30
	1	-0.5	3.69	0.53	-2.9	0.36	<0.30	-2.1	0.68	<0.30
	2	-2.8	0.44	1.28	-1.7	0.60	0.65	-2.8	0.24	<0.30
	3	-2.6	1.32	0.90	-1.2	0.32	0.47	-1.3	1.40	0.30
challenge	4	-1.8	0.08	0.83	-1.9	0.48	0.50	-1.0	0.84	0.60
	5	2.1	3.73	>2.55	-2.5	0.12	>2.55	-2.4	0.20	1.65

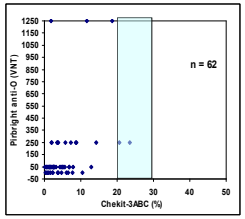
Vaccine strain:
O1 Manisa

Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC

Screening of sera from vaccinated pigs

pv (weeks)	3ABC (%) ⌀	n = 5 STD	A_{24} Cruzeiro VNT	O_1 Manisa VNT	C_1 Detmold VNT
0	5.2	2.69	0.09	0.09	0.09
1	7.8	1.42	0.42	0.99	0.45
2	5.2	4.78	0.60	1.23	0.84
3	5.0	1.19	1.11	1.80	1.35
4	4.4	1.92	1.44	2.01	1.74

Vaccine strains:
A24 Cruzeiro
O1 Manisa
C1 Detmold



Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC

Summary:

- **CHEKIT-3ABC-ELISA validated for cattle, sheep and swine**
- **high specificity (99.7 - 100%) with samples from negative populations of different European countries**
- **no false positive results with samples from vaccinated animals tested so far**
- **seroconversion of experimentally infected animals can be detected from day 11 on**

Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC

DIFFERENTIATING INFECTION FROM VACCINATION IN FOOT-AND-MOUTH-DISEASE: DISCRIMINATIVE POTENTIAL OF STRUCTURAL AND NON-STRUCTURAL VIRUS PROTEINS

U. Bruderer[a)], M. van der Linden[b)], G. Lozano[a)], and C. Schelp[a)]

a) Bommeli Diagnostics, Stationsstr. 12, CH-Liebefeld-Bern, Switzerland
b) Intervet International BV, Wim de Körverstraat, Boxmeer, Netherlands

Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC

Introduction

Outbreaks of foot-and-mouth disease (FMD) are responsible all over the world for tremendous economic loss. Until now measures have been based on stamping out in Europe and/or vaccination in other parts of the world. Recent outbreaks in Europe suggest that new more efficient measures for the control and eradication of this highly contagious viral disease are urgent (1-4). Here we describe the potential of the non-structural protein 3ABC as a serological marker for the monitoring of vaccinated animals.

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Fig.1. Structural and non-structural proteins in foot-and-mouth disease

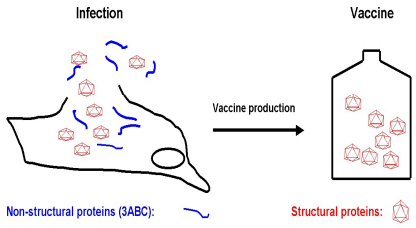

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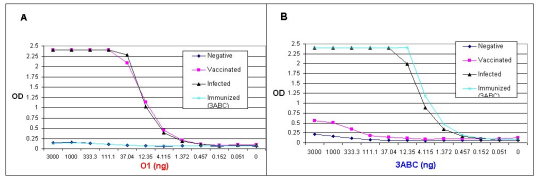
Fig. 2. Binding of serum antibodies to structural proteins (A) and 3ABC (B)



Serum	structural proteins	3ABC (non-structural protein)
Negative	-	-
Vaccinated	+	-
Infected	+	+
Immunized (3ABC)	-	+

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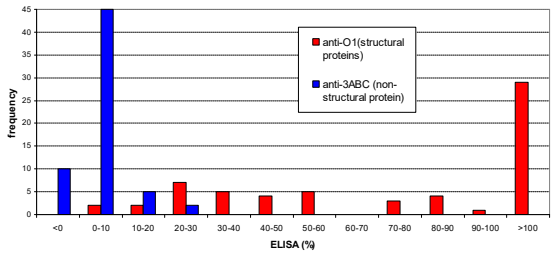
Fig. 3 Screening of sera from vaccinated pigs



Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC

Materials & Methods

CHEKIT-FMD-3ABC was performed as described by the manufacturer. Briefly, sera are incubated in microtiter plates coated with purified, recombinant 3ABC. Bound antibodies are visualized with enzyme conjugated monoclonal species specific anti-IgG antibodies.
Serotype O specific antibodies were detected as described above for 3ABC with the exception that microtiter plates were coated with purified structural proteins.

Results

The preparation of modern FMD vaccines results in the depletion of non-structural proteins such as 3ABC (Fig. 1). As a consequence, sera from vaccinated animals contain antibodies against structural proteins (Fig. 2A) but not against the nonstructural protein 3ABC (Fig. 2B). In contrast, infection results in antibodies against structural and nonstructural proteins (Fig. 2). Furthermore, these data formally demonstrate that the tested vaccine preparations do not contain serologically detectable contamination of 3ABC. The screening of sera (n=62) from vaccinated pigs is shown in Fig. 3. Results are expressed in relation to a negative control (0 %) and positive control (100 %). The majority of the sera (41/62) contain anti-O1 antibodies with levels of >30% of the control. None of the sera contain anti-3ABC antibodies with levels of >30% of the control. Analysis of sera from negative animals and from animals vaccinated or infected with different serotypes is shown in Slide 13. The results demonstrate that the induction of anti-3ABC antibodies is serotype independent, and that independent of the serotype, vaccination does not elicit anti-3ABC antibodies.

Bommeli DIAGNOSTICS CHEKIT®-FMD-3ABC

Summary

Our results confirm that a) the analyzed vaccines do not contain serologically detectable 3ABC contamination, b) vaccination does not elicit significant amounts of anti-3ABC antibodies, c) infections results - independent of the serotype - in the generation of anti-3ABC antibodies. These results demonstrate the potential of 3ABC as a marker for discrimination between vaccination and infection.

References

1. Y. Leforban (1999) Vaccine 17, 1755.
2. M. De Diego et al. (1997) Arch. Virol. 142, 2021.
3. D.K.J. MacKay et al. (1998) Vaccine 16, 446.
4. I. E. Bergmann et al. (2000) Arch. Virol. 145, 473.

New Approaches To Differentiate FMD Vaccinated From FMDV Infected Animals

Proof of principle

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Developments in vaccine technology

- Demand for Marker Vaccines in disease control

Employed Methods

- Deletion mutant virus strains
- Virus subunits
- Vector vaccines

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Marker Vaccine and FMD?

Is it possible to transfer the concept of Marker Vaccines to FMD?

Problems:

Ongoing high variability of FMDV
Limited number of virus proteins that can be deleted
Limited success with peptide vaccines

Bayer BAYER Animal Health

Progress in FMD research

- Analysis of the FMD genome and identification of function
- Identification of variable and conserved regions
- Analysis of the host specific immune response to selected structures of the FMDV
- Differentiation of B-cell and T-cell epitopes

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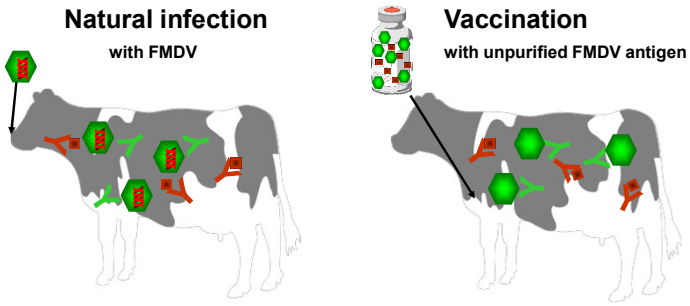
Use of conventional FMD vaccines



Antibodies against both structural Y and NSP Y
→ no differentiation is possible

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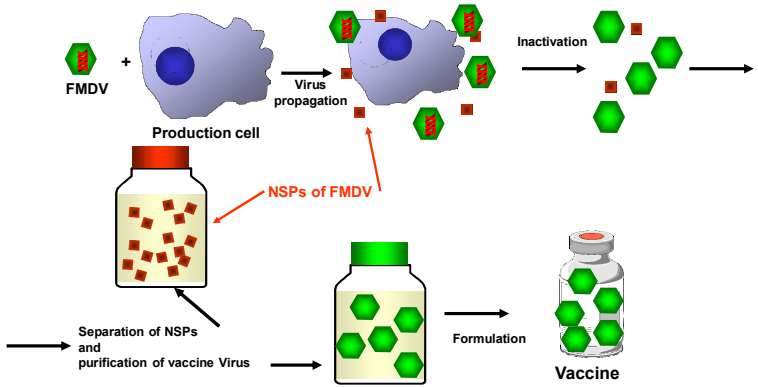
Animal Health

Production of purified FMD vaccines



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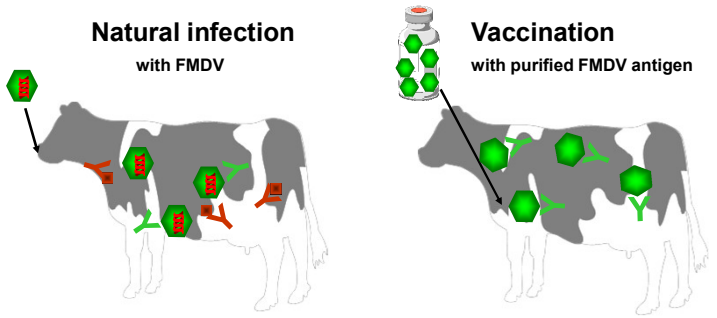
Use of purified FMD vaccines as marker vaccines



Antibodies against structural Y and NSP Y

Antibodies after vaccination Y

→ A differentiation is possible

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Basic steps for the development of a Marker Test

- Sequence analysis
- Synthesis of overlapping peptides
- Analysis of synthetic peptides for immunological function
- Selection of appropriate peptides

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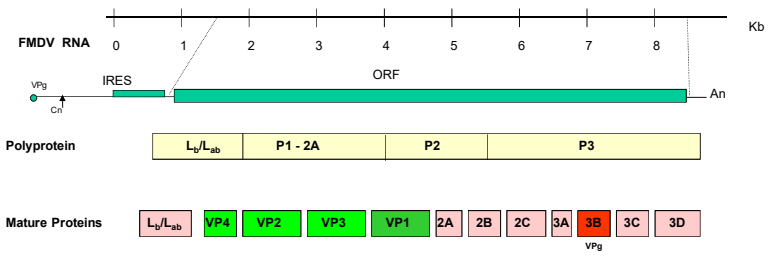
Scheme of FMDV Genome



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Peptides used for the test establishment

Positive control peptides out of VP1

- pep32
- pep266 (part of 32, including RGD)

Test peptides out of 3B

- pepA
- pepB
- pepA and pepB

Negative control peptide

- peptide from the Hepatitis C-Virus

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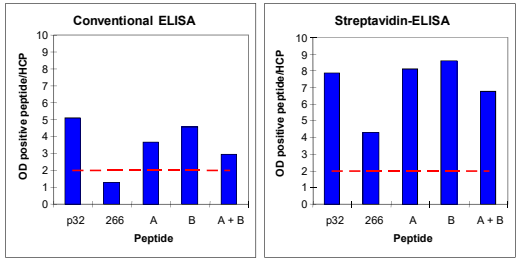
Selection of ELISA System



A post challenge serum tested in 2 ELISA systems

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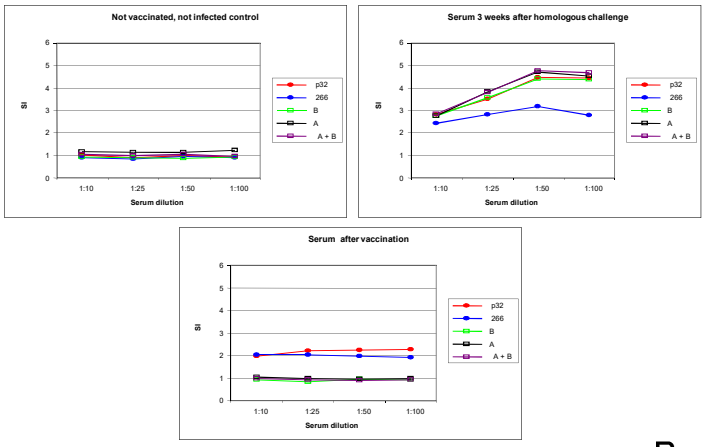
Establishment of optimal serum dilution



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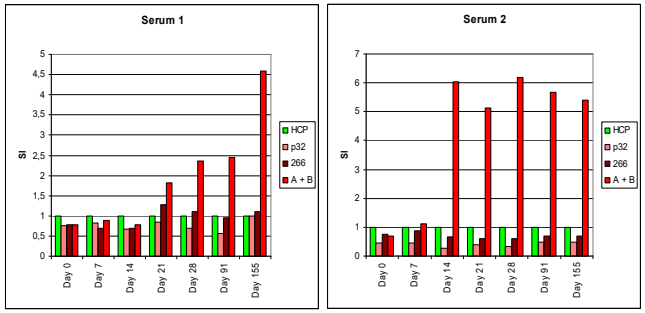
Analysis of sera after infection with A_5



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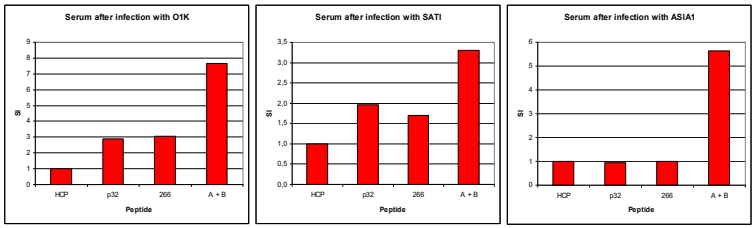
Analysis of sera after infection with O_1K, SAT1 and ASIA1



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Dr. Grunmach

Dr. Friederichs

Dr. Wolfmeyer

Laboratory Dr. Glatthaar

in co-operation with the Federal Research Institute
for Virus Diseases, Tübingen

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Appendix 18

Repeated administration of maximum payload emergency vaccines made from inactivated purified antigen concentrates do not induce significant titres of antibodies against non-structural proteins of Foot-and-Mouth disease virus

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Departamento de Virologia, Instituto de Microbiologia, UFRJ, CCS, Rio de Janeiro, Brazil;
Pan American Foot and Mouth Disease Center (PAHO/WHO), PO Box 589, 20001, Rio de Janeiro, Brazil.

Introduction

At the present time, there is not an officially (Office International des Epizooties) prescribed procedure for the routine discrimination of animals which have been infected with foot and mouth disease virus (and have otherwise fully recovered) from those which have received vaccination only. The official serological methods measure only antibodies against the structural proteins of the virus and, under normal circumstances, where there is no supporting clinical picture or other evidence, are unable to differentiate between those antibodies induced by vaccine or previous exposure to live virus. All of this has significant implications for the export of livestock or livestock products from countries which are either free of FMD with vaccination or decide to use vaccine as a control measure for a recent introduction of the disease.

The deficiency with the officially prescribed serological methods led to the examination of other techniques to discriminate vaccinated from infected animals and considerable work has been done over several decades to quantify antibodies against the so-called non-structural (NS) proteins of the virus because these would be particularly prevalent following virus infection. The NS proteins are coded by the FMDV genome and are involved in the replication of the virus within the host cell.

The earliest work concentrated on the VIAA antigen which contains the 3D NS protein (RNA polymerase) of the virus and was for many years incorrectly considered as an indicator of a past virus infection, either as a consequence of field challenge or improperly inactivated vaccine. However, Pinto and Garland (1979) showed that fully inactivated FMDV vaccines induced antibodies against VIAA but their data was to some extent overlooked until relatively recently when studies by a number of groups essentially dismissed VIAA as an exclusive indicator of infection (Bergmann et al, 1993, Mackay, 1998).

Vaccines made from relatively impure but fully inactivated FMDV antigens contain large quantities of some of the NS proteins and, in particular, VIAA (3D) antigens. Indeed, even the purified FMD virus particle contains small amounts of the 3D protein integrated into the viral capsid and absolute purity from this protein is not possible (Newman and Brown, 1997).

Because of the limitations of the assays based on VIAA (3D), more recent studies have focussed on techniques such as immunoblotting or ELISA with other NS proteins, expressed in recombinant systems, and have demonstrated that titres of antibodies against other NS proteins are much more valuable indicators of previous vaccination or infection (Bergmann et al, 1993; Lubroth and Brown, 1995; Mackay et al, 1998).

Thus, research workers and diagnosticians within the public domain are in a substantially stronger position in terms of discriminating between vaccinated and previously infected animals and, indeed, an ELISA based version of the procedure described in this paper and developed by Bergmann and colleagues is now used quite extensively in South America. The success of the public sector in this regard places a responsibility on the commercial FMD vaccine production sector to produce higher purity vaccines which will enable even more the capability of NS protein diagnostic kits to discriminate the infected from the vaccinated animal. In this respect, the focus of Merial has been the development of FMD vaccines made from highly purified antigens such that the induction of antibodies to non-structural proteins is abolished.

One of the most critical scenarios where reliable discrimination would be invaluable would be that of a country, previously free of FMD, and needing to use vaccine to prevent the spread of a new incursion of the disease. Under these circumstances, the unfortunate country would probably use one or two doses of high potency vaccines to limit the spread of the disease from the initial focus(i) and would be subsequently faced with the need to know the clinical status of vaccinated cattle which had apparently resisted infection but could be 'carrier' animals. The persistence of FMDV in the upper respiratory tract of otherwise healthy and immune ruminants is a well known phenomenon.

With this scenario in mind, we decided to repeatedly vaccinate cattle with extremely high payloads of purified FMD antigens greatly exceeding those which would be prepared for emergency banks such as the European Union FMD Antigen Bank. While 'ring' or zonal vaccination would only be used once or at most twice during an eradication campaign we chose a worst case approach of three rounds of vaccination. Although pigs do not become persistently infected with FMD, we also examined sera from animals given repeated doses of high potency vaccine.

Sera were prepared from the vaccinated cattle at frequent intervals and subjected to analysis of antibodies against the NS proteins using the procedure described by Bergmann et al (1993).

Materials and Methods

Vaccine and Vaccinations. Vaccines were prepared from chromatography purified inactivated antigens of 4 strains of FMDV (Asia1 Shamir, A22 Iraq, O Manisa, C Philippines) using aluminium hydroxide and saponin or mineral oil (double oil emulsion, DOE) as adjuvants. Each 2ml cattle dose of vaccine contained 16 µg of each of the 4 strains. Thus, a single dose of vaccine contained 64 µg of purified FMDV antigens. Groups of cattle were vaccinated by the subcutaneous route in the

case of aqueous vaccine or the intramuscular route in the case of oily vaccine with initially either one dose or two doses. All animals were boosted with a single dose at 21 days and 42 days after the initial vaccinations. Serum samples were taken at regular intervals. All of the cattle were shown to be seronegative for FMDV prior to the start of the experiment and had never been vaccinated with FMD vaccine or exposed to FMD virus.

EITB test. Essentially, this was as described by Bergmann et al (1993). Briefly, purified recombinant NS proteins, 3A, 3B, 2C, 3D, 3ABC of FMDV were mixed, electrophoretically resolved using 12.5% SDS PAGE and the protein bands electrophoretically transferred to nitrocellulose sheets. Strips of the nitrocellulose sheets were soaked in buffer containing 5% non-fat dry milk to block non-specific adsorption of antibodies and then immersed in 1/200 dilutions of test and control sera, the latter representing positive, weakly positive, cut-off and negative serum samples. After incubation, the strips were washed and bovine or porcine antibodies specifically bound to the recombinant NS proteins detected by alkaline phosphatase-conjugated rabbit anti-bovine or anti-porcine antibodies. After further washing to remove unbound enzyme conjugate, the presence of bovine or porcine antibodies bound to NS proteins was determined by alkaline phosphatase substrate colour development. A serum sample was considered negative if all the NS bands were less than the reactivity of the cut-off control (negative control) or no more than 2 bands were greater than the reactivity of the cut-off control. A serum was considered positive if all 5 NS bands had a reactivity equal or greater than the cut-off control. A sample was deemed indeterminate if the criterion for negativity or positivity was not met.

Virus Neutralisation Test. The titres of virus neutralising antibodies in the serum samples were determined in a microneutralisation test in IB-RS2 cells (renal swine). Titres were expressed as the $\log_{10}$ of the reciprocal of the serum dilution which neutralised 50% of the infectivity of 100 $TCID_{50}$ of each homologous test virus.

Results

The figures (Fig. 1 and 2) show the summarised data from the EITB test analysis of the sera of cattle vaccinated with aluminium hydroxide and saponin adjuvanted vaccine. To facilitate interpretation, we used the following 'positivity' scale to allow graphical presentation: Where there was no evidence of antibody against a given NS protein, we scored –1.0; for NS protein bands slightly below or above the cut-off serum controls, we scored +0.1; for NS protein bands significantly above the cut-off serum controls, we scored +1.0. In the figures shown here, a negative serum sample is indicated by 'neg' and one serum sample, which was indeterminate, is indicated by 'ind'.

It can be seen that after 1 administration of a single or double dose of aluminium hydroxide and saponin vaccine there is no evidence of antibody induction against non-structural proteins of the virus and even after the first booster vaccination, only one animal in the double dose group showed evidence of antibody against 3D protein. After the third vaccination of the two groups there was some boosting of the anti 3D response but essentially no reactivity in any of the cattle sera against the other non-structural proteins.

Essentially identical data was observed with the oil adjuvanted vaccines in cattle as shown by Fig. 3 which is the equivalent experiment to that shown in Fig.1. After three doses of vaccine, two animals did show low levels of antibodies against the 3B protein although the overall scoring was still negative.

It is important to stress that these results are all the more significant when the payload of the vaccine used is considered. Whereas conventional monovalent vaccines made at Pirbright contain between 2 and 10 µg of 146S particles per dose, the quantity depending on the strain, the vaccines used in the present study contained 64 µg of 146S particles per dose and two groups of cattle received twice this concentration at day 0. That is, with the double dose initial group, each animal received the approximate equivalent of 20 monovalent cattle doses at time 0, and 10 monovalent cattle doses at days 21 and 42. Such antigen payloads are considerably greater than would be used even for high potency emergency vaccines and provide a considerable safety margin in the interpretation of the data. Fig.4 and 5 show the mean virus neutralising antibody titres of the cattle used in the aqueous vaccine experiments (Fig. 1 and 2) and illustrate the very high potency of the vaccines in the current study. Similar data was obtained with the oil adjuvanted vaccines in cattle.

The minimal NS antibody responses of cattle administered high concentrations of purified FMD antigens contrasts with the EITB results seen with non-vaccinated control cattle following live virus challenge. Two of the lanes in Fig. 6 show high levels of antibodies against all of the NS proteins of the virus and correspond to two cattle challenged 8 days previously by intradermolingual injection of A_{24} Cruzeiro strain of FMD. Six of the lanes immediately above the two control animals correspond to cattle which had been vaccinated twice, each time with approximately 16 µg of A24 viral antigens in aluminium hydroxide/saponin adjuvant, followed by A_{24} challenge. Eight days after the vaccination, it is clear that the vaccinated animals were completely negative in terms of antibodies against NS proteins. This was supported by the virus neutralising antibody titres which indicated that the immune response to the A_{24} vaccine was not boosted by the live virus challenge (results not shown).

We have also examined the induction of neutralising antibodies against NS proteins based on more conventional vaccines and vaccination regimens. Sera taken from cattle vaccinated at day 0, day 56 and day 238 with an aqueous vaccine containing 4 µg of 146S particles of each of 4 vaccine strains showed no evidence of antibodies against NS proteins, except for a very weak response to the 3D protein, and were considered to be negative according to the criteria described in this paper (results not shown).

Conclusions

The antigenic payloads of the vaccines prepared for the present study greatly exceeded those of vaccines routinely made at Pirbright and were also substantially greater (five to ten fold) than those used for high potency emergency vaccines typical of antigen banks. Furthermore, each vaccine contained 4 strains of FMD virus, all of which would potentially contribute to non-structural antibody responses because of the high level of sequence conservation among the non-structural proteins of the different serotypes (in contrast to the antigenic and sequence diversity among the

structural proteins of the viruses). Clearly, emergency vaccines such as those used recently in South East Asia (South Korea) are exceptionally unlikely to be anything other than monovalent.

Given the repeated use and the very high payloads of antigens within the vaccines, it is noteworthy that the antibody responses to the non-structural proteins of the FMD virus are primarily confined to the 3D (VIAA) protein which, it is widely accepted, is no longer indicative of virus replication without the presence of significant titres against other non-structural proteins of the virus. None of the EITB profiles indicated a positive status for cattle given high potency vaccines in aluminium hydroxide/saponin vaccines (cattle) or oil (DOE) adjuvants . ***It is concluded that repeated application of high potency, high purity vaccines in an emergency situation would not be expected to induce antibodies against non-structural proteins of the virus other, possibly, than the 3D protein and would not therefore confuse the status of the herd in terms of discrimination between infected and vaccinated animals.*** This conclusion is further supported by the absence of significant antibody titres against non-structural proteins in serum samples from cattle repeatedly vaccinated with more conventional payloads of 146S particles.

There is also evidence that the use of a high potency monovalent vaccine prevents replication of the virus following challenge and is consistent with a previous report from Doel et al (1994) that high potency vaccines can reduce or even prevent the establishment of the carrier state.

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SYNTHETIC PEPTIDE-BASED ENZYME IMMUNOASSAYS FOR DIFFERENTIAL DIAGNOSIS OF FOOT-AND-MOUTH DISEASE

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Goals for FMDV Diagnostics

- Develop peptide-based immunoassays for improved performance over existing tests.
  - Sensitivity and specificity
  - Stability
  - Convenience
  - Reproducibility
  - Availability
  - Cost
- Convenient ELISA format.
- Use conserved NS protein peptide fragment as the antigen to detect animals of convalescent and potential carrier status (by any FMDV serotype), in the presence of vaccination.
- Use peptides containing VP1 neutralizing epitopes to evaluate effectiveness of vaccination.
- Develop protocols to expedite eradication of FMDV and verification of FMD-free status, using ccombined vaccination and serological surveillance programs.

Superiority of Synthetic Peptide-Based FMDV EIAs

- More site-specific, and less cross-reactivity.
- Immunoassays based on recombinant proteins (i.e. r2C, r 3ABC) performed poorly in specificity, due to presence of animal antibodies against vector or *E. coli* antigens.
- Specific epitopes capable of distinguishing infected animals from vaccinated ones.
- Peptide antigen is easier to manufacture and control.
- UBI has a distinguished track record in design, development, and manufacturing of peptide-based EIAs (HIV, HCV, HTLV, …etc.).

Foot-and-Mouth Disease Virus NS/VP1 EIAs

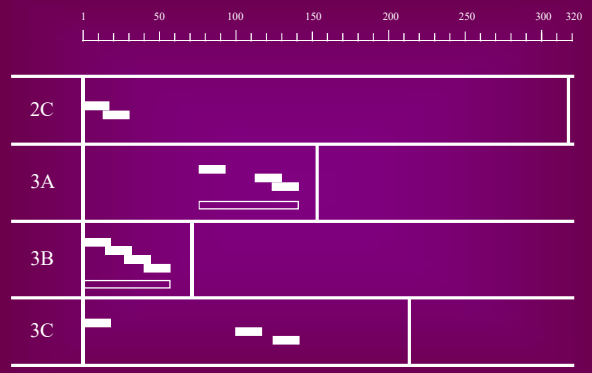
- Qualitative, indirect EIA
- Internal quality control feature:

  Non-reactive and Reactive Controls provided for each assay run
- Antigen is well-defined, representing immunodominant regions of FMDV NS 3B or VP1 protein; highly site specific, minimal potential for cross-reactivities, highly concentrated on the solid phase
- TMB chromogenic substrate
- Non-biohazardous constituents:

  - Chemically synthesized peptide antigens

  - Reactive control sera from FMDV peptide-immunized ruminants and swine, not from infected animals
- Controlled manufacturing assures reproducibility and reliability in specificity and sensitivity

Mapping Antigenic Sites of FMDV Nonstructural Proteins



Differentiation of convalescent animals from those vaccinated against foot-and-mouth disease by a peptide ELISA. F. Shen, P. D. Chen, A. M. Walfield, J. Ye, J. House, F. Brown, and C. Y. Wang. Vaccine 17 (1999) 3039-3049

The solid bars represent antigenic NS peptides. The open bars represent long 3A and 3B peptide asntigens used in the EIAs shown on the next slides.

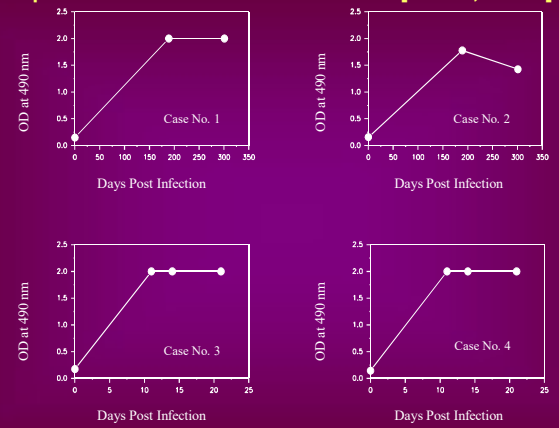
Bovine Serum Panel #2 (CS=convalescent, VS=vaccinee serum, NEG=normal) for serological validation of FMDV-NS differential peptide EIAs

Sample ID	Description	Infecting or Vaccine virus	Absorbance 3A	3B	3A+3B
1	VS	O1 BFS 1860	0.236	0.049	0.217
2	CS	O1 BFS 1860	>2.000	>2.000	>2.000
3	VS	O India 53/79	0.253	0.071	0.298
4	CS	O India 53/79	>2.000	>2.000	>2.000
5	VS	O Turkey 1/78	0.218	0.048	0.212
6	CS	O Turkey 1/78	>2.000	>2.000	>2.000
7	VS	O1 Campos	0.179	0.132	0.233
8	CS	O1 Campos	>2.000	>2.000	>2.000
9	NEG		0.135	0.075	0.145
10	NEG		0.163	0.033	0.151
11	NEG		0.126	0.082	0.185
12	NEG		0.054	0.031	0.069
13	NEG		0.143	0.070	0.279
14	VS	A22 Iraq 24/64	0.262	0.051	0.242
15	CS	A22 Iraq 24/64	>2.000	>2.000	>2.000
16	VS	A Renya 42/66	0.249	0.096	0.219
17	CS	A Renya 42/66	>2.000	>2.000	>2.000
18	VS	A24 Cruzeiro	0.495	0.058	0.558
19	CS	A24 Cruzeiro	>2.000	>2.000	>2.000
20	NEG		0.249	0.161	0.226
21	NEG		0.079	0.053	0.102
22	NEG		0.047	0.031	0.070
23	NEG		0.113	0.112	0.132
24	NEG		0.095	0.039	0.114

Sample ID	Description	Infecting or Vaccine virus	Absorbance 3A	3B	3A+3B
25	VS	C Pando	0.575	0.196	0.610
26	CS	C Pando	>2.000	>2.000	>2.000
27	VS	C Noville	0.949	0.031	0.862
28	CS	C Noville	>2.000	>2.000	>2.000
29	NEG		0.036	0.164	0.223
30	NEG		0.086	0.074	0.090
31	NEG		0.083	0.047	0.082
32	NEG		0.168	0.123	0.152
33	NEG		0.092	0.089	0.093
34	VS	SAT 1 Bot 1/68	0.294	0.113	0.291
35	CS	SAT 1 Bot 1/68	1.949	0.639	>2.000
36	VS	SAT 2 R183/74	0.398	0.118	0.401
37	CS	SAT 2 R183/74	>2.000	>2.000	>2.000
38	VS	SAT 3 BEC It65	0.234	0.101	0.242
39	CS	SAT 3 BEC 1/65	1.984	1.172	2.000
40	NEG		0.178	0.052	0.175
41	NEG		0.063	0.044	0.075
42	NEG		0.280	0.214	0.343
43	NEG		0.206	0.126	0.182
44	NEG		0.378	0.044	0.344
45	VS	Asia 1 India	0.272	0.123	0.287
46	CS	Asia 1 India	>2.000	>2.000	>2.000
Pos			1.638	>2.000	>2.000
Neg			0.134	0.049	0.134

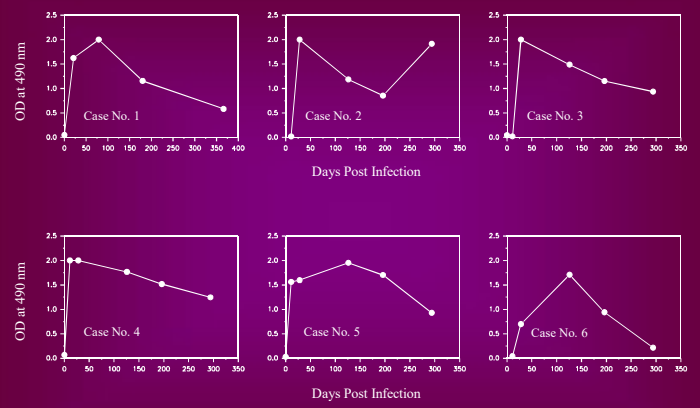
Conclusion: The 3B EIA detects only the CS but not the VS sera. The 3A and 3A+3B EIAs have poor differential specificity, reactive with both CS and VS sera.

Seroconversion by UBI FMDV NS EIA Experimental FMDV Infection in Swine [PIADC, USDA]



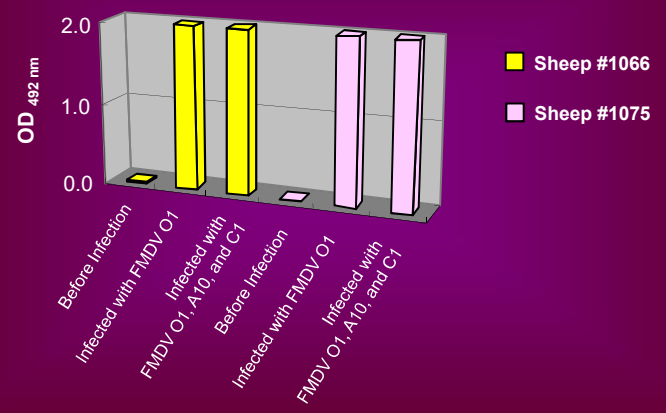
Seroconversion was detected at 10days post-exposure, the earliest sample drawn, from swine experimentally infectedc with O_1 Taiwan. Seroreactivity persisted through day 300 on swine experimentally infected with A_{24}.

Seroconversion by UBI FMDV NS EIA
Experimental FMDV Infection in Cattle [PIADC, USDA]



In 5 out of 6 experimentally-infected cattle, seroreactivity persisted through day 300, and through day 200 in one.

The UBI FMDV NS EIA Detects Infection in Sheep



Sensitivity and Specificity of UBI® FMDV NS EIAs

Study Date	Kit	Places	Animals	Samples	Specificity	Sensitivity
09/1997	UBI-NS	PIADC USDA	Cattle	n=1202 (naive) n= 31 (exp infected) n= 13 (exp infected) n= 13 (vaccinated)	98.8 % 100.0 %	 96.7 % 100.0 %
02/1998	UBI-NS	TARI Taiwan/ UBI-USA	Goat Cattle	n= 500 (naive) n= 26 (exp infected)	99.4 %	 96.1 %
			Swine Swine Swine	n= 155 (naive) n= 30 (exp infected) n= 90 (vaccinated)	98.7 % 98.9 %	 100.0 %
07/1999	UBI-NS	PIADC USDA	Cattle	n= 81 (convalescence) n= 61 (exp infected) n= 42 (naive)	 97.8 %	100.0 % 83.6 %
			Swine	n= 109 (naive) n= 42 (exp infected)	98.2 %	 85.7 %
09/2000	UBI-NS	CAHIC	Swine	n= 521 (naive) n= 310 (exp infected) n= 121 (vaccinated)	99.6 % 96.7 %	 100.0 %

Sensitivity and Specificity of UBI® FMDV VP1 EIAs

Study Date	Study Site	Animals	Samples	Specificity	Sensitivity
02/1998	TARI, Taiwan	Goat	N = 12 (vaccinated)		100 %
		Swine	N = 155 (naïve) N = 90 (vaccinated)	98.7 %	 95.5 %
03/1999	TARI, Taiwan	Goat	N = 36 (vaccinated)		83.3 %
		Swine	N = 50 (naïve) N = 10 (vaccinated)	100 %	 90 %
09/2000	CAHIC	Cattle	N = 149 (naïve)	99.3 %	
		Goat	N = 97 (naïve)	96.9 %	
		Swine	N = 521 (naïve)	99.8 %	

Range and Reproducibility of Assay Cut-Off Values

Cut-Off Value	NS Swine EIA n = 27	NS Ruminant EIA n = 20	VP1 Swine EIA n = 27	VP1 Ruminant EIA n = 7
Min.	0.358	0.317	0.360	0.323
Max.	0.388	0.372	0.389	0.378
Avg	0.371	0.352	0.375	0.351
SD	0.009	0.017	0.008	0.016
%CV	2.42	4.83	2.13	4.56

The cutoff values of reactive sera in both swine and ruminant EIAs are highly reproducible.

The Confirmatory Tests

- In order to minimize the already low false reactivity, confirmatory tests for UBI® FMDV NS EIA (SWINE) are developed for supplemental use.
- Two methods can be used:
  - A. Method A: NS 3A plus Blank Strips
    - NS 3B EIA
    - NS 3A EIA
    - Blank EIA
  - B. Method B: Analyze the Ratio of VP1 EIA Signal/Cut-off vs. NS 3B EIA Signal/Cut-off to distinguish over-vaccination from infection
    - VP1 EIA
    - NS 3B EIA

:

Method A of the Confirmatory Tests

FMDV-NS (3B)	Confirmatory Test		Interpretation
	Strip A (3A)	Strip B (Blank)	
+	+	-	Infected
+	+	+	negative
+	-	-	negative
+	-	+	Negative*

*Infected samples have higher reactivities for the 3A NS peptide than for the 3B NS peptide antigen(see next slide).

Method B of the Confirmatory Tests

FMDV-NS (3B)	FMDV-VP1	VP1 s/c / NS 3B s/c	Interpret.
+	+	> = 1.7	Vac/(Infected)
+	+	< 1.7	Infected

* Determination of this ratio provides for differentiation of even multiply-vaccinated animals from infected animals and distinguishes infected vaccinees from uninfected vaccinees. Infected/vaccinated animals will invariably have more reactivity for NS peptide than for VP1 peptide while vaccinated animals always have greater seroreactivity for VP1 peptdie.

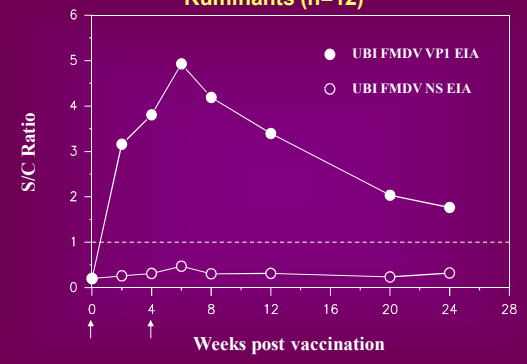
Conclusions

- From the 18 Repeatably reactive (RR) sera shown above, it was found that the 3A Strip in combination with the Blank Strip can be very useful for confirmation of FMDV infection in either non-vaccinated animals or vaccinated ones. Although the 3A along does not provide 100% confirmation, most of the false positives can be excluded by the test.

- The VP1 test can be very useful for the confirmation of FMDV infection in vaccinated animals, especially the sows that have been vaccinated multiple times. The S/C of VP1, if larger than 1.7X of the S/C of NS 3B, can be indicative of multi-vaccinations.

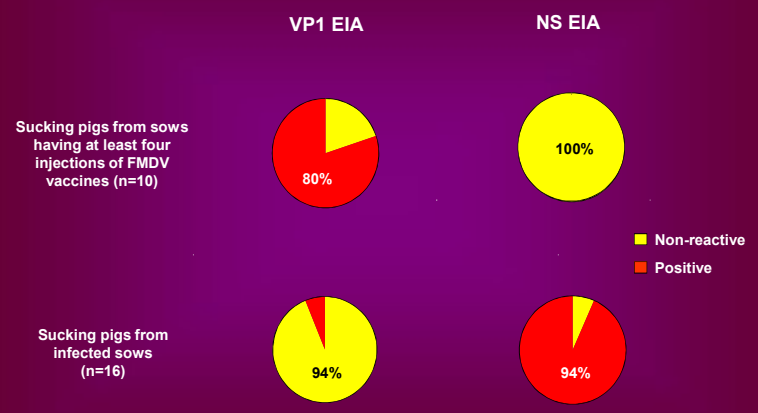
Response to Decivac* FMDV O1 and Asia1 Vaccination

Ruminants (n=12)



Seroconversionto VP1 EIA reactivity appears following vaccination with Decivac. The vaccinated animals remain non-reactive by the NS EIA.

Passively Transmitted Maternal Antibodies Detected by VP1 or NS EIAs



The NS and VP1 swine EIAs have high sensitivity for maternally transferred antibodies.

Dr. W. Linchongsubongkoch FMD Centre, Pakchong, Thailand

In cattle, the results compare UBI and 3ABC Mab trapping ELISAs are as follows:

Sample type	n	UBI NS peptide EIA		Mab trapping ELISA	
		% Pos	% Neg	% Pos	% Neg
No vaccine	141	0.7	99.3	1.4	98.6
Single vaccine	112	0	100	4.5	95.5
Multiple-vaccine	120	0	100	2.5	97.5

Dr. B. Verin, DVM PAHC, Manila, Philippines

- UBI FMDV NS peptide EIA — 70% sensitivity on PAHC panel
- Pirbright/Brescia 3ABC Mab Trapping ELISA — 50% sensitivity on PAHC panel
- Danish Vet. Inst. 3AB Blocking ELISA — 30% sensitivity on PAHC panel

C. Sanchez Martinez ICA, Bogota, Colombia

- Samples FMDV free without vaccination: n=184 UBI Specificity=100%
- Samples FMDV free with Vaccination: n=400 UBI Specificity=99.7%
- UBI test detected seroconversion at Day 6 pi and reactive through out 30 day test period, in both experimentally infected (serotype O) cattle.
- UBI test detected seroconversion at Day 7 pi and reactive through out 120 day test period, in both experimentally infected (serotype O) swine.
- UBI test detected seroconversion at Day 6 pi and reactive through out 22 day test period, in one experimentally infected (serotype O) sheep.

Based on the performance characteristics of the UBI FMDV EIAs, the tests have high potential as a method for:

- The rapid detection of infectious animals in the presence or absence of vaccination
- The detection of potential carriers among vaccinated herds
- Monitoring the progress of FMDV vaccination and eradication programs
- Epidemiological surveys in regions which practice vaccination
- Encouraging more extensive vaccine coverage for control of FMD
- Widespread serological surveys in all countries regardless of OIE status
- For import / export controls and for expedited evaluation of FMDV-free status in countries that retain immunized animals

Summary

- UBI is an experienced biopharmaceutical company with well-developed core technologies in peptide antigen and immunogen designs. UBI is committed to quality, innovation, and scientific excellence.
- UBI® FMDV NS EIAs and UBI® FMDV VP1 EIAs are two of UBI's most recent products designed for scientific management of FMD.
- UBI® FMDV NS EIAs and UBI® FMDV VP1 EIAs showed excellent specificity, sensitivity, and reproducibility in field trials and evaluation studies.
- Confirmatory tests can further improve the specificity of UBI® FMDV NS EIAs, and drastically reduce false positives.
- UBI® FMDV NS EIAs and UBI® FMDV VP1 EIAs are manufactured according to cGMP standards, with stringent QC/QA measures.

Appendix 20

EXPERIENCE OF IAEA

J. R. Crowther, Joint FAO/IAEA Division, Vienna

Extacts(*) from comments on the use of non-structural antigens of FMD virus to assess antibodies in vaccinated and infected livestock.

This document should be regarded as summarizing experiences with some tests and some on-going data. No interpretation favouring one kit, or set of reagents, against another, is intended. Tests have not always been made at the same time to allow proper harmonization, nor in some cases, has quality control been confirmed. It is meant to promote discussion and lead to better, more efficient ways of managing the developments. The data quoted comes from a continuing Co-ordinated Research Program D3 20.20, with the Joint FAO/IAEA Division on Vienna. The cooperation of many of the test developers as Agreement holders is instrumental in evaluating the assays.

Some General Observations

1. Reagents have been put together and form basis of tests to differentiate vaccinated and infected animals.
2. Various systems have been examined then changed.
3. Some systems can be regarded as approaching kits, some not.

Note. As from October 2001, there are only three viable "kits" available from the point of view of costings, sustainability and distribution, namely,
the kit from S. America (bovine, caprine, murine);
the kits from UBI (bovine, caprine, murine as well as porcine)and
the new kit from Intervet (Pirbright an Brescia reagents (assumed to be bovine etc., and porcine). See definition of kits below.

4. The internal quality control (IQC) aspects have not been addressed well.
5. Commercial considerations are important and complicating with regard to supply and cost for developing countries.
6. The purpose of the tests needs to be clearly defined and tests "fit for purpose" are needed with appropriate activities defined to arrive at the required test performance. This requires agreement on diagnostic sensitivity and specificity criteria.
7. Reference sera are badly needed.

Definition of kits ?

A 'true' kit is:-

1) Available in bulk. (Assessment of likely need here worldwide).
2) Available and distributed on demand.
3) Costed - high costs will prohibit use in developing countries.

(*) full text can be obtained from the author on request

4) Quality controlled in terms of day to day running. IQC and EQA needed.
5) Robust (stable reagents with defined performances).
6) Validated in terms of diagnostic sensitivity.
7) Validated in terms of diagnostic specificity.
8) Fit for purpose (linked to estimates of sens/spec).
9) Containing control sera, agreed reference standards.

Issues

Diagnosis covers different scenarios of infection:

- Multi species and variation of response within species
- Vaccines (induction of antibodies against NS proteins).
- Carriers.

1. Species

Work has concentrated on pigs and cattle. Sheep not so much. (qualification needed with respect to possible work (or potential) y in terms of the UK outbreak. Indirect assays suffer from the need to use a specific conjugate. The three commercial kits are all Indirect and this is expected to throw up problems when kits are used widely. Note that there have been complications noted on the ability of some bovine kits to measure antibodies from certain species (e.g. Philippine buffalo).

Needs then are to cover:

Cows
Pigs
Sheep/goats
*Deer etc e.g. zoo animals
*Buffalo
*Wild animals

more difficult to deal with and can only be addressed where kits are examined on a wider basis worldwide.

2. Vaccines do pose a problem!

Are antibodies against NS proteins produced against some vaccines?
Evidence in cattle -YES. Forms of vaccine are important. Data limited, although it is available from S. America. Intervet kit (recent press release) in fact indicates that kits are valid when used in conjunction with purified vaccines (this indeed needs strong clarification and data to substantiate the claim).

Areas relevant to vaccines include:

Standard formulation quality controlled vaccines commercial reputable
Not so reputable vaccines (local)?
Oil-reputable
Oil-not reputable
Other approaches e.g. attenuated

Note: methods in S. America for estimating concentration of NS proteins pre-formulation helps to eliminate post vaccination responses in some vaccines. They have set up a standard assay for this (information from Ingrid Bergman-PANAFTOSA)

3. Carrier animals

Some questions which have been looked at.

In genuine carriers- we get long term isolation of infectious virus/PCR products. Antibody and virus detection is intermittent! What is the risk of carrier to animals with no antibodies? (Sheep to cow?)

Passive carriers (shorter term).

Both situations may give rise to antibody positive or antibody negative (in time) animals. Question. How does Ab wane in various animals? (Note pig data saying that Abs maintained- carrier?)

Antibody positive animals where Ab may wane (extent of antibody production?)

Risk assessment of carriers**.** Is it a problem?

Problems associated in assessing immune/infectivity status of animals

This highlights:

a) Research needs (particularly with reference to animal experiments).

b) Improvements in sensitivity/specificity of tests needed.

Table highlights situations which can be found.

Animal status possible

	NOT I	**Non immune then I to 50 d**	**Non immune then I post 50 d**	**Post V first 50 d**	**Post V after 50 d**	**Re-V first 50 d**	**Re-V post 50 d**	**V then I first 50 d**	**V then I post 50 d**
1. Cow	- L, -NS	+L, +NS (at 7d)	+L +NS (?)	+L -NS	+/-L -NS	+L -NS (*)	+/- L -NS (+/-)	+L +NS	+L +NS
2. Pigs	- L, -NS	+L, +NS (at 7d)	+L +NS (?)	+L -NS	+/-L -NS	+L -NS (*)	+/- L -NS (+/-)	+L +NS	+L +NS
3. Sheep	- L, -NS	+L, +NS (?)	+L +NS (?)	+L -NS	+/-L -NS	+L -NS (*)	+/- L -NS (+/-)	+L +NS	+L +NS
4. Buffalo	- L, -NS	+L, +NS (at 7d)							
Possible Carrier State									
Cows	-		C					C	C
Pigs	-								C???
Sheep	-		C?					C?	C
Buffalo			C					C	C

L = liquid phase blocking ELISA measurement of antibodies
NS is result of non structural ELISA measurement of antibodies.
V = Vaccination
I = Infected
C = Carrier state established

Conclusions

- Available tests need more validation and need to build in better IQC control and have an EQA element. Quality control and distribution as well as robustness of reagents may not have been addressed.

- There are differences in the relative analytical sensitivity and diagnostic sensitivity/ specificity of the assays.Some of the Indirect ELISAs suffer from problem of the anti-species conjugate and individual serum samples backgrounds.There is too much test-to-test variation in controls for the assays.There is little data on sheep/goat seraSupply of large numbers of kits and costs will be a limiting factorCompetitive assays should be developed rather than Indirect assays to allow any species to be tested.Vaccinated and recovering animals pose major problems since they may allow carrier state. Results suggest that Ab against replicating virus is present at "good" levels in cattle and sheep, in carrier state, and can be detected by NS tests. The detection varies between tests. Further work is needed on pigs/sheepThe sampling frames for animals in various epidemiological situations has to be considered from the point of view of testing (kit needs, cost, manpower) with assays. This will determine the needs for tests (capacity needed) and is linked to rules of trade.It would be provident to sample vaccinated herds at some time to continuously establish the prevalence of Abs against NS associated with vaccination. The figures would add greatly to any studies where vaccinated animals are challenged by infection.

- Reference sera needed for all species.

- Surveillance documentation needed.

- Agreement on defining sensitivity and specificity needed.

- The most validated and used assay is from S. America where it has been used in the face of vaccination. Attention to this assay must be made along with strategies devised and interpretation of the results.

- Work to evaluate the antigenicity of the NS proteins and complexes should be made.Vaccines should be assessed for NS contamination.

- There should be agreement on the validation factors and documentation needed to establish a test. Estimation and agreement on the diagnostic sensitivity and diagnostic specificity in all tests (with reference to panels) should be encouraged. Measurement of analytical sensitivity of all tests should also be made with reference samples.Inclusion of full charting IQC protocols in test kits should be encouraged.There should be a Central reference center for EQA management.

- Development of a reference bank of sera characterizing different epidemiological situations. Cow, sheep, goat and pig serum has to be made and held in suitable quantities in a bank for reference as well as developmental purpose.

- Development of competitive assays for the estimation of antibodies for all species should be made suitable for wild life and zoo animal species, as well as various breeds of buffalo etc.

- Detection and quantification of NS proteins in vaccines immediately before formulation might be standardized. Estimation of antigenicity of NS proteins in cows, sheep, goats and pigs might be made.

- Examination of sheep/goats in terms of antibody production after vaccination and contact in developing antibodies against NS proteins.

- Titration of titres of antibodies produced against NS proteins at different stages following infection should be made.

- Further studies correlating the antibody production, virus isolation, PCR, NS testing, VNT in carriers. This should also be examined in pigs.

- Measurement of antibodies produced in vaccinated then challenged pigs should be made, particularly those showing no clinical signs.

- Estimation of performance of the tests in SE. Asia, Africa, Middle East, Russia and ex CIS countries should be made.

- Training in basic principles of FMD serology. There is a need for a practical guide here to facilitate the best use of NS and all other kits.

Appendix 21

THE RESULTS OF SEROLOGICAL SURVEILLANCE FOLLOWING THE VACCINATION IN THRACE REGION IN 2000

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The trivalent FMD vaccine (O1 Manisa, A22 Mahmatli and Asia1) donated by the EU was used in the Turkish Thrace including the Anatolian part of Istanbul and Canakkale provinces for the Autumn 2000 campaign. A serosurvey was conducted after this vaccination. This surveillance was carried out in four different groups;

1st group- a total of 35 villages and 30 large and small ruminants from each group were selected and sera were collected at days 0., 28., and 120. post vaccination,
2nd group- the same amount of animals but from different 35 villages were selected and the blood sera collected 60 days post vaccination.
3rd group- was selected to measure the protective level of vaccine in the field experimentally. For this purpose 30 seronegative cattle and 30 seronegative sheep were vaccinated and sera were collected at days 28. and 120. and were tested by LPB-ELISA.
4th group- the sera from the first two groups were tested by MAT-ELISA for detecting NSP antibodies.

In the first two groups, sera were tested in single dilution (1/100) which was accepted as protective level by LPB-ELISA. In the third group, LPB-ELISA was carried out with two fold dilutions of the sera. In the last group, sera were tested by MAT- ELISA to detect antibodies against non-structural FMD proteins.
Since the vaccination had begun before the sampling, only 279 sera were tested as the prevaccination sera. The ratio of positive sera were 37% for type O, 41% for type A and 2% for type Asia1. The tables below show the results as total (Table 1a), by animal species (Table 1b), and by age (Table 1c).

At day 28, a total of 989 sera from 523 cattle and 466 sheep were tested. The positive antibody titres for O1, A, and Asia1 were 75%, 82%, and 70% respectively (Table 2a, 2b,2c, 2d).

However, in the same group, at the day of 120, the protection rate for O1, A, and Asia-1 serotypes was decreased down to 22,5%, 54,6% and 13,53% respectively (Table 3a,3b, and Figure 1).

Sera from the second group were tested to detect the protective level 2 months after vaccination. The percentage of the protection against O, A, and Asia-1 were 35.25%, 60%, and 18,5% respectively (Table 4a, 4b, 4c).

In the third group, 60 large and small ruminants were vaccinated and sera collected at days 28, and 120 was tested by LPB-ELISA. Although the protective level was 99% for all three types at day 28, in the 4th month it was 16% for O, 60% for A and 10% for Asia-1 (Figure 3).

The results of the MAT-ELISA carried out with the sera from the 4th group will be discussed in the next item.

According to the results, protective level were low particularly in type O and type Asia-1 in the second month, although high protection rates were observed initially.

Table 1a: Cumulative results of the sera collected from the animals in group1 before vaccination

FMD Types	Positive	(%)	Negative	(%)
O	87	31	192	69
A	115	41	164	59
ASIA-1	6	2	273	98

Table 1b: LPB-ELISA results of the sera collected from the animals in group 1 before vaccination: Distribution of results by animal species

	Large Ruminant (119)				**Small Ruminant (160)**			
FMD Types	**Positive**	**(%)**	**Negative**	**(%)**	**Positive**	**(%)**	**Negative**	**(%)**
O	61	51	58	49	26	16	134	84
A	79	66	40	34	36	23	124	77
ASIA1	2	2	117	98	4	2.5	156	97.5

Table 1c: LPB-ELISA results of the sera collected from the animals in group 1 before vaccination: Distribution by ages

	Large Ruminant (119)												Small Ruminant (160)											
FMD Types	0-1 (42)				1-2 (46)				>2 (31)				0-1 (57)				1-2 (54)				>2 (49)			
	+	%	-	%	+	%	-	%	+	%	-	%	+	%	-	%	+	%	-	%	+	%	-	%
O	18	43	24	57	21	46	25	54	22	71	9	29	7	12	50	889	5	9	49	91	14	29	35	71
A	22	52	20	48	30	65	16	35	27	87	4	13	5	9	52	91	13	24	41	76	18	37	31	63
ASIA1	0	0	42	100	2	4	44	96	0	0	31	100	1	2	56	98	1	2	53	98	2	4	47	96

Table 2a: Cumulative results of the sera collected from the animals in group1 28 days postvaccination

FMD Types	Positive	(%)	Negative	(%)
O	736	75	253	25
A	811	82	178	18
ASIA-1	696	70	293	30

Table 2b: LPB-ELISA results of the sera collected from the animals in group 1, 28 days postvaccination: Distribution of results by animal species

	Large Ruminant (523)				**Small Ruminant (466)**			
FMD Types	**Positive**	**(%)**	**Negative**	**(%)**	**Positive**	**(%)**	**Negative**	**(%)**
O	428	82	95	18	308	66	158	34
A	454	87	69	13	357	77	109	23
ASIA-1	391	75	132	25	305	65	161	35

Table 2c: LPB-ELISA results of the sera collected from the animals in group 28 days postvaccination: Distribution by ages

	Large Ruminant (523)												**Small Ruminant (466)**											
FMD Types	**0-1 (189)**				**1-2 (150)**				**>2 (184)**				**0-1 (126)**				**1-2 (166)**				**>2 (174)**			
	+	%	-	%	+	%	-	%	+	%	-	%	+	%	-	%	+	%	-	%	+	%	-	%
O	150	79	39	21	132	88	18	12	146	79	38	21	91	72	35	28	110	66	56	34	107	62	67	38
A	144	76	45	24	140	93	10	7	170	92	14	8	99	79	27	21	124	75	42	25	134	77	40	23
ASIA1	147	78	42	22	116	77	34	23	128	70	56	30	94	75	32	25	107	64	59	36	104	60	70	40

Table 2d: LPB-ELISA results of the sera collected from the animals in group 28 days postvaccination: Distribution of the results by sex groups

	Female (842)				**Male (147)**			
FMD Types	**Positive**	**(%)**	**Negative**	**(%)**	**Positive**	**(%)**	**Negative**	**(%)**
O	635	75	207	25	101	69	46	31
A	702	84	141	16	109	74	38	26
ASIA1	590	70	252	30	106	72	41	28

Table 3a: Cumulative results of the sera collected from the animals in group1, 120 days postvaccination

FMD Types	**Positive**	**(%)**	**Negative**	**(%)**
O	223	22,5	766	77,5
A	540	54,6	449	45,4
ASIA-1	135	13,53	854	86,47

Table 3b: LPB-ELISA results of the sera collected from the animals in group 1, 120 days postvaccination: Distribution of results by animal species

	Large Ruminant (523)				**Small Ruminant (466)**			
FMD Types	**Positive**	**(%)**	**Negative**	**(%)**	**Positive**	**(%)**	**Negative**	**(%)**
O	157	30	366	70	66	15	400	85
A	298	57	225	43	242	52	224	48
ASIA-1	79	15	444	85	56	12	410	88

Figure 1: The positive percentage rates of sera collected from the animals in group1, 0., 28. and 120. days

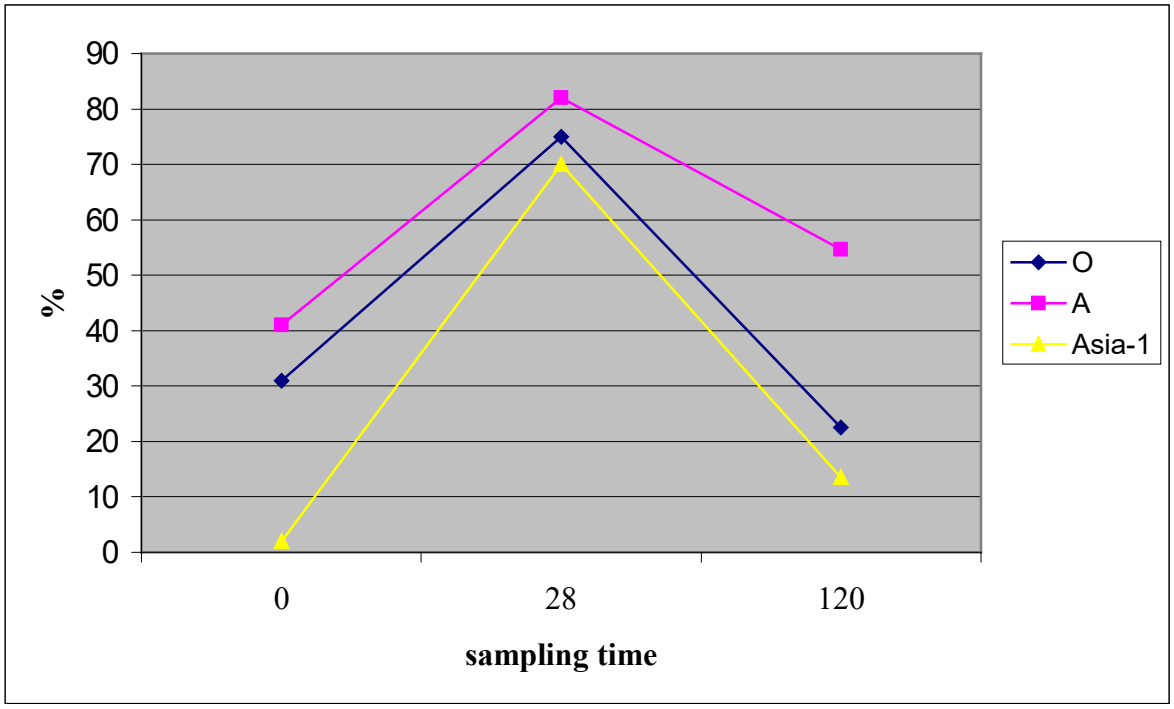


Table 4a: Cumulative results of the sera collected from the animals in group 2, 60 days postvaccination

FMD Types	Positive	(%)	Negative	(%)
O	275	35,25	505	64,75
A	515	60	265	40
ASIA-1	145	18,5	635	81,5

Table 4b: LPB-ELISA results of the sera collected from the animals in group 2, 60 days postvaccination: Distribution according to animal species

	Large Ruminant (405)				**Small Ruminant (375)**			
FMD Types	**Positive**	**(%)**	**Negative**	**(%)**	**Positive**	**(%)**	**Negative**	**(%)**
O	162	40	243	60	113	30	262	70
A	279	69	126	31	236	63	139	37

ASIA-1	73	18	332	82	72	19	303	81

Table 4c: LPB-ELISA results of the sera collected from the animals in group 2, 60 days postvaccination: Distribution of the results by provinces

	O				**A**				**ASIA-1**			
Provinces	**Positive**	**(%)**	**Negative**	**(%)**	**Positive**	**(%)**	**Negative**	**(%)**	**Positive**	**(%)**	**Negative**	**(%)**
Canakkale (60)	21	35	39	65	40	66	20	34	11	18	49	82
Edirne (225)	73	32	152	68	149	66	76	34	39	17	186	83
Istanbul (135)	40	30	95	70	60	45	75	55	34	25	101	75
Kirklareli (360)	141	40	219	60	266	74	94	26	60	17	300	83

Figure 2: The percentage of the positive sera collected from the animals in group 2, 60 days postvaccination by LPB-ELISA: Distribution of the results by provinces

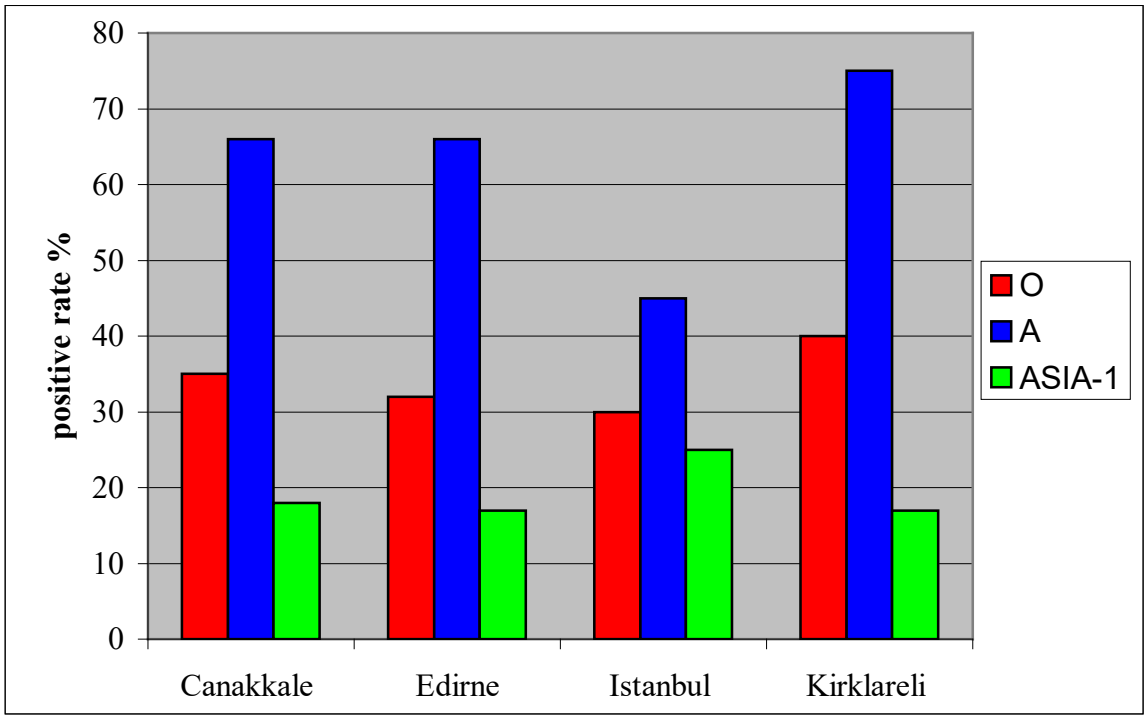
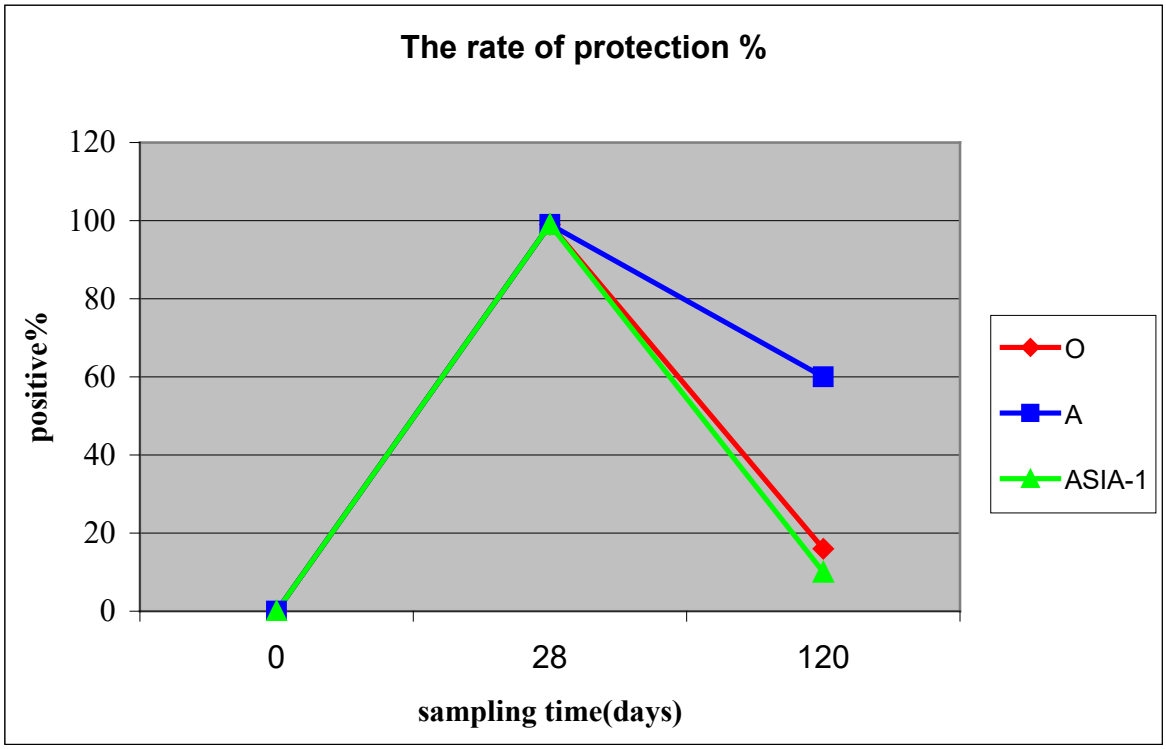


Figure 3: Cumulative results of the sera collected from the animals in group 3 in Bala, 0., 28, and 120 days postvaccination



Appendix 22

THE RESULTS OF THE 3 ABC ELISA SEROSURVEY CONDUCTED WITH THE SERA FROM THRACE

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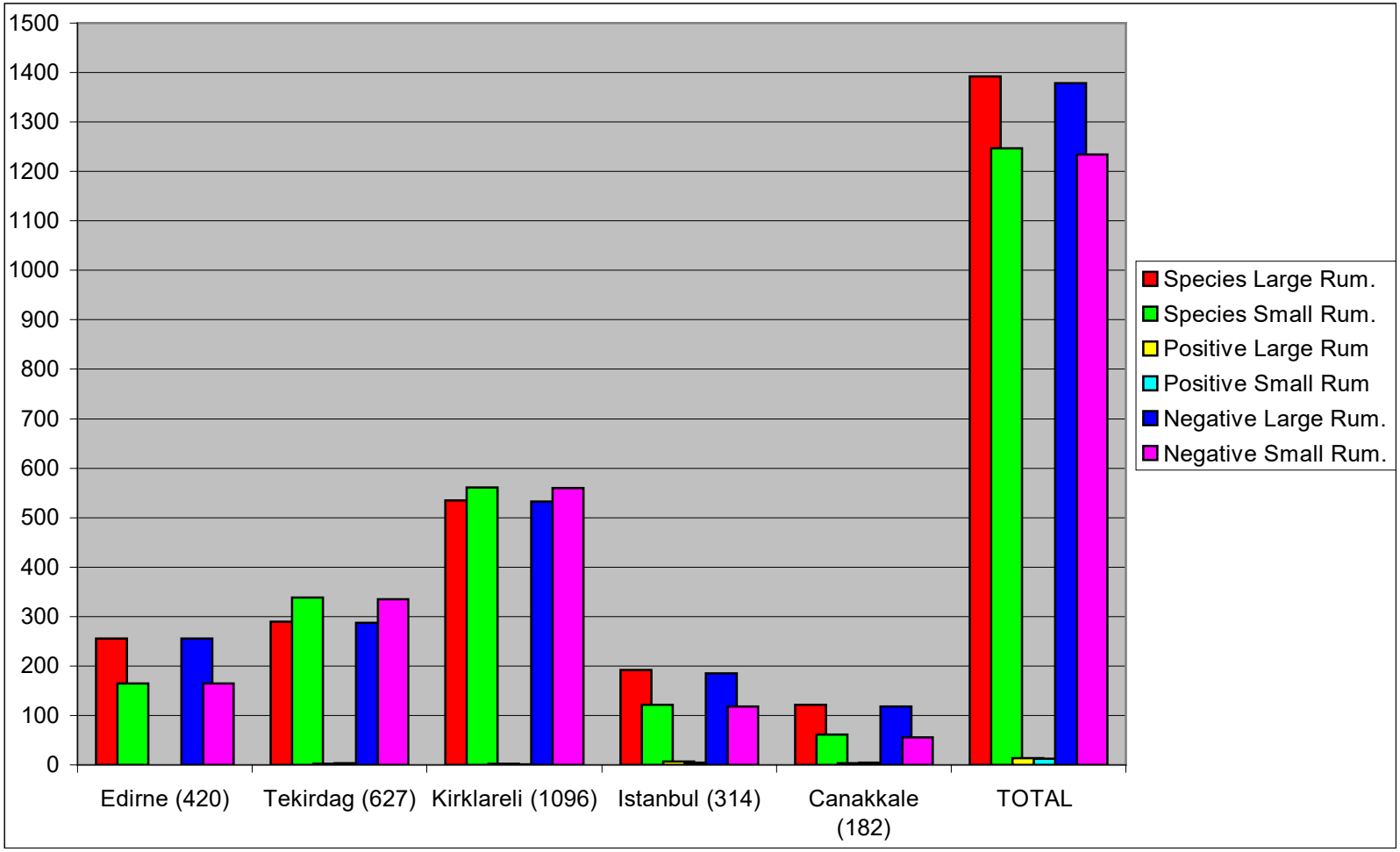
A total of 2639 sera collected from 1392 large ruminants and 1247 small ruminants were tested by MAT-ELISA against 3ABC proteins in Thrace Region including Edirne, Tekirdag, Kirklareli, Istanbul and Canakkale Provinces. The ratio of positive samples in total was 1.02 % (1.0 % bovine and 1,04 % ovine sera).

These positive animals might have been infected and recovered from the disease and possibly been introduced into Thrace from Anatolia in the past during the Kurban or for the purpose of slaughter but not slaughtered and sold in the animal market. The results are given in Table 1.

Table 1: The results of the MAT-ELISA

District	**Species**		**Positive**				**Negative**			
	Large Rum.	**Small Rum.**	**Large Rum.**		**Small Rum.**		**Large Rum.**		**Small Rum.**	
	No.	**No.**	**No.**	**%**	**No.**	**%**	**No.**	**%**	**No.**	**%**
Edirne	255	165	0	0	0	0	255	100	165	100
Tekirdag	289	338	2	0.69	3	0.88	287	99.3	335	99.11
Kirklareli	535	561	2	0.37	1	0.17	533	99.62	560	99.82
Istanbul	192	122	7	3.64	4	3.27	185	94.27	118	96.72
Canakkale	121	61	3	2.47	5	8.19	118	96.35	56	91.80
TOTAL	**1392**	**1247**	**14**	**1.0**	**13**	**1.04**	**1378**	**98.99**	**1234**	**98.95**
	2639		**27 (1.02%)**				**2612 (98.97%)**			

Figure 1: The results of the MAT-ELISA



Appendix 23

PERSISTENCE OF FMDV AND THE ROLE OF CARRIER ANIMALS : WORKSHOP ORGANISED BY ID-LELYSTAD, THE NETHERLANDS

Aldo Dekker and Kris De Clercq

1. Scientific Programme

Overview of the FMDV carrier problem (Dr. S. Alexandersen, ENG)
Past and Future of carriers in FMD (P.Sutmoller, NL)

Diagnostic possibilities for detection of carrier animals (B. Haas, GER)
Diagnosis of persistent FMDV infection in cattle (P. Moonen, NL)
The NS-Elisa is a diagnostic tool to control FMDV (E. Brocchi, IT)

Molecular epidemiology of FMDV (N. Knowles, UK)
Species specificity and strain variation in Asian FMD-viruses (P. Mason, USA)

Mechanism of persistence of Polio virus (T. Kimman, NL)
Immunological aspects of FMDV infections and persistence (K. McCullough, UK)
Molecular aspects of FMDV persistence (Z. Zhang, UK)
Adaptability of FMDV quasispecies in vitro and in vivo (E. Domingo, Esp)
FMDV epitope recognition and persistence (F. Sobrino, Esp)
Evolution of FMDV antigenicity and receptor usage (E. Baranowski, ESP)

The North American decision tree for Foot-and-Mouth disease vaccine use (D. Geale, Can)
The 2001 Foot-and-Mouth Disease epidemic in the Netherlands:an overview (A. Bouma, NL)
Economic impact of FMDV carriers (M. Nielen, NL)
Role of FMDV persistence in EU control measures (K. De Clercq, Bel)

Round table discussion (K. De Clercq, EUFMD)

2. Conclusions workshop

Non-vaccination policy is the strive for a disease free population.

* No vaccination when disease free
* Emergency vaccination when necessary

Use of emergency vaccination is dependent on: (based on the EU report on FMD vaccination of 10-3-1999)

* Population density of susceptible animals
* Clinically affected species
* Movement of potentially infected animals or products
* Predicted airborne spread
* Suitable vaccine available
* Origin of outbreaks
* Incidence slope of outbreaks

* Distribution of outbreaks
* Public reaction to total stamping out policy

There is need for further *analysis of all epidemiological* (mostly circumstantial evidence) *available data* on transmission from carriers to susceptible animals.

- A meta-analysis on the basis of all existing data
- Identify whether there is a difference in risk between carriers from vaccinated carriers and non-vaccinated carriers.
- Development of good sampling schemes which should be used after an outbreak which is stopped with the use of vaccination. Data from South-America would be very helpful.

For *risk analysis* a better understanding on the *mechanism of transmission* from carrier to susceptible animals is necessary

- Sexual transmission
- Exchange of rumenal content
- Use quantitative data on virus concentration in different samples from carriers and the number of possible contacts, and the amount of material transferred. All data should be put in a mathematical model

Knowledge on *the pathogenesis of carriers* will help to stop the carrier problem

- Current vaccines have been developed without knowledge of the pathogenesis and are not capable to stop the development of carriers.
- Difference between pigs and cattle may help to clarify the mechanism of carriers
- Induction of mucosal immunity may help to prevent the development of the carrier state
- Dexamethasone treatment influences the carrier state, insight in this process might help to elucidate methods to prevent carriers
- Development of effective antivirals for FMD might prevent the (development) of the carrier state

From a scientific point of view the current *NS-ELISAs* are useable to check for carriers after emergency vaccination

- The test should be accepted by politicians
- The present test is definitely usable at a herd level and probably also on a individual basis
- The test is already mentioned in the proposed text for the new OIE manual
- Test kits should be produced under standard conditions
- The test has been used testing over 600.000 sera in South-America
- New information on the immunogenic sites of the protein will help to develop better tests

3. Research subjects identified

* Diagnostics
* Mucosal immunity
* Identification of other disease associated factors that might be used for diagnosis
* Improvement of the NS-ELISA using other proteins (e.g.. baculo expressed) or synthetic peptides

* Epidemiology and risk analysis
* Statistical analysis of all currently reported cases of transmission by carriers (compared to the number of cases a contact from a possible carrier to susceptible animals occurred)
* Economic impact of different disease control strategies based on the objective risk of carriers
* How does a FMD infection persist at a herd level, do carriers play an important role or is spread from acutely infected animals most important
* Development of sampling schemes to be used when testing sera in the NS-ELISA
* Treatment / Vaccination
* Block persistence (e.g. dexamethason, antivirals)
* Peptide vaccines
* Vaccines that induce mucosal immunity
* Mechanisms of persistence
* Role of specialised (epithelial or tonsilair) cells
* Animal model for persistence
* Cell model for persistence
* Difference in cells (in vitro and in vivo between species)
* Difference in virus strains
* Role of the immune system
* Antibodies
* CTL and NK cells
* Cytokines
* Site of persistence

Appendix 24

CARRIER SHEEP DISCOVERED DURING THE DUTCH FOOT-AND-MOUTH DISEASE EPIDEMIC: A CASE REPORT

A. Dekker, J.M.A. Pol

The foot-and-mouth epidemic 2001 in the Netherlands started in one of the most densely populated livestock areas in Europe. Therefore, it was necessary to control the disease using vaccination in a large area in the centre of the Netherlands. Approximately 200.000 animals, cattle, pigs, sheep and goats were vaccinated. On each herd, serum samples were collected before vaccination. In only one the 1,120 farms all sera collected before vaccination contained high titres of neutralising antibodies. This concerned a small sheep farm containing 16 ewes and approximately 25 lambs. All 16 ewes had been sampled and were serological positive at the time of vaccination. The ewes had entered the stable on March 14. On the farm of origin, 12 sheep were also sampled before vaccination but serologically negative. The sheep on both farms were killed on April 26, at this time probang samples were collected from the 16 serological positive ewes and from 5 ewes on the farm of origin.

At post-mortem examination in almost all sheep at the farm with serological positive results had a fault in the hoof 1 to 1½ cm from the coronary band, which could be consistent with a FMD infection a few weeks previously. 12 out of the 16 sputum samples taken from the serological positive ewes were positive by RT-PCR and 4 out of these 12 probang samples were positive by virus isolation. None of the probang samples taken at the farm of origin were positive, nor were hoof lesions observed.

This report clearly shows that a foot-and-mouth disease infection in sheep can easily be overlooked and therefore additional control measures in sheep, like serological screening during quarantine, seem necessary in areas where a foot-and-mouth outbreak has occurred.

Appendix 25

EVALUATION OF AUTOMATED RT-PCR SYSTEMS TO ACCELERATE FMD DIAGNOSIS

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Summary

Automated 5'-nuclease probe-based RT-PCR procedures have been evaluated for FMD diagnosis using tissue epithelium, serum/blood, milk and oesophageal-pharyngeal fluid ("probang") samples submitted to the OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease (WRL for FMD), Pirbright, from the current United Kingdom (UK) epidemic and from experimentally infected animals. The RT-PCR results were directly compared to the results of the routine diagnostic tests of ELISA and virus isolation in primary calf thyroid cell culture. A MagNA Pure LC was programmed to automate the nucleic acid extraction and reverse transcription (RT) procedures with PCR amplification carried out by 5'-nuclease probe-based assay on a 5700 thermal cycler. The PCR amplification was also automated later in the evaluation. Automated RT-PCR successfully detected FMD virus in suspensions of tissue epithelium (ES) and in cell cultures following their inoculation with ES. The results of RT-PCR and virus isolation/ELISA on original serum/blood and milk samples were in broad agreement but the positive-negative acceptance criteria for the RT-PCR testing of probangs has to be fully optimised. This evaluation, however, demonstrated that automated RT-PCR has the potential to accelerate FMD diagnosis as positive results were achieved on first passage cell culture supernatant fluids from samples not showing an observable cytopathic effect until second passage.

1. Introduction

Foot-and-mouth disease is a highly infectious and contagious disease with the potential for rapid diffusion in susceptible animal populations. Effective control and eradication require that suspected cases are quickly reported, diagnosed accurately and that infected animals are slaughtered without delay. The main policy adopted by the UK government to control the 2001 epidemic is to slaughter the animals on infected premises within 24 hours and those in dangerous contact and contiguous premises within 48 hours. Since this policy was introduced on 29 March the majority of the outbreaks during the epidemic have been diagnosed on clinical grounds and so by the time the results of laboratory investigations have been available the animals have already been slaughtered. If laboratory investigations are to play a part in diagnosis under such control policies then the time to perform tests will have to be considerably accelerated. In the OIE/FAO World Reference Laboratory for Foot-and-Mouth Disease (WRL for FMD), Pirbright, an evaluation is being made of the application of PCR technologies to achieve this objective.

In the WRL for FMD, epithelial suspensions (ES) are routinely tested by ELISA (Ferris and Dawson, 1988) which enables positive samples to be reported within hours of receipt. However, as the sample may contain concentrations of virus lower than the ELISA detection limit, a negative result cannot be confirmed until the suspensions have been inoculated in primary calf thyroid cell cultures in attempts to isolate virus. Serum/blood and probangs cannot be tested directly by ELISA and have to be inoculated onto cell culture. Virus isolation in cell culture may be slow if the multiplicity of infection is low and may take up to four days (two passages each of two days duration) before the appearance of a cytopathic effect (CPE) thus delaying the issue of a final diagnostic result which may hinder control measures in the field.

Existing reverse transcription polymerase chain reaction (RT-PCR) procedures can supplement, but not replace, the routine procedures for diagnosis of FMD virus (Reid et al., 1998; Reid et al., 1999; Reid et al., 2000) and are limited in the number of samples that can be tested in a single assay without increased risk of contamination. Moreover, the PCR products are conventionally analysed by gel electrophoresis which is time-consuming, insensitive and non-quantitative.

Fluorogenic polymerase chain reaction (5'-nuclease probe-based) methodology has been provisionally evaluated in our laboratory as an FMD diagnostic tool on ES and cell culture virus preparations. This method, combining the total RNA extraction and RT procedures of our conventional RT-PCR with PCR amplification using a fluorogenic primer/probe set in a GeneAmp® Sequence Detection System 5700 thermal cycler (Applied Biosystems, UK), has been used successfully to detect virus genome in all seven serotypes of FMD virus to provide quantitative results (Reid et al., manuscript in preparation). We have developed the 5'-nuclease probe-based RT-PCR further by using automated procedures for the nucleic acid extraction, RT and PCR amplification stages. This increased the speed and capacity of the assay by providing a more convenient means of testing larger panels of ES, serum/blood, probang and milk samples simultaneously by RT-PCR. The results of our initial evaluation of these procedures on samples arising from the current UK FMD epidemic and from animals experimentally infected with the contemporary FMD virus serotype O UKG 2001 are presented.

2. Materials and Methods

2.1. Sample preparation, ELISA and virus isolation

ES of samples submitted from the UK 2001 FMD epidemic (along with three ES and two ES of samples from the Republic of Ireland and Senegal respectively) were prepared, tested by ELISA and inoculated onto primary calf thyroid cell cultures (Ferris and Dawson, 1988). Serum/blood, probang and milk samples from the epidemic and sera from animals experimentally infected with the FMD virus serotype O UKG 2001 were similarly inoculated onto primary calf thyroid cell cultures. Samples showing a CPE were harvested and the FMD virus specificity of the supernatant fluids was confirmed by ELISA. Supernatant fluids of a selection of inoculated cultures not showing a recognisable CPE on first and second cell culture passage were collected. Another batch of cell culture supernatant fluids was collected after first passage without a recognisable CPE but in which a CPE was observed later on second passage.

2.2. Total nucleic acid extraction and reverse transcription

Prior to the extraction procedure, 0.2 ml of ES was added to 1 ml TRIzol® reagent (Life Technologies, UK). Serum/blood, probang and milk samples and cell culture supernatant fluids were added to an equal volume of lysis/binding buffer (Roche, UK) and each sample mixed for 10-15 sec. Samples were placed in batches of 32 inside a MagNA Pure LC (Roche, UK) programmed to extract total nucleic acid to a final elution volume of 100 µl. The MagNA Pure LC was also programmed to mix 6 µl of each nucleic acid with 9 µ RT mix in a PCR plate immediately after the extraction procedure. RT was completed by placing the plate in a PTC-100™ thermal cycler (MJ Research, Inc.) and incubating successively at 48^0C for 45 min, 95^0C for 5 min and 20^0C for at least 20 min.

2.3. PCR amplification by 5'-nuclease probe-based reaction

Redundant primers and a fluorogenic 5'-nuclease probe were designed from the 5'-untranslated region of the virus genome for the intended detection of all seven FMD virus serotypes. PCR amplification of the majority of sample cDNAs was carried out by a 5'-nuclease probe-based procedure in which 3 µl cDNA was pipetted manually to 22 µl PCR mix containing 0.9 pmol/µl each of the forward and reverse primer and 0.3 pmol/µl of probe. PCR amplification was carried out in a 5700 thermal cycler using the programme: 50^0C for 2 min, 1 cycle; 95^0C for 10 min, 1 cycle; 95^0C for 15 sec, 60^0C for 60 sec, 50 cycles.

Other cDNA samples prepared from ES, serum/blood and probangs were added to the PCR mix by an automated process in which the MagNA Pure LC was programmed to add 7 µl cDNA to 18 µl PCR mix containing the same concentrations of the primers and probe as above. This was followed by PCR amplification in the 5700 thermal cycler as before.

After each amplification a C_T value (detection threshold; the cycle at which target sequence is detected) was assigned to each PCR reaction (Oleksiewicz et al., 2001). A C_T value of 40.00 was selected as the positive/negative cut-off but samples with C_T values from 39.00 - 41.50 were designated 'borderline' and require retesting by the PCR to determine their status. The majority of negative samples should have a C_T value close to 50.00.

3. Results and Discussion

The results from the automated 5'-nuclease probe-based RT-PCR procedures, ELISA and virus isolation on the ES, serum/blood, milk and probang samples from the UK 2001 epidemic and on sera from animals infected experimentally with the FMD virus serotype O UKG 2001 are summarised in Table 1. Using automated programmes for nucleic acid extraction and RT, the 5'-nuclease probe-based RT-PCR detected FMD viral RNA in the ES of nine samples which were negative by ELISA but positive in cell culture (FMD virus specificity confirmed by ELISA). Seven ES samples were borderline with this RT-PCR procedure (one NVD by ELISA/virus isolation, the other viruses amplifying in cell culture) and require repeat testing. However, two ES samples were negative by RT-PCR but positive by ELISA and virus isolation. These ES samples will have to be re-tested by RT-PCR to establish the cause of this discrepancy. The 5'-nuclease probe-based RT-PCR with automated pipetting for PCR amplification detected three

positive ES samples testing negative by ELISA on primary diagnosis. One ES sample was borderline by this RT-PCR but NVD by ELISA and virus isolation.

Five serum/blood samples, submitted from a single premises, were positive by virus isolation (FMD virus specificity confirmed by ELISA) and four of these were positive by RT-PCR (Table 1). Table 1 also shows that all sera from the experimentally infected animals were positive by RT-PCR and virus isolation (FMD virus specificity confirmed by ELISA). With automated pipetting, two anomalous results were seen where the RT-PCR was borderline with one sample which tested positive on virus isolation (FMD virus specificity confirmed by ELISA) and borderline on another testing negative by virus isolation. This latter sample was submitted in a batch containing positive FMD virus sera so it may have contained FMD virus genome.

Testing of probangs by automated RT-PCR is very preliminary. The virus isolation results of twenty one probangs were compared to those of RT-PCR using automated nucleic acid extraction and RT programmes (but not automated PCR amplification [Table 1]). One probang was positive by virus isolation (FMD virus specificity confirmed by ELISA). This sample was negative (just below the borderline level with a C_T value of 46.3) by the RT-PCR assay acceptance criteria based on testing of ES samples but one probang was borderline and three other probangs were close to the borderline level (C_T values ranging from 43.5 to 46.5) by RT-PCR. Only seven probangs were tested by the RT-PCR with automated PCR amplification and the results compared with those from virus isolation (Table 1). One sample was borderline and two others just below the cut-off level (C_T values of 42.6 and 44.2) by the RT-PCR. No virus was detected by virus isolation in the seven probangs. It is likely that the acceptance criteria for testing of probangs by automated RT-PCR will have to be adjusted and more positive samples are required to fully validate the RT-PCR for the diagnosis of FMD based on probang tests. As more data from probang testing becomes available the RT-PCR cut-off will be optimised.

Table 2 shows the results obtained by automated RT-PCR and ELISA on primary calf thyroid cell cultures inoculated with ES. RT-PCR detected FMD virus in 8 cell culture supernatant fluids collected after first passage without a recognisable CPE; this being observed in the cell cultures on second passage.

The automated programmes used in our evaluation enabled the 5'-nuclease probe-based RT-PCR to provide FMD diagnostic results in a shorter time scale than either of our conventional RT-PCR or non-automated 5'-nuclease probe-based RT-PCR methods as larger panels of ES, serum/blood, milk and probang samples could be tested simultaneously in single assays. The data also demonstrated the ability of the RT-PCR to detect FMD viral RNA in positive cell culture supernatant fluids on first passage (without a recognisable CPE) which routinely undergo a second passage for up to two more days. In a normal working day, results from 64 test samples can realistically be obtained by the automated RT-PCR procedures currently described.

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Table 1. Comparison of the 5'-nuclease probe-based RT-PCR procedures using automated nucleic acid extraction and RT programmes (with or without automated PCR amplification) with ELISA and virus isolation for the testing of epithelial suspensions (ES), serum/blood, milk and probang samples from the UK 2001 epidemic and on sera from animals infected experimentally with the FMD virus serotype O UKG 2001

Procedure	Sample source	Number of samples positive, NVD or borderline (to repeat test) per source of antigen[a]											
		ES			Serum/blood[b]			Milk			Probangs		
		O	NVD	repeat	O	NVD	repeat	O	NVD	repeat	O	NVD	repeat
5'-nuclease probe-based RT-PCR[c]	UK 2001 epidemic	56	38[d]	7[e]	4[f]	104	0	0	20	0	0	20[g]	1
ELISA	UK 2001 epidemic	49	52	0	5[b]	0[h]	0	0	20	0	1[b]	0[h]	0
Virus isolation	UK 2001 epidemic	64	37	0	5	103	0	0	20	0	1	20	0
5'-nuclease probe-based RT-PCR[c]	Experimentally infected animals	NT[i]	NT	NT	55	0	0	NT	NT	NT	NT	NT	NT
Virus isolation/ELISA	Experimentally infected animals	NT	NT	NT	55[b]	0	0	NT	NT	NT	NT	NT	NT
5'-nuclease probe-based RT-PCR[j]	UK 2001 epidemic	15	2	1	1	22	2[k]	NT	NT	NT	0	6[l]	1
ELISA	UK 2001 epidemic	12	6	0	2[b]	0[h]	0	NT	NT	NT	NT	NT	NT
Virus isolation	UK 2001 epidemic	15	3	0	2	23	0	NT	NT	NT	0	7	0

[a] All samples submitted from the UK epidemic reported as FMD virus serotype O or NVD (no virus detected) by ELISA/virus isolation. Eight ES and two serum/blood samples tested borderline for FMD virus by RT-PCR (recommend repeat RT-PCR test).
[b] Serum/blood, milk and probang samples are not tested directly by ELISA but inoculated onto primary calf thyroid cell culture. ELISA then used to test supernatant fluids from cell cultures showing a CPE.
[c] 5'-nuclease probe-based RT-PCR procedure using automated nucleic acid extraction and RT programmes.
[d] Two ES samples negative by RT-PCR but positive by ELISA and virus isolation.
[e] One sample gave a borderline result by RT-PCR but NVD by ELISA and virus isolation. Six samples borderline by RT-PCR, negative by ELISA but positive in cell culture.
[f] One serum/blood was negative by RT-PCR but positive by virus isolation/ELISA (the five positive samples by virus isolation/ELISA and the four positives by RT-PCR respectively were submitted from the same premises).
[g] RT-PCR result from four probangs just below the borderline level.
[h] Only the supernatant fluids of cell cultures with a recognisable CPE after inoculation with serum/blood or any other source of antigen are tested by ELISA.
[i] NT, not tested.
[j] 5'-nuclease probe-based RT-PCR procedure using automated programmes for nucleic acid extraction, RT and PCR amplification.
[k] One sample positive by virus isolation/ELISA and the other NVD by virus isolation. The latter sample was submitted from a batch containing other FMD virus positive sera (not tested in this evaluation).
[l] RT-PCR result from two probangs just below the borderline level.

Table 2. The results of the 5'-nuclease probe-based RT-PCR procedure using automated nucleic acid extraction and RT programmes (PCR amplification not automated) and ELISA on primary calf thyroid cell cultures inoculated with epithelial suspensions (ES)

Procedure	Ratio of number of samples positive (FMD virus serotype O) or NVD[a] in passages of primary calf thyroid cell culture following inoculation with ES			
	First passage (0-48 hr)		Second passage (48-72 hr)	
	O	NVD	O	NVD
5'-nuclease probe-based RT-PCR	8/8	0/24	NT [b]	NA[c]
ELISA	0[d]	0[d]	8/8	NA
Virus isolation	0/8	0/24	8/8[e]	0/24

[a] NVD, no virus detected.

[b] NT, not tested.

[c] NA, not available for testing. Cell culture supernatant fluid collected after second passage without a recognisable CPE is routinely discarded.

[d] Only the supernatant fluids of cell cultures with a recognisable CPE after inoculation with ES or any other source of antigen are tested by ELISA.

[e] FMD virus specificity confirmed by ELISA.

A NOVEL METHOD FOR DETECTION OF FMDV FROM CULTURE AND CLINICAL SAMPLES BY RT-PCR AND RESTRICTION ENZYME ANALYSIS

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A reverse transcription-PCR (RT-PCR) method has been developed for the highly sensitive and specific detection of all seven serotypes of Foot-and-mouth disease virus (FMDV). A primer pair flanking a region of the viral polimerase gene (3D) corresponding to the C-terminus of the protein was designed and a single step RT-PCR reaction was performed. The assay was validated for detection of viral RNA from a variety of animal samples and from a wide range of FMDV isolates of different origin and serotype. The presence of a conserved *Ahd* I restriction site within the amplicon allows an additional confirmation step of the positive reactions by a simple digestion yielding characteristic fragment sizes. The set of primers described here was suitable for direct sequencing of the PCR product (290 bp), and the nucleotide sequences corresponding to SAT 1 and SAT 3 strains were determined. The segment amplified, when used in phylogenetic studies, allowed the clustering of SAT isolates and the rest of FMDV strains as two separate lineages.

VALIDATION OF A LIGHTCYCLER BASED RT-PCR FOR THE DETECTION OF FOOT-AND-MOUTH DISEASE

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The quick diagnosis of a foot-and-mouth disease infection is very important. In most cases the antigen present in vesicular material is abundant and can be detected by ELISA, but in a few cases, virus isolation is necessary. Primary bovine thyroid cells are considered the best substrate for growing foot-and-mouth disease virus. Other primary cells, or BHK or IBRS-2 cells are also susceptible to the virus. Culturing primary cells and making sure cells are available all the time is very time consuming. Therefore, new techniques like RT-PCR are much easier, and probably cheaper. The disadvantage of PCR techniques is that they have a predisposition to contamination. In most cases caused by the product produced. The LightCycler system, however, detects the product real time in a closed capillary. An RT-PCR for foot-and-mouth disease was developed using the LightCycler system and validated using samples collected during the outbreak and samples from experimentally infected animals.

Analytical sensitivity was determined by diluting a positive sample and testing the dilutions by virus isolation and RT-PCR. To check whether the RT-PCR was able to detect all 7 serotypes, we selected 26 different FMDV isolates covering all 7 serotypes and one Swine Vesicular Disease virus and one Coxsackie B5 virus isolate and tested them in the LightCycler RT-PCR for FMD. Almost every vesicular sample submitted during the last FMD epidemic in the Netherlands was tested by both virus isolation and RT-PCR. Plasma samples collected on two outbreak farms were tested by virus isolation and RT-PCR.

Compared to virus isolation the RT-PCR was 1 to 10 times more sensitive, and detected all 26 different isolates. Both the Swine Vesicular Disease virus and the Coxsackie B5 virus isolate were negative in the RT-PCR. All vesicular samples submitted during the outbreak and found positive by virus isolation were also positive by the RT-PCR. In the RT-PCR, however, we found a positive reaction in the negative control in 12 of the 113 runs. In almost all cases caused by a high positive sample in the same run or the previous run. Many of those high positive samples were not tested by virus isolation because they were already positive by ELISA. Therefore, the chance on false positive results in virus isolation was limited. In a few cases, however, problems with contamination in virus isolation were observed, which led to repetition of the procedure.
60 out of 92 plasma samples collected on outbreak NET 3/2001 were positive by RT-PCR where only 11 samples were positive by virus isolation. On this farm, most animals had high titres of neutralising antibodies; those animals were negative by virus isolation. On outbreak NET 4/2001, however, only 1 animal was positive in the virus neutralisation test, still 23 out of 48 plasma samples were positive by RT-PCR an only 14 by virus isolation.

This validation shows that the LightCycler RT-PCR is a sensitive and reliable technique. False positive reactions are mainly caused by cross-contamination with high positive samples. This problem can be controlled by excluding ELISA positive samples from testing in the RT-PCR.

VALIDATION OF A MONOCLONAL ANTIBODY-BASED ELISA FOR MULTI-SPECIES DETECTION OF ANTIBODIES IN SERUM DIRECTED AGAINST TYPE O FOOT-AND-MOUTH DISEASE VIRUS

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A quick and simple monoclonal antibody based ELISA for the detection of antibodies directed against type O Foot and mouth disease virus (FMDV) and was developed. The ELISA was validated using field sera from cattle, pigs and sheep collected from FMDV-infected and non-infected Dutch farms, reference sera obtained from the World Reference Laboratory for Foot and mouth disease at the Institute for Animal health, Pirbright laboratory U.K., and sera from experimentally infected animals. Testing 2664 sera collected from non-infected cattle, pigs and sheep resulted in a specificity of 96.4%. A sensitivity, relative to the virus neutralisation test (VNT), of 98% was achieved when testing cattle, pig and sheep sera collected from FMDV infected Dutch farms. All international reference sera scored consistently correct. The ELISA scored 459 of 484 VNT positive experimentally derived sera correctly (95%).

Figure 1 shows that setting the cut-off at 50% seems a conservative value, but because several neutralisation test positive sera have a very low percentage inhibition, the sensitivity and specificity are almost equal. Repeated testing of 577 positive and negative sera resulted in a Kappa of 0.94.

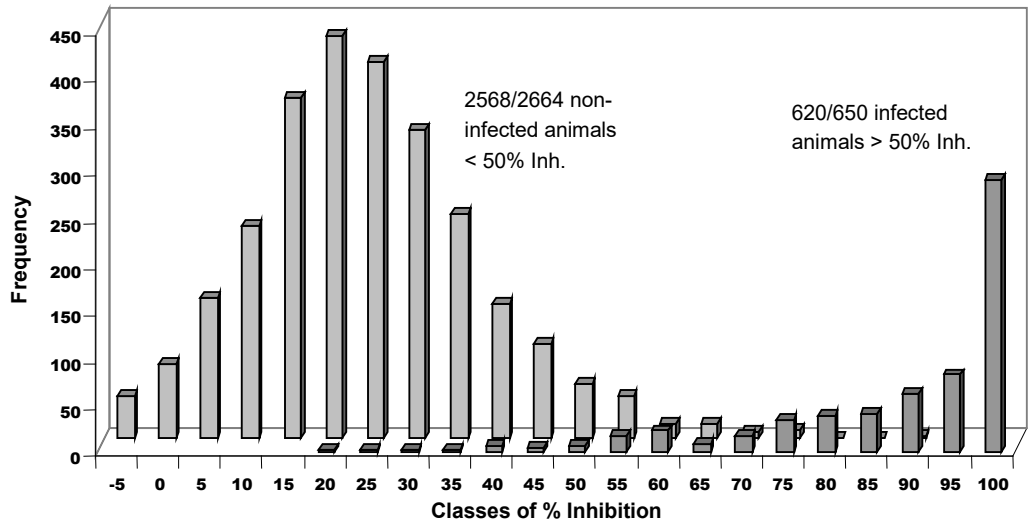

Figure 1. Frequency distribution percentage inhibition of sera (n = 2664) collected from herds free of FMDV serotype O neutralizing antibodies and neutralizing antibody positive sera (n = 650) from infected herds or from animals experimentally vaccinated and /or infected.

The sensitivity, specificity and repeatability of this monoclonal antibody-based ELISA for detection of FMDV type O antibodies is sufficient for the use as a screening ELISA. ELISA positive sera have to be confirmed using the virus neutralisation test.

Appendix 29

QUANTITIES OF INFECTIOUS VIRUS AND VIRAL RNA RECOVERED FROM SHEEP AND CATTLE EXPERIMENTALLY INFECTED WITH FOOT-AND-MOUTH DISEASE VIRUS O UK 2001

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SUMMARY

Foot-and-mouth disease virus (FMDV) can be spread by a variety of mechanisms. The objective of the current study was to obtain aerosol excretion data for the O UK 2001 isolate in sheep and in cattle and to measure the time course of virus load (infectivity and viral RNA) in nasal swabs, rectal swabs and serum to formulate a viral load framework for assessment of transmission risks. Oesophageal-pharyngeal (probang) samples were collected from the sheep at 28 days after exposure to establish whether any of them became persistently infected.

Virus replicated rapidly in inoculated sheep from which a peak infectivity of airborne virus of $10^{4.3}$ $TCID_{50}$ per sheep per 24 hours was recovered. Around 24 hours later contact-infected sheep excreted airborne virus maximally, also around $10^{4.3}$ $TCID_{50}$ per sheep. The peaks of airborne excretion of the inoculated and contact sheep were 24 hours apart, lasted for 24 hours and then fell to below detection limits. Similar peak amounts of airborne virus were recovered from cattle, however, they maintained this level of excretion for about 3 days. The excretion of virus by the sheep fell into four phases. Firstly, a period of high excretion of airborne virus as described above. Secondly, a highly infectious period of 5-7 days, when excretions (nasal swabs and rectal swabs as well as serum) had significant levels of infectivity. Thirdly, a period of a few days (1-3 days) just after the infectious period when low amounts of viral RNA were recovered in nasal and rectal swabs as well as in serum. Fourthly, at 4 weeks when oesophageal-pharyngeal samples showed that 50% of the sheep were carriers.

This data provides a basis for developing a more comprehensive picture of the various transmission risks from livestock, especially sheep, at various stages of the infectious process.

INTRODUCTION

FMD is a viral disease of domesticated and wild ruminants and pigs characterised by the development of vesicles in and around the mouth and on the feet. FMD virus is a member of the *Aphthovirus* genus within the *Picornaviridae* family [4]. FMD is feared by farmers and veterinary authorities because of its highly contagious nature, its ability to cause persistent infection in ruminants (carriers) and the difficulties inherent in eradicating the

virus following outbreaks. Even vaccinated animals may become carriers when exposed to live virus [23]. FMD is most often spread by the movement of infected animals. Next in frequency is spread by contaminated animal products, e.g. milk and meat and infection may also be spread by mechanical means, for example via animal contact with virus on the surfaces of transport vehicles, milking machines or on the hands of animal attendants. An additional mechanism is the spread of FMD virus by the wind[3;12].

The objective of the present investigation was to study the aerosol excretion from sheep infected by the FMDV type O UK 2001 and to provide a quantitative framework for a more detailed analysis of transmission risks from sheep in particular and to a lesser extent from cattle.

METHODS

Animals

Ten female cross-bred sheep weighing around 30 kg were used. The sheep were shorn of their fleeces and placed in a single room in a biosecure animal building. Six "inoculated" sheep, i.e. animals selected at random from the group, were infected by injection of the coronary band. Four "contact" sheep were kept in the same room throughout the experiment. In a subsequent experiment two heifers were used (Holsteins).

The Inoculated sheep received 0.5 ml of FMDV O UKG 2001 inoculated intradermally/subdermally in the coronary band of a left fore foot [5]. Titration of the inoculum showed that each animal had received around $10^{7.5}$ TCID50. The two heifers were inoculated with the same virus by subdermo-lingual injection [19;20].

The animals were examined clinically each day for signs of FMD. Rectal temperatures were recorded daily until 10 days after inoculation (dpi). Blood samples (serum tubes) and nasal and rectal swabs were taken daily for the first 2 weeks after inoculation (only for day 0 to day 3 for the cattle). The blood samples were immediately transported to the laboratory, kept at 4° C for 16-24 hours and the serum separated. An aliquot of serum from each sample were immediately diluted 1:1 with a commercial RNA stabilizer solution (Roche Lysis Solution) and stored at room temperature until nucleic acid extraction and subsequent analysis by real time 5'-nuclease RT-PCR (to be described in detail elsewhere). The rest of the serum was immediately frozen and stored at –80° C. Swabs were taken in duplicate, one swab was placed in 2 ml of maintenance medium and stored at –80° C while the other one was placed in 1 ml of TRIzol (Life Technologies, Paisley, UK) and stored at –80° C. Probang samples were taken from the sheep at 28 days after exposure. These samples were shaken with a buffer and stored frozen at –80° C until analyzed.

The sheep were killed at 28 and the cattle at 3 days post exposure (dpe).

Virus

The virus used was prepared as an original suspension of vesicular epithelium collected from a pig at Brentwood Abattoir, Essex, UK during the 2001 epidemic in the UK. The virus isolate is denoted FMDV O UKG 34/2001. A 10% (w/v) suspension of foot vesicular epithelial tissue lesion was made in MEM-HEPES and stored in aliquots at –80°

C. The titres of this stock virus were $10^{8.8}$ and $10^{7.6}$ $TCID_{50}$ per ml in BTY and IB-RS-2 cells, respectively. Each inoculated sheep received approximately $10^{7.5}$ $TCID_{50}$ (BTY).

Measurement of aerosol excretion of FMDV from sheep and cattle infected with UKG 2001

Samples of the air in the room were collected with a cyclone sampler on the first and second day (sheep) and first and third day (cattle) after inoculation. In addition, a series of pairs of sheep were selected on the same days and placed in a 610 litre cabinet [10] and multiple air samples collected with a May sampler. The inside of the cabinet and the walls and ceiling of the room were sprayed with water before commencing the air sampling to ensure that the relative humidity was high and therefore suitable for the survival of airborne virus [10]. After measurement, the sheep were returned to the box.

Air sampling methods

Air samples collected by Cyclone sampling as well as by May sampling af the exposure cabinet were performed as described previously [1]

The peak of virus excreted was expressed as the total amount of airborne TCID50 per sheep/ or heifer per 24 hours (as measured at 1 and 2 dpi (sheep) and 1 and 3 dpi (cattle).

Assay for virus

The infectivity in the collection fluid from air samplers and in other samples were assayed by inoculation of monolayer cultures of primary bovine thyroid (BTY) cells in roller tubes [25]. The specificity of the cytopathic effect observed in cell cultures was confirmed by antigen ELISA [15;17;22].

Quantitative RT-PCR. Quantitative reverse transcription polymerase chain reaction (RT-PCR) was used to determine the amount of FMDV RNA in extracts of total nucleic acid from blood and swab samples. The assay method was similar to that used previously [2;21] to quantitate the amounts of FMDV RNA in tissues from pigs infected with the O_1 Lausanne virus. The method used in the current studies involved an almost identical protocol, however, the primers and the probe (patent pending) were changed so that the assay was able to detect all serotypes of FMDV (Reid and others, This meeting!). The specific conditions used will be published in detail elsewhere. All extractions involved 0.100 ml of sample and the nucleic acid were finally eluted in a volume of 0.1 ml. Thus, the nucleic acid was more dilute than in earlier investigations [2;21], however, the extraction method had several advantages over the manual method. Firstly, the extraction was very consistent and gave highly reproducible results. Furthermore, because the samples were more dilute, and of a much higher purity than our previously extractions, only a single dilution was assayed (i.e. an amount corresponding to 0.003 ml of initial sample in a single RT-PCR).

All estimations included standard reactions using samples with a known content of FMDV (as determined by virus titration in cell culture), and furthermore all quantitations were based on a comparison with standard curves based on dilution series of infected cell culture supernatant as described in detail previously [2;21]. The method is influenced minimally by sample type

Assay for antibodies
Serum samples were tested by an enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to FMD virus [14;18].

RESULTS

Airborne virus recovery and estimated aerosol excretion and exposure doses
The amount of virus in air samples are summarized in Table 1 (sheep) and Table 2 (cattle).

In brief, the data shown can be summarized as follows:
Excretion of airborne virus was calculated as the average excretion from infected sheep at 1 and 2 dpi after inoculation and from infected cattle at 1 and 3 dpi. Thus, excretion of FMDV O UKG was up to $10^{4.3}$ TCID50 per 24 hour per sheep (weighing about 30-40 kg) and up to $10^{4.3}$ TCID50 per heifer (150 kg).

Therefore the amounts of airborne virus emitted by the two species were very similar, although the heifers were probably around 150 kg and the sheep were only around 30-40 kg. However, the data for the sheep may indicate that peak aerosol excretion lasts for only a single day, while in cattle significant amounts of virus could be detected for 3 days.

Clinical signs, virus load in serum and swabs, and seroconversion
Among the inoculated sheep, 4 animals showed signs of local vesicular lesions (unruptured) at the injection sites as well as increased temperature of one or more additional feet (generalization) by 1 dpi. One sheep had local lesions on day one and generalisation to several feet by day 2. Fever, defined as a temperature above 40^0 C, lasted from 1-2 days and up to 5 days and was detected in 2 sheep at day 1, 6 sheep at day 2, 4 sheep at day 3, 3 sheep at day 4 and a single sheep on 5 dpi. The average time for development of fever was at day 2 in the inoculated group. At day 2 one or two sheep from the contact group showed signs of lameness while on day 3 a third sheep had increased temperature of a foot and became lame on day 4. Fever (temperature above 40° C) lasted from 1 to 3 days in this group, one animal having fever at day 3 only, one on days 3-5, one on day 4 and the last sheep on day 5 only (after inoculation of the injected sheep). The average time for fever was at day 4 in this contact group. The first time that lesions, increased temperature or clinical signs of disease were observed was on average 1.4 days for the inoculated group and around 3 days for the contact group.
The cattle showed only mild signs of disease and a minor temperature increase. At 3 days after inoculation the 2 heifers were killed. Examination showed severe local lesions on the tongue (ruptured vesicles) but only mild additional lesions, in the form of small, ruptured vesicles on the dental pad. In addition one animal had 3 feet and the other animal had 2 feet with ruptured, small vesicles.

Viraemia in the inoculated sheep was detectable from day 1 and reached peak values of around $10^{4.5}$ TCID50/ml at 2 to 3 dpi. All of the inoculated sheep ceased to have a viraemia by days 5-8. Among the contact animals a single sheep had a very low level of viraemia on day 1 and this animal and one more had a slightly increased level at day 2, however, high levels could not be detected in the contacts before day 3 and peaked, again at around $10^{4.5}$ TCID50/ml, on day 4. All of the contact sheep ceased to have a viraemia

by day 8. However, in both groups, but most pronounced in the inoculated group, low levels of FMDV RNA were detected in sera between day 9 and 11. None of the 10 sheep had detectable viraemia at day 12, 13 or 28. However, the signals in the RT-PCR on the day 9-11 serum samples corresponded to very low FMDV genome levels, most likely below the detection limit of cell culture. The results were repeatable and could be seen in both serum and swab samples.

Peak viraemias in both groups correlated strongly with elevated body temperatures above 40^0 C, which peaked at day 2 and at day 4 for the inoculated and the contact sheep, respectively.

Initially the correlation between the virus titres of swab samples on BTY cell cultures was compared to TaqMan RT-PCR by testing 40 nasal swabs (taken at 3, 4, 5 and 8 dpi). Correlation was obvious in samples with more than $10^{2.0}$ TCID of virus per ml, however, samples with little or no detectable live virus were often positive in the RT-PCR and this assay appears to deliver the most accurate quantitation of viral load, even though the virus genome detected may not necessarily be infectious. Nevertheless, infectivity and RT-PCR reactivity were strongly correlated for the day 3, 4 and 5 samples. On day 8 all samples were negative for infectivity (below detection limit) but several had a low reaction in RT-PCR. This indicated that the correlation between the assays was strong and that samples being weak positive in RT-PCR but negative in cell culture are just below the detection limit of that particular assay. For the rectal swabs a linear correlation between infectivity and RT-PCR signal was moderately strong, however, the infectivity of these samples was clearly much lower than the nasal swabs, even for samples having a comparable signal in RT-PCR. This suggested, that the alimentary passage of the virus had partially inactivated its infectivity, or alternatively, that the fecal swabs contain some material reducing the sensitivity of the cell culture system.

Samples from the two cattle were also examined. Nasal swabs were positive on day 1-3 after infection at a level corresponding to 10^4 to 10^5 TCID50 of virus per ml. This value fits well with the findings for the day 3 and 4 sheep samples and is at a level similar to that found in probang samples or in serum from the same heifers at day 1-3 after infection (data not shown).

Antibodies could be detected in inoculated sheep from day 4 and reached high levels by day 7. Contact sheep were positive from day 6 and reached high levels by day 8.

Carriers The 10 sheep were tested for persistent infection at 4 weeks after exposure. Three sheep were positive (virus isolated from OP-fluid and positive in RT-PCR) while 2 sheep had a low reaction in the RT-PCR but were negative by virus isolation. Thus, by virus isolation 3 sheep were definitively carriers, and the more sensitive technique of RT-PCR indicated that a total of 5 sheep had detectable levels of FMDV RNA in probang samples taken at 4 weeks after inoculation or exposure. Interestingly, all the serum samples and all the nasal swabs were negative at his time point, however, one out of 10 sheep had a rectal swab positive by RT-PCR at 4 weeks. This particular animal was positive by both virus isolation and RT-PCR on the OP-fluid and thus was a carrier. Interestingly, dividing the results of the RT-PCR analysis of the10 sheep into two groups of 5 sheep (5 carriers and 5 non-carriers) indicate, that the average number of days detected positive were higher in the carrier group for both nasal and rectal swabs and

furthermore, that the average peak levels in these swabs also were higher for the carriers. The differences between these two groups in regard to the levels in the serum were similar although much less pronounced. Average viraemia was only slightly longer in the carriers, however, the average peak viraemia was increased by approximately 4-fold.

DISCUSSION

The experiments described in the current study confirmed that sheep excrete airborne FMDV at maximal levels early in the infection (approximately one day after inoculation and for contact sheep the day afterwards). The maximum amount of virus excreted for the UKG 2001 FMDV corresponds to around $10^{4.3}$ TCID50 in a 24 hour period per sheep (approximately 30 kg). However, peak excretion apparently only lasts for 1 day. The aerosol excretion from infected cattle was similar, i.e. a maximum of $10^{4.3}$ TCID50 in a 24 hour period per heifer (approximately 150 kg), however, in cattle excretion continued at high level for another 2 days. The maximum level of $10^{4.3}$ TCID50 in a 24 hour period is significantly less than what is excreted from pigs infected with this isolate of FMDV. In pigs, excretion up to $10^{6.1}$ TCID50 in a 24 hour period per 90-100 kg pig has been described for this particular isolate [8;9], equivalent to an aerosol excretion from pigs approximately 60-fold higher than for sheep and cattle.

Investigations during the UK 2001 epidemic (R. P. Kitching, S Alexandersen and others, unpublished results) have shown that the disease progressed slowly in sheep and that evidence from the field may indicate, that only about 5% or less of the sheep in a flock were infected after several weeks, while in pig herds and cattle herds up to 40-50% of the animals had disease and were excreting FMDV at the same time. The total aerosol excretion from an affected pig farm can be estimated by thorough clinical investigations and calculated on the basis of the excretion values for the UKG 2001 FMDV. The same could be done with cattle premises – although excretion is much lower. For sheep, it is more difficult to make estimates of the maximum excretion levels by sheep flocks due to the cryptic nature of FMD in that species, but the amounts are likely to be very low due to the slow progression of the disease and the short, sharp aerosol excretion period.

This relatively low level of airborne excretion from sheep and cattle confirm previous work [6;7;11;13;16;24] suggesting that these species only play a minor role in airborne spread between farms. However, the experiments also showed, that although unlikely to be involved in distance transmission, sheep may easily cause aerosol transmission under local conditions especially when high density housing conditions is used.

For the sheep the peak aerosol excretion apparently only lasted a single day (then fell to levels below our detection limit). This will of course have an impact on the ability to transmit disease. Thus, if an infected animal is not in relatively close contact with other sheep at that particular day, risk of aerosol transmission is greatly reduced. However, as the day of peak excretion is very early in the infection, and before any clinical signs can be noticed, it is very difficult to control this mode of spread in sheep.

In order to explain the variable nature of FMDV transmission in sheep, we propose a dual mechanism to be part of the explanation for the variable spread of FMDV in sheep. An amount of virus responsible for infection may alternatively to coming from airborne virus, come from excretions and be internalized by close direct or indirect contact to infected

sheep, as an amount of infectious virus in the period from 3 to 7 days after inoculation or contact of 10^3-10^4 TCID50 may equal the amount of nasal fluid being taken up by a single swab. However, this amount may need to somehow enter by the respiratory/aerosol route instead of the oral route, in order to be sufficient to cause infection. However, it may be possible, that nasal fluid as well as saliva or perhaps OP-fluid, which all are produced and drooled in large amounts during acute infection, can cause infection if directly or indirectly deposited onto traumatized skin. Nevertheless, as the rectal swabs contained significantly less infectious virus it is considered unlikely to be a major vehicle of spread during an epidemic, however, feces can not be excluded as a potential risk considering the large amounts produced and the finding of, albeit low levels, live virus. Taken together, the findings indicate, that sheep excrete airborne FMDV very early in the infectious process, under the conditions described here the day after inoculation or approximately one day after contact exposure, furthermore, aerosol excretion is only measurable on a single day. Thus, efficient spread of the disease will only occur when contact among excreters and non-infected sheep are widespread and close and are likely to be enhanced by housing, i.e. by maximizing the concentration of aerosolized virus. Rather high amounts of virus infectivity is found in the nasal swabs even at times where airborne excretion has stopped. However, it seems possible, that in situations with occasional close contact, sheep may be infected by close physical contact to infected sheep, most likely in the period from 2-7 days after infection. If spread by indirect means (indirect contact) is to be considered during this period we expect it to be of a relatively low risk unless the contact involve physical handling of a susceptible animal, resulting in exposure to damaged skin. From day 8 and later, we did not isolate any live virus (below detection limit) although low levels of virus RNA could be detected in a number of samples from day 8 to 14. On day 28 virus was isolated from the OP-fluid of 3 sheep and these three samples as well as two additional samples were positive in RT-PCR.

The results on viral loads in the sheep indicated that viraemia were detectable from day 1 and reached peak values of around $10^{4.5}$ TCID50/ml at day 2 to 3 after direct inoculation. Of the contact animals a single sheep had a very low level of viraemia on day 1 and this animal and one more had a slightly increased level at day 2, however, high levels could not be detected in the contacts before day 3 and peaked, again around $10^{4.5}$ TCID50/ml, at day 4. Thus, the level of aerosol excretion can not be correlated to the viraemia levels, apparently the aerosol excretion peaks before viraemia while the data suggest that the nasal excretion (swabs) may peak after the viraemia. In both groups, but most pronounced in the inoculated group, positive reactions could be found using the RT-PCR . It should be mentioned, that the signals on the day 9-11 serum samples corresponded to very low FMDV genome levels, most likely below the detection limit of cell culture. We conclude that a low level viraemia (copies of FMDV RNA) can be seen in infected animals after the first peak is cleared by the antibody reaction. Similar minor peaks could also be observed in the swabs. The mechanisms and potential importance is currently unknown.

The virus load found in the nasal and rectal swabs indicated that especially the nasal tract contained significant amounts of virus while the rectal swabs albeit often being positive by RT-PCR, often were negative or very low regarding infectivity. Interestingly, as we have suggested before [2] the exact correlation of signal in our quantitative RT-PCR assay is only directly proportional to samples taken in early infection, i.e. up to about day 5 after exposure, when the host reaction, including antibody being produced, diminish infectivity

with a comparable slower fall in RT-PCR reactivity. However, evidently the correlation of the two methods on rectal swabs indicated a more intense decrease in infectivity in such samples.

The development of antibodies at days 4 and 6 in inoculated and contact sheep, respectively, fits well with the observed sharp decrease in viraemia. However, it appears, that the decrease caused by antibodies is more pronounced in the blood (serum) than observed in for instance the nasal swabs. A reduced decrease in virus load despite development of antibodies has previously been suggested for epithelial lesions in pigs [2].

As mentioned above, virus isolation showed that 3 sheep were definitively carriers, and the more sensitive technique of RT-PCR indicated that a total of 5 sheep had detectable levels of FMDV RNA in probang samples taken at 4 weeks after inoculation or exposure. Interestingly, all the serum samples and all the nasal swabs were negative at his time. Interestingly, dividing the results of the RT-PCR analysis of the 10 sheep into two groups of 5 sheep (5 carriers and 5 non-carries) indicate, that the average number of days with swabs detected as positive were significantly higher in the carrier group for both nasal (increased with 3.5 days) and rectal swabs (increased with around 2 days) and furthermore, the average peak levels in these swabs were also higher for the carriers. The differences between these two groups in regard to their serum samples was less pronounced than for the swabs. Average viraemia was only slightly longer in the carriers (increased by less than 1 day), however the average peak viraemia was increased as for the swab samples (an average increase of about 4-fold). Thus, these studies indicate, that there is a direct correlation between peak viral load and duration of the viral load in serum and swabs on the subsequent development of carrier sheep.

The profile of infectiousness of FMD in sheep has been established based on a quantitative real time RT-PCR combined with virus titration of selected samples. Under our experimental conditions the curve of infectiousness was short and showing that when there is a high contact rate between sheep, transmission will occur rapidly, most likely by inhalation of aerosolized virus. The evidence that this does not always occur under field conditions is probably a reflection of the management conditions. Management activities which will increase the direct contact rate, and therefore transmission are for example housing for lambing or in connection with transport. Other conditions increasing direct contact are shearing and de-worming, the highest risk in this setting probably being the contamination of potential virus containing material onto damaged epithelium. Thus, transmission in sheep may have very different outcomes depending on the specific husbandry of a certain premises or a whole area. Thus, under intensive husbandry, sheep are likely to be kept at a high density and often the sheep spend at least part of their time inside. Thus, in such a system the possibilities for FMD transmission are maximal. However, in less intensive systems this may be very different. Stocking density is lower and most sheep will spend all/almost all time outside without solid housing. In such a system significant aerosol transmission is not very likely, because the potential concentration of FMDV in air never reach the minimal infective dosis (MID) level. Thus, it appears more likely, that in such cases, transmission will be much slower, and that the mechanism will involve contact transmission via infected excretions, as for instance vesicular fluid, nasal fluid or saliva/drool. However, it should be pointed out, that at the time of for example peak viral levels in nasal fluid the sheep, described in this experiment, had shown clinical signs of FMD, including lameness, and have had

detectable vireamia for several days. In other words, while sheep to sheep aerosol transmission (short range) is difficult to control because airborne excretion occur before clinical signs; it is most likely that transmission by virus-containing excretions later in the infectious process could in fact be minimized/controlled provided thorough examination of all sheep before and after movement.

In conclusion, available evidence suggests that conditions facilitating aerosol transmission, i.e., high animal density and closed housing with limited air-change, favor fast transmission in sheep, while, in contrast, conditions decreasing aerosol transmission, i.e., low density, out-door sheep herds with little contact among different groups of sheep, favor slow transmission among sheep.

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TABLE 1. Doses of airborne virus excreted by the infected sheep.

Group	Air sample	TCID/litre air	Excretion/amount present 24 hours TCID50
UI 97 UJ 03	May 1 Sample at 1 day pi	0.7	4.74 for 2 sheep
UJ 01 UJ 00	May 2 Sample at 1 day pi	0.27	4.34 for 2 sheep
UI 97 UJ 03	May 3 sample at 2 day pi	neg	ND
UI 96 UI 99	May 4 sample at 2 day pi	neg	ND
All 10 sheep	Cyclone 1 Sample at day 1 pi	neg	ND
All 10 sheep	Cyclone 2 Sample at day 2 pi	0.2	4.7 (likely from 4 contacts)

Average aerosol excretion of $10^{4.3}$ TCID50/24 hour/sheep at 1 day after inoculation and below detectable levels on day 2.

$10^{4.7}$ TCID50 for the box with 4 contact sheep (likely to excrete virus) equals $10^{4.1}$ TCID50 per (contact) sheep as measured in the box (reduced by air filtration) and thus to be equivalent to about $10^{4.5}$ TCID50 per 24 hour per contact sheep around day 2 (i.e. 1 day after the inoculated sheep).

TABLE 2. Doses of airborne virus excreted by the infected cattle.

	Collected in 20 min	in litre air	Estim. 24 hours	Average per cattle with Box added (0.5)	
Both cattle Cyclone 1 (PID 1):	252 TCID	3400	4.3		
Both cattle Cyclone 2 (PID 1):	100	3400	3.9	4.35	logs tcid/24h/cattle
Both cattle Cyclone 3 (PID 3):	252	3400	4.3		
Both cattle Cyclone 2 (PID 3):	<30	3400	<3.3	4.24	logs tcid/24h/cattle

Cyclone average per cattle is 4.24-4.35 logs per 24 hour per cattle, highest on day 1, i.e. 4.3 logs as for sheep, and slightly lower on day day 3, i.e. around 4.2 logs per 24 hours.

Appendix 30

FURTHER STUDIES TO QUANTIFY THE DOSE OF NATURAL AEROSOLS OF FOOT-AND-MOUTH DISEASE VIRUS FOR PIGS

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SUMMARY

Foot-and-mouth disease (FMD) can be spread by a variety of mechanisms, including, under certain climatic and epidemiological circumstances, by the wind. While the quantities of airborne virus excreted by animals infected with historical strains of the virus have been determined there is relatively little information for contemporary strains and furthermore, the aerosol MID for pigs needs to be more accurately quantified. The objective of the study was to obtain data for the O_1 Lausanne (1965), O SKR 2000, O UKG 2001, and C Noville (1973) strains of FMDV to enhance the capability of airborne virus simulation models.

The collection of air samples near pigs infected with these strains has shown that the amount of virus (in $TCID_{50}$) emitted per pig per 24 hours was: $10^{5.8}$ for O SKR 2000; $10^{6.1}$ for O UKG 2001; $10^{6.4}$ for O Lausanne; and $10^{7.6}$ for C Noville.

The results indicate that the previous estimate of "above" 800 $TCID_{50}$ as the MID50 for the O_1 Lausanne strain was a considerable under-estimate and that the actual dose may be as high as 6000 $TCID_{50}$. A dose of around 650 $TCID_{50}$ of the O SKR 2000 strain failed to infect any pigs. Pairs of recipient pigs kept physically separated from donor pigs and exposed to aerosol doses of around 50 $TCID_{50}$ per minute of the O UKG 2001 strain or 130 TCID50 per minute of the C Noville virus over 24-48 hour periods failed to infect any of eight pigs exposed to O UKG 2001 and only resulted in a transient antibody reaction (subclinical infection) in one out of 8 pigs exposed to C Noville. These results confirm previous findings that pigs, compared to cattle and sheep, are relatively resistant to infection by airborne FMDV.

INTRODUCTION

FMD is most often spread by the movement of infected animals. Next in frequency is spread by contaminated animal products, e.g. milk and meat. Infection may also be spread mechanically, for example by virus on vehicles, milking machines or on the hands of animal attendants. An additional mechanism is the spread of virus on the wind. This occurs infrequently as it requires particular climatic and epidemiological conditions [2;12].

The determination of the biological parameters of the airborne spread of FMD such as virus excretion, airborne virus survival, the quantitation of minimal infectious doses and the marrying of those factors with the physical determinants of airborne particle diffusion has provided the basis for the development of models which can predict the risk of airborne spread of FMD [6;9;11-13;16;17;21;24;25;27]. A parameter which has not been quantified in sufficient detail, although the subject of recent preliminary findings [1;7], is the minimal infectious dose 50% (MID_{50}) of airborne FMD virus needed to infect pigs.

The objective of the present investigation was to expand data for the MID50 of airborne virus using additional strains of FMD virus delivered to pigs as natural aerosols as well as modified exposure arrangements making it possible to deliver high doses of virus to recipient pigs. We have extended the previous studies with the O_1 Lausanne strain and added two contempory strains of FMD virus, the O SKR 2000 and the O UKG 2001 strains (both members of the type O PanAsia group of strains) as well as the historical serotype C Noville (Swiss 73), known to cause excretion of airborne virus at high levels [10].

METHODS

Animals

The pigs were Landrace cross-bred Large White weighing between 20 and 30 kg. Four separate experiments were done. Three "donor" pigs, i.e. animals selected from a group of four inoculated animals as a source of natural aerosols of FMD virus, and eight or ten "recipient" pigs, i.e. animals exposed to airborne FMD virus, were used in each of Experiments 1 and 2. In Experiment 3, a total of 5 pigs were inoculated and then transferred each to a cubicle containing an uninoculated pig in a series of rooms. In the other cubicle in each of the rooms were 2 recipient pigs. Thus, there was direct contact between the inoculated and contact pigs, while the recipient pigs were exposed to aerosol virus generated within the room. Four pigs located in room 3 of Experiment 3 were excluded from the results because on two occasions a recipient pig managed to escape from its cubicle and climb into the cubicle with the donor pigs. Thus, this animal was potentially exposed to direct transmission. Therefore, the results from Experiment 3 consist of the results from 4 donor pigs, 4 direct contacts and 8 recipient pigs. Experiment 4 was performed identical to experiment 3, although using the C Noville inoculum. Also for this experiment a single pig in one of the groups managed to escape from the cubicle, and thus that box were excluded from the experiment. Therefore, the results from Experiment 4 also consist of the results from 4 donor pigs, 4 direct contacts and 8 recipient pigs.

All pigs were housed within cubicles in isolation rooms of a biosecure animal building and inoculated as described previously [1] with approximately 0.5 ml of stock virus O_1 Lausanne for Expt. 1, stock virus O SKR 2000 for Expt. 2, stock virus O UKG 34/2001

for Expt. 3 and stock virus C Noville (Swiss 73) for exp. 4. All the inocula were diluted 1:10 in MEM-HEPES (Eagle's Minimal Essential Medium with 20 mM HEPES buffer and x2 antibiotics). Titration of the inocula showed that each animal received around $10^{5.5}$ BTY $TCID_{50}$ of the O Lausanne inoculum, around $10^{5.5}$ $TCID_{50}$ of the O SKR 2000 inoculum or around $10^{7.5}$ $TCID_{50}$ of the O UKG 34/2001 isolate and the C Noville virus.

A clinical examination of the donor pigs for signs of FMD was carried out at least once and sometimes twice per day. Rectal temperatures were recorded daily. When early signs of generalised vesicular disease were present (2 or 3 days after inoculation) three pigs (Expt. 1 and 2) were selected as donors, removed and placed in an aerosol production chamber located in the corridor outside the room. Donor pigs were killed soon after they had been removed from the aerosol production chamber (Expt. 1 and 2) or for 24 to 48 hours after showing the first vesicular lesions (Expt. 3 and 4).

Recipient pigs were housed singly (Expt. 1 and 2) or in pairs (Expt. 3 and 4) in cubicles constructed within biosecure isolation rooms as described previously [1].

After each recipient pig had been exposed to airborne virus (Expt. 1 and 2) it was returned to its cubicle and examined daily for signs of FMD over a three-week period. For Expt. 3 and 4 the recipient pigs were not exposed in the chamber, instead they were exposed to the virus emitted over a 24 to 48 hour period by the inoculated and contact donor pigs in the other cubicle in the room. The pigs were not handled except on the occasions when blood samples were being collected. Any animal which developed clinical signs of FMD was killed immediately, otherwise they were killed at the end of the experiments i.e. at 20 or 21 days post exposure (dpe).

Virus

The O_1 Lausanne Sw/65 strain of FMD virus was used. It had been passed in cattle and then grown in IB-RS-2 cells [4;5]. The titre of this stock virus was $10^{6.7}$ $TCID_{50}$ when assayed in primary bovine thyroid (BTY) cells and $10^{5.7}$ $TCID_{50}$ in IB-RS-2 cells. This stock virus was used for Expt. 1 and is the same O_1 Lausanne inoculum as used previously [1].

The virus used for Expt. 2 was prepared by passing an original epithelial suspension of isolate O SKR 1/2000 three times in pigs. The titres of this stock virus were $10^{6.45}$ and $10^{5.7}$ $TCID_{50}$ per ml in BTY and IB-RS-2 cells, respectively.

The virus used for Expt. 3 was prepared as an original suspension of vesicular epithelium collected from a pig at Brentwood Abattoir, Essex, UK during the 2001 epidemic in the UK. The virus isolate is denoted FMDV O UKG 34/2001. A 10% (w/v) suspension of foot vesicular epithelial tissue lesion was made in MEM-HEPES and stored in aliquots at –70° C. The titres of this stock virus were $10^{8.8}$ and $10^{7.6}$ $TCID_{50}$ per ml in BTY and IB-RS-2 cells, respectively.

The virus used for experiment 4 was original 1st cell culture passage (BTY cells) of field material (Swiss 73) kept frozen for many years. Approximately 10^4 TCID50 were inoculated into a single pig, and at day 3 severe disease was evident. A virus stock was prepared from foot epithelial lesions. The titer of this C Noville inoculum was 10^9 TCID/ml.

Exposure of pigs to natural aerosols of FMD virus

The procedures used were modifications of those described previously for both pigs, cattle and sheep [1;11;16]. In brief, three donor pigs were selected at 2 to 3 dpi when they had signs of early generalised FMD and placed in the aerosol production chamber [10]. The chamber was then disinfected on the outside and moved to the other end of the corridor where 2 exposure masks connected to 30 cm long, 2.5 cm wide tubing were attached to its side.

Before exposure to airborne virus a pair of recipient pigs were sedated by injection with Propofol as descibed previously [1]. The pair of sedated recipient pigs were then connected to the chamber via the exposure masks and allowed to inhale airborne virus for 5 min. During the exposure period the transmission tunnel used in previous experiments [1] was disconnected from the cabinet so the only fresh air drawn into the cabinet was that which entered through a small hole in one side of the chamber. The resulting challenge concentrations of airborne virus were much higher than in the previous experiments. After exposure to virus the recipient pigs were transferred to individual cubicles in biosecure isolation rooms [1]. Two experiments (1 and 2), using a series of 8 and 10 pigs in each, respectively, were performed. In the interval between the exposure of each pair of recipient pigs fresh air was drawn through the cabinet by connecting it to wide-bore ducting secured just beneath the filter housing of an extractor air vent in the ceiling of the corridor.

The amount of air inspired during the exposure period was based on previous experiments, which showed that the average volume of air inspired by a pig under these experimental conditions (measured by an ultrasonic flowmeter) was around 0.6 liter air per kg pig per minute. This estimate was based on the individual measurement of 39 pigs of 20-30 kg of weight [1].

The experimental design for Expt. 3 and 4 was different. Recipient pigs, two per cubicle, in a series of 4-5 isolation rooms were exposed to airborne virus generated by a pair of inoculated/direct contact pigs in the other cubicles in the rooms. The inoculated/direct contact pigs were present in the rooms from when the donor pigs were inoculated until 24 to 48 hours after they had developed clinical signs. Both donor pigs were then removed and killed. The amounts of virus in the air to which recipients were exposed were estimated by collecting air samples using a cyclone sampler as well as by placing donor (inoculated and contact) pigs in the cabinet described above and collecting multiple air samples with a 3-stage (May) sampler.

After exposure, each recipient pig was returned to its cubicle (Expt. 1 and 2) or left in the cubicle (Expt. 3 and 4) and observed daily for signs of FMD. In order to avoid mechanical transfer of virus the pigs were only handled when blood samples were collected or when they developed signs of FMD. Any recipient pig which developed signs of FMD was removed from its cubicle and killed. Blood samples were collected from recipient pigs at 14 and 20 or 21 dpe.

Air sampling methods
Cyclone sampling of the animal boxes and May sampling af the exposure cabinet were performed as described previously [1]. In Expt. 3 and 4 air samples were also collected from 2 isolation rooms each containing exposed animals. Sampling was done with all-glass cyclone samplers operating for 2 min (Expt. 1 and 2) or 20 min (Expt. 3 and 4) at a sampling rate of around 170 litres/min [16].

During the exposure of each pair of recipient pigs (Expt. 1and 2) an air sample was collected from the aerosol production cabinet using a 3-stage liquid impinger [22]. In Expt. 3 and 4 two samples were collected from the cabinet with the same sampler when 3 donor pigs taken from each of two isolation rooms were placed in it.

Assay for virus
The infectivity in the collection fluid from air samplers and in blood samples were assayed by inoculation of monolayer cultures of primary bovine thyroid (BTY) cells in roller tubes [26]. The specificity of the cytopathic effect observed in cell cultures was confirmed by antigen ELISA [15;18;23].

Assay for antibodies
Serum samples were tested by an enzyme-linked immunosorbent assay (ELISA) for the presence of antibodies to FMD virus [14;19]. Positive samples were confirmed by virus neutralization test.

RESULTS

Airborne virus recovery and estimated respiration and exposure doses
The average concentration of virus in the air, the average dose inhaled by the pigs and the dose excreted as airborne virus per pig in each experiment are shown in Table 1.

Based on the excretion of airborne virus from the donor pigs, we have calculated that the average excretion of FMDV O_1 Lausanne equals $10^{6.4}$ $TCID_{50}$ per 24 hour period per adult pig (NB, calculated as a pig of around 90-100 kg, which equates to three small donor pigs). The excretion of O SKR 2000 averaged $10^{5.8}$ and the O UKG strain $10^{6.1}$ $TCID_{50}$ per 24 hour per pig (90-100kg). The airborne excretion of the C Noville virus was clearly the highest, i.e. $10^{7.6}$ TCID50 per pig per 24 hours. In Expt. 3 the amount of virus to which recipient pigs were exposed equated to $10^{5.5}$ $TCID_{50}$ and in expt. 4 to $10^{6.5}$ $TCID_{50}$ per 24 hour per room. This lower challenge dose was due to the continued operation of the ventilation in the rooms which was around 3-5 air-changes per hour.

Clinical signs, viraemia and seroconversion
The only recipient pig which developed clinical FMD was No. UG 77 in Expt. 1. At 4 dpe it was lame and showed vesicles on the snout and on the coronary bands of the feet. It was killed immediately. Post-mortem examination showed that it had vesicular lesions on all four feet, the gingival mucosa, the tongue and snout.

None of the other 7 recipient pigs in Expt. 1 nor any of those in Expt. 2 or 3 developed signs of disease. Blood samples taken at 7, 10, 14 and 21 dpe (Expt. 1) showed antibodies to FMD virus in 4 out of the remaining 7 recipients, specifically at 10 or 14 dpe . Thus, of 8 pigs exposed to a very high dose of virus (Table 1), one developed

typical signs of FMD and four were subclinically infected. Interestingly, by 21 dpe those pigs were negative for serum antibody, indicating, as seen previously [3], that they had experienced an infection of very short duration.

Antibodies were not detected in any of the recipient pigs in Expt. 2 and 3 (data not shown), except for the excluded single pig (UJ 28) in Expt. 3 which had been in direct contact with the inoculated donor pig.

In expt. 4 a single recipient pig had a transient, weak antibody reaction at 14 dpe. This pig did not show any clinical signs of disease and had no vesicular lesions and thus was subclinically infected.

In all, the Results can be summarized as follows:

Expt. 1: 8 pigs receiving an average dose of 1700 $TCID_{50}$ during a 5 min exposure period (340 $TCID_{50}$ per minute). One pig developed clinical FMD, 4 pigs were subclinically infected and 3 remained normal. Thus, the MID_{50} dose to subclinically infect the pigs in this experiment with the O_1 Lausanne strain of virus is even higher than the dose reported in an earlier study [1] and may be around 1500 $TCID_{50}$ (calculated after Kärber (as described in [20])). The dose to cause clinical disease may be as high as 4000 to 6000 $TCID_{50}$ when given during a 5 min period.

Expt. 2: 10 pigs received an average dose of 650 $TCID_{50}$ during a 5 min exposure period (130 $TCID_{50}$ per minute). None of the pigs developed FMD nor detectable antibodies. Since none of the pigs developed infection or disease, it is difficult to calculate an accurate MID for the O SKR 2000 strain. However, from the limited data, it appears that the MID_{50} dose to cause either subclinical infection or disease is likely to be more than 1000 $TCID_{50}$ for this strain and is likely to be as high or higher than the O_1 Lausanne isolate.

Expt. 3: 8 pigs receiving an average dose of 50 $TCID_{50}$ per min for at least 24 hours which equates to an accumulated dose of more than 70 000 $TCID_{50}$. None of the pigs developed FMD or detectable antibodies. Thus, when accumulated over a 24 hour period, it seems that the MID_{50} dose to infect pigs with the UKG isolate may be higher than 70 000 $TCID_{50}$. Thus, a concentration of around 2500 $TCID_{50}$ per m^3 (as found in this experiment) is apparently not sufficient to infect pigs with this strain, even when they were exposed for 24 hours or more.

Expt. 4: 8 pigs receiving an average dose of 130 $TCID_{50}$ per min for at least 24 hours which equates to an accumulated dose of more than 200 000 $TCID_{50}$. Only one of the pigs developed a transient antibody response, but no clinical disease. Thus, when accumulated over a 24 hour period, it seems that the MID_{50} dose to infect and cause disease in pigs with the C Noville isolate may be higher than 200 000 $TCID_{50}$. Thus, a concentration of around 6000 $TCID_{50}$ per m^3 (as found in this experiment) is apparently only sufficient to subclinically infect a low proportion of pigs with this strain, even when they were exposed for 24 hours or more.

DISCUSSION

The primary objective of this study was to define more accurately the minimal infectious dose (MID) for pigs of FMDV inhaled as a natural aerosol. Although we have previously determined the dose for the O_1 Lausanne strain, [1] only one pig in those studies developed clinical disease so more studies were needed with higher challenge doses and with different strains of virus.

The results show that of the 26 pigs exposed to airborne virus in Expt. 1 to 3, four were subclinically infected and only one developed typical signs of FMD. The infected pigs were in the group exposed to the O_1 Lausanne strain (Expt. 1) and calculated to have inspired around 340 $TCID_{50}$ per minute for 5 min. This dose is 5 to 10 times greater than in earlier experiments with the same strain when pigs inspired around 30 $TCID_{50}$ per minute for 10 min [1]. Thus, the previously estimated MID_{50} value of above 800 $TCID_{50}$ may have been a considerable underestimation and the real value could be much higher, perhaps as high as 6000 $TCID_{50}$. Pigs exposed to the O SKR 2000 strain and calculated to have inspired about 130 $TCID_{50}$ per minute for 5 min did not develop clinical disease or subclinical infection. However, because donor pigs infected with the O SKR 2000 strain excreted relatively little virus ($10^{5.8}$ log $TCID_{50}$ per 24 hour per pig compared to $10^{6.4}$ $TCID_{50}$ for the O_1 Lausanne virus) we were unable to increase the exposure concentration for the O SKR 2000 strain. In Expt. 3 donor pigs infected with the UKG 34/2001 strain excreted around $10^{6.1}$ $TCID_{50}$ per pig per 24 hours, however none of the recipient pigs in cubicles exposed to this strain at a concentration of around 50 $TCID_{50}$ per minute i.e. , an accumulated dose of approximately 70 000 $TCID_{50}$ per pig in a >24 hour period were infected. Furthermore, expt.4 showed that recipient pigs exposed to around 200 000 TCID50 of C Noville only one of 8 recipient pigs got subclinically infected. This indicates that the respiratory clearance mechanisms for FMDV inhaled by pigs are very efficient and that aerosols of FMDV virus have to be at very high concentrations to infect pigs. In contrast, contact pigs, even after very brief contact, easily got infected.

The findings in the present paper is supported by our previous study on the O1 Lausanne strain [1]; experimental findings using the A_5 Parma strain of FMDV (G O Denney, unpublished results); and by another experiment with O UKG 2001 strain performed at IAH, Pirbright (N. Aggarwal and R.P. Kitching, unpublished results). Also relevant are field observations by veterinarians in the Philippines who have reported that FMD rarely spreads from one pig premises to another when the possibility of direct contact can be excluded (Carolyn Beningo, personal communication).

We conclude from the present and previous findings [1] that pigs, compared to sheep and cattle, are relatively resistant to infection by airborne FMDV. The doses required to cause infection and disease in pigs may be as high as 300 to 2000 and 800 to 6000 $TCID_{50}$, respectively. Furthermore, these doses need to be delivered within a very short period. By contrast, cattle and sheep can be infected by a dose of only 10 $TCID_{50}$ [11;16]. Therefore, although a pig excrete as much virus as 60 sheep or cattle [7;8], it is very unlikely that an infected pig premises will generate a virus plume of sufficient concentration to cause aerosol infection of pigs located on separate farms. In fact our calculations indicate, that even though the excretion from pigs is about 60-fold higher than from sheep and cattle (for the UKG 2001 isolate [8], pigs are also at least 60 times (and probably more) as resistant to aerosol infection as sheep and cattle. Thus, the risk of airborne transmission

from infected pigs to other pigs is probably low and only likely to occur at very short distances, similar to what we expect for ruminant to ruminant transmission by aerosol [8]. However, the combination of high excretion of aerosol virus from pigs with the high sensitivity of cattle and sheep by this route, makes this the main mode of airborne transmission of FMDV.

In this context, the relatively large difference in maximal airborne excretion for the various isolates in pigs, will most likely have a significant influence on the ability to spread to distant cattle and sheep premises.

It is theoretically possible that the exposure of pigs to a fraction of a MID_{50} could result in a proportion of the animals becoming infected [20;28]. Those animals could then amplify the virus and transmit it to others either directly or indirectly. However, none of the 10 pigs exposed to 650 $TCID_{50}$ of the O SKR 2000 strain (Expt. 2) or the 8 pigs exposed to 50 $TCID_{50}$ per minute for 24 hours (O UKG 2001 strain, Expt. 3) became infected, which suggests that there is a threshold level below which infection does not occur, or more likely, where the respiratory clearance of the pig can prevent the establishment of FMDV infection.

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TABLE 1. Type of virus, dose inhaled by recipients, and dose excreted as airborne virus from donors*

	Average $TCID_{50}$/litre air	Average dose inhaled $TCID_{50}$	Amount airborne Virus excreted $TCID_{50}$
O_1 Lausanne virus	35	1700	$10^{6.4}$
O SKR 2000	8	650	$10^{5.8}$
O UKG 2001	2.5	70000	$10^{6.1}$
C Noville	6	200000	$10^{7.6}$

* Dose inhaled is estimated per recipient pig (body weight of 25-35 kg) over the period exposed (5 min or 24 hours), while airborne virus excreted is estimated per 90-100 kg pig, i.e. the excretion from 3 donor pigs for 24 hours.

Appendix 31

RISK ASSESSMENT REGARDING TRANSIT QUARANTINE OF EXOTIC RUMINANTS ORIGINATING FROM COUNTRIES HARBORING FOOT-AND-MOUTH DISEASE

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Background

Conservation programs for endangered species (Species Survival Plans) depend on input of new genetics. For this reason the USDA-APHIS informally explored the possibility to establish a transit quarantine for antelopes and deer from the Philippines and Zaire in Switzerland. The Swiss Federal Veterinary Office (FVO) initiated a formal risk assessment in order to estimate the risk of introducing foot and mouth disease virus (FMDV) into Switzerland by means of temporary import (transit quarantine) of cloven-hoofed animals from countries or regions that are not free from FMDV.

Risk policy of the Swiss Federal Veterinary Office

Based on international standards and guidelines, the FVO recently developed and implemented a framework for dealing with risk analysis regarding animal health, food safety and international trade issues.
Risk analysis is defined as an iterative process consisting of interrelated steps which comprise (i) risk management, (ii) risk assessment and (iii) risk communication.
Risk management is the process of deciding and implementing measures in order to reduce the risk to an acceptable level considering the results of risk assessment and other legitimate factors. Risk assessment is the process of estimating the risk of an identified hazard based on a scientific approach. Incomplete data and uncertainty must be clearly documented. The paragraphs below describe the steps of the risk assessment: description of the risk network, release assessment, exposure assessment and estimation of the risk. Risk communication means an open and transparent exchange of information between all involved parties and those who will be affected in the outcome of the decision.

Risk network for transit quarantine

When released from pre-export quarantine in the country of origin, animals are shipped by air to Switzerland. After border clearance at the airport the animals are immediately forwarded by truck to the quarantine station of the zoological garden of Zurich. The distance between airport and quarantine station is approximately 12 kilometres leading partly through rural area. After termination of the quarantine, the animals are transported again to the airport and shipped by air to their final destination.

Release assessment

According to the OIE, the Philippines are not entirely free from FMD (268 outbreaks have been reported from 1.1.2000 to 31.12.2000). There are no official records of the presence of FMD in Zaire and therefore the FVO assumed that the country is not free from FMD.
Deer and antelopes may be infected naturally by FMDV. Clinical disease, however, may vary from inapparent to severe depending on the species. Experimental studies have failed so fare

to provide solid evidence of viral persistence in antelopes but it cannot be excluded that it can occur depending on the serotype with which the animals are infected. Little data is available on the incubation period and virus excretion in deer and antelopes. Considering the uncertainty of the diagnosis of FMDV in deer and antelopes during the pre-export quarantine it was concluded that the release of FMD virus is possible due to false-negative diagnosis.

Exposure assessment

After arrival of the animals in Switzerland, they are immediately transported to the quarantine station by truck. Although the distance is only 12 kilometres, the airborne spread of FMD cannot be prevented completely when animals excrete the virus. The following critical risk factors were identified: (i) the duration of the journey, (ii) the number of excreting animals and (iii) the (air/water) tightness of the vehicle. The magnitude of release of FMDV and the airborne spread from trucks carrying potentially FMDV excreting animals cannot be predicted. Therefore the transmission of FMDV and infection of susceptible domestic animals along the route cannot be neglected. Within a 3 km corridor along the route there are approximately 800 cattle, 300 small ruminants and 900 pigs (National livestock database) which could potentially be exposed.

The quarantine station of the zoo of Zurich is approved by the USDA-APHIS and meets the Swiss requirements. Based on diagnostic results of the national reference laboratory for FMD (Institute of Virology and Immunoprophylaxis, IVI), the Swiss Veterinary Service can lift the quarantine measures. Due to the quality assurance systems of the Veterinary Service and the reference laboratory (ISO 17025 certified), the probability of false-negative diagnoses is estimated to be very low. Therefore, an exposure of the domestic animals during and after suspension of the quarantine is considered to be very unlikely.

Estimation of the risk and conclusions

FMD is among the economically most important livestock disease. The last major outbreaks in Switzerland were recorded in 1965/66. Based on the risk assessment we conclude that the import of infected deer or antelope shedding virus from Zaire or from the Philippines cannot be completely prevented and that the transmission of FMDV during the transport from the airport to the quarantine station and a following outbreak of FMD cannot be excluded. Therefore the risk assessors recommend that exotic animals should be handled the same way as for a definite import. As a rule of thumb, the probability of importing infected animals should not exceed one in 10^6 (acceptable probability). This level of safety must be achieved through measures applied by the exporting country complying with internationally accepted standards.

Appendix 32a

THE STUDIES ON THE POSSIBLE NEW TYPE "O" FMDV VARIANT IN TURKEY

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Every year some of the field samples collected from several FMD outbreaks in Turkey are sent to Pirbright IAH for confirmation of the Sap Institute's results and for further investigation of the circulating virus as the results of the typing with modern methods. In 2000, 9 field samples were sent and these viruses were reported in the same year.

This year, on 18 June 2001 the Secretariat of EUFMD sent a message regarding to the results of samples from Turkey which were sent to Pirbright, IAH in 2000. In the message it was mentioned that, O1 Manisa strain (vaccine strain of Ankara Sap Institute) may not be able to provide enough protection against these field viruses. Afterwards, in another message dated on 17th of July, the r values of the same samples which were reported between 0,3-0,5 against O1 Manisa and some protection could be provided. Whereas, some samples which were sent to Pirbright, IAH in 1999, were O1 and their r values was 0,4, and it was still acceptable with O1 Manisa (Rep. of Sess. of the Res. Gr. of the Stand. Tech. Comm., 29 Sep. 1999, Appendix, 7).

Following the first warning from the Secreteriat of EUFMD, 18 samples from infected animals in the fields were sent to Pirbright IAH for strain identification, and also tests were conducted at Sap Institute to control situation in the field. r values of the some samples were calculated by ELISA at Sap Institute. They were close (r=between 0.5- 1.0) to the O1 Manisa strain so, these results are going to be checked with further duplicated tests (ELISA and nucleotide sequencing) in Ankara Sap Institute (Table 1).

An in vivo challenge test of TUR/00/2 sample was carried out, but we couldn't have enough titre to calculate and to grow. After calculating r values, in the case of an antigenic difference with the vaccine strain other samples identified as O1 type will be tested by in vivo challenge.

The r values of the O type field samples obtained in ELISA tested at the WRL, Pirbright and Sap Institute in the last 3 years are shown in Table 2. All of figures are either 0,4 or higher.

There is no indication in the field showing the lack of the protection of the vaccine produced with O1 Manisa. In the Table2 the total number of the O type outbreaks in Turkey since 1999 are listed. Those figures are also confirm that idea.
Despite all, the in vivo and in vitro tests conducted to control the antigenic differences of O1 Manisa vaccine strain will be completed at Ankara, Sap Institute. We also hope receive the ELISA results of the last 10 samples sent to WRL on June 2001 in that period.

Table:1 The r values of the O type field samples obtained in ELISA tested at Sap Institute in 2001

Item	Referens No.	Serotype	r values
1	272-01	O	1
2	417-01	O	1
3	499-01	O	0.5
4	100-01	O	0.5
5	39-01	O	>1
6	11-01	O	0.5

Table:2 The r values of the O type field samples obtained in ELISA tested at the WRL, Pirbright and Sap Institute in the last 3 years

r values					
WRL				**Sap Institute**	
1999		**2000**		**2001**	
TUR1/99	0,4	TUR2/00	0.5	272-01	1
TUR2/99	0,4	TUR5/00	0.3	417-01	1
TUR3/99	0,4	TUR7/00	0.5	499-01	0.5
TUR7/99	0,4			100-01	0.5
				39-01	>1
				11-01	0.5

Table 3: Cumulative results of O type outbreaks in Turkey since 1999

Years	O1 Manisa
1999	32
2000	39
2001	46

Appendix 32b

FMD VIRUS STRAINS CIRCULATING IN TURKEY

Sinan Aktas

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FMD remains endemic in Turkey over the years. FMD virus types O, A and Asia 1 have been currently present in Turkey. Some studies have routinely been carried out at FMD Institute, Ankara, to characterize these viruses and to determine suitable vaccine strains. Besides every year some of the field samples collected from several FMD outbreaks in Turkey are sent to Pirbright IAH for confirmation of the Sap Institute's results and for further investigation of the circulating viruses with modern methods. Sequencing results of these viruses is shown in Figures 1 and 2. The r values by ELISA of these viruses are given in Tables 1 and 2.

In 2000, 9 field samples were sent to Pirbright and a report for these viruses was received in the same year. This year, on 18 June 2001 the Secretariat of EUFMD sent a message regarding to the results of samples from Turkey which were sent to Pirbright, IAH in 2000. In the message it was mentioned that, O1 Manisa strain (vaccine strain of Ankara Sap Institute) may not be able to provide enough protection against these field viruses. Afterwards, in another message dated on 17th of July, the r values of the same samples were reported between 0,3-0,5 against O1 Manisa and some protection could be provided (Table 3).

Following the first warning from the Secreteriat of EUFMD, 18 samples obtained from infected animals belonging to different regions of Turkey were sent to Pirbright IAH for strain identification, and also tests were conducted at Sap Institute to understand the situation in the field. Some of the type O samples were studied at WRL and the results were reported recently (Table 4). Six of those samples were also tested at Sap Institute and the results are given in Table 5. These results showed that although there are some differences for some samples determined, O Manisa would give enough protection against these viruses.

There is no indication in the field showing the lack of the protection of the vaccine produced with O1 Manisa.

FMD type A viruses obtained in 2001 were also tested by ELISA to determine the r values against A 22 Mahmatli and A Aydin 98 (Table 6). Results showed closer relationship against A Aydin 98.

Due to some problems have been faced in supplying some reagents on time we had some problems with the sequencing studies. Only recently we were be able to sequence one type O virus isolated in 2000 by manuel RT-PCR cycle sequencing. This virus found to be closely related with the group "Panasia". Hopefully we will be able to sequence more isolates soon. We have also obtained an automatic sequencer through an FAO TCP project. As soon as we receive the consumables, which we are expecting to receive soon, we will start sequencing with this sequencer as well. We hope this will help us to speed up sequencing studies.

Figure 1. Dendogram depicting the relationships between FMD type O viruses isolated from Turkey

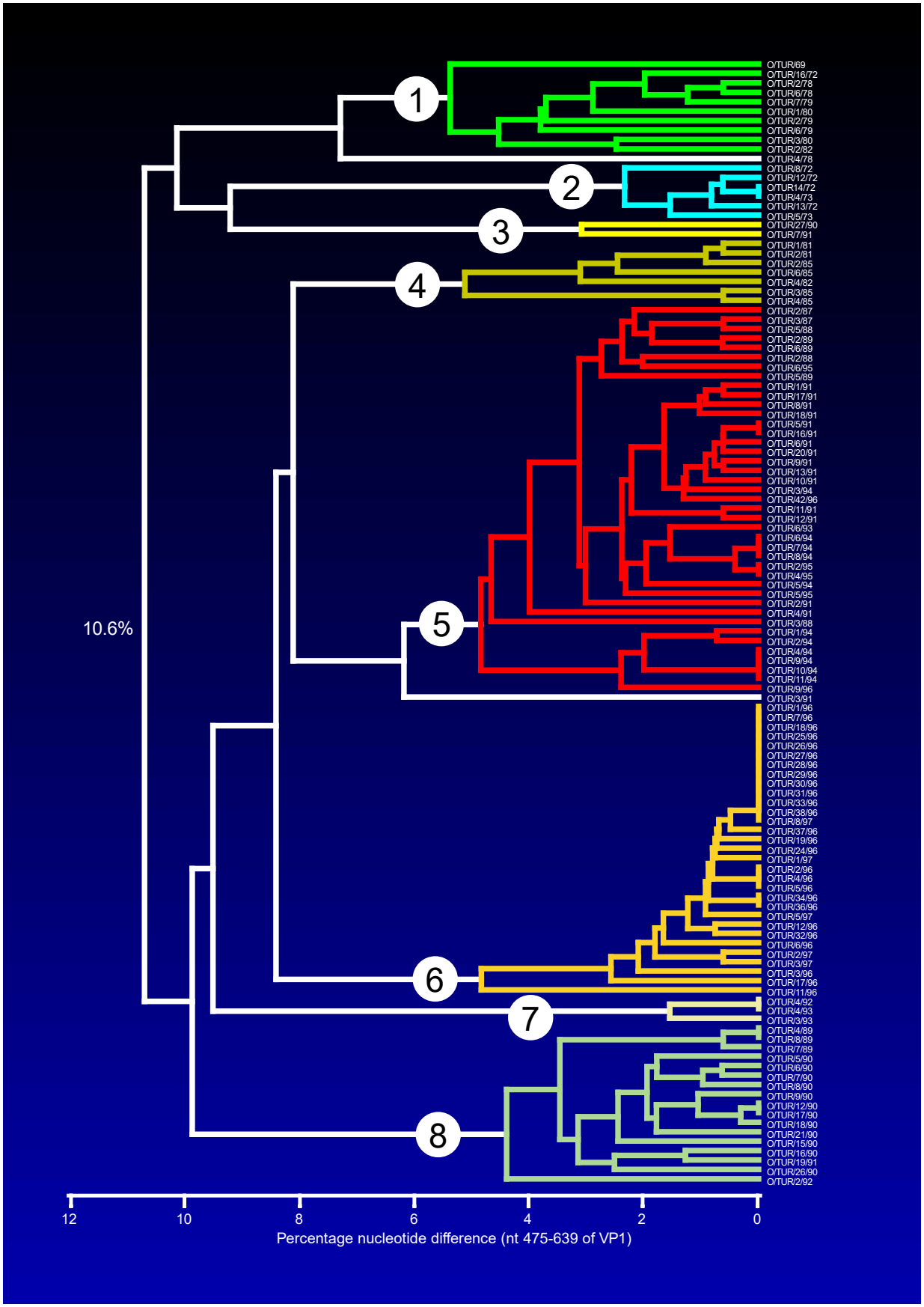

Figure 2. Dendogram depicting the genetic relationships between FMD type A viruses isolated from Turkey.

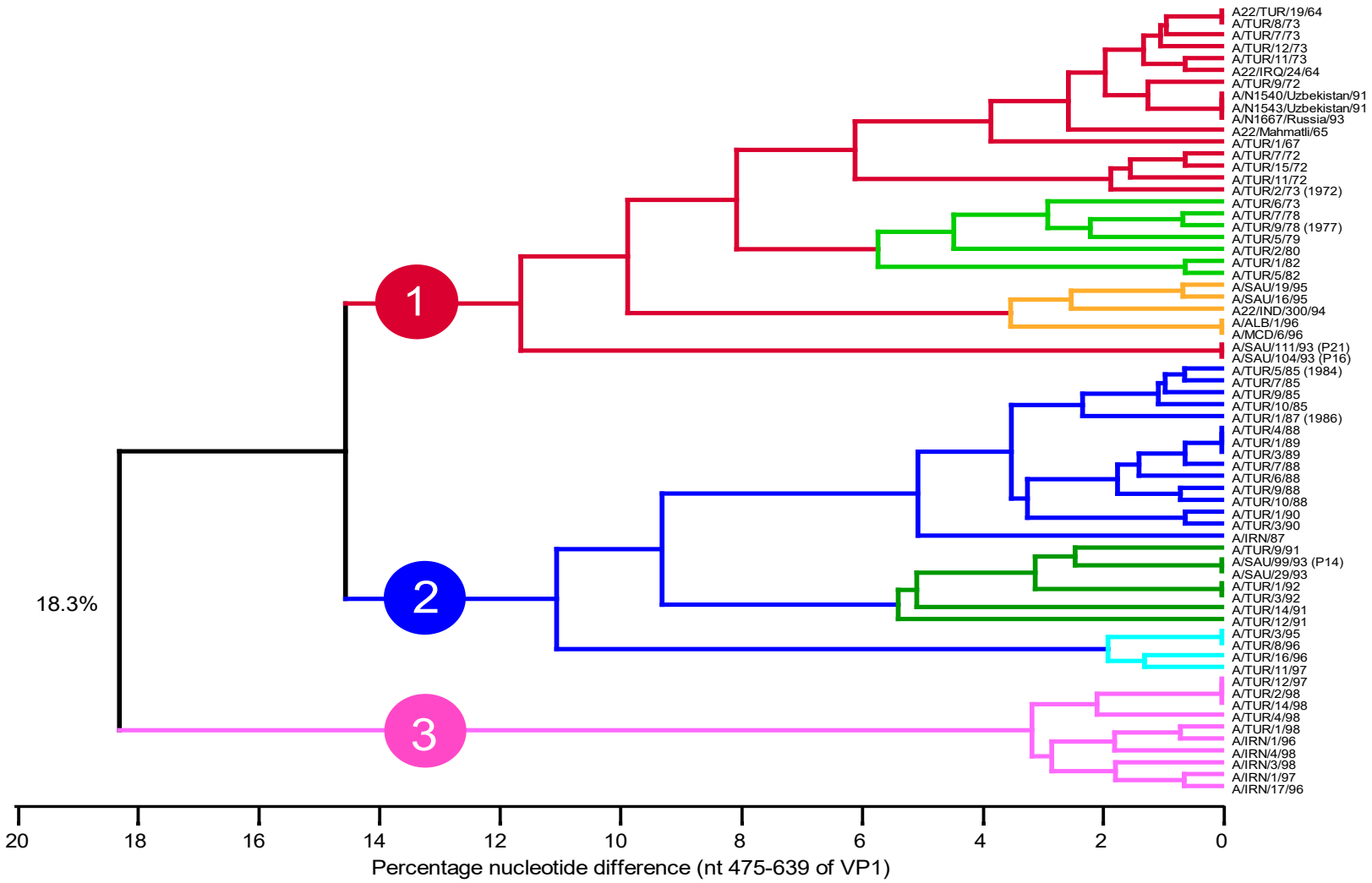

Table 1. r values of type O FMD viruses against several vaccine strains.

WRL NO	BFS	MANISA	DALTON	IND/53/79
TUR/2/87	1.0	ND	ND	ND
TUR/2/88	0.1	0.3	ND	ND
TUR/8/89	0.3	0.1	<0.1	ND
TUR/6/90	1.0	0.3	ND	ND
TUR/7/90	1.0	0.3	ND	ND
TUR/14/90	0.5	>1.0	ND	ND
TUR/17/90	1.0	0.5	ND	ND
TUR/18/90	1.0	0.5	ND	ND
TUR/19/90	>1.0	0.8	ND	ND
TUR/20/90	1.0	1.0	ND	ND
TUR/21/90	0.4	1.0	ND	ND
TUR/22/90	0.5	0.4	ND	ND
TUR/23/90	0.4	0.4	ND	ND
TUR/24/90	0.3	1.0	ND	ND
TUR/27/90	0.8	1.0	ND	ND
TUR/2/91	0.5	>1.0	0.5	ND
TUR/3/91	0.5	>1.0	ND	ND
TUR/4/91	0.8	0.8	ND	ND
TUR/7/91	0.5	>1.0	ND	ND
TUR/8/91	1.0	1.0	ND	ND
TUR/10/91	1.0	>1.0	0.5	ND
TUR/11/91	1.0	>1.0	1.0	ND
TUR/13/91	1.0	>1.0	1.0	ND
TUR/15/91	1.0	>1.0	1.0	ND
TUR/16/91	0.5	>1.0	0.5	ND
TUR/18/91	0.4	0.5	0.3	ND
TUR/20/91	0.8	1.0	0.5	ND
TUR/2/92	0.2	0.5	1.0	ND
TUR/4/92	0.5	0.8	0.4	>1.0
TUR/1/93	0.3	>1.0	0.3	>1.0
TUR/2/93	0.2	1.0	0.2	1.0
TUR/4/93	0.1	0.8	<0.1	1.0
TUR/1/94	0.3	1.0	0.3	ND
TUR/2/94	0.2	0.3	0.2	ND
TUR/3/94	0.3	1.0	0.2	ND
TUR/5/94	0.5	1.0	0.3	0.7
TUR/7/94	0.4	1.0	0.2	0.3
TUR/9/94	0.3	0.7	<0.2	0.5
TUR/11/94	<0.2	0.4	<0.1	0.2
TUR/2/95	ND	1.0	ND	ND
TUR/5/95	0.5	>1.0	ND	ND
TUR/1/96	0.5	0.7	ND	ND
TUR/4/96	>1.0	0.3	ND	ND
TUR/9/96	0.5	1.0	ND	ND

Table 2. r values obtained for FMD type A viruses isolated from Turkey

WRL NO	A_{22} Iraq	A_{22} Mahmatli	A 4164	IND/57/79	Sau/23/86
TUR/1/87	1.0	1.0	ND	ND	0.5
TUR/4/88	0.4	0.8	ND	0.3	ND
TUR/7/88	0.3	0.7	ND	0.3	ND
TUR/9/88	0.4	1.0	ND	0.3	ND
TUR/1/89	0.1	<0.1	ND	ND	0.1
TUR/3/89	<0.2	<0.2	ND	ND	ND
TUR/3/90	0.5	0.5	ND	0.5	ND
TUR/9/91	0.3	ND	ND	ND	0.3
TUR/12/91	0.3	ND	ND	ND	0.1
TUR/14/91	0.5	0.4	ND	ND	0.3
TUR/1/92	0.5	0.4	ND	ND	0.2
TUR/3/92	0.1	0.5	ND	ND	0.2
TUR/3/95	0.8	0.8	0.4	ND	ND
TUR/8/96	0.8	0.7	0.3	ND	ND
TUR/12/97	ND	<0.2	ND	ND	ND
TUR/1/98	<0.2	<0.2	<0.2	ND	<0.2
TUR/4/98	<0.2	<0.2	<0.2	ND	<0.2
TUR/17/98	ND	<0.2	ND	ND	ND
TUR/26/98	ND	<0.2	ND	ND	ND
TUR/40/98	ND	<0.2	ND	ND	ND

Table:3 The r values of the O type field samples isolated in 1999 and 2000 from Turkey against O Manisa obtained by ELISA in WRL.

WRL			
1999		**2000**	
TUR1/99	0,4	TUR2/00	0.5
TUR2/99	0,4	TUR5/00	0.3
TUR3/99	0,4	TUR7/00	0.5
TUR7/99	0,4		

Table 4: The r values obtained by ELISA at WRL, Pirbright for the latest outbreaks of 2001 against different vaccine strains

WRL Reference No	BFS	Manisa	Phil-95	3039	4174	IND R2/75
TUR 2/01	0.3	1.0	>1.0	0.9	0.2	1.0
TUR 5/01	0.4	0.3	>1.0	0.9	0.4	0.9
TUR 10/01	0.3	1.0	1.0	0.9	0.2	1.0

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Table 5. The r values obtained by ELISA at Sap Institute for type O samples isolated in 2001 against O Manisa.

Item	Origin	Serotype	r values
1	Nevşehir	O	0.5
2	Erzurum	O	1
3	Kars	O	0.5
4	Adıyaman	O	1.0
5	Sakarya	O	0.5
6	Kastamonu	O	>1.0

Table 6. The r values obtained by ELISA at Sap Institute for type A samples isolated in 2000 and 2001 against A/Aydin/98

Item	Origin	Serotype	r values
1	Manisa	A	0.5
2	Amasya	A	0.75
3	Bingöl	A	0.66

Appendix 33

ACTIVITIES REGARDING THE IMPROVEMENT OF THE FMD VACCINE'S QUALITY

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To improve the quality and the quantity of the FMD vaccine a number of changes have been realized as recommended by the national and international experts in years 2000 and 2001.

These are summarized as below:

1. The management organisation of the Institute was re-structured as follows;
    a. Cell and Virus Bank
    b. Diagnosis
        i. Typing
        ii. Serology
        iii. Molecular Epidemiology
        iv. Monoclonal Antibodies
    c. Vaccine Production
        i. Cell culture
        ii. Medium preparation
        iii. Virus culture
        iv. Vaccine preparation
    d. Quality Control
        i. Raw Material Control
        ii. In-Process Control
        iii. Final Product Control
        iv. Test Calibration
    e. Laboratory and Large Animal Testing

2. For security, use of electronic cards were introduced for entrance and exit.

3. The physical separation between the non-FMD- infected (Unrestricted) areas and the potentially FMD infected (Restricted) areas was completed. So, the free movement of staff between restricted and unrestricted areas was prohibited.

4. Cell Bank and Master Cell Bank Laboratory was rebuilt in the non-FMD infected area.

5. For the production of large scale cell culture up to 700L, use of commercial sera was introduced.

6. Also studies regarding the use of commercial, pre-mixed powder media have been in progress.

7. Experimentally, the oil adjuvanted vaccine was produced and tested. Initial studies to install a purification and concentration system for vaccine virus have been started and to be completed soon.

8. The studies to decrease the dose of FMD vaccine from 5 ml to 3 ml was completed and field trials will be conducted within the next 3 months.

9. In addition to the safety and potency test in the laboratory, the vaccine is regularly being tested in the field for herd immunity levels.

10. Regarding the independent vaccine control laboratory, the test standardisation studies have already been started at Bornova Vaccine Control Centre (Bornova Veterinary Research and Control Institute, Izmir).

STRATIFIED AND CRYOGENICALLY STORED SACS VACCINES, A NOVEL FORMULATING PROCEDURE FOR EXTENDING THE SHELF-LIFE OF EMERGENCY FOOT-AND-MOUTH DISEASE VACCINES

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Summary

Strategic reserves of foot-and-mouth disease (FMD) antigen have become an integral part of FMD control policy for many countries. They are based on two principles, ready formulated vaccine stored at +4^{0}C, or concentrated antigen preparations held at ultra-low temperature for later formulation. However, the latter is more economical, since ready formulated vaccine, based on oil or aluminium hydroxide/saponin adjuvants, requires regular replacement. This is primarily the result of the vaccine's limited shelf-life, nominally 18 months at +4ºC.

Montanide ISA 206 and ISA 25, two >ready-to-formulate= oil adjuvants which can be used in all target species, are ideal for emergency vaccination.

A novel approach of layering the individual components of FMD vaccine in the same primary container and then storing the product at ultra-low temperature is described. This avoids the detrimental effect on potency, normally observed with frozen formulated FMD vaccine. The implications of substantially extending the products shelf-life for emergency vaccination strategy are discussed.

Introduction

The principle of storing concentrated FMD antigen over liquid nitrogen for later formulation was originally established by Denmark, a non-FMD vaccinating country. The United States set up a similar reserve of FMD antigen concentrates in 1980, to which Canada and Mexico later subscribed and the antigens held by this North American Vaccine Bank (NAVB), if required, would be formulated by the commercial sector. In 1985 the International Vaccine Bank (IVB) was established at Pirbright in the United Kingdom (UK) by a consortium consisting of the UK, Australia, New Zealand, Finland, Ireland, Norway and Sweden. Malta later joined the IVB as an associate member in 1995. However, unlike the NAVB, the IVB has the convenience of its own manufacturing facility allowing vaccine to be formulated and despatched within days of a request. Indeed, early in the 2001 FMD outbreak in the United Kingdom, and at the request of the UK Ministry of Agriculture, Fisheries and Food, the IVB was, for the first time, called upon to produce 500,000 bovine doses of alumium hydroxide/saponin adjuvanted 01 Manisa vaccine over a 12 day period. The more recent establishment of a European Community FMD antigen reserve and many other examples of individual countries assigning their own FMD reserves, which are maintained commercially or through government support (Ryan, 1999; Garland, 1997; Callis, 1994), underline the increasing popularity of antigen banks.

Conventional formulated FMD vaccine, either oil or aqueous, have a limited shelf life, normally 18-24 months at +4ºC and it has been demonstrated that aqueous vaccines prepared from

commercial antigen concentrates are considerably less stable when stored at +4ºC (Doel and Pullen, 1990).

Work at the Institute for Animal Health, Pirbright, has shown that a reduction in potency occurs when oil adjuvanted vaccines are stored at either –20ºC and –70ºC (unpublished data), and therefore neither type of formulation can be frozen under these conditions without detrimental effect.

Montanide ISA 206 and ISA 25 are two >ready-to-formulate= oil adjuvants (SEPPIC, France) which are effective in cattle, pigs and sheep and capable of promoting early protective responses (Doel et al., 1994; Cox et al., 1999; Salt et al., 1995; Salt et al., 1998) making them ideal for use as emergency vaccines. Their potential is enhanced by the simplicity of formulation into oil emulsion vaccines, requiring no complicated high-shear emulsification equipment. We have been monitoring the oil adjuvant component (Montanide ISA 206), which, in an attempt to extend its shelf-life (nominally 2 years at +4ºC), has unconventionally been stored at –20ºC. This batch, lot 3001, currently in its eighth year of storage, is still a viable component, as quality control tests undertaken by the commercial suppliers, SEPPIC, have shown that two critical parameters, the acid and peroxide values, are still within acceptability limits.

Recent studies on the IVB's antigen concentrates have also established that the shelf-life of these preparations are likely to be well in excess of 15 years (Barnett and Statham, 1998).

Given that both the oil adjuvant and the antigen component can be maintained appropriately at low temperature, and that the 'ready-to-formulate' adjuvant readily forms a stable emulsion, we examined the possibility of extending the shelf-life of the final product by a novel process. Here we describe a procedure applying the main components of FMD vaccine as stratified layers in the same primary container and storing at ultra-low temperature.

Materials and Methods

Vaccine preparation

Vaccine formulations, incorporating FMDV O_1 Lausanne inactivated antigen as either water-in-oil-in-water (W/0/W) emulsion with Montanide ISA 206, or as a oil-in-water (O/W) emulsion with Montanide ISA 25, were prepared conventionally (Barnett et al., 1996), or by a novel procedure, using antigen concentrate held by the IVB over liquid nitrogen with a PD_{50} value of 41 per bovine dose. The formulated vaccine contained 5.62 []g of 146S antigen per 2ml bovine dose.

The novel formulation procedure (see Figure 1) involved 4 main steps as follows:-

1. Oil adjuvants Montanide ISA 206 or 25, at the required volume, were aliquoted into the desired primary container, placed in the ultra-low temperature gaseous phase of liquid nitrogen, and snap frozen.

2. The frozen oil adjuvant is then momentarily removed from the low temperature environment and the prerequisite volume of aqueous buffer is carefully layered onto the top of the frozen oil adjuvant to form two distinguishable layers or stratifications. This is immediately and carefully returned to the ultra-low temperature gaseous phase of liquid nitrogen to snap freeze the aqueous buffer.

3. The frozen oil adjuvant and aqueous buffer layers are again momentarily removed from the low temperature environment and the prerequisite volume of concentrated antigen is then layered on top

of the frozen buffer. This is immediately returned to the ultra-low temperature environment to snap freeze the antigen concentrate.

4. When required, the stratified and cryogenically stored (SACS) vaccine are thawed at room temperature, mixed by simply agitation, and administered into the target host.

In vivo potency tests

Vaccine preparations were tested in female Duncan-Hartley guinea pigs, approximately 400-500 gm in weight. Each group of five animals received a specific volume of vaccine of either 1ml, 0.33ml or 0.11ml. administered subcutaneously. Animals were challenged 28 days postvaccination with 3 x 10^3 ID_{50} of the homologous guinea pig adapted virus, injected by the intraplantar route. All animals were monitored closely for 7-10 days, and immunised guinea pigs were considered protected if the virus failed to be generalised beyond the challenge site.

Later experiments incorporated dilutions of vaccine instead of the reduced volume dose described previously. Essentially vaccines were diluted in a similarly formulated vaccine that did not contain the antigen component so that the antigen but not the adjuvant was diluted. The dilution range used was three-fold from neat to 1/81. Again animals were challenged 28 days post-vaccination with 3 x 10^3 ID_{50} of the homologous guinea pig adapted virus, injected by the intraplantar route and monitored as described previously. This dilution range allowed the potency (PD_{50}) of the vaccine to be calculated by the method of Karber (Karber, 1931).

Results

In the first trial, SACS vaccines based on either Montanide ISA 206 or ISA 25 were examined for their stability at ultra-low temperature over a 40 month period. Using a divided dose regime results were encouraging showing that in the absence of any loss in vaccine potency the procedure was not detrimental to either adjuvanted formulation (Table 1).

Table 1 Potency of SACs vaccines based on Montanide ISA 25 (oil-in-water) and 206 (water-in-oil-in-water) adjuvanted vaccines following long term storage at ultra-low temperature

Vaccine	0 day			5 months			7 months			40 mouths		
	1.0ml	0.33ml	0.11 ml	1.0ml	0.33ml	0.11m1	1.0ml	0.33ml	0.11 ml	1.0ml	0.33 ml	0.11 ml
SACs ISA 206	100*	100	100	100	100	100	100	100	90	100	100	100
SACs ISA 25	100	100	100	100	100	100	100	100	100	ND	ND	ND

*** Percentage of guinea-pigs protected per dosage group.**
ND - Not determined

In a second trial, SACS vaccine's based on Montanide ISA 206 or ISA 25 were diluted in similarly treated vaccine without the antigen component and compared to the PD_{50} value of conventionally formulated vaccines (Table 2).

Table 2 Potency (PD50) estimation of SACs ISA 206 and ISA 25 vaccines

Vaccine	Dilutions						
	1/1	1/3	1/9	1/27	1/81	Control	PD_{50} value
SACs ISA 206	100	100	100	100	75	0	**106.5****
SACs ISA 25	100	100	100	60	60	0	**58.2**

*** Figures show the percentage of guinea-pigs protected per dosage group.**

***This compares with conventionally made oil vaccine using the same batch of Montanide ISA 206 with a PD_{50} value of 46.71, which was performed on a separate occasion (data not shown).*

Using the two mineral oil adjuvants in a third trial, SACs vaccine when thawed mixed and subsequently stored at $+4^0$C were shown to still remain potent after 7 months (Table 3). This compared well to previous observations on conventionally formulated emergency vaccines composed of the same adjuvants (Barnett et al., 1996)

Table 3 Potency of SACs vaccines based on Montanide ISA 25 (oil-in-water) and 206 (Water-in-oil-inwater) adjuvanted vaccines following thawing, mixing and storage at +4°C for up to 7 months

Vaccine	0 day			4 months			7 months		
	1.0ml	0.33ml	0.11ml	1.0ml	0.33ml	0.11ml	1.0ml	0.33ml	0.11ml
SACs ISA 206	100	100	100	100	100	100	100	100	100
SACs ISA 25	100	100	100	100	100	100	100	100	100

*** Figures show the percentage of guinea-pigs protected per dosage group.**

Conclusion

In order to be credible, countries free of FMD that maintain the option to vaccinate in the event of an outbreak, must be able to access sufficient FMD vaccine within days of making such a request. This is only possible if they are members of an antigen or vaccine bank underlining there important supporting role in the control of FMD. These banks are based on a) concentrated antigen which can be rapidly formulated into vaccine, and/or b) formulated vaccines for immediate use. Of vital importance is the locality of stored antigens, since the need to formulate may require antigen to be returned to the original manufacturer, which in an emergency would further delay its production. Even if the antigens are held in the commercial sector, delay following a request for the supply of emergency vaccine might still occur if the manufacturer is currently in production, and should the facility be available for manufacture the time to produce the vaccine may be at best 24-48 hours. Such delays in the production and despatch of emergency vaccine to control an outbreak inevitably leads to wider spread of the disease and further difficulty in its control. Formulated vaccine would of course allow for immediate access. However, beside the wasteful and uneconomic implications resulting from regular replacement of the vaccine, it may not always contain the most suitable strain

to deal with an outbreak. Even when an appropriate vaccine is produced in an emergency, such as the 500,000 bovine doses of O_1 Manisa vaccine formulated by the IVB from a commercial antigen concentrate, a major disadvantage is the short shelf-life. Given these considerations a fully formulated vaccine that can be stored for an indefinite period of time has many benefits including:-

1. Readily available vaccine on request, alleviating delays in despatch.

2. No requirement once formulated for accessibility to a **manufacturing facility.**

3. Quality control, sterility and efficacy, at the required standards, performed well in advance of its possible use.

4. Cold-chain requirement less critical in transit as the vaccine could be shipped as it is thawing and hand mixed prior to application.

5. Economical, alleviating the need to replace vaccine components such as adjuvant on a regular basis.

6. Full drawing rights or dose requirement could be available in >one-hit= without the need to do further manufacturing runs.

7. Accessibility, could be strategically stored in various locations ready for immediate use.

Using a novel approach of layering the individual components of FMD vaccine in the same primary container and then storing at ultra-low temperature, these so-called stratified and cryogenically stored (SACS) vaccines appear to make these list of benefits more realistic. The results of experiments indicate that applying this methodology to Montanide 25 or 206 oil based vaccines has no detrimental effect to the potency or stability of the final product or indeed its shelf-life following reconstitution and storage at +4ºC. More significantly the process maintained the potency of fully formulated FMD vaccine after several years of storage at ultra-low temperature, well in excess of the 12-18 month shelf-life period of conventional FMD vaccine kept +4ºC .

Consideration will of course have to be undertaken on the optimum manufacturing approach for this type of vaccine, including the most suitable primary containers and method of labelling. Nevertheless, this approach offers many advantages over the existing system, not only in the context of FMD but also other vaccines including those based on attenuated strains, in order to improve there shelf-life characteristics and immediate accessibility.

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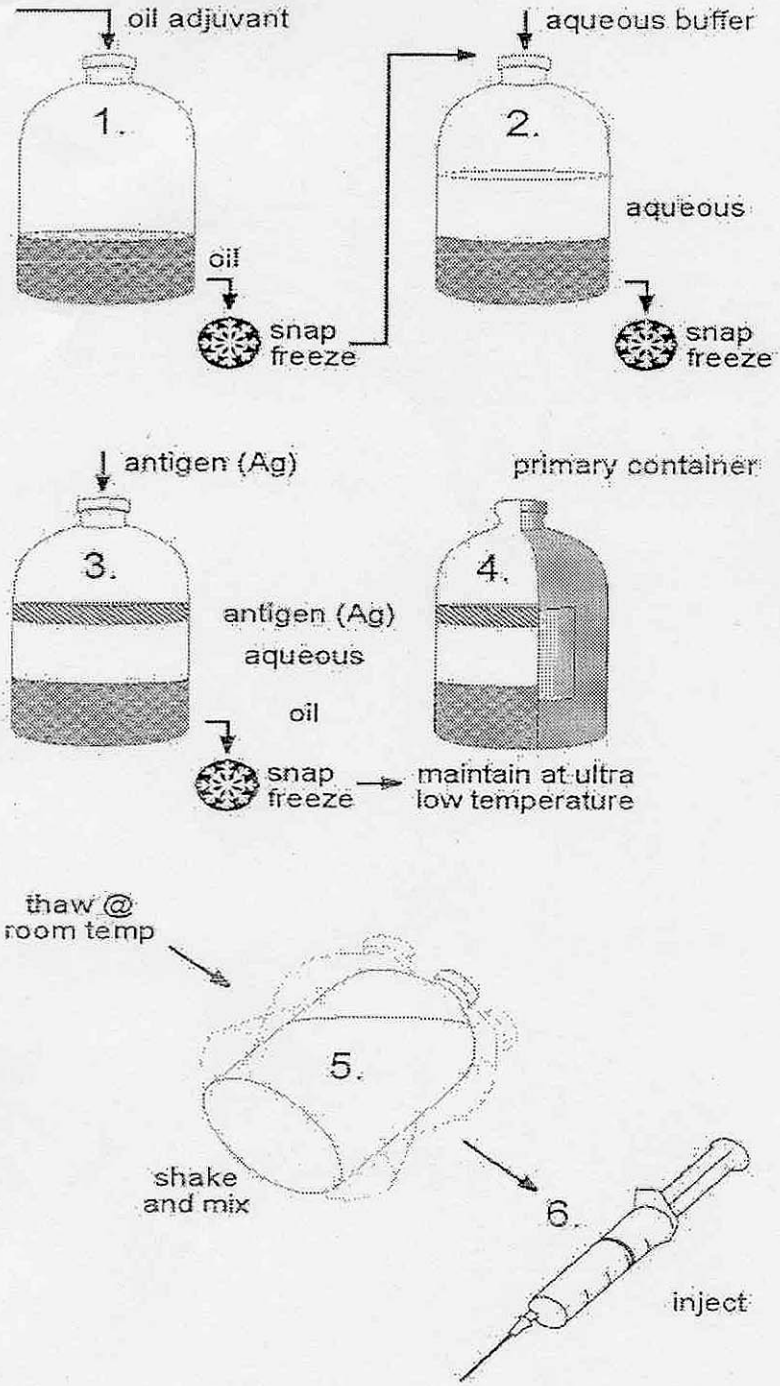
Figure 1: Formulation procedure for the production of stratified and cryogenically stored (SACS) foot-and-mouth disease vaccine.



Appendix 35

PROVISIONAL RECOMMENDATIONS FROM THE WORLD REFERENCE LABORATORY ON FMD VIRUS STRAINS TO BE INCLUDED IN FMDV ANTIGEN BANKS

High Priority

O Manisa *(covers panasian topotype)*
O BFS or Lausanne
A22 Iraq
A24 Cruzeiro
Asia 1 Shamir
A Iran '96
SAT 2 Saudi Arabia *(or equivalent)*

(not in order of importance)

Medium Priority

SAT 2 Zimbabwe
A15 Bangkok related strain
A87 Argentina related strain
A Saudi Arabia 23/86 *(or equivalent)*
SAT 1 South Africa
A Malaysia 97 *(or Thai equivalent such as A/NPT/TAI/86)*
A Eritrea 98
C Noville
O Taiwan 97 *(pig-adapted strain or Philippine equivalent)*
A Iran '99

(not in order of importance)

Low Priority

SAT 2 Kenya
SAT 1 Kenya
SAT 3 Zimbabwe
A Kenya

(not in order of importance)

Appendix 36

REVISION OF THE EUROPEAN PHARMACOPOEIA MONOGRAPH FOR FOOT-AND-MOUTH DISEASE VACCINE AND EUROPEAN GUIDELINES ON REQUIREMENTS FOR FMD VACCINE PRODUCTION

Kris De Clercq
on behalf of the FAO EUFMD EurPhar Working Group

In the year 2000 the FAO EUFMD EurPhar Working Group was represented at meetings with Group 15V of the EurPhar and with the CVMP/Immunologicals Working Party of EMEA. Based on the discussion at the EurPhar meeting it was proposed that a revision of the FMD Monograph would be prepared by Dr. L. Bruckner. Based on the discussion at the EMEA meeting it was proposed that an ad hoc group would be established to harmonise existing guidelines on FMD vaccine production and control. Prof. PP. Pastoret (Chairman) would prepare the terms of reference of the group. Both documents (added to this report) came available in 2001. The revision of the FMD Monograph was discussed at a meeting of Group 15V of the EurPhar in Gent-Belgium on June 6, 2001. The EMEA proposal will be discussed on September 19, 2001 in London-UK under the chairmanship of Dr. David MacKay.

The comments of the FAO EUFMD EurPhar Working Group were summarised in a letter addressed to Dr. Bruckner and the EurPhar (added to this report). The comments were then presented at the EurPhar meeting on June 6. During the following four hours discussion the proposed revision was amended (see hand written comments). As a result a new revision proposal will be made by Mr. Castle, Secretary of Group 15V.

The comments of our group were focused on:

The safety test: it was not clear whether or not this was a test to perform once or with every batch. It was made clear by Dr. Bruckner that this was not a batch test.

The batch potency test: The criteria for using an alternative test were unclear. The meeting concluded that there is a need for manufacturers information on the criteria of batch acceptance using an alternative test and for peer reviewed publications covering the criteria used or to be used.

Different FMD strains now and in the future: the meeting referred to future guidelines. EurPhar, FAO and EU will be present at the EMEA meeting.

A monograph for pigs: monographs are published by species. A proposal for pigs will be worked out by Dr. Bruckner in future. This will also be discussed at the meeting organised by EMEA.

Prof. Pastoret proposed to have a discussion with all partners involved: EMEA, OIE, EUFMD, EurPhar, EU, Vaccine manufacturers.
The aim of the meeting would be:

- to review the existing requirements for FMD vaccines from the different organisations;
- to propose draft guidelines on quality, safety and efficacy requirements for FMD vaccine productions as well as for the addition or replacement of FMD strains;
- to discuss the possibility of a marker vaccination programme based on the absence of NS proteins;
- to evaluate the impact of the quasi species status of FMD virus populations.

Appendix 37

REPORT ON THE JOINT EUFMD/EC WORKSHOP ON FOOT-AND-MOUTH DISEASE SIMULATION EXERCISES

5 – 7 June 2001 - Brno, Czech Republic

John Ryan, EUFMD

Introduction

A workshop, jointly organised between EUFMD and the EC, was held in Brno, Czech Republic on the 5-7 June 2001. Experts from EUFMD, the EC and from the following member countries were present: Belgium, France, Germany and The Netherlands. The workshop was very well attended with 49 participants representing 23 countries that included every Eastern European country (with the exception of Slovakia), the Baltic States, Cyprus, Malta and Iceland.

Timetable

Day 1 of the workshop was a desk-based session where the invited experts presented the FMD situation in Europe and other regions; the major risks of FMD introduction to Europe and the lessons to be learned from the 2001 outbreaks in Western Europe; the situation in UK, the Netherlands and France; the measures taken to prevent the introduction of the disease in Germany, Belgium and Austria; the FMD Legislative Measures taken by the EC; Contingency planning in EC; and Austria and the Czech Republic presented their Contingency plans.

Day 2 of the workshop was an on-farm exercise where the Czech Veterinary and Emergency Services demonstrated all the practical steps involved in responding to a suspicion of Foot-and-Mouth Disease. On the farm in the district of Znojmo that was chosen for the simulation, the following practical demonstrations took place:
the correct bio-security procedures for entering and exiting a suspect farm;
how to conduct clinical examinations of animals (with live cattle, sheep and pigs);
the correct procedures for taking and packaging samples for laboratory submission;
the techniques and equipment for slaughtering animals on-site (the animals examined above were humanely slaughtered on site with captive bolt pistols, and electrical stimulation);
the techniques and equipment for transporting the carcasses to a rendering plant while maintaining biosecurity en route;
the special equipment designed for the disinfection and cleaning of personnel, vehicles and equipment.
In an afternoon desk-based session, a very detailed presentation and Questions-and-Answers (Q&A) session took place. The measures to be taken (i) in the protection and surveillance zones, (ii) in the country as a whole and (iii) in co-operation with neighbouring countries (particularly Austria, because the farm chosen for the simulation was very close to the Austrian border) if the samples taken in the morning session proved to be positive were discussed in great detail.

On *Day 3*, the participants made presentations detailing the contingency plans of their home country and in addition there were presentations on emergency vaccination, the management and structure of the outbreak response, carcass disposal, modelling airborne spread and computers as an aid to disease management.

Conclusions

The FMD situation outside and inside Europe and the control measures applied in relation with outbreaks within the EU were reviewed.
The rapid movements of live animals and products of animal origin between different regions of the world and within countries and regions add to the risks of unexpected FMD outbreaks.
Contingency planning was reviewed and many individual contingency plans were presented. The key aspects of contingency planning were consistently highlighted, in particular:
Disease awareness - the importance of the relationship between farmers and veterinarians and the degree of education and training of the veterinarians.
Disease preparedness –including the legal basis for action, the importance of the contingency plan and the importance of using other national organisations such as the army and civil defense.
Rapid response – to disease outbreaks with abilities to eradicate FMD including provisions for emergency slaughter and emergency vaccination
Communication – the importance of communications
The simulation exercise carried out on a farm situated in the district of Znojmo was very well prepared and implemented. The control measures to be established in the event of an FMD outbreak linked to the exercise scenario were presented in a comprehensive way and the time table drawn up for the establishment of and enforcement of measures indicated that measures would be in place within hours.

Recommendations

1. It should be guarantied in all European/participating countries, that they have:
   - Equal abilities to detect and to control FMD,
   - An emergency plan where all necessary activities, funds, manpower, heavy machinery etc. are written down in such a way that the plan is a reliable document to organise control measures for FMD,
   - Either the country concerned based on national laboratory capacity or based on a contract with an other country have the possibility to get within a short period a positive diagnosis and a confirmed negative result in accordance with the protocol of the laboratory.
   - Awaiting results from the laboratory the authority sending the sample should arrange for appropriate preventive and control measures.
2. To reach and to keep the ability to detect and to control FMD the veterinary service must make clear that combat against the disease is a task for the whole society (industry, government). An outbreak of FMD may destroy the competitiveness of the national economy of every country involved for a long time. As learned from the FMD events of this year a sufficient number of trained experts in the public veterinary services and support by appropriate authorities such as the police/army are decisive.
3. It is necessary to have regular training of official veterinarians in the field of FMD control. This training should be done in two ways:
   - To train the strategy of control for leading veterinarians and staff from other governmental authorities concerned how to organise things in a region and how to co-operate with the industry and local authorities,
   - How to do the control on a farm or village level. Simulation exercise must be carried out to train staff in the procedures for carcass disposal. This exercise must train staff in decision-making for carcass disposal by including a process to accurately measure the real-life capacity of different disposal options and compare these measurements to the disposal needs generated by different scenarios.

4. Measures to reduce the free movement of people in the case of FMD seems to be an important point to improve the control regime.

5. Funding should be found to support the recommendations of this meeting by:
   - Organising seminars and workshops on topics about implementation of contingency plans. The exchange of practical information about the implementation between the pre-accession countries is highly valuable,
   - Organising regularly simulation exercises.

6. There is no reason to come back to a policy of preventive vaccination against FMD. But every country should be prepared (vaccine, syringer, other equipment, veterinarians) to do emergency vaccination if necessary.

7. Contingency plans should include a section on information which ensures that a clear communication message is given the whole society, including the media, about the content and the challenges of the plan.

Appendix 38

INFORMATION ON THE NEW ITALIAN REFERENCE LABORATORY FOR VESICULAR DISEASES (CERVES)

F. De Simone, IZSLER, Brescia, Italy

The CERVES ("Centre of Reference for Vesicular Diseases") is devoted to diagnosis (and every other related subject) of vesicular diseases all over Italy; it is the only laboratory authorised to handle infectious vesicular viruses.
The location of the CERVES is at the IZSLER (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia), Brescia, Italy. CERVES was established in 1968.

In 1991 the production of FMD vaccine was stopped and the restricted area was moved to the first floor of a building formerly in use for vaccine production. Laboratories dealing with diagnosis and control of vesicular diseases continuously increased their work and collaborative research with other European reference laboratories.

Due to the increasing activities which also include those not related to vesicular diseases and the non-adaptability of the plant in use, the IZSLER decided to assign a new plant for CERVES which is a part of another building formerly used for rolling bottles.

The renovation project required years for preliminary and bureaucratic fulfilment and work started only one year ago.

The Building in which the new CERVES will be located was used as a plant for the production of BHK cells rolling bottles until 1991.

It is composed of 4 floors:
U.ground (570 m^2) and ground (1320 m^2) floors, entirely devoted to the CERVES.
First (420 m^2) and second (420 m^2) floors, partly devoted to the CERVES.
Total area of the CERVES: 2730 m^2.

Renovation will be accomplished in two phases.

1st Phase:

Will include the masonry, utilities and fittings

U.ground floor
- Plants for air conditioning
- Plant for treatment of waste waters
- Room of 170 m^2 (ceiling 5.5 meters). This room at present is provided with utilities only. It can be adapted as a unit for pilot plants, small scale productions, inner core of a lab with higher containment level, etc.
- Lift

Ground floor
- Entrance
- Showers
- Hall
- three air locks
- Double door Autoclave
- Incubator at 37°C and cold rooms (+4° and –20°C)
- 1st Group of Laboratories and offices (240 m^2) with hall.

First floor
- Canteen
- Laundry

Second floor
- Air Filtration Plants

2nd Phase:
Will include masonry, utilities and fittings

Ground floor
- 2nd Group of Laboratories and offices (185 m^2)

First floor
- small animal room.

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