AGA: EUFMD/RG/81

R E P O R T

of the

Session of the Research Group of the Standing Technical Committee

of the

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

Tübingen, Federal Republic of Germany

29 September - 1 October 1981

Food and Agriculture Organization of the United Nations

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APPENDICES

INTRODUCTION

A Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth Disease was held at the Federal Research Institute for Animal Virus Diseases, Tübingen, Federal Republic of Germany from 29 September to 1 October 1981.

The Session was chaired by Dr. J.G. van Bekkum, Netherlands, Chairman of the Research Group. The members of the Research Group present were:- Dr. M. Eskildsen, Denmark, Dr. G. Kubin, Austria, Dr. M. Mussgay, Federal Republic of Germany, Dr. G.F. Panina, Italy, and Dr. R.F. Sellers, United Kingdom. Dr. R. Strobbe attended in place of Dr. J. Leunen, Belgium who was unable to be present.

The following observers attended:- Dr. S.J. Barteling, Central Veterinary Institute, Lelystad, Dr. F. de Simone, Istituto Zooprofilattico Sperimentale, Brescia, Dr. T.R. Doel, Animal Virus Research Institute, Pirbright, Dr. H. Favre, IFFA-Mérieux, Lyons, Dr. U. Kihm, Federal Vaccine Institute, Basel, Dr. J.C. Lei, State Veterinary Research Institute, Lindholm, Dr. M. Lombard, IFFA-Mérieux, Lyons, Dr. T.W.F. Pay and Dr. M.M. Rweyemamu, Wellcome FMD Laboratory, Pirbright, Dr. P. Sütmoller, Pan-American Centre for FMD, Rio de Janeiro and Drs. R. Ahl, H. Bohm, H.D. Matheka, E. Straube (guest scientist, Australia), K. Strohmaier and G. Wittmann, staff members of the Federal Research Institute, Tübingen.

Dr. P. Stouraitis and Miss Joan Raftery of the European Commission for the Control of Foot-and-Mouth Disease, FAO Rome, provided the secretariat and the rapporteur for the Session was Dr. G.N. Mowat, Animal Virus Research Institute, Pirbright.

Dr. H. Pittler welcomed the participants to Tübingen on behalf of the Federal Government and the Minister of Agriculture. He said the work of the Research Group was much appreciated in Europe and would continue to be needed since there was still much to do in the field of FMD control.

The agenda for the Session comprised the following items:-

1. Further information on preparation and shelf-life of concentrated FMD vaccine.
2. Procedures for elution and innocuity testing of FMD vaccines.
3. FAO Collaborative International Study for the Standardization of Laboratory Methods in FMD Research. Discussion of the results of Phase V.
4. Topics raised by the Commission, Twenty-Fourth Session, Rome 1981.
5. Further information on cross-protection between European and South American A-viruses.
6. Other business.
7. Final Conclusions and Recommendations.

The texts of most of the papers presented were distributed in advance of the meeting. This was much appreciated by the participants.

Item 1 - Further information on preparation and shelf-life of concentrated FMD vaccine

Three papers were presented in this section of the agenda. The first (Appendix A-1) from Lindholm, was concerned with improving the safety with which larger volumes of infective material can be handled during the preparation of vaccines. The innovation in the Lindholm process has been to introduce an extra inactivation step by the addition of formalin to the unclarified virus culture prior to its harvest. This results in a 3 - 4 log unit drop in infectivity. Subsequently all steps in the formulation of the vaccine are made with largely inactivated virus. It has been shown that when carried out on an industrial scale this modification has not reduced the cf activity, the 146s antigen content nor the apparent potency of the vaccine as measured in PD_{50} tests with guinea pigs. The two-stage inactivation

procedure has been in successful operation in Denmark since late 1979 and has been used, not because of any difficulties in passing the innocuity tests, but as an improvement towards safer procedures during large-scale vaccine preparation.

In another contribution from the Danish laboratory (Appendix A-2) it was shown that the two-stage inactivated antigen could be precipitated with polyethylene glycol (PEG) 6000. Preliminary small-scale experiments have shown, however, that recoveries of antigen were disappointingly low depending upon the virus strain involved. For type A recoveries of 140s ranged from 36-65% and for type O virus the corresponding figures were 51-61%. In contrast, quantitative recovery of type C virus was obtained. Tests to compare the potency of standard vaccines with those formulated with antigen which had been PEG concentrated showed that in general the latter type of preparation was not significantly inferior despite the antigen assay results. This process is in line with previously reported work and could offer significant advantages in the storage of concentrated antigens as a strategic reserve against field emergencies.

A report from IFFA-Mérieux (Appendix A-3) showed that working with PEG 100,000 and ethyleneimine inactivated cell culture virus, antigens which had been concentrated 500-800 times could be recovered quantitatively. The amount of 146s antigen available varied from strain to strain. The type O strain produced the greatest amounts of immunogen in culture but despite this, larger than average amounts were required for vaccine preparation. Data presented showed that O, A and C antigens after storage in concentrated form at -70^{o}C for one year, were stable and vaccines prepared from them were of the same potency as conventional preparations. During the general discussion opinions were expressed that inactivated concentrated antigens would keep well in liquid nitrogen and this included the viruses exotic to Europe. Classical vaccines prepared directly from freshly harvested virus resulted in preparations that could be stored for one year - the period required by most countries. In Holland this has been extended to 18 months. Some doubts were raised that some concentrated preparations might not store as well as others or as compared to conventional vaccines and the buffers used to reconstitute the stored antigen might be critical. However, there were still relatively little data on this matter as yet.

From the reports and the ensuing discussion it is clear that there is continued interest in the concept of highly concentrated antigens and that research will continue in the various centres into establishing the optimum conditions for their preparation and storage.

Item 2 - Procedures for elution and innocuity testing of FMD vaccines

Due to the inclusion in the European Pharmacopoeia of a procedure for the safety testing of FMD vaccines by elution of adsorbed antigen, this topic and the allied one of the efficiency of different safety tests has continued to be of considerable importance.

Six papers (Appendix B1 - 6) were presented at the Session on various aspects of safety testing of vaccines. Data were presented from two laboratories which showed that with formaldehyde inactivated vaccines it was possible by means of elution and testing in unweaned mice or tissue culture systems, to find infective virus for up to 30 days after the start of inactivation. Subsequently, in tests at 60 days infectivity was not found and such vaccines are only used after 3-4 months storage. Residual infectivity in this type of vaccine has long been recognised and it has been found that by altering the conditions under which the inactivation is carried out, a more satisfactory product is obtained. Increasing the pH of the virus suspension to pH8.0 - pH8.7 appears to be important as does increasing the concentration of formaldehyde. Clarification of the virus suspension prior to inactivation was also thought to be essential.

It was clear both from the papers presented and the subsequent discussion, that there are still serious doubts about the efficiency of the elution method for the detection of small amounts of infective virus. It has been shown that up to 70 per cent of 146s particles of most strains of FMD virus may be recovered from saponin-free aluminium hydroxide vaccine by elution with 0.3M phosphate at pH7.6. However, the validity of this procedure, when applied

to vaccines prepared with both aluminium hydroxide and saponin, is questionable. Some viruses appear to bind to the aluminium hydroxide much more firmly than others. C Noville virus is eluted to much higher levels than most other strains. The SAT3 strain Bec 1/65 is very firmly bound and the SAT2 strain Bot 3/77 degrades on elution to 12s particles. Experience with the fraction of saponin known as Quil A and used as an adjuvant, was variable. In some hands it appears to bind virus as firmly as saponin whilst in others the reverse was true. One point universally accepted was that it was difficult to compare the results from different laboratories in view of the fact that the vaccines under test were prepared from antigens prepared and inactivated by different methods and that different saponins and aluminium hydroxide gels were used in their preparation.

A useful adjunct to the elution techniques was described by two laboratories. This involved the concentration of the eluted material by ultrafiltration and enabled proportionately larger samples of the production batch of antigen to be tested. A disadvantage of the technique however, was that the concentration of inactivated particles may become so high that they can mask the presence of traces of infectivity by an interference effect. It was suggested that dilution of the concentrated material and prolongation of the adsorption period on cell cultures might help to overcome this problem. Another concentration method involving the use of polyethylene glycol was described but this was of variable efficiency due to the high salt concentration in the buffers used to elute the virus.

The first order kinetics of the inactivation of virus infectivity by the aziridine compounds was also discussed. It was shown that by adopting a double dosing system at 24 hours and 48 hours it is possible to arrive at probable confidence levels for innocuity that are many orders of magnitude below that capable of being detected in any form of innocuity test. Acetylethyleneimmine (AEI) is now being replaced by binary ethyleneimmine (BEI) which can be formed within the inactivation vessel. The half-life of virus inactivated with this agent is similar to that obtained with AEI and AEI and BEI vaccines have comparable potencies in animals. If inactivation is performed with aziridines the use of inactivation curves will help to ensure final innocuity.

In summary there is still much interest in methods for the inactivation of FMD virus for vaccines and the optimum methods for testing to ensure that only safe products reach the field. Due to the obvious deficiencies in the system of testing vaccines by elution of antigen at present, it is clear that there is no standard method that could be applied universally to the testing of all FMD vaccines though, under defined circumstances, it could be valuable as an 'in house' quality assurance test. As a result of the variability of results obtained with different vaccines, doubts were expressed as to whether it was appropriate for inclusion in the methods for safety testing quoted in the European Pharmacopoeia.

Item 3 - FAO Collaborative International Study for the Standardization of Laboratory Methods in FMD Research. Discussion of the Results of Phase V.

Following the discussion of the results of Phase IV of this study at the Session in Vienna last year, it was concluded that the next Phase should be directed to investigating the reliability of the assay methods used to estimate the weight of FMD virus in a given preparation.

To this end, Phase V was devoted to comparing the sensitivity and linearity of response of measuring equipment (i.e. spectrophotometers) by means of a distributed standard preparation whose characteristics were sufficiently precise and also stable to be useful for this purpose. Preliminary work at both Lelystad and Pirbright compared various candidate materials including ferritin but eventually it was decided to use Adenine Hydrochloride as a suitable standard. Also it was decided to examine further the estimation of virus weight by sucrose density gradient fractionation. This investigation was aimed at gaining some impression of the reproducibility and reliability of the method at various virus concentrations. This last point was considered important since doubts were raised at last year's Session about the linearity of the response of the measuring systems. For this purpose a virus - MS2 Phage - regarded as more structurally stable than FMD virus was included in the study. The two FMD viruses used were O BFS 1860 and C Noville. For each virus three samples were provided there

being a three-fold difference in virus concentration between the samples. Participants were requested to measure each preparation at least three times.

Eleven laboratories participated in Phase V and the results of this work were presented.

Adenine Standards

Shortly after the sets of materials had been despatched from Pirbright it was found that the apparent concentration of the series of Adenine standard preparations was dropping significantly. It was thought this might be due to the adsorption of the Adenine to the plastic containers and participants were provided with a further set of dilutions in glass bottles and a concentrated preparation from which to make their own dilutions.

Results with the preformed dilutions have shown that at all three wavelengths the response of the instruments was reasonably good although in the case of some laboratories there was some deviation from a straight line at the most sensitive end of the range. In terms of absolute values there was some difference between instruments. Freshly prepared dilutions in each laboratory gave a linear response in some cases whilst others showed very great deviations from the expected. There was sometimes a considerable variation in values between instruments.

Virus Preparations

Examination of the crude data shows that within each laboratory the variation in the results is remarkably small and this appears to be true for both the FMD materials and the MS_2 phage. In contrast the degree of variation in the results from different laboratories, i.e. the between lab. variation is quite considerable. Rather surprisingly the results with MS_2 phage, the most stable of the two viruses under test, showed, if anything, the greatest degree of between lab. variation. However, overall mean values calculated for each of the three preparations of each virus showed that within the limitations of this type of assay system, the linearity of response was remarkably good. The observed values of the dilution intervals between preparations in any of the series was very close to the theoretical value i.e. three-fold.

Statistical analyses of the results obtained with the three type C preparations were shown. The conclusion was that each set of results included several groups which were different and this confirmed statistically the magnitude of the between laboratory variations. One rather disconcerting feature of the results overall was that variation between labs. appeared to be randomly distributed, i.e. there did not appear to be a consistent and predictable difference between laboratories when comparing the results from one virus series with the next.

Much of the discussion that followed the presentation of the results centred on the possible reasons for differences in the results from various laboratories. Factors such as the pH of materials, the manner of storage and the length of time after receipt before tests were made, were all extensively discussed as was the finding in some laboratories of more than one virus peak in some gradient preparations. It was agreed that several factors contributed to the level of variation observed including differences in instruments and also the gradient method used. The method of calculating the results could also contribute to the variation. It was suggested that instead of giving results in terms of absolute values it might be more meaningful if they were quoted as arbitrary or relative units. It was agreed that there was some merit in this and that it should be pursued further.

The universal opinion of the meeting was that the Collaborative Study was still considered to be a very worthwhile exercise. Whilst it was recognised that complete standardization of procedures in all the laboratories was probably never going to be possible, nevertheless, the collaborative work with common materials would undoubtedly bring about a better understanding of each others problems and could result in some measure of standardization between laboratories.

It was agreed that the Study should continue into a sixth phase and that it should be concerned with the further investigation of the possibility of finding better standards for measuring equipment and improving the methods of gradient analyses.

Item 4 - Topics raised by the Commission, Twenty-Fourth Session, Rome 1981

This Session was devoted to consideration of topics which had been raised at the Twenty-Fourth Session of the Commission held in Rome during April 1981 and which had been passed to the Research Group for advice.

(a) Seed Virus Stocks: The Commission wished to know whether the keeping of seed stocks against certain exotic virus strains should be continued and which strains should be kept. Dr. Mowat reported that the seed stocks of viruses kept at AVRI on behalf of the Commission were the same as had been quoted at the last meeting of the Research Group. Dr. Sellers' opinion regarding which strains should be kept was that there was little variation against the A_{22} strain therefore there was no need to change the present stock. For the ASIA$_1$ type the recent Middle East strains would be most appropriate and it was really the SAT 1, 2 and 3 strains which required reappraisal. It was probably better to have strains which were known to make good vaccines than have an homologous field isolate which only produced a poor vaccine. During the discussion the question of how much effort should be put into assessing seed viruses was raised. It was pointed out that the full development of a new vaccine could cost anything up to £50,000 - the bulk of this cost arising from the need to test the new vaccine in cattle. In view of this it was thought inappropriate to recommend the full development of new strains. It was mentioned that the International Association of Biological Standardisation had compiled a list of vaccine strains against which field isolates could be compared and it was suggested that the exotic viruses held at AVRI might be added to that list. Dr. Sütmoller on behalf of the Pan American Centre for Foot-and-Mouth Disease, Rio de Janeiro, offered to provide seed viruses for some of the South American strains should they be required. After considerable discussion which included points concerning the suitability or otherwise of BHK adapted viruses for growth in the cells available in various laboratories, it was decided that a continuation of the present policy of maintaining stocks of viruses exotic to Europe would be recommended.

(b) Formalin inactivated vaccines: The Commission wanted an opinion on whether the use of formalin as an inactivant for the preparation of FMD vaccines should be discontinued.

The FMD situation in Europe during the last seven years was reviewed. It was apparent that there were a number of outbreaks in which there was a close association with the use of vaccine. All of the vaccines involved were prepared by inactivation of the virus with formalin. It was the opinion of the meeting that if these outbreaks were due to vaccines still containing infective virus, this had happened as a result of either faulty equipment or the particular method of using formalin. It was pointed out that in the past there had been very satisfactory results from the use of formalin inactivated vaccines. However, it was of some significance that most of the major European manufacturers had now ceased to use formalin and had changed over to one of the aziridine compounds. It was considered important that the safety testing of vaccines should be as effective as possible. The Research Group felt that it was important that manufacturers should accept the responsibility of ensuring their products were safe and should be in close consultation with their own National Control Authority on matters relating to this. The Research Group felt it would be inappropriate for the European Commission to be involved in specifying the exact methods by which vaccines are prepared.

(c) Minimum acceptable potency of FMD vaccines

The monograph for FMD vaccines of the European Pharmacopoeia at present reads: "The vaccine contains at least 3 PD_{50} per dose for cattle" and the Commission required an opinion as to whether this was acceptable.

The Chairman of the Research Group pointed out that the precise definition of vaccine potency had proved to be extremely difficult and this had been a problem over the last 10 years. The difficulty lay in the wording and it had proved impossible so far to arrive at an entirely un-ambiguous definition. It was not clear as to what the stated numerical value related. Most workers would prefer that some indication of the expected confidence limits for this type of assay should be given. There was a considerable amount of discussion on the embiguity of some of the tests in use and the interpretation of the results. Concern was expressed that

there should not be a decline in standards. It was pointed out that under certain circumstances where vaccines were being used routinely for prophylactic purposes, up to 30 percent of the livestock population could be unprotected and it was important that vaccines of high potency be used. A statistical study showed that to have a 95% probability that vaccines would satisfy a 3 PD_{50} pass mark the average value of such vaccines would have to be approximately 6.8 PD_{50}. It was eventually agreed that it would be better to raise the expected pass mark to 7.0 and not to quote the confidence limit value.

(d) Movement of livestock and meat from areas where inactivated exotic strains have been applied

At present the recommendations of the Commission are that movement of slaughter stock or meat from those regions where cases of FMD caused by virus strains exotic to Europe have occurred (and vaccines against such viruses have been used e.g. in buffer zones in southeastern Europe), should not be permitted for a period of six months from the last overt case. Also where vaccination is practised in the absence of disease there should be a restriction of three months from the last vaccination. The Commission wished to know if the intervals quoted in these recommendations could be reduced. Dr. Stouraitis explained the situation and stated that this rule had been in operation for 10 years and affected not only Greece but also those other countries in which buffer zones were situated. During the discussion that followed, the question of whether there was danger from residual infection in the form of carrier animals or whether there was a possibility of infection from incompletely inactivated vaccines, was raised. After careful consideration of the risks to European agriculture that might be involved in reducing restrictions in those areas where exotic strains of FMD have occurred or inactivated exotic vaccines were applied, the members of the Research Group concluded that they could recommend that:-

(a) the rule restricting movement of slaughter stock or meat from areas where exotic strains have occurred for a period of six months from the last case should continue.

(b) where vaccination is practised a similar restriction should apply for two months from the date of vaccination without prejudice to the requirements established in para. (a) above.

Item 5 - Further information on cross-protection between European and South American A viruses

At that time the question was whether European countries should continue to vaccinate with the now classical A_5 vaccine strain or should switch to more recent field strains, for instance those of South American origin. Information on protection obtained with A_5 vaccine against recent South American field strains was lacking and it was hoped that during the year it would be possible for studies on cross-protection between the European and South American strains to be made at the Plum Island Laboratory. Unfortunately this has not proved to be the case. However some information on the serological and immunological characteristics of some of these strains was available from other sources and was presented to the Session.

In a study comparing A_5 Allier and A_{24} Argentina 1968, it was shown by cross-neutralisation tests that these strains are significantly different and that the A_{24} may be regarded as dominant to the A_5. This was also confirmed in cross-protection tests in cattle. Further, it was shown that the A_5 strain was not very effective in protecting against the A_{24} after one vaccination but that two vaccinations gave much better results though not complete protection. Conversely, A_{24} vaccine gave much better protection against the A_5 strain. (Appendix C-1).

A most useful paper giving recent information on the types O and A strains active in South America was presented by Dr. Sütmoller on behalf of the Pan American Center at Rio de Janeiro. Cross-neutralisation studies have shown that the strains A Bage, A Argentina 79 and A Brazil 79 are closely related. Strains with serological characteristics similar to them are among those most frequently recovered at present and these strains are more or less replacing the A_{24} strain which was previously widespread. The A Brazil/79 strain is tending to replace the A Bage strain

but there appears to be a spectrum of strains and, to a minor extent, these strains are all different from all of the foregoing, A RS-Brazil/81 was identified in an outbreak in the State of Rio Grande do Sul during 1981. The incidence of this strain is now decreasing although the majority of isolates are closer to A Brazil/79.

In relation to the O strains in South America, the number of field strains with characteristics similar to O_1 Campos is increasing. There was, however, an outbreak in Rio Grande do Sul in 1980 caused by a strain O RS-Brazil/80 which is obviously different from O_1 Campos. This strain is declining relative to the O_1 Campos which may have been spread by an infective vaccine. It has been shown in cross protection tests that the O_1 Campos strain does not give full protection against the O RS Brazil/80 strain and it is important that the spread of this latter virus should be carefully monitored. (Appendix C-2).

The opportunity was also taken in this Session to present information relating to the O strain which had been isolated in Austria during 1981. This strain and also one from an outbreak in Israel this year, were shown to have similar profiles when tested against a series of nine type O_1 strains. They were not closely related to any of the nine strains involved but within the limitations of a one way test were thought to be similar to each other. In addition, a brief presentation from AVRI Pirbright showed that by means of one of the newer techniques - iso-electric focusing of viral polypeptides - the O Austria 81 strain was unlike any of a series of some 47 strains with which it had been compared. One characteristic of the Austrian strain which was emphasised by Dr. Kubin, was its predilection for growth in pig cells and the difficulty of growing it in bovine tissues. This was confirmed by Dr. Lombard. (Appendix C-3).

The remaining two contributions in this Session were devoted to reports of studies comparing the titrations of FMD antibodies in cattle sera by means of the ELISA technique and sero-neutralisation in cells or mice. In both studies the titres obtained with the ELISA technique were of a similar order to those found with the neutralisation tests. There appeared to be more cross-reactivity with the ELISA tests and this, in the case of the Italian work, was most pronounced with type A reagents. It was also apparent from studies in which the responses to a single vaccination and also after revaccination were followed, that the ELISA test was detecting non-neutralising antibodies after the revaccination which did not diminish at the same rate as neutralising antibody. (Appendix C4 - 5).

It is clear that these newer test systems (ELISA) still require further investigation and modification to obtain the optimum results; nevertheless, the technical advantages to be obtained from no longer having to depend upon cells which may be variable in sensitivity, and the speed of obtaining results make such investigations very worthwhile.

Item 6 - Other business

(a) Transfer between laboratories of materials for genetic engineering

This topic was raised by Dr. Kihm. With the increasing amount of work in this field it was felt that the risks involved in manipulating and transferring infectious materials or their products between laboratories might not be fully understood by some workers. Dr. Kihm was of the opinion that the Research Group Session was an appropriate forum for discussion on this subject and possibly a proposal recommending a code of good practice could be included in the proceedings of the Session.

The Chairman pointed out that there were two main aspects to the problem. Firstly, with the advent of a new approach to FMD vaccine there was the possibility of previously uninvolved private companies starting to produce such vaccines. There was, therefore, the possibility of FMD virus or its derivatives being introduced into new areas previously free of FMD. There was also all the other points of concern associated with the use of genetically manipulated materials. Dr. van Bekkum was of the opinion that it was only in the area of disease security in relation to FMD that the Research Group should make recommendations.

During the considerable discussion this topic provoked, many opinions were expressed on what should be allowed in the way of transfer of material. Several members thought that this should be restricted to c-DNA alone, whilst others thought that if it was possible to transfer infectious FMD virus as happens at present, the regulations governing this should be adequate for genetically manipulated materials. All were of the opinion, however, that adequate safety testing of any materials before they were transferred was of paramount importance. It was agreed that a proposal relating to this topic should be included in the proceedings and a small group of members was given the task of drafting this statement. The latter is included in Item 7.

(b) Arrangements for the next Session of the Research Group

The Chairman announced that Dr. Sellers had very kindly agreed to the proposal that the next Session should be held at the Animal Virus Research Institute, Pirbright, U.K. The intended dates for the Session are 20 to 22 September 1982 and these have been suggested so as not to coincide with those of the O.I.E. Meeting in Paris due to take place during the previous week.

The following provisional agenda for the Session at Pirbright was proposed:-

Item 1: Information on strains current in the Middle East especially in relation to differences with European vaccine strains.

Item 2: Further studies on the safety testing of FMD vaccines.

Item 3: FAO International Collaborative Study for the Standardization of Laboratory Methods in FMD Research. Discussion of the results of Phase VI.

Item 4: Studies to assess the quality of antigens for the preparation of FMD vaccines.

Item 5: Alternative methods to the conventional cattle tests for assessing the immunising potency of FMD vaccines.

Item 6: Demonstration of the facilities and some of the current research at the Animal Virus Research Institute.

Item 7: Any other business.

Item 8: Final conclusions and recommendations.

Since the distribution of papers in advance of the Session had been so much appreciated, Dr. van Bekkum proposed that this arrangement should be continued for future meetings and requested that the texts of papers for next year's meeting should be sent to Dr. Sellers at least one month beforehand to allow time for their distribution.

During the closing Session Dr. van Bekkum thanked the speakers and contributors to the discussions, the FAO Secretariat, Dr. P. Stouraitis and Miss Joan Raftery and the rapporteur Dr. G.N. Mowat. He paid particular tribute to Professor Manfred Mussgay for the splendid arrangements which he and his staff had made on behalf of the Research Group. It was 10 years since the last Session in Tübingen and it had been a great pleasure to return to such a warm and generous reception. Dr. van Bekkum extended his sincere thanks on behalf of all the participants.

Item 7: Final Conclusions and Recommendations

These were as follows:-

Item 1: Further information on preparation and shelf life of concentrated FMD vaccine

In working with vaccine production systems which are less than completely secure, preliminary application of formaldehyde reduces the infectivity of large-scale cultures to a much safer level for handling. This additional treatment did not significantly reduce the immunogenicity of vaccines in comparison to standard preparations.

Preliminary results show that although full recovery of 146S antigen (especially types O and A) was apparently not obtained with PEG concentration of formaldehyde inactivated antigen, vaccines prepared from the concentrated material and formulated similarly to standard vaccines were at least the same potency for guinea pigs.

Ethyleneimine inactivated FMD antigen concentrated by use of PEG 100,000 had recoveries of 146S antigen which depended upon the strain involved. After storage of this antigen for one year at $-70^{o}C$ there was no apparent loss of 146S material and a vaccine prepared with the stored material was as potent as a vaccine prepared from the original concentrated antigen.

The papers presented indicated that although concentrated material can be stored and make successful vaccines, undesirable losses of antigen may occur in the preliminary concentration procedure. In addition, there are indications that vaccines prepared from stored concentrated antigen may be less stable than conventional vaccines. However, the work presented was of considerable practical importance and it was recommended that further investigation of these topics should continue.

Item 2: Procedures for elution and innocuity testing of FMD vaccines

The following recommendations regarding the safety testing of FMD vaccines according to the European Pharmacopoeia were made:-

1. The safety test in cattle formulated in the European Pharmacopoeia has the probability of failing to detect traces of non-inactivated virus in the vaccine. Therefore the test should be complemented by a tissue culture test, employing cells with the greatest available sensitivity for the vaccine strain.

2. The tissue culture test may be carried out on either unadsorbed antigen or on adsorbed antigen after elution. In both cases the volume to be tested of the unadsorbed antigen or of the eluted antigen should be equated with a certain number of cattle doses of final vaccine. It is recommended that an appropriate statistical assessment should be made to define this number.

3. In the present test for the control of inactivation, the procedure for elution is not effective in all cases and may be less so in the presence of saponin. The present method of concentration by poly-ethyleneglycol 6000 can give poor recoveries. Alternative methods such as ultrafiltration should be considered.

4. The interference effect of inactivated virus in the tissue culture test should be excluded by the use of appropriate techniques.

Item 3: FAO International Collaborative Laboratory Study

The Laboratory Group recommends a continuation of the current FAO collaborative study of the measurement of 146S particles by the density gradient procedure.

Although the work from Phase V has shown an improvement in "between" laboratory variations it is felt that the differences which still exist can be reduced further.

To this end, it is suggested that a pilot study by the Animal Virus Research Institute, the Wellcome FMD Vaccine Laboratory, Pirbright, and the Centraal Diergeneeskundig Instituut, Lelystad, be made in order to further standardise procedures prior to distribution of samples for Phase VI.

For the pilot study it is suggested that the following be undertaken:-

1. Studies on the stability of spectrophotometer standards.
2. Studies on the stability and reliability of the MS2 reference virus.

Subject to the findings of this preliminary investigation, the following types of samples would be distributed:-

1. A series of spectrophotometer standards including one standard of certified optical density to be used to calibrate the instrument.

2. A series of virus preparations to be evaluated by the laboratories. These will probably include 0 BFS1860 and MS2 viruses.

It is recommended that an attempt is made to standardize the experimental procedures to be used in Phase VI with particular reference to gradient conditions. This should take account of the materials used to make the gradients. Protocols should be distributed in advance of the samples giving precise information relating to gradient design, instrument calibration and extinction coefficients to be used in the calculation of the data.

It is considered important to send to A.V.R.I. Pirbright sufficient information so that all the results may be presented after calculation by the same method, at the next Session of the Research Group.

Item 4: Topics raised by the Commission, Twenty-Fourth Session, Rome 1981

(a) Virus seed stocks

The Research Group recommended that the Animal Virus Research Institute, Pirbright, should continue to hold seed viruses of some of the sub-types of A_{22}, SAT_1, SAT_2, SAT_3, and $ASIA_1$ as at present. It was also recommended that AVRI should monitor the appropriateness of these strains for emergency use in Europe. It was noted that the Pan American Center for FMD, Rio de Janeiro, offered to make available appropriate South American virus strains on official request.

(b) The continued use of formalin as an inactivant for the preparation of FMD vaccines

The Members of the Research Group recognised that there may be many different aspects of importance in obtaining innocuous FMD vaccines. Experience suggests that while the use of some formalin inactivated vaccines has occasionally been associated with outbreaks of disease, other FMD vaccines prepared with this inactivant have been fully satisfactory in this respect.

The Research Group is of the opinion that vaccine safety is primarily the responsibility of vaccine producers and National Control Authorities. These agencies should ensure that only those procedures which result in a completely innocuous preparation should be used in routine vaccine production.

The Research Group also considers that it would be inappropriate for the European Commission for the Control of Foot-and-Mouth Disease to make specific recommendations regarding the formulation of vaccines.

(c) European Pharmacopoeia Monograph on Standards for FMD Vaccines

The problem of defining a standard was extensively discussed. The majority opinion of the Members of the Research Group was that a minimal requirement of at least 3 PD_{50} as defined or stated in the Monograph was not acceptable. The term "at least 3 PD_{50}" was ambiguous, since it could be read either as an observed value or as a minimum value. Consideration of the statistical aspects of defining a precise value showed this to be very difficult if not impossible if that value is to satisfy all circumstances.

Members were concerned that a value which had previously been put forward as a minimum acceptable standard should not now be allowed to become the norm. Since the National Authorities of several countries in Europe at present require significantly more than 3 PD_{50} as the minimal requirement for vaccines, to safeguard against fall in such standards and to remove the difficulties associated with defining limits of confidence, it was recommended that an observed value of 7 PD_{50} without stated confidence limits should be the value for vaccines to conform to the requirements of the Monograph.

(d) Movement of slaughter stock and meat from areas where exotic strains of FMD have occurred or inactivated exotic vaccines were applied

After careful consideration of the risks to European agriculture that might be involved in reducing restrictions in those areas where exotic strains of FMD have occurred or inactivated exotic vaccines were applied, the members of the Research Group concluded that they could recommend that:-

(a) The rule restricting movement of slaughter stock or meat from areas where exotic strains have occurred for a period of six months from the last case should continue.

(b) Where vaccination is practised a similar restriction should apply for two months from the date of vaccination without prejudice to the requirements established in para. (a) above.

Item 5: Further information on cross-protection between European and South American A-viruses

The Group discussed the desirability of recommending changes from A_5 to current extra-European strains of type A for vaccine production. The information presented by different participants indicated that the protection by A_5 vaccines against A_{24} strains could not be considered acceptable after one vaccination and also that the epidemiological picture in South America is much more complicated than had previously been thought. The Group concluded therefore that at present they were not able to recommend any current field strain for incorporation in European vaccines.

In order to meet problems that might arise due to the introduction of non-European strains of the classical types of virus, European laboratories might equip themselves with reference sera against the various extra-European viruses and the World Reference Laboratory, Pirbright was asked to coordinate the distribution of such sera with the Pan American Center, Rio de Janeiro. It was also thought desirable that the FMD Group of the International Association for Biological Standardization should be included in future discussions on the distribution of reference material. The World Reference Laboratory, Pirbright and the Pan American Center, Rio de Janeiro, should stimulate and coordinate further investigations on the relationships of South American virus strains to European strains.

It was considered desirable that national laboratories should submit samples of materials from outbreaks with unusual characteristics to the WRL at the earliest possible date.

It is recommended that techniques to evaluate the relationships between new outbreak strains and available vaccine strains involving the use of inactivated antigens should be further investigated.

Item 6: Other business

(a) Transfer of genetically manipulated materials

With regard to recombinant DNA work on FMD virus, in general the Group was concerned about the release of possibly infective materials from FMD laboratories. Materials leaving such laboratories should routinely be tested for the absence of infectivity. As recent developments open the door to a much wider circulation of genetic material derived from FMD virus than was previously the case, the opinion was further expressed that National Authorities

should develop legislation covering the importation of FMDV derived materials used in recombinant DNA work. Adequate tests for the absence of infective virus should be required.

Several groups have succeeded in cloning FMD viral genome fragments by the recombinant DNA technique. This success now offers the possibility of studying the molecular basis for differences between serotypes and subtypes. Furthermore, it has been demonstrated that an inserted viral genome fragment coding for the major viral protein is expressed in the manipulated bacteria. The product of this expression can induce immunity after repeated application. The Research Group considers this development as a great achievement in FMD research especially in its immediate application to the determination of the nature of the genome. However, the Group recognizes that much more work is required before the possible value of this type of product can be established in relation to the control of FMD in the field.

Appendix A-1

Formalin-treatment of crude BHK suspension cell-derived FMDV as a safety measure in antigen processing

by

J.C. Lei*

The classical approach in foot-and-mouth disease virus (FMDV) vaccine preparation involves processing of large volumes of highly infectious material from harvest of the virus to the start of inactivation, irrespective of the final inactivation procedure used. Whereas the virus growth step with the increasing use of suspension culture techniques is virtually devoid of manipulations with infectious material and can be easily contained, the processing of virus harvests into final vaccines involves several manipulative steps which are not always safeguarded with ease and efficiency.

The present report deals with the treatment of crude FMDV harvest, while still under containment in the fermentor, with 0,05% formalin for 24 hours at 26°C to improve the safety of vaccine production.

Experimental part

Virus

The serotypes and strains of FMDV used in this study have been described previously (1). The virus was propagated in BHK suspension cells as reported by Jensen et al (2).

Chemicals etc.

All chemicals used were of analytical grade quality. Formalin (min. 35%) was applied as a 1:20 dilution in redistilled water. An aqueous solution of bisulfite (0.35 M sodium sulfite, 0,35 M sodium bisulfite, pH 6.4) was used in the neutralization of formaldehyde before titration of infectivity. The composition of the glycine buffer has been given previously (3). The aluminium hydroxide gel used was Alhydrogel (1.3% as Al_2O_3) available from Superfos a/s, Denmark. The Kieselguhr (KG) was Hyflo Super Cel from Johns-Manville, USA.

Preparation of vaccines

The preparation of vaccines was carried out as presented in fig. 1.

Assay procedures

a) Antigen

Titration of infectivity and complement-fixing (CF) activity was done as previously described (3). This reference also gives information on the assay of protein by a modified Biuret method. A complete description of the 140 S particle determination can be found in reference 4.

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b) Vaccines

Formaldehyde was quantitated by the Hantzsch reaction as described by Nash (5) and carried out on the supernatant liquid of vaccines. The potency in terms of PD_{50}-content of the one-stage and two-stage inactivated vaccines was estimated in a comparative guinea pig test. A series of 4-fold dilutions in dummy vaccine of each of the two vaccines were prepared and 1.0 ml of vaccine dilution was injected subcutaneously into each of 10 guinea pigs. Three weeks after vaccination, all vaccinated animals together with 10 non-vaccinated ones were challenged by inoculation in one plantar pad with homologous guinea pig adapted cattle virus (titer of about 10^3 guinea pig ID_{50} per ml). Symptoms of generalization of the disease were recorded three times with final inspection taking place 7 days after challenge. The PD_{50}-content was estimated according to the method of Kärber (6).

Results and conclusions

Treatment of crude FMDV harvest with formalin was first investigated under laboratory conditions. The data of all three serotypes have been collected in table I.

Conclusions drawn from table I

a) Heat treatment alone:

1. Generally, a drop in titer of infectivity of about 0.4 log_{10}-units can be observed.
2. The protein content is reduced by about 7% on an average.

b) Combined effect of heat and formalin:

1. A drop in infectivity of about 5 log_{10}-units is obtained.
2. No significant reduction in CF-activity occurs.
3. No significant reduction in content of 140 S particles can be observed.
4. Formalin in itself does not appear to affect the protein content.

The data concerning the influence of fomalin-treatment on antigen parameters during large-scale processing of the three serotypes are presented in tables II, III and IV, respectively.

Conclusions drawn from the tables

1. The drop in titer of infectivity ranging from average values of 3.2 log_{10}-units for type C to 4.0 log_{10}-units for type A is lower than that observed under laboratory conditions. However, the reduction in infectivity under fermentor conditions is in good agreement with literature data (7, 8, 9).

2. No significant reduction in CF-activity can be observed.

3. No significant reduction in content of 140 S particles occurs.
   Item 2 and 3 are in agreement with references 8, 10 and 11.

4. Contrary to laboratory conditions, no reduction in protein content occurs.

From the data of table V it is concluded that formaldehyde does not appear to be consumed during treatment or to be lost during processing of crude FMDV harvest.

The one-stage and two-stage inactivated vaccines have been evaluated in a comparative potency test in guinea pigs. The results which have been grouped according to serotype are found in tables VI, VII and VIII, respectively. From the tabulated data it is concluded that the two-stage inactivation procedure does not appear to impair the immunogenicity of FMDV to any greater extent than the traditional one-stage inactivation procedure.

Concluding remarks

From a practical point of view, the treatment of crude virus harvests with inactivants before processing of antigen into FMDV vaccines offers a significant improvement of safety. It is common experience that leakages in the form of liquid or aerosol do occur during vaccine preparation. The contamination of the environment during these accidental leakages may be expected to be reduced proportionally to the reduction in infectivity. During the 24 hours treatment with formalin used in this study, a reduction in infectivity of at least 3 $\log_{10}$-units could be achieved.

The two-stage procedure reported here has been in successful operation in the Danish production of FMDV vaccines since late 1979 with the modification that the second period of inactivation was prolonged from 24 hours to 48 hours, irrespective of the pretreatment with formalin. This modification has not been prompted by failure to pass the vaccine innocuity test but was introduced as an extra safety margin in inactivation of aluminium hydroxide gel containing vaccines with formalin. The immunological performance of bulk vaccines prepared by the two-stage procedure over this period of time has not been found to differ significantly from the previous one-stage inactivated vaccines.

Acknowledgement

The author thanks the Department of Serology (head: Dr. K. Schjerning-Thiesen) for titration of CF-activity and handling of experimental animals, and the Department of Cell Culture and Virus Production for titration of infectivity and adaptation of the formalin-treatment to fermentor operating conditions. Likewise, the technical assistance of Lene Haarby Hansen and Kit Frederiksen, Department of Chemistry, is highly appreciated.

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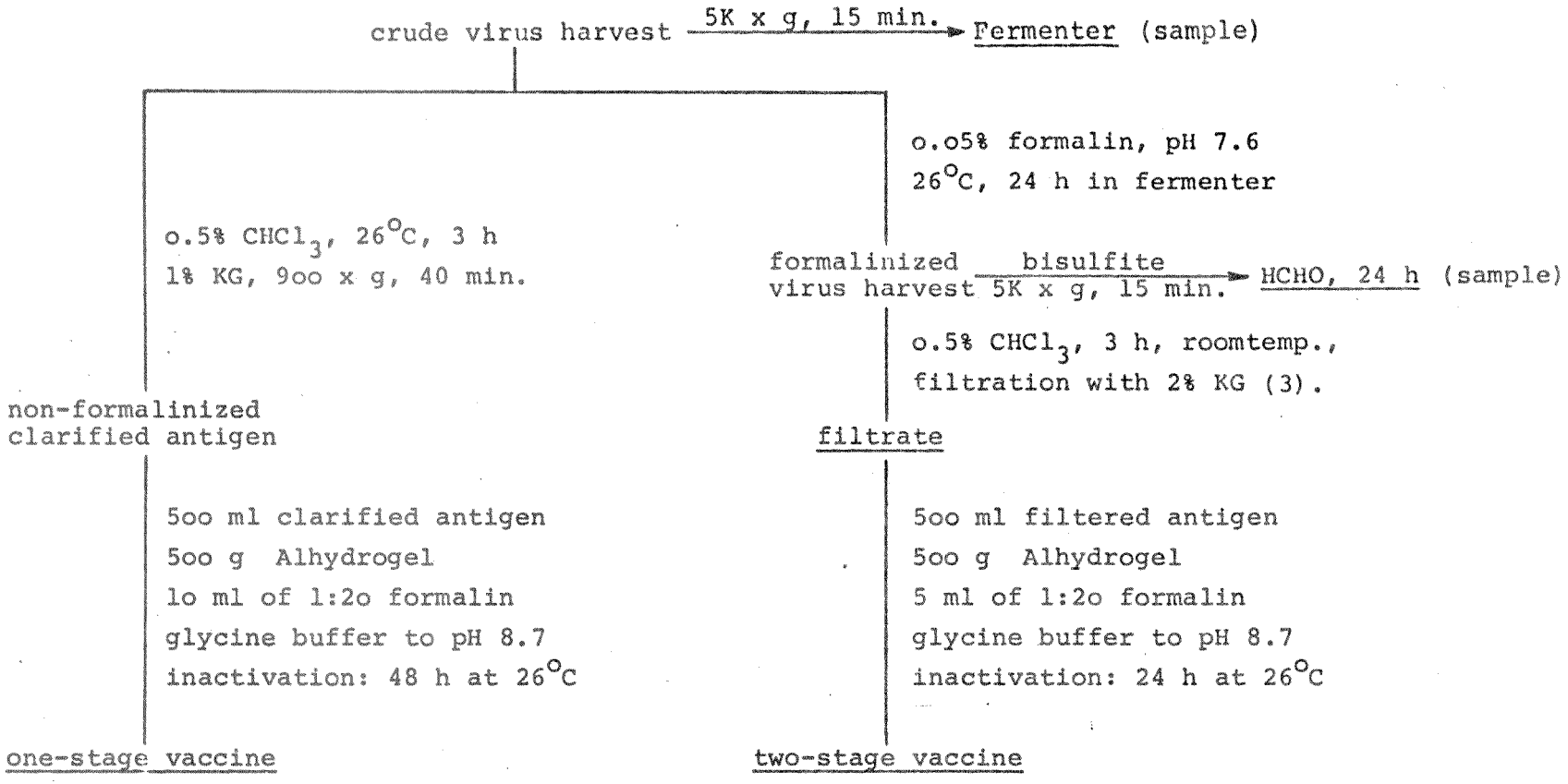

Fig. 1. Schematic presentation of the preparation of one-stage and two-stage inactivated vaccines, respectively. Samples and vaccines appearing in the tables are marked by underlining.

Table I.

Formalin-treatment of FMDV harvested from BHK suspension cells.

Effect of treatment on various antigen parameters.

Conditions of treatment: 0.05% formalin, 26°C for 24 hours, laboratory scale

Batch no.	Sero-type	Treat-ment	Inf. titer	CF-titer	140 S µg/ml	Protein
		None	6.9	4	1.8	0.614 a)
350279	A	26°C, 24h	6.1	4	n.d.	0.560
		HCHO,26°C,24h	1.7	4	2.0	0.557
		None	5.7	4	1.8	0.614
350179	A	26°C, 24h	6.3	4	n.d.	0.575
		HCHO,26°C,24h	1.3	4	1.6	0.588
		None	6.1	8	2.2	0.573
360279	O	26°C, 24h	6.1	8	n.d.	0.546
		HCHO,26°C,24h	1.7	8	2.5	0.552
		None	6.9	4	2.2	0.579
370179	O	26°C, 24h	6.5	4-8	n.d.	0.529
		HCHO,26°C,24h	1.7	4	2.5	0.529
		None	6.7	8	2.3	0.575
370279	O	26°C, 24h	6.7	4-8	n.d.	0.544
		HCHO,26°C,24h	1.5	4-8	2.3	0.544
		None	6.5	4-8	2.9	0.645
380179	C	26°C,24h	6.1	4-8	n.d.	0.583
		HCHO,26°C,24h	1.5	4-8	2.9	0.580
		None	6.7	4-8	2.5	0.566
380279	C	26°C, 24h	5.9	4-8	n.d.	0.531
		HCHO,26°C,24h	1.7	4-8	2.6	0.529
		None	6.9	4-8	2.7	0.625
390179	C	26°C,24h	6.5	4	n.d.	0.598
		HCHO,26°C,24h	1.9	4-8	2.9	0.596
		None	6.7	4	2.4	0.605
390279	C	26°C,24h	6.3	4-8	n.d.	0.555
		HCHO,26°C,24h	1.7	4-8	2.8	0.572

Titer of infectivity is in $\log_{10}$-units per 0.1 ml.
Titer of complement fixation is the reciprocal of the highest antigen dilution estimated to cause complete binding of 4 units(100%) of complement in the presence of a surplus of guinea pig hyperimmune serum.

a) Mean of 2 determinations, expressed in OD-units.

n.d. - not done

Table II.

The influence of formalin-treatment on antigen parameters during large-scale processing of FMDV from BHK suspension cells.

Conditions of treatment: 0.05% formalin, 26°C for 24 hours.

Serotype of virus: A

Batch no.	Sample marked	Inf. titer	CF-titer	140 S µg/ml	Protein
	Fermenter	6.1	4	2.0	0.642 a)
480279	HCHO, 24h	2.1	4-8	2.0	0.641
	Filtrate	n.d.	4-8	1.7	0.602
	Fermenter	6.3	4-8	2.4	0.583
030280	HCHO, 24h	2.1	8	2.5	0.564
	Filtrate	n.d.	4	2.8	0.550
	Fermenter	7.1	4-8	1.6	0.568
040281	HCHO, 24h	3.5	8	1.7	0.564
	Filtrate	n.d.	4-8	1.8	0.547
	Fermenter	6.9	8	2.1	0.602
120281	HCHO, 24h	2.7	8	2.1	0.599
	Filtrate	n.d.	4-8	2.0	0.592

Titer of infectivity is in $\log_{10}$-units per 0.1 ml

Titer of complement fixation is the reciprocal of the highest antigen dilution estimated to cause complete binding of 4 units(100%) of complement in the presence of a surplus of guinea pig hyperimmune serum.

a) Mean of 2 determinations, expressed in OD-units.

n.d. - not done.

Table III.

The influence of formalin-treatment on antigen parameters during large-scale processing of FMDV from BHK suspension cells.

Conditions of treatment: 0.05% formalin, 26°C for 24 hours.

Serotype of virus:O

Batch no.	Sample marked	Inf. titer	CF-titer	140 S µg/ml	Protein
410179	Fermenter	6.3	4-8	2.1	0.640 a)
	HCHO, 24h	2.3	4-8	1.8	0.632
	Filtrate	n.d.	4	1.8	0.603
090280	Fermenter	7.1	4	1.6	0.555
	HCHO, 24h	2.7	8	1.7	0.555
	Filtrate	n.d.	8	1.7	0,541
060281	Fermenter	7.1	8	1.5	0.629
	HCHO, 24h	3.7	16	1.6	0.623
	Filtrate	n.d.	8	1.8	0.583
130281	Fermenter	6.7	8-16	1.9	0.612
	HCHO, 24h	3.9	8-16	2.0	0.606
	Filtrate	n.d.	8	2.2	0.556

Titer of infectivity is in $\log_{10}$-units per 0.1 ml.

Titer of complement fixation is the reciprocal of the highest antigen dilution estimated to cause complete binding of 4 units(100%) of complement in the presence of a surplus of guinea pig hyperimmune serum.

a)Mean of 2 determinations, expressed in OD-units .

n.d. - not done.

Table IV.

The influence of formalin-treatment on antigen parameters during large-scale processing of FMDV from BHK suspension cells.

Conditions of treatment: 0.05% formalin, 26°C for 24 hours.

Serotype of virus: C

Batch no.	Sample marked	Inf. titer	CF-titer	140 S µg/ml	Protein
400179	Fermenter	6.5	4	2.9	0.604 a)
	HCHO, 24h	3.3	4-8	2.7	0.591
	Filtrate	n.d.	4-8	2.8	0.548
070180	Fermenter	6.5	4	3.4	0.582
	HCHO, 24h	3.3	8	3.0	0.581
	Filtrate	n.d.	4-8	3.1	0.552
080281	Fermenter	6.9	16	2.7	0.580
	HCHO, 24h	3.7	16	3.2	0.584
	Filtrate	n.d.	8-16	3.1	0.553
100281	Fermenter	6.5	8-16	3.0	0.574
	HCHO, 24h	3.1	8	3.3	0.574
	Filtrate	n.d.	8	3.3	0.542

Titer of infectivity is in $\log_{10}$-units per 0.1 ml.
Titer of complement fixation is the reciprocal of the highest antigen dilution estimated to cause complete binding of 4 units(100%) of complement in the presence of a surplus of guinea pig hyperimmune serum.
a) Mean of 2 determinations, expressed in OD-units.
n.d. - not done

Table V.

Comparative determination of final concentrations of formaldehyde in vaccines prepared by one-stage and two-stage inactivation, respectively.

Batch no.	Sero-type	HCHO in µg/ml	
		one stage inactivation	two-stage inactivation
400179	C	140.0	134.8
420179	A	124.4	118.4
460179	A	124.8	120.8
460279	A	126.0	117.2
470179	A	121.0	113.2
470279	A	132.4	127.8
480279	A	135.0	129.8

Table VI.

Comparative potency testing in guinea pigs of one-stage and two-stage formalin-inactivated FMD vaccines.

	Serotype A			
	one-stage inactivation		two-stage inactivation	
Batch no.	PD_{50}/ml	Range	PD_{50}/ml	Range
4201/79	3.5	2.6- 4.5	2.6	2.0- 3.4
4601/79	2.0	1.4- 2.9	1.0	0.8- 1.3
4602/79	3.0	2.2- 4.2	4.6	3.4- 6.3
4701/79	3.5	2.4- 5.2	5.3	3.8- 7.3
4702/79	1.5	1.1- 2.1	1.5	1.2- 2.0
4802/79	2.6	2.1- 3.4	3.0	2.3- 4.1
0301/80	2.0	1.4- 2.8	2.3	1.7- 3.0
0302/80	0.8	0.6- 1.0	4.6	3.2- 6.1
0401/80	9.2	6.3-13.4	4.6	3.4- 6.3
0402/80	6.1	4.3- 8.6	9.2	6.3-13.3
0501/80	16.0	10.5-24.5	35.6	24.5-51.9
0502/80	64.0	50.8-80.6	10.6	7.3-15.2

Table VII.

Comparative potency testing in guinea pigs of one-stage and two-stage formalin-inactivated FMD vaccines.

Serotype O.

	one-stage inactivation		two-stage inactivation	
Batch no.	PD_{50}/ml	Range	PD_{50}/ml	Range
4101/79	15.8	10.8-23.3	18.2	12.8-25.9
0901/80	48.5	35.1-67.0	13.9	10.2-19.1
0902/80	18.4	14.3-23.7	8.0	5.9-10.9
1001/80	27.9	20.0-38.8	24.3	17.0-34.7
1002/80	19.0	14.1-25.5	12.1	8.7-17.0

Table VIII.

Comparative potency testing in guinea pigs of one-stage and two-stage formalin-inactivated FMD vaccines.

Serotype C.

	one-stage inactivation		two-stage inactivation	
Batch no.	PD_{50}/ml	Range	PD_{50}/ml	Range
4001/79	5.5	4.0- 7.5	10.4	7.7-14.1
0701/80	7.0	4.7-10.2	9.2	6.9-12.2
0702/80	4.6	3.2- 6.6	12.1	8.2-18.0
0801/80	10.6	7.9-14.1	16.0	12.1-21.2
0802/80	12.1	9.1-16.2	12.1	8.4-17.5

Appendix A-2

Introductory study on the storage of PEG-concentrated antigens from formalin-treated crude harvests of FMDV

by

J.C. Lei

In another paper presented at this meeting, the formalin-treatment of crude FMDV harvests from BHK suspension cells was investigated with a view to improvement of safety in vaccine preparation. In the present report, the safety aspect of treatment with formalin is taken one step further to the storage of PEG-concentrated antigen from formalinized crude virus harvests.

Experimental part

Materials

The serotypes and strains of virus (1), the method of virus propagation (2) and the chemicals etc. were as reported in the paper on formalin-treatment presented at this meeting. PEG 6000, in flakes, was obtained from Hoechst, Germany. The Kieselguhr (KG) used for the isolation of PEG-precipitated material by filtration was Standard Super Cel from Johns-Manville, USA. The preparation of the modified Earle's medium used to resuspend the PEG-precipitated material has been given previously (1).

Methods

The preparation of vaccines has been outlined in fig. 1. The assay procedures applied to virus samples have been described (4,5). The comparative potency test of the vaccines was similar to that described in the paper on formalin-treatment (presented at this meeting), except that a dose volume of 0.33 ml was used due to concentration of the antigen. In the preliminary test of the vaccines, only undiluted vaccine and vaccine diluted 1:9 in dummy vaccine were given. The dose volume, the route of vaccination, the method of challenge and the subsequent recording of diseased animals were as used in the above potency test.

Results and discussion

The analytical data describing the main points of the procedure are shown in table I. In terms of CF-activity, the recovery of antigen after precipitation with PEG, storage and resuspension was found to be modest. In most cases, only from 1/3 to 2/3 of the original activity was recovered. However, this does not necessarily indicate insufficient recovery of immunogen as has been observed repeatedly in our laboratory (1,4). More alarming were the recoveries in terms of 140 S particles in the case of serotypes A and O. For serotype A, a recovery ranging from 36 to 65% was found. The corresponding figure for type O ranged from 51 to 61%. Contrary to these recoveries, serotype C showed quantitative recovery of 140 S antigen. These results are quite different from corresponding results of technical scale experiments with non-formalinized virus harvest (1). In the latter experiments there was no pronounced difference in recovery of 140 S particles between the three serotypes and the recoveries rated from 101 to 129%. The present recovery of 140 S material of serotype C from formalinized virus therefore appears to be within the expected range, whereas type A and type O fail in this respect.

Confronted with a possible loss of immunogen, the PEG-vaccine and the corresponding standard vaccine were subjected to a preliminary comparative evaluation of immunizing effect in guinea pigs. The results are shown in table II. This preliminary comparison of the two vaccines did not reveal significant differences in performance. It is of particular

interest to note that the PEG-vaccines prepared from virus of serotypes A and O did not differ significantly from the corresponding standard vaccines in spite of the apparent low recovery of 140 S antigen.

The test program was therefore extended by selection of two vaccine sets of each serotype for estimation of PD_{50}- content. The results of this trial are shown in Table III. As can be seen from the figures, only one of the vaccine sets indicated a significant difference in potency in favour of the standard vaccine. However, compared with the outcome of the preliminary testing presented in table II, it was concluded that in general the PEG-vaccine functions as well as the standard vaccine.

On the basis of these findings it is our intention to proceed to technical scale experiments. With the reduction in infectivity of 3 to 4 log_{10}- units prior to precipitation with PEG, achieved by treatment with formalin (paper presented at this meeting), the present Danish strategic stock of concentrated FMDV antigen would correspond to storing about 10 litres of native infectious virus harvest. This represents a major improvement of safety in storage and subsequent processing of PEG-concentrates of FMDV. A disadvantage of the method is that titration of infectivity is no longer meaningful as a monitor during processing, a situation which is further complicated by a possible failure in the quantitation of 140 S particles of serotype A and O.

Acknowledgement

The author thanks the Department of Serology (head: Dr. K. Schjerning-Thiesen)for titration of CF-activity and handling of experimental animals, and the Department of Cell Culture and Virus Production (head: Dr. M.Jensen) for titration of infectivity. Likewise, the technical assistance of Lene Haarby Hansen and Kit Frederiksen, Department of Chemistry, is highly appreciated.

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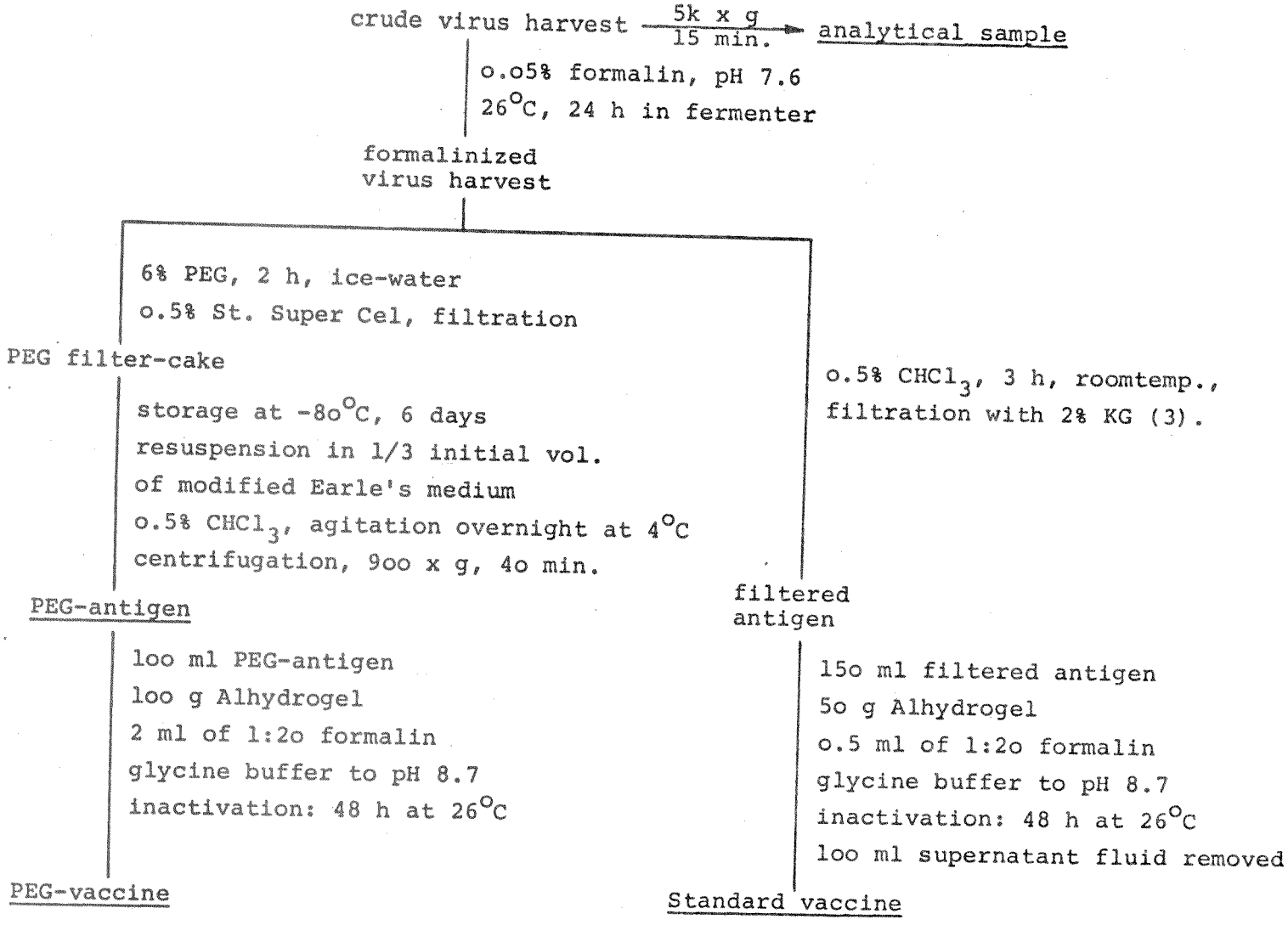

Fig. 1. Schematic presentation of the preparation of PEG-vaccine and Standard vaccine. Samples and vaccines appearing in the tables are marked by underlining.

Table I.

Antigen parameters of crude virus harvest and corresponding PEG-antigen obtained after formalin-treatment, PEG-precipitation and storage of precipitated antigen.

Batch no.	Sero-type	Crude virus harvest: Inf. titer	Crude virus harvest: CF-titer	Crude virus harvest: µg/ml 140 S	Supernatant after PEG-precipitation: CF-titer	PEG-antigen: CF-titer	PEG-antigen: µg/ml 140 S	Apparent 140 S antigen recovery in %
0301/81	A	6.7	4	1.6	1-2	8	2.7	56.3
0401/81	A	7.1	4	1.5	2	4-8	2.4	53.3
1101/81	A	6.5	8	1.9	1	4-8	3.7	64.9
1201/81	A	6.7	4	1.3	2	8	1.4	35.9
0501/81	O	6.5	4	1.8	4	4	3.3	61.1
0601/81	O	7.1	4-8	1.3	8	4-8	2.0	51.3
1401/81	O	6.9	8	2.7	4	8	4.5	55.6
0701/81	C	6.7	8	2.8	4	16	7.9	94.0
0801/81	C	7.1	16	2.3	8-16	8-16	6.8	98.6
0901/81	C	7.7	4	2.6	8-16	8-16	7.7	98.7
1001/81	C	6.9	4	2.3	2-4	16	7.5	108.7

Infectivity titers are given as $\log_{10} ID_{50}/0.1$ ml.

CF-titers given are reciprocals of the highest antigen dilution estimated to cause complete binding of 4 units(100%) of complement in the presence of a surplus of guinea pig hyperimmune serum.

Table II

Preliminary comparative testing of immunizing effect in guinea pigs of standard FMD-vaccine and PEG-vaccine.

		Standard vaccine		PEG-vaccine	
Batch no.	Serotype	undil.	1:9	undil.	1:9
0301/81	A	9/10[a)]	7/10	10/10	7/10
0401/81	A	8/10	8/10	10/10	6/10
1101/81	A	9/10	4/10	10/10	9/10
1201/81	A	8/10	6/9	10/10	8/10
0501/81	O	9/10	9/10	9/10	9/10
0601/81	O	9/10	6/10	9/10	5/10
1401/81	O	10/10	5/10	10/10	8/10
0701/81	C	9/10	7/10	10/10	5/10
0801/81	C	10/10	4/10	8/10	5/10
0901/81	C	10/10	6/10	10/10	7/10
1001/81	C	8/10	3/10	10/10	7/10

a) number of immune animals/total number of animals in group

Table III

Comparative testing of potency in guinea pigs of standard FMD vaccine and PEG-vaccine.

		Standard vaccine		PEG-vaccine	
Batch no.	Serotype	PD_{50}/ml	Range	PD_{50}/ml	Range
4001/81	A	63.3	42.9- 93.5	13.8	9.8- 19.4
1101/81	A	27.6	19.1- 39.8	36.4	24.6- 53.7
0501/81	O	48.0	32.9- 70.1	63.3	47.1- 85.1
1401/81	O	15.8	10.6- 23.8	24.0	16.1- 35.7
0801/81	C	89.6	61.8-129.9	167.2	122.2-228.7
1001/81	C	41.8	29.8- 58.7	48.0	33.9- 68.0

PD_{50} according to Karber

Appendix A-3

Conservation of Antigenic and Immunological Properties of Concentrated FMD Virus after Storage at -70°C

by
A. Rey - A. Brun*

It appeared to be of interest to study the effect that the length of the storage time had on concentrated FMD virus when kept at -70°C, by studying the value of its antigenic and immunological properties after thawing. This relatively simple work required a great deal of time and a great deal of cattle.

Materials and Methods

1) Virus

The three types of virus (O, A and C) currently used in France in the production of vaccine with $IFFA_3$ cell culture were used. The virulent fluid was inactivated with ethyleneimine and then concentrated as much as 800 times before being deep frozen. It was then rapidly thawed.

2) Vaccines

The vaccines were produced using the industrial manufacture method, in an aqueous form, with aluminium hydroxide and saponine. The concentrated virus was thawed and then diluted in a saline medium so that for each final dose of vaccine, an antigen concentration equivalent to that used in current industrial production was obtained.

3) Cattle

Usual test animals were used once the absence of a.b. had been checked.

4) Measurement of the concentration of 146 S particles

This was measured after ultracentrifugation on sucrose gradient (15 -45%) according to ROULET's technique (1).

5) Complement fixation

The Technicon continual flow method as described by M. Roumiantzeff (2) was used.

6) Potency tests

Vaccinated cattle were challenged by applying the Bovine Potency method (3) and their a.b. titrated by seroneutralisation (4).

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RESULTS AND CONCLUSIONS

Results can be seen in tables I and II.

Table I

Virus Serotype	Concentration (F. C. L.)	Time / Tests	T_0	1 month	12 months
O	800 x	concentration in 146 s : µg/ml	536	516	510
		FC/0.8 ml	55 090	52 400	50 600
A	500 x	concentration in 146 s : µg/ml	203.1	172.8	196
		FC/0.8 ml	10 835	9 000	12 550
C	800 x	concentration in 146 s : µg/ml	92,6	104.2	147
		FC/0.8 ml	13 084	12 737	15 100

Table II

Virus used : type C_1 - 800 x concentrated			
Storage period.....		T_0	12 months
Number of animals protected/challenged	dose	5/5	5/5
	1/4 dose	4/5	5/5
Bovine potency.....		≥ 5.57	≥ 8
SN a. b.....	dose	$\overline{m}$ = 1.85 (± 0.21)	$\overline{m}$ = 2,06 (± 0.19)
	1/4 dose	$\overline{m}$ = 1.08 (± 0.32)	$\overline{m}$ = 1.26 (± 0.35)

In table I it can be seen that results of studies on the concentration of 146 S particles and complement fixation were found to be encouraging for the three types of virus after one year.

Table II gives the result of potency tests on a type C vaccine which was prepared from virus kept for 12 months. It is hoped that these first encouraging results can be repeated with other types of virus.

Appendix B-1

Quantification and Safety of FMD Virus Antigen Eluted from Vaccine

by

F. De Simone, M. Bugnetti, G.F. Panina, S. Barei and D. Mellano *

The European Pharmacopoeia suggests, as standard procedure for testing the safety of FMD vaccines the elution method as described by Matheka (1959).

We have controlled the suitability of the above test by eluting and concentrating the virus antigen from FMD vaccines produced in our industrial plant (Ubertini et al., 1969). Virus grown in BHK cells was chloroform treated (2%), filtered through diatomites and microfibre cartridges (0.3 µ) and adsorbed to aluminum-hydroxide gel (1.42% of Al_2O_3).

The elution was performed on 400 ml samples. Concentration was made by precipitation of the antigen with ammonium sulphate (Brown, 1963). Precipitate was resuspended in a final 10 ml volume. Tests for the quantification of the eluted antigen and for the safety of the vaccine were performed on:

a. "not inactivated" samples collected after absorption of the virus to the gel (2hs at + 26°C), immediately before the addition of formaldehyde;

b. "inactivated" samples, collected at the end of the inactivation process (48 hs at + 26°C, formalin 0.08% pH 8.0-8.2) and at 30 and 60 days during the storage of the vaccine at + 4°C.

Quantification of the eluted virus antigen was accomplished in terms of infectivity, 146 S particles and CF titres. Safety was tested by detecting the residual infectivity in IBRS-2 cell cultures and in baby mice. All the eluted material (10 ml) was tested.

Results and Conclusions

The quantification of the eluted antigens shows (tables 1-2-3) that only a 30% (11% - 50%) of the original antigen was eluted from the aluminum-hydroxide gel. Therefore, when the safety of the FMD vaccines is tested by eluting and concentrating the virus antigen, a considerable part of the absorbed virus escapes the control.

Safety tests performed on 8 batches of formaldehyde-inactivated vaccine show (table 4) that the most part of them, i.e. 6 batches, were not safe at the end of the standard process of inactivation. In addition, infectious antigen was eluted from a batch of vaccine after 30 days, but not after 60 days, of storage at + 4°C. The above vaccines were tested intradermolingually in susceptible cattle 3 months after the preparation: no one was found infectious.

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Table 1 Quantification of the virus antigen eluted from " not inactivated " samples. Mean values of infectivity tests in IBRS-2.

Number of tested batches	Type of FMD virus	$\log_{10}$ TCD_{50}/ml		%
		Virus preparation	Eluted virus	
2	O	8.2	7.6	25
1	A	7.9	7.6	50
2	C	7.9	6.8	12

Notes: Figures of the eluted virus refer to the original volume of the virus preparation.

% = percentage of the original infectivity detected in the eluted virus.

The supernatant of the preparations and the washing buffer contained less than 1% of the original virus.

Table 2 Quantification of the virus antigen eluted from "inactivated" samples Mean values of 146 S tests.

Number of tested batches	Type of FMD virus	146 S content							
		Virus preparation		0 day		30 days		60 days	
		µg/ml	%	µg/ml	%	µg/ml	%	µg/ml	%
3	O	2.23	100	0.40	18	0.44	20	0.38	17
5	A	2.58	100	1.03	40	0.93	36	0.8	31

Notes: Figures of the eluted antigen refer to the original volume of the virus preparation.

The "0 day" samples were collected at the end of the inactivation process (48 hs at + 26°C). The "30 days" and"60 days" samples were collected after storage of the vaccines at + 4°C.

Values are expressed as µg/ml of 146 S and as percentage of the original content.

No detectable 146 S was found in the supernatant of the vaccine and in the washing buffer.

Sucrose gradients 10-25% were used.

Table 3 Quantification of the virus antigen eluted from "inactivated" samples.

Mean CF titres.

Number of tested batches	Type of FMD virus	CF titres							
		Virus preparation		0 day		30 days		60 days	
		titre	%	titre	%	titre	%	titre	%
3	0	6.8	100	0.8	12	0.9	14	0.9	14
5	A	8.8	100	0.9	11	1.1	13	1.6	19

Notes: Figures of the eluted antigen refer to the original volume of the virus preparation.

"0 day", "30 days" and "60 days" as in table 2.

Values are expressed as CF titres (reciprocal of the dilution of the antigen giving 50% haemolisis) and as percentage of the original CF titre.

No detectable CF titre was found in the supernatant of the vaccine and in the washing-buffer.

Table 4 Safety tests on the "formaldehyde inactivated" vaccines.

Vaccine batches	Type of FMD virus	Infectivity					
		0 day		30 days		60 days	
		IBRS-2	Mice	IBRS-2	Mice	IBRS-2	Mice
1	O	+	+	-	-	-	-
2	O	+	+	-	-	-	-
3	O	+	-	-	-	-	-
4	A	-	-	-	-	-	-
5	A	+	+	-	-	-	-
6	A	+	+	+	+	-	-
7	A	+	+	-	-	-	-
8	A	-	-	-	-	-	-

Notes: "O day", "30 days" and "60 days" as in table 2.

Infectious antigen: +

Not infectious antigen: -

Appendix B-2

Elution of Foot-and-Mouth Disease Virus from Aluminium Hydroxide Vaccines

by

T.R. Doel and R.F. Staple*

Introduction

Formulation of virus suspensions with aluminium hydroxide gel represents one of the final steps in the production of foot-and-mouth disease virus (FMDV) vaccine. If the virus has not been previously inactivated with acetylethyleneimine (AEI) or ethyleneimine (EI), the adsorbed virus is inactivated with formaldehyde and the final product supplemented with an adjuvant such as saponin.

Experiments with adsorbed virus particles, other than animal protection tests, necessitate their elution from the aluminium hydroxide gel. Thus innocuity testing of formaldehyde inactivated virus is usually carried out with concentrated preparations of eluted material.

In our laboratory we are interested in the influence of both vaccine production procedures such as AEI-inactivation and adsorption to aluminium hydroxide gel and materials such as saponin on the integrity of 146 S particles. It is well known that protective immunity is conferred by 146 S particles (6) and that their degradation by heat or acid treatment to 12 S particles reduces considerably the potency of vaccine (2).

In the work reported here we have examined the elution of different strains from conventional vaccines with particular reference to the influence of saponin on the elution process. Experiments were also conducted with radioactive preparations of 146 S and 12 S particles in order to assess the degree of aggregation or degradation of eluted virus.

Methods

Production of Viruses

Viruses were grown in BHK 21 cell monolayers maintained in Eagles medium supplemented with 10% tryptose phosphate broth and 1% ox serum. Radioactive viruses were also prepared by growing in the presence of 35 S-methionine. All virus preparations were inactivated with 0.05% AEI for 24 hours at 37°C.

Purification of Viruses

All virus suspensions were clarified by low speed centrifugation. Purified radioactive 146 S particles were prepared by sucrose density gradient centrifugation.

Preparation of Vaccine

Adsorbed vaccine was prepared by mixing two volumes of 2% aluminium hydroxide with ten volumes of clarified virus suspension. Selected vaccines were supplemented with either saponin (Food Industries Ltd, P3 grade, kindly provided by the Wellcome Foundation Laboratory, Pirbright) or Quil A (Superfos Export Company a/s, Denmark).

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Elution Procedure

An elution procedure described by Matheka (5) was used as a basis for most of the experiments described here. Briefly, 10 ml of washed vaccine was eluted with 3.3 ml of 0.3 M sodium phosphate pH 7.6. In some experiments three cycles of elution were used.

Quantification of 146 S Particles

This was as described by Doel et al.(4). Radioactive virus was fractionated in sucrose density gradients and detected by liquid scintillation spectrometry. A CsCl cushion was used to allow the measurement of virus aggregates.

Results

Elution of 146 S Particles from Conventional Vaccines

The results in Table 1 indicate the quantities of 146 S particles eluted from vaccines with 0.3 M phosphate buffer. With the exception of C Noville, the use of saponin inhibited significantly the elution of 146 S particles. This was particularly marked with SAT 3 Bec 1/85. Experiment 3 demonstrated significant levels of inhibition with two other batches of saponin.

If saponin was omitted from the formulation, recoveries of 146 S particles of all of the viruses usually exceeded 50%. The use of 1.5 mg/ml Quil A in vaccines had little or no inhibitory effect on the elution of 146 S particles. The three-stage elution procedure allowed the recovery of slightly higher concentrations of 146 S particles from saponin treated vaccines.

Elution of Radioactive 146 S, 75 S and 12 S Particles

Because the sucrose gradient procedure used in the above work only detects soluble 146 S particles, a series of experiments was conducted with 35 S-methionine labelled viruses to assess degradation or aggregation of 146 S particles in vaccine eluates. Radioactive 146 S particles of seven viruses and 75 S particles of A Cruzeiro were purified and mixed with appropriate non-radioactive clarified virus suspensions.

Three types of vaccine were prepared. These were i) virus adsorbed to aluminium hydroxide, ii) virus adsorbed and supplemented with 1.7 mg/ml saponin (Batch 3089/79), iii) virus heated at 56°C for 90 mins to convert 146 S particles to 12 S particles and adsorbed and supplemented with saponin as before. Non-adsorbed control preparations were included to allow the measurement of eluted versus bound radioactivity and the detection of spontaneous degradation of 146 S particles to 12 S particles. Elution was carried out by the one-stage procedure and both eluates and control preparations centrifuged in sucrose density gradients incorporating a CsCl cushion.

Fig. 1 shows the results of the elution experiments. With the type of gradient used, aggregated 146 S or 75 S particles would be detected at the sucrose-CsCl interface (fraction 2) degraded particles would be detected in fractions 9 to 10 and soluble 146 S or 75 S particles would be detected usually in fractions 4 to 6. The relative levels of radioactivity among the four gradients of each virus may be taken as a reliable indication of the quantities of radioactivity adsorbed to and eluted from the vaccines. With the exception of the SAT 2 Bot 3/77, the gradient profiles in Fig. 1 indicate that the only eluted virus products from 146 S vaccine were soluble 146 S particles. The eluate from each of the 12 S vaccines gave a radioactive peak in the predicted position in the gradient. The eluate from the Bot 3/77 aluminium hydroxide/saponin 146 S vaccine contained, in addition to soluble 146 S particles, a significant though relatively small amount of degraded virus. The gradient profile of the non-adsorbed Bot 3/77 showed no evidence of degradation of 146 S particles. Both results were confirmed in a duplicate experiment.

Table 2 summarises the elution characteristics of the radioactive viruses calculated from the areas of the peaks depicted in Fig. 1. Although saponin inhibited the elution of

146 S particles and 75 S particles, C. Noville, SAT 1 Bot 1/68 and Asia 1 Iran 1/73 were least affected. Although the results in table 2 were derived from single sucrose gradients whereas other elution work in this paper was conducted with duplicate or triplicate gradients, there is reasonable agreement with the data in table 1. With the exception of SAT 3 Bec 1/65 12 S vaccine, the results in table 2 emphasize the very low levels of radioactivity which eluted from the 12 S vaccines. This last observation has important implications for the Bot 3/77 146 S aluminium hydroxide/saponin vaccine in which 2% of the 10% radioactivity eluted was detected at the top of the gradient in the 12 S position. Thus, even low levels of eluted, degraded 146 S particles with this vaccine are probably indicative of higher levels of strongly bound degraded 146 S particles.

Further experiments with radioactive O BFS 1860 confirmed the results in Fig. 2 and indicated that 12 S particles were strongly bound to aluminium hydroxide in the absence of saponin. This series of experiments also indicated that the inhibitory effect of saponin on elution was exacerbated by omission of serum from the medium (results not shown).

Experimental Elution Procedures

The low levels of 146 S particles recovered from some aluminium hydroxide/saponin vaccines prompted a series of elution experiments. In the first experiment, 1% ox serum was adsorbed to aluminium hydroxide gel and the elution of serum proteins by various solutions measured by a modified Lowry procedure (1). Table 3 indicates that similar levels of protein were recovered from the saponin free gel, regardless of the elution conditions. In contrast to elution of 146 S particles, saponin had a relatively insignificant influence on the elution of serum proteins, although the level of elution with sodium phosphate was slightly lower than with ammonium phosphate.

A range of buffer solutions was used to elute O BFS 1860 from a saponin containing vaccine (Table 4). The highest level of eluted virus was obtained with 0.3M sodium phosphate containing 0.01M EDTA and 1% mercaptoethanol. An interesting feature of the experiment was the cooperative nature of the components of the elution buffer. Replacement or omission of one of the components, for example 0.3M sodium phosphate replaced by 0.04M sodium/potassium phosphate, gave a significantly reduced recoveries of virus. Experiments with 35 S methionine labelled O BFS 1860 indicated that the highest concentration of mercaptoethanol used had no significant effect on the integrity of 146 S particles. The poor recoveries of virus with the higher concentrations of EDTA (table 4) were probably due to degradation of 146 S particles during elution.

A number of other reagents and procedures were also evaluated in an attempt to elute larger quantities of 146 S particles. These included elution with detergents such as sodium dodecyl sulphate and Nonidet NP40, pre-treatment of the vaccine with trichloro-ethylene, ether, aqueous ethanol and acetone solutions prior to elution with 0.3M sodium phosphate, and cycles of freeze-thawing combined with ultrasonication. None of these experimental conditions produced levels of eluted virus comparable with conventional 0.3M sodium phosphate elution (results not shown).

Discussion

In this study we have shown that up to 70% of 146 S particles of most strains of FMDV may be recovered from saponin-free aluminium hydroxide vaccine by elution with 0.3M phosphate pH 7.6. Experiments with radioactive 146 S and 12 S particles indicated that, with the exception of Bot 3/77, 146 S did not elute in a degraded or aggregated form. These results lend credence to the method of innocuity testing used by some workers in which virus is first adsorbed to aluminium hydroxide, inactivated and finally eluted and tested for residual infectivity. However, the validity of this procedure when applied to saponin treated vaccines is questionable. Whereas the recoveries of C Noville and Asia 1 Iran 1/73 from saponin/aluminium hydroxide vaccines would probably be high enough to assure a statistically representative sample for innocuity testing, the often poor levels of recovered 146 S particles of viruses such as SAT 3 Bec 1/65 make the procedure of doubtful value.

An interesting result which has emerged from the experiments reported here is the relative insensitivity of C Noville to saponin. It seems reasonable to speculate that a virus such as C Noville which elutes relatively easily from vaccine would stimulate a different immune response compared with a virus such as SAT 3 Bec 1/65 which elutes with considerable difficulty. Experiments are in progress to examine the possible relevance of 'elutability' to vaccine potency.

Finally, the results in this paper have shown with one virus, Bot 3/77, that formulation with aluminium hydroxide and saponin results in degradation of 146 S particles to 12 S particles. The instability of Bot 3/77 during formulation is consistent with thermal stability data from our laboratory (3). In view of the relatively low thermal stability of O BFS 1860 virus (3) the possibility cannot be ruled out that formulation with this virus also results in degradation on the aluminium hydroxide. The failure to detect radioactive degradation products may be due simply to their more avid binding to the aluminium hydroxide.

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Acknowledgements

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Table 1. Elution of 146S particles of five strains of foot-and-mouth disease virus from aluminium hydroxide vaccines

	Number of Elutions	Percentage of 146S particles eluted from vaccine containing		
		No Saponin	1.7 mg/ml Saponin	1.5 mg/ml Quil A
Experiment 1				
O BFS 1860 (Serum free)	3	58	<10	50
O BFS 1860	3	64	16	54
C Noville	3	51	54	52
A Cruzeiro	3	47	<10	58
SAT 3 Bec 1/65	3	55	0	76
Experiment 2				
O BFS 1860	1, 3	59, 72	11, 19	-
O BFS 1860	1	59	19	-
O Lausanne	1	68	21	-
C Noville	1, 3	46, 67	46, 52	-
C Noville	1, 3	51, 47	31, 39	-
Experiment 3				
O BFS 1860	3	69	30, 25[a], 30[b]	60

Key Saponin used throughout was Batch 3089/79 except for a - Batch 1220, b - Batch 308/79.

Table 2. Elution of radioactive 146 S and 12 S particles of seven strains of foot-and-mouth disease virus from aluminium hydroxide vaccines

Virus	Percentage* of particles eluted from vaccines containing		
	No Saponin 146S	1.7 mg/ml Saponin 146S	12S
A Cruzeiro	61(51)+	30(17)	15
Asia 1 Iran 1/73	62	48	11
C Noville	54	43	19
O BFS 1860	54	28	6
SAT 1 Bot 1/68	33	26	7
SAT 2 Bot 3/77	39	8 as 146S 2 as 12S	1.5
SAT 3 Bec 1/65	59	10	19

Key *Expressed as a percentage of the radioactivity in control (non-adsorbed) gradients and calculated from the areas under the radioactive peaks in Figure 1.

+ Values in brackets refer to 75 S particles of A Cruzeiro.

Table 3. Elution of ox serum proteins from aluminium hydroxide gel

Elution Buffer	Protein eluted from*	
	$Al(OH)_3$	$Al(OH)_3$ + 1.7mg/ml Saponin
0.3M sodium phosphate pH 7.6	24	23
" pH 9.0	25	20
" pH 9.5	24	21
" pH 10.0	24	21
0.3M ammonium phosphate pH 9.0	23	24
" pH 9.5	25	25
" pH 10.0	25	27

Key * Expressed as a percentage of total protein absorbed by $Al(OH)_3$ gel measured by the TCA-Lowry procedure.

Single step elution procedure.

Table 4. Elution of 146S particles of O BFS 1860 from aluminium hydroxide vaccine containing 1.7 mg/ml Saponin

Elution Buffer*	Percentage of 146S particles eluted
0.3M PO_4	12.5
" + 1% mercaptoethanol	17.0
" + 2% mercaptoethanol	19.5
" + 4% mercaptoethanol	17.0
" + 8% mercaptoethanol	17.0
" + 0.01M EDTA	13.0
" + 0.02M EDTA	10.0
" + 0.04M EDTA	0
" + 1% mercaptoethanol + 0.01M EDTA	26.0
0.04M PO_4 + 2% mercaptoethanol	0
" + 0.02M EDTA	0
" + 1% mercaptoethanol+ 0.01M EDTA	0

Key *0.3M PO_4 was prepared from sodium salts and was pH 7.6.

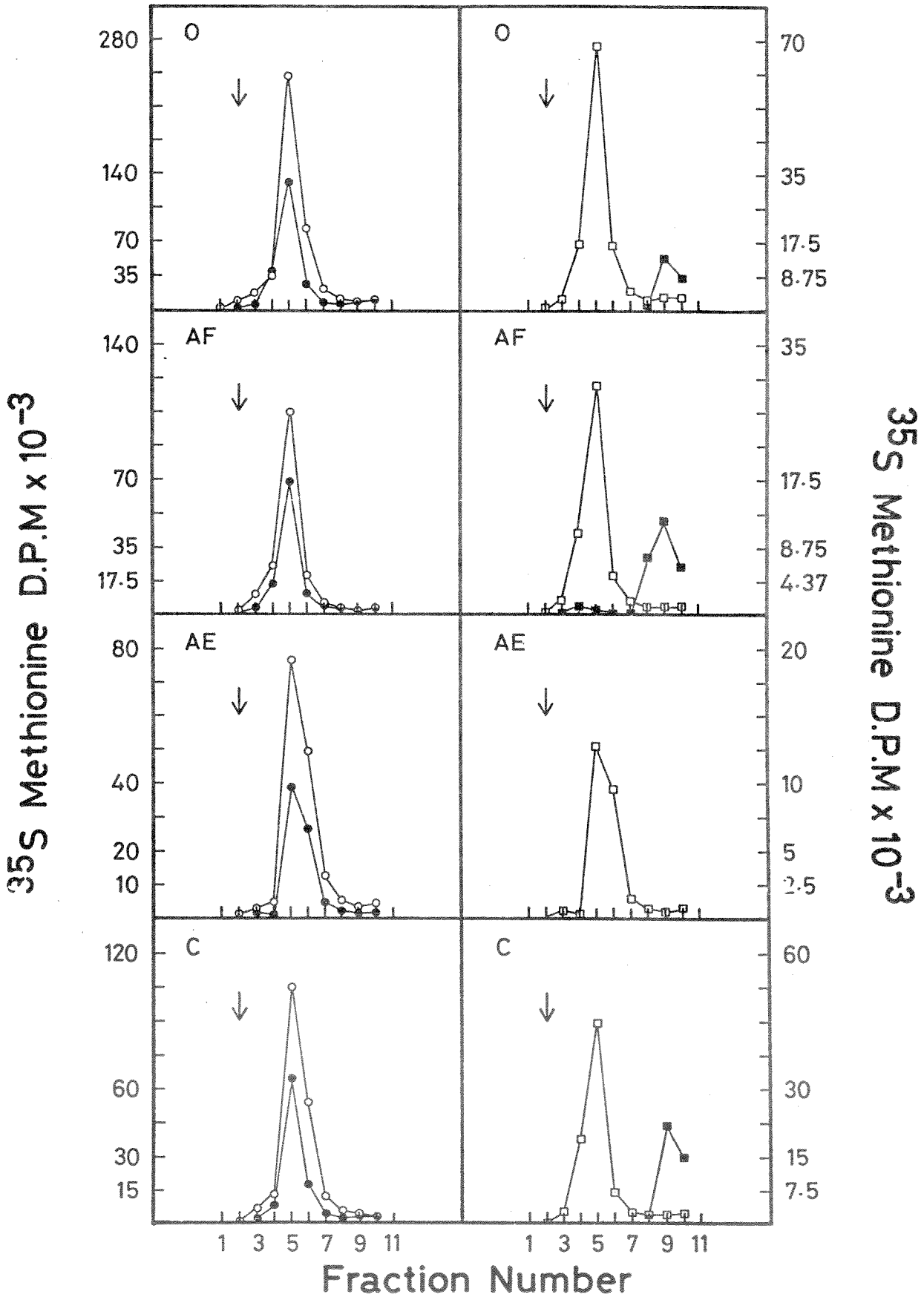
0.04M PO_4 was mixed potassium and sodium salts, pH 7.6.

Single step elution procedure.

Figure 1. Sucrose density gradient analysis of 146 S, 75 S and 12 S particles of seven strains of FMDV eluted from aluminium hydroxide vaccines.

Viruses were C BFS 1860 (O), A Cruzeiro 146 S particles (AF) and 75 S particles (AE), C Noville (C), Asia 1 Iran 1/73 (Asia 1), SAT 1 Bot 1/68 (SAT 1), SAT 2 Bot 3/77 (SAT 2), SAT 3 Bec 1/65 (SAT 3).

O——O control, non-adsorbed 146 S particles;
●——● aluminium hydroxide adsorbed 146 S particles;
■——■ aluminium hydroxide/saponin adsorbed 12 S particles;
□---□ aluminium hydroxide/saponin adsorbed 146 S particles.
The position of the CsCl cushion is indicated by the arrow.


O
AF
AE
C
280
140
70
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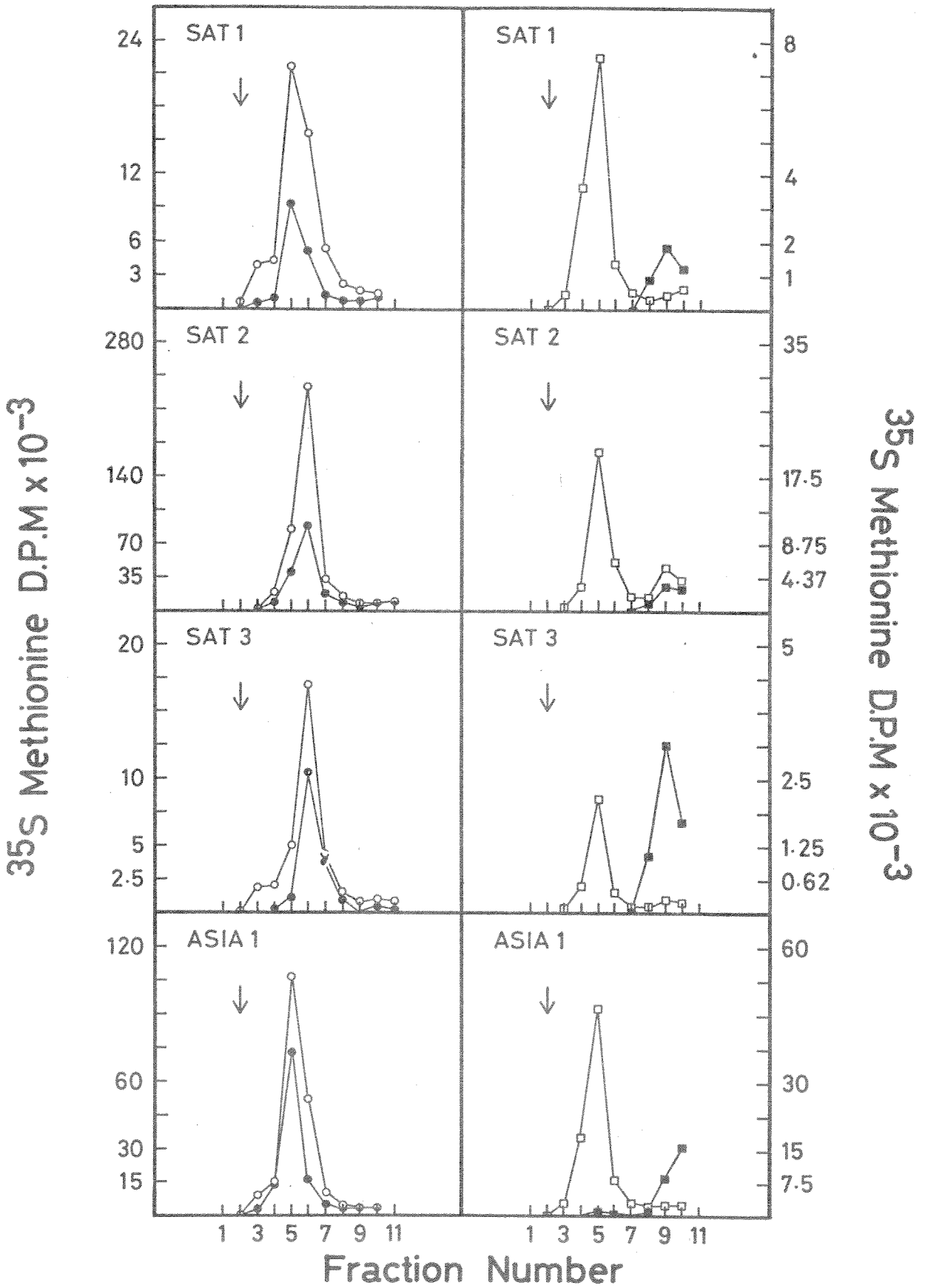
2·5
1 3 5 7 9 11
Fraction Number
^{35}S Methionine D.P.M x 10^{-3}
^{35}S Methionine D.P.M x 10^{-3}


SAT 1
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280
140
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35
35
17·5
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120
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7·5
1 3 5 7 9 11
1 3 5 7 9 11
Fraction Number
35S Methionine D.P.M x 10-3
35S Methionine D.P.M x 10-3

Appendix B-3

Elution and Innocuity of FMD Vaccines

by

R. Strobbe, J. Debecq and J. Leunen *

1. Introduction

Elution was introduced, about ten years ago, as a routine laboratory test for the evaluation of FMD vaccines (1). Besides the determination of antigenicity, particle concentration and density of eluted virions, the technique also proved useful for the detection of eventual residual infectivity.

The vaccine procedure was adapted following elution data, to increase the safety.

2. Material and methods

a. Vaccine

All the vaccine batches considered in the present study were of FMD O, A and C trivalent type, produced in Frenkel cultures. The virus was filtered on Schleicher and Schull 1121 cellulose filter pads, adsorbed on aluminum hydroxide gel and formol inactivated for 42 or 66 hours at 26°C, with a pH of 7.6 to 8. The volume of Formol added was calculated to obtain from 0.5 to 0.85:1000 at the preparation of the vaccine.

b. Formol titer at elution

The formol titer at the time of elution was determined in the supernatant of the vaccines by the TANENBAUM method (2).

c. Elution technique

Four hundred ml (40 doses) of well resuspended vaccine are centrifuged in 4x100 ml tubes at 4000 rpm, +4°C for 20 minutes. The supernatant is discarded.

Step I: 20 ml of elution buffer at +4°C are added to each tube. Elution buffer (HOBOHM) (3) is a solution of 50 g di Ammonium phosphate and 5 g. Ammonium chloride in 1 liter distilled water, pH is adjusted at 7.5 with 1N hydrochloric acid. The sedimented part of the vaccine is resuspended in the elution buffer and stirred for 20 minutes at moderate speed, using a Heidolph RZR type mixer. The suspension is centrifuged and the supernatant is collected.

Step II, III and IV are identical to step I except that only 10 ml of elution buffer are added to each tube. The final volume of eluate is about 200 ml. After clarification by centrifugation, the eluted virus is sedimented by ultracentrifugation (R40 type rotor, 16000 rpm, 16 h., +4°C). To detect any transitory defect of the temperature control system that could cause thermal inactivation of the virus, the rotor temperature is continuously recorded during the overnight run. The pellets are suspended in 6 ml buffered phosphate saline pH 7.6, using an Ultraturrax N-10 mixer. The final concentration rate is 67:1. Parts of the eluted virus suspension are used for CF-test, analytical ultracentrifugation and residual infectivity test.

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d. Residual infectivity test

Detection of eventual residual infectivity was made by injecting ip 0.1 ml volumes of eluted virus suspension in 4 to 7 days old baby mice and in cell cultures of different cell types. Positive reactors were tested for specific reaction by CF. Results were noted up to 120 hours.

e. Innocuity test

The one vaccine dose method was used for all the 42 h inactivation vaccine group and for part of the 66 h inactivation group. The 3 dose method was used for the other part of the 66 h group.

All the cattle for the tests was imported from Eire. The duration of the tests was of 10 to 15 days.

3. Results

In a first period, elution was carried out immediately after the inactivation, and many vaccines showed residual infectivity between 2 and 14 days post-inactivation.

When such vaccines were tested for innocuity in cattle, usually 2 or 3 months post-inactivation, nothing abnormal was detected. Repeated elution tests showed that in a few vaccines residual infectivity persisted for relatively long periods.

To exclude the possibility of cross contamination between eluted inactivated virus and virulent virus during the manipulations, additional precautions such as extended sterilisation of all the material and use of separate apparatus for virus cultures and vaccines were arranged.

In the other direction, to make sure no thermal inactivation could occur during the overnight ultracentrifugation, the rotor temperature was continuously recorded. This precaution showed that even if transient failures of the temperature control system of the centrifuges are exceptional, they are not inexistent: 2 cases were noted. The pH of the solutions was also carefully verified.

Becoming more confident in the elution test, the inactivation time of the vaccines was increased from 42 to 66 hours and the formol concentration adjusted to have a titer of at least 0.5:1000 at the time of elution. Furthermore a delay of at least 30 days was fixed between inactivation and elution test, and result of elution test must be obtained before the vaccine may be tested for innocuity in cattle.

The results of these decisions were very positive: vaccines with residual infectivity became extremely rare, they were held in on the base of elution result and were not allowed for the innocuity test.

Elution is now considered a sifting test and the innocuity test as a confirmatory test. The vaccines must pass the two safety tests to be accepted. About 170 batches of vaccine were tested this way: no one of the vaccines that passed the elution test was found infectious by the cattle innocuity test. The potency of the vaccines with prolonged inactivation was not significantly reduced.

4. Summary

Elution proved a very valuable technique for the detection of eventual residual infectivity in FMD vaccines. Certain precautions were introduced to avoid false positive or false negative results. This test is now used as a sifting test before the cattle innocuity test.

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Appendix B-4

Safety Tests of Foot-and-Mouth Disease Vaccine

Concentration of samples of inactivated antigens

by

M. Lombard, H. Mougeot, H. Favre*

Safety tests done on batches of ready-to-use vaccines which represent the last stage of a series of specific safety tests (or not, as the case may be) carried out by the producer at all stages of manufacture. This series of tests starts off with the testing of the satisfactory inactivation of the industrial virus and then continues by tests to prove that the animal tolerates the different constituents of aqueous or oily vaccines.

The European Pharmacopoeia has fixed regulations on this subject which are well known by specialists and manufacturers. However, for a manufacturer, it is absolutely essential to be able to ensure, as early as possible and as safely as possible, that the virus that is to lead to the ultimate vaccine is properly inactivated. This is why we have perfected an original testing technique for the inactivation of viruses, which calls upon the ultrafiltration technique, and which we present here below:

Principle and Statistical Aspects

- The presence of residual fully virulent virus after inactivation is revealed by tests for possible virus multiplication in susceptible cells. The existence of a possible interference phenomenon was studied by Wittmann (1964), Danacher (1970), Anderson (1970) and Fayet (1971). All of these authors conclude that inactivated virus does not interfere with the detection of virulent virus.

- It is known that statistically speaking, it is impossible to prove the total absence of virulent particles in a batch of hundreds of litres of inactivated virus or vaccine, unless the whole batch is tested. However, the credit accorded to the safety test in cells is reinforced by the repetition of negative results obtained during industrial production and by the total mastery of the inactivation process (Lucam 1957).

- In practice, in order to have valid industrial tests, there is always a risk to be taken as the volume of the sample taken for testing purposes only represents a low proportion of the volume of the batch. This problem has been considered in different ways by several authors and statistics experts and was the object of an entire review made by Danacher (3). If we want to conform to the results of studies made by Lorenz (5) and Anderson (1), it would be necessary, to obtain a satisfactory test, to take samples of volumes ranging from 100 ml to 500 ml, which would make the safety test far out of proportion. This was the reason that we avoided the difficulty by concentrating the samples by ultrafiltration, the advantages of which are as follows:

  a) being able to examine larger samples in reduced volumes;

  b) to eliminate, when an inactivating agent of Order 1 type reaction is used, the molecules of this product whose toxicity for the cells harms the sensitivity of the test.

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Materials and Methods

a) Materials

- sample to be tested whose volume is of 200 ml;
- AMICON ultrafiltration cell with MW 300.000 membrane (capacity 250 ml);
- culture in Baxter 2000-ml roller bottles of lamb kidney secondary cells in MEM medium enriched in lactalbumin hydrolysat.

b) Methods

This is a two-stage process:

- First stage

The 200 ml of samples are concentrated by ultrafiltration down to 40 to 60 ml, taking all precautions necessary when loading the ultrafiltration cell which has been previously sterilised. With pressure at one bar, concentration requires about 2 hours and a final volume of about 30 to 40 ml of product is obtained. To decrease even further the inactivation agent molecules, the cell is reloaded with 150 ml of sterile MEM medium and concentration is carried on for a further hour to obtain samples of 60 ml. This molecule washing operation further removes the inactivating agent and enables the observation, in the test in cells, of only discreet signs of toxicity.

- Second stage

The sample is put to incubate. The 60 ml are placed at 37°C in a roller bottle (690 m^2 of monolayer cell culture surface). After a 30-minute adsorption period, the sample remains in the bottle where 100 ml of serum-free MEM medium are added. The cultures are examined after 24 & 48 hours; the bottles are then frozen, thawed and a second culture or subculture is done in a roller bottle using the 60 ml of thawing supernatant in the same way as the previous culture. Microscope observations are then made up until the 72nd hour.

c) Results

- The inactivation test is considered as being satisfactory if no cytopathic effect is observed during the two successive cultures.
- Should a cytopathic effect appear, the nature of the virus is identified by using a seroneutralisation index against monospecific sera. The batch of industrial virus recognised as not being satisfactory is discarded.

Discussion

Concentration by ultrafiltration of voluminous virus samples enables two opposing requirements to be fulfilled: the sensitivity and the level of the method and the ease of execution and handling of a few bottles of cell culture.

In this way, the ratio sample volume: volume of industrial batch is considerably improved, as with this technology, 1 ml of sample corresponds to 6 to 11 litres of inactivated industrial virus batch.

The ratio sample volume: sensitive cell surface is 1 ml for 11.5 cm^2 of sensitive cells per culture. This figure is to be multiplied by the number of subcultures used.

When calculated according to the figures published by Anderson (1), the theoretical sensitivity of the method reveals that, in a test considered as being good, the upper limit

of possible virulence is ten CCID 50% units per litre of product, at P = 0.01 (i.e. 64 times more sensitive than the testing method in animals, Dannacher (3). This sensitivity has been tested during experimental contamination of samples with very dilute virulent viruses.

In our industrial productions, we now systematically use this technique, which enables us to know the value in relation to the European Pharmacopoeia, which is also systematically applied, i.e.:

- the test in susceptible cattle of the finished vaccine,
- the test in cells after virus elution of the aluminium hydroxide or oily vaccine.

No contradiction was noted in either of the methods.

Conclusion

- Safety testing techniques of finished vaccine are sensitive methods which are very important.
- However, it is possible to implement simple specific safety testing techniques as early as the end of the inactivation stage.
- These simple methods use larger samples and eliminate the toxic effects of inactivating agents on cells.
- Consequently, vaccine manufacturers can now offer better guarantees to consumers by practising the safety test on the constitutive antigens of the vaccines, which are more sensitive (50 to 100 times more) and of a much lower detection level.

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Appendix B-5

Safety tests of FMDV-vaccines

by

N. Visser, R. Woortmeijer and S.J. Barteling

Summary

Conditions that contribute to efficient innocuity testing are considered. Statistically significant results require the testing of samples of several liters (1,2). In vitro safety testing of formaldehyde-inactivated alhydrogel (Frenkel-) vaccines as such is not possible because the gel is toxic for culture cells. In cattle only limited volumes can be applied. Methods for the elution and subsequent concentration of FMD antigen have been reported (3,4). These tests were compared with two other methods for the elution and concentration of the antigen. No dramatic differences were observed in the efficiency of elution by different methods (table 1). However, under our conditions the yields were always very poor if the European Pharmacopoeia prescriptions(5) with respect to antigen concentration were followed (method I).

Relatively good recoveries were obtained by method II (4) in which the antigen is concentrated by ultracentrifugation, but the best yields were observed if ultrafiltration by an Amicon diaflo (XM300) apparatus was applied. Thus method IV (elution with 1.2 M K-phosphate in combination with ultrafiltration) became the method of choice (6).

With vaccine samples of 2 l mean final recoveries of approx. 50% (1-3 µg of 140S) were obtained. However, if small amounts of live virus (100 p.f.u. in PK_2) were added to these concentrates the virus could only occasionally be detected if inoculated intramuscularly (i.m.) or intradermolingually (i.d.l.) into cattle. If tested in primary foetal calf thyroid cells (7) the scores were positive even although the sample volumes were 100 times smaller (table II), containing only about one p.f.u. per tube. The low score in cattle could be due to interference by the highly concentrated (approx. 150 µg/ml) antigen. This was further studied in separate tests. As can be concluded from the experiments presented in table III, the presence of 150 µg of inactivated antigen (per ml) reduced the i.d.l. virus titres of types O, A and C with 0.8 to 2.6 logs. Thus concentration of eluted antigen can affect the efficiency of detection of infective virus in cattle. Interference in cattle was also observed by others with non-concentrated antigen (1). Conflicting results were reported in this respect for the detection of live virus in the presence of inactivated antigen in cell culture (8,9).

The interference by (BEA-) inactivated virus that was produced in BHK-suspension cultures, was studied with the agar-cell suspension (Cooper) plaque titration using standard BHK cell suspensions from batches that were stored frozen over liquid nitrogen (10). Both cattle and thyroid cells have low susceptibility for this virus (2, own observations). Plaque formation by $A_{Holland}$ and $C_{Detmold}$ virus was strongly inhibited if samples, that were added to the cells, contained over 2.5 µg 140S/ml. With BHK-adapted O_1BFS-virus over 25 µg/ml of AEI-inactivated virus was necessary to reduce the plaque formation completely (fig. 1). If virus was first concentrated by the PFE-method (11) and successively inactivated with AEI or BEA, we sometimes observed incomplete inactivation even although the preparations had been first filtered through a 0.2 µ membrane. If inactivation was incomplete, plaques developed often only in the 10^{-2} or 10^{-3} dilutions but in general not in the 10^{0} or 10^{-1} plates. Since these preparations in general contained approx. 100-200 µg/ml of 140S antigen this masking of residual active virus in the nondiluted and 10^{-1} diluted samples maybe due to interference.

Conclusions

1. Probably due to local production circumstances the European Pharmacopoeia prescriptions for elution and concentration of antigen from formaldehyde inactivated alhydrogel vaccines may not be applicable to every vaccine.

2. An alternative method is described which gave us good recoveries of antigen from locally produced vaccines.

3. The outcome of safety tests in cattle on concentrated antigens may be affected by interference.

4. Interference was not observed in primary calf thyroid cell cultures. Since these cells are extremely sensitive for Frenkel culture virus this system can be recommended for safety tests on Frenkel vaccines.

5. BEA-inactivated virus produced in BHK-cells may interfere with the detection in BHK-cells of non-inactivated virus.

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Table 1

Outline of 4 elution procedures

All methods consisted of an elution and a concentration step. In each case 250 ml of vaccine were eluted in 3-4 cycles. Recoveries are expressed as percentage of the amount of 140S antigen incorporated in the vaccine.

Method:	I*	II**	III	IV***
Starting vol.:	250 ml	250 ml	250 ml	250 ml
Elution with:	0.3 M Na-phosph.	0.3 M NH_4-phosph.	4M CsCl	1.2 M K-phosph.
volume:	250 ml	25 ml	150 ml	100 ml
average recovery: (140S)	*34%*	*39%*	*50%*	*44%*
Concentration:	PEG-precipitation	Centrifugation (2h 3 x 10^5 g)	Dehydration (PEG 20M)	Ultrafiltration (Amicon XM 300)
Final. vol.: (conc. factor)	4.2 ml (60x)	1.5 ml (167x)	5 ml (50x)	2.5 ml (100x)
average recovery: (140S)	*1%*	*14%*	*1%*	*27%*

* Ref. 3 and 5

** Ref. 4

*** Recoveries at the 2 l scale: 70 and 48% resp. (mean of 10 exp.)

Table II

Comparison of susceptibility of cattle and thyroid cells.

Approx. 100 p.f.u. (PK_2) of type $A_{Holland}$ were added to 20.5 ml of eluted inactivated (homologous) antigen (150 µg 140S/ml). Cattle were daily examined for lesions.

exp. nr.	host system	inoculation	score
I	cattle	20 ml i.m.	-
	thyroid	3 x 0.2 ml	+ + +
	2nd passage		+ + +
	(after acid treatment)		- - -
II	cattle	8 ml i.m.	-
		5 ml i.d.l. (20 sites)	1/20*
	thyroid	3 x 0.2 ml	+ + +
	2nd passage		+ + +
	(after acid treatment)		- - -

* one small lesion was observed at day 3 p.i.

Table III

Influence of (homologous) eluted antigen upon i.d.l. titration in cattle. Each tongue was inoculated with three dilutions (7 inoculation sites/dilution). Lesions were scored at 28 hr p.i.

A. Experimental results

	inoculated	lesion score	
Type	ID_{50}/0.1 ml	virus only	+ el. antigen (15 µg/0.1 ml)
$A_{Holland}$	0.6	2/7	0/14
	6	6/7	2/14
	60	7/7	6/14
O_1BFS	0.25	2/7	3/14
	2.5	4/7	6/14
	25	7/7	6/14
$C_{Detmold}$	16	7/7	0/14
	160	7/7	6/14
	1600	7/7	10/14

B. Estimated titres

	log ID_{50}/ml		log reduction
	contr.	+ el. antigen	
Type A	6.3	4.5	1.8
Type O	7.5	6.6	0.9
Type C	8.0	5.4	2.6

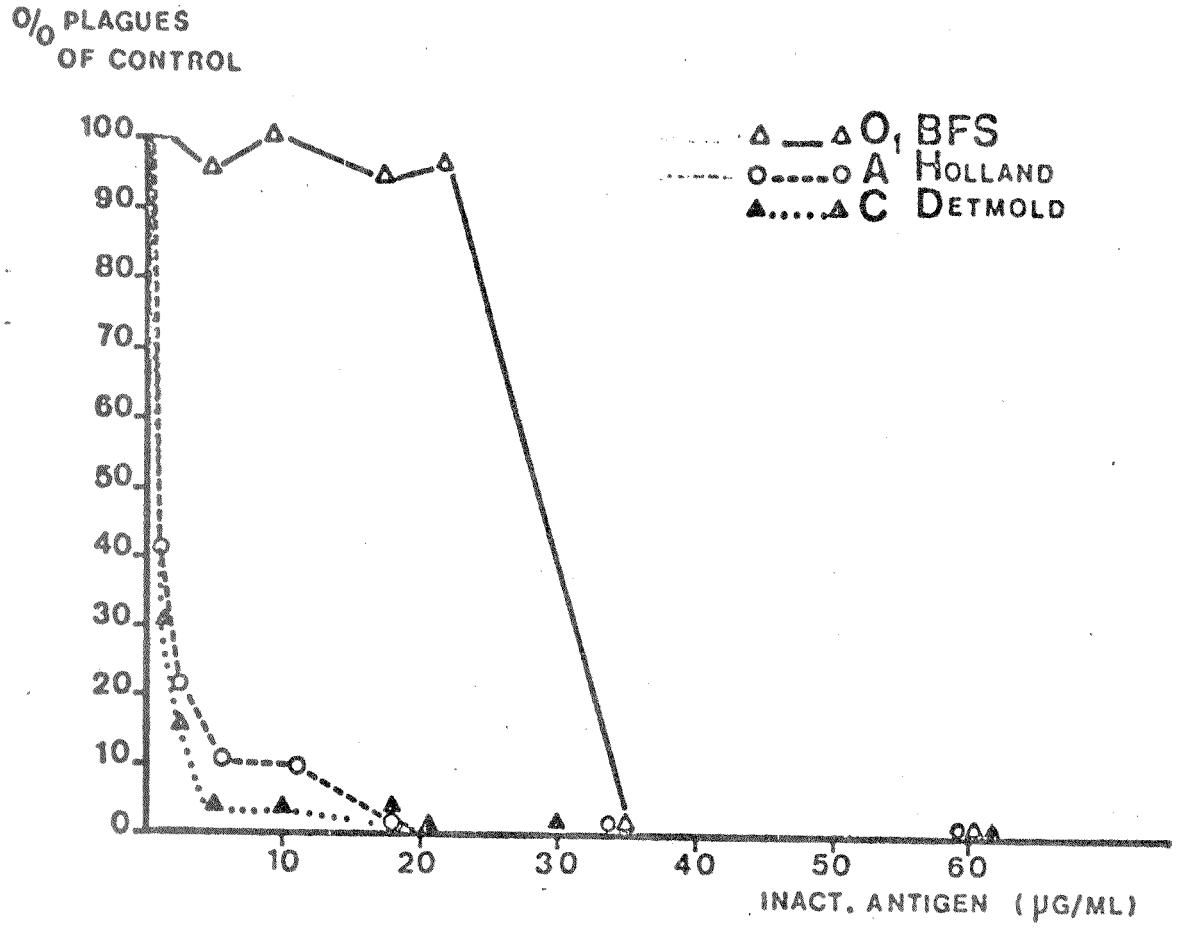

Fig. 1: Inhibition of plaque formation by inactivated antigen.

FMDV of type $A_{Holland}$, O_1 BFS and $C_{Detmold}$ produced in BHK-suspension cultures were inactivated with binary ethylene immine and concentrated/purified by two precipitation (PEG) - filtration - elution cycles. Different amounts of inactivated 140S antigen were added to approximately 50 p.f.u. of virus before inoculation into standard (frozen) suspended BHK-cells.

Appendix B-6

Inactivation of FMD Virus with Aziridines

by

T.W.F. Pay, R.C. Telling and A.C. Thorne*

1. The inactivation process

At the 1971 Session of the Research Group of the Standing Technical Committee of the European Commission for the Control of FMD held in Tübingen, we presented communications describing the use of an aziridine compound, acetylethyleneimine (AEI), for the inactivation of FMD virus and the use of tissue cultures for the safety testing of FMD vaccines inactivated with either formalin or AEI (Pay et al, 1971).

A concept we introduced, which at that time seemed novel, was the use of a double dosing regimen for the inactivant. An initial dose (0.05% v/v = 5.9 mM) was given and then 24 hours later a second, identical dose of inactivant was added to the antigen. Originally, the second period of inactivation was continued for a further 6 hours, but after 1971 we extended the time of the second inactivation period to 24 hours, making a total of 48 hours incubation at 26°C at pH 7.6. The purpose of the double dosing regimen is sometimes assumed to be merely a means of minimizing the likelihood of committing some dosing error during the inactivation process. While it does, of course, do that, it offers far greater advantages.

From the information that is retrievable from the tests carried out on samples taken during the inactivation process, a composite picture can be built up which indicates levels of residual infectivity corresponding to different levels of probability.

1.1 Thus, if both the first (24 hour) and the second (48 hour) 100 ml. samples were negative in their innocuity tests, we can say that there is only a 5% probability of the residual infectivity level being higher than $10^{-1.8}$ ID_{50}/ml. or 1 infectious unit in 66 ml. of antigen.

1.2 If the first innocuity test (24 hour) was negative, we can say that there is only a 5% probability of the residual infectivity level being higher than $10^{-1.5}$ ID_{50}/ml. If the starting titre had been, say, $10^{7.5}$ ID_{50}/ml. it will have been demonstrated that at least 10^{9} ID_{50}/ml, has been effectively inactivated in the 24 hour period in that particular virus lot, using that particular batch of inactivant. Now, by adding a second identical dose from the same batch of inactivant to the antigen and treating for a further 24 hours, it is not unreasonable to have expected that a further reduction of 10^{9} ID_{50}/ml in infectivity will have been obtained, making a reduction of 10^{18} ID_{50}/ml in total. The slope is drawn through the point ($10^{-1.5}$) indicated by the 1st innocuity test and at 48 hours the intercept represents the "highest probable level of residual infectivity".

1.3 In the 1971 paper we stressed the value of carrying out an estimation of the inactivation kinetics, based on at least four serial samples taken during the first 6 to 8 hours of the inactivation, as an "in-process" control of the consistency of the process. It is possible to extrapolate the calculated slope of the inactivation regression line, if one assumes linearity over each of the 24 hour periods and ignores the fact that some residual inactivant will still be present at the time of making the second addition of inactivant.

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The intercept at 48 hours will then give a "lowest probable level of residual infectivity".

1.4 It can be seen that by using a double-dose regimen with a first-order inactivant it is possible to arrive at "probable" confidence levels for innocuity that are many orders of magnitude below that capable of being detected in any form of innocuity test.

1.5 Reference was made in the 1971 paper to the fact that up to that time on only one out of 17 occasions was a 24 hour sample taken from an AEI inactivation run positive in the tissue culture innocuity test, and that related to an antigen lot which showed an abnormally high half life in the inactivation kinetics assay. Since that time more than 400 further antigen lots have been prepared at Pirbright using AEI and not one of these has given rise to a positive tissue culture innocuity test result with either the 24 hour or the 48 hour sample. Furthermore, during the fifteen years that AEI has been used for vaccine production at the Wellcome Laboratory at Pirbright not one batch of vaccine has given a positive result in the cattle intradermolingual innocuity test. This represents a level of safety which is some orders of magnitude above that previously obtained with the classical formalin inactivation method in the laboratories of the Wellcome Foundation Ltd., when a high proportion of antigens would retain infectivity at the end of the nominal 48 hour inactivation period, and would drop to levels below that of detectability in the cattle innocuity test only after further undefined periods of storage at $4^{o}C$.

2. Inactivation with 'Binary' Ethyleneimine (BEI)

2.1 Bahnemann et al (1974) described the use of ethyleneimine prepared from 2-bromoethylamine hydrobromide (BEA), either by an 'external' or by an 'in situ' cyclisation process. Girard et al (1977) and Abaracon et al (1979) have reported the use of the BEI process for the preparation of satisfactory FMD vaccines.

2.2 Both the 'external' and the 'in situ' processes have been extensively evaluated within the Wellcome Foundation's FMD vaccine laboratories. The overall results obtained may be summarised as follows:-

2.3 With both processes, a final regimen was arrived at which employed two doses, each yielding final concentrations of 1.0mM BEI, at $37^{o}C$ and with a 24 hour interval between doses.

2.4 The BEI 'in situ' process offers considerable advantages from the point of view of producing a reduced hazard to the process operators. A solution of 2M sodium carbonate was shown to be as effective as a 1M solution of sodium hydroxide for the cyclisation of BEA, and this reduces even further any toxicity hazard to the operator.

2.5 The mean half life (T 1/2) values obtained at the industrial scale have been:-

AEI. 5.9mM at $26^{o}C$ = 38 minutes
BEI. 'external' 1.0mM at $37^{o}C$ = 27 minutes
BEI. 'in situ' 1.0mM at $37^{o}C$ = 36 minutes

The differences in the T 1/2 values for the 'external' and 'in situ' processes with BEI is probably due to differences in the efficiency of the cyclisation of BEI. The 'external' process has an efficiency of greater than 90%, but the 'in situ' process may be less efficient due to the presence of competitive substances in the culture medium. In addition, cyclisation in the 'in situ' requires time to come to completion and the maximal rate of inactivation will not be obtained until this has happened.

2.6 Recoveries of 140S antigen after inactivation by the BEI 'in situ' process were predicted at 95% which, although higher than the recoveries after AEI inactivation (78%) were not statistically different ($p=0.06$).

2.7 Vaccines of excellent potency have been made from antigens inactivated by either the BEI 'external' or 'in situ' process.

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Appendix C-1

Etudes sérologique et immunologique des souches vaccinales A_5 Allier France 1960 et A_{24} Argentine 1968

par

H. Favre* - M. Lombard* - J. Juve** - G. Lopez**

A la réunion de Vienne en 1980 le Groupe de Recherche de la Commission européenne de lutte contre la fièvre aphteuse de la FAO avait souhaité qu'une étude soit faite pour apprécier la couverture immunologique réciproque entre les virus A_{24} et A_5. Ce souci était motivé par l'apparition fréquente ces 10 dernières années, en Europe, de foyers dont les virus ont été trouvés relativement proches des souches sud-américaines.

Une étude comparative sérologique et immunologique a été entreprise entre les virus de type A_5 et A_{24} utilisés respectivement pour la préparation des vaccins en France (IFFA-Mérieu) et en Argentine (Estrella-Mérieux).

Ce sont les résultats les plus significatifs acquis dans ces deux pays qui sont présentés ici.

Matériel

a) Virus Ce sont les virus officiellement choisis par les Autorités Vétérinaires pour le contrôle des vaccins.

A_5 Allier France 1960 et A_{24} Argentine 1968 ont été multipliés sur explants linguaux en survie (Frenkel) sur cellules (études sérologiques et séroneutralisation) et sur bovins (virus d'épreuve titrés Henderson).

b) Vaccins Ils ont été fabriqués à partir de virus industriels inactivés par l'éthylènéimine en France ou par le formol en Argentine; ils sont adsorbés sur hydroxyde d'alumine et adjuvés par la saponine.

Après avoir satisfait aux épreuves d'innocuité, ils ont été utilisés dans les études immunologiques croisées à la dose monovalente de 1,66 ml (dose trivalente O A C = 5 ml).

c) Animaux Des bovins reconnus exempts d'anticorps spécifiques avant les essais sont hébergés en stabulation pendant toute la durée de l'expérience.

d) Sérums Pour les études sérologiques, ce sont des sérums de cobayes qui furent utilisés (normes IABS - RIO 1979).

Pour les études immunologiques, les sérums de bovins soumis à vaccination ont été recueillis au moment de l'épreuve virulente, soit 21 jours après vaccination.

Méthodes

1) Les parentés sérologiques ont été établies par la méthode de fixation du complément à 50% d'hémolyse (ROUMIANTZEFF M.).

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2) Les méthodes immunologiques directes ont été engagés selon un protocole d'immunisation croisée qui est le seul test direct complet bilatéral et comparatif pour l'étude de deux souches (JOUBERT L. et MACKOWIAK C.).

Après échange de vaccin, chaque laboratoire a testé par èpreuve de 10,000 D.I.B. 50% de la souche officielle, les bovins vaccinés, soit avec le vaccin local, soit avec le vaccin importé. Outre l'utilisation d'une dose complète, des dilutions vaccinales (1/3 et 1/9è de dose) ont été faites en tampon carbonate immunoligiquement neutre.

3) Les méthodes immunologiques indirectes sont des microméthodes de séroneutralisation sur culture de tissus (cellules secondaires de reins de porcs ou de reins de moutons) utilisant des doses variables de sérum par rapport à une dose constante de virus (200 DECP/ml).

Résultats

Ils sont rassemblés selon les rubriques "Sérologie et Immunologie" (directe et indirecte) dans les tableaux de 1 à 5.

1) Sérologie

VIRUS / SERUMS	A_{24} Argentine	A_5 Allier	A_{24} Philippines	A Santander 72
A_{24} ARGENTINE	1	0,34	0,80	NT
A_5 ALLIER	0,37	1	0,33	0,30
A_{24} PHILIPPINES 75	0,71	0,29	1	0,36
A SANTANDER 72	0,40	0,22	0,19	1
A MAROC 77	0,10	0,09	0,11	0,18

TABLEAU 1 : Valeurs sérologiques r

A_{24} ARGENTINE	100			
A_5 ALLIER	35%	100		
A_{24} PHILIPPINES	75%	30%	100	
A SANTANDER	NT	25%	26%	100
	A_{24} ARGENTINE	A_5 ALLIER	A_{24} PHILIPPINES	A SANTANDER

TABLEAU 1 bis : Valeurs sérologiques R

2) Immunologie directe

VIRUS / VACCIN	EPREUVE A_5	EPREUVE A_{24}
Vaccination A_5	Pb = 16,50 99,50%	Pb = < 1 40%
Vaccination A_{24}	Pb = 2,10 79%	Pb = 4,57 94%
Double vaccination A_5 (full dose)	-	4/5

TABLEAU 2 : Relations immunologiques entre A_5 Allier France 1960 et A_{24} Argentine 1968

Pourcentage de protection croisée (Pb - dilutions en tampon neutre)

3) Immunologie indirecte

VACCIN OAC FRANCAIS

Titres séroneut. homologues	Dose	1/3	1/9
bovins français	$\bar{m}$ = 1,70	$\bar{m}$=1,37	$\bar{m}$=0,79
bovins argent.	$\bar{m}$ = 1,48	$\bar{m}$=1,03	$\bar{m}$=0,70

TABLEAU 3

VACCIN OAC ARGENTIN

Titres séroneut. homologues	Dose	1/3	1/9
bovins français	$\bar{m}$=1,71	$\bar{m}$=0,84	$\bar{m}$=0,60
bovins argent.	$\bar{m}$=1,65	$\bar{m}$=1,05	$\bar{m}$=0,96

TABLEAU 4

1- Titrages témoins de la bonne conservation des vaccins lors de l'échange entre la FRANCE et l'ARGENTINE. (Valence A)

SERUMS \ VIRUS	A_5 ALLIER	A_{24} ARGENTINE
Sérums bovins primovaccinés A_5 ALLIER	dose $\bar{m} = 1,70$ 1/3 dose $\bar{m} = 1,37$ 1/9 dose $\bar{m} = 0,79$	dose $\bar{m} = \leq 0,46$ 1/3 dose $\bar{m} = \leq 0,26$ 1/9 dose $\bar{m} = \leq 0,24$
Sérums bovins bivaccinés A_5 ALLIER	$\bar{m} = 2,34$	en cours
Sérums bovins primovaccinés A_{24} ARGENTINE	dose $\bar{m} = 0,84$ 1/3 dose $\bar{m} = 0,76$ 1/9 dose $\bar{m} = \leq 0,48$	dose $\bar{m} = 1,71$ 1/3 dose $\bar{m} = 0,84$ 1/9 dose $\bar{m} = 0,60$

3.2- TABLEAU 5 : Titres séroneutralisants croisés.

Discussion

1) La parenté sérologique entre le virus A Allier 1960 et le virus A Argentine 1968 est de 35%; ils appartiennent bien à deux sous-types différents.

   Les réactions sérologiques accessoirement effectuées avec d'autres virus confirment la classification A_{24} du virus isolé aux Philippines en 1975.

   Le virus A Maroc 1977 est, quant à lui, éloigné de tous les virus testés.

2) Le pouvoir immunogène homologue des vaccins est conforme aux normes de la pharmacopée européenne.

   Il est de plus vérifié que la conservation des vaccins pendant leur acheminement dans les deux pays est correcte (les séroneutralisations effectuées dans les deux laboratoires donnent des résultats identiques).

3) Le pouvoir immunogène hétérologue après une seule vaccination est faible lorsqu'il s'agit de la valence A_5 et correcte pour la valence A_{24} (elle est cependant inférieure au résultat homologue).

4) Un double vaccination, à l'aide du vaccin A_5, ne protège pas l'ensemble du groupe d'animaux éprouvés avec un virus A_{24}.

5) Les titres obtenus lors des épreuves de séroneutralisation croisée confirment les résultats des épreuves directes: le vaccin A_{24} confère une immunité meilleure vis à vis du virus A_5 que le vaccin A_5 vis à vis du virus A_{24}.

Conclusion

- Les souches A_5 ALLIER et A_{24} ARGENTINE engagées dans un processus croisé de comparaisons sérologique et immunologique sont différentes entre elles.

- La souche isolée en ARGENTINE en 1968 et classée A_{24} dommine immunologiquement la souche A_5 représentée par le virus A ALIER 1960.

Appendix C-2

Information on South American FMD Strains of Types O and A

by

Albino Alonso Fernández*; Yderzio Luiz Vianna Filho**;
Luis A. E. Durini *** and Paul Sutmöller*

The following information relates to collaborative work of the Center and the official vaccine control units of Argentina and Brazil on the cross reactivity and protection of South American vaccine and field strains.

Table 1 lists the A, O and C strains presently used in South America for vaccine production. In general, homologous strains are used by the official vaccine control laboratories with the exception of Argentina and Colombia. In these countries, the vaccine contains an O_1 Campos component but the control tests are done with O_1 Caseros and O Magdalena, respectively. In Uruguay, the vaccines contain C_3 Resende and C_2 Pando while the cattle challenge tests are performed with C_2 Pando. In Venezuela, the O_1 Campos modified live virus vaccine is tested against O_1 Cura.

The O_1 Campos, isolated in Brazil in 1952, is widely used in the Continent. O_1 Caseros O_1 Urubamba and O_1 Cura are close relatives (table 2).

O Magdalena was identified in Colombia in 1964 but presently the number of field strains with characteristics of O1 Campos is increasing. The strain O RS-Brasil/80 caused an extensive outbreak in the state of Rio Grande Do Sul (RS), Brazil during 1980. Presently, the number of isolations of this virus is decreasing relative to the number of O_1 Campos identifications, which most likely is a consequence of a batch of vaccine from one producer, which contained infectious O_1 Campos virus.

Table 3 shows that similar results are obtained in neutralization tests using sera from vaccinated cattle with reasonable good cross neutralization between the O_1 strains. The O Magdalena and O RS-Brasil/80 sera only react well with homologous strains. The results of a cattle protection test at the official vaccine control laboratory in Rio Grande do Sul, Brazil, are shown in table 4. The cattle were vaccinated with a commercial aluminium hydroxide saponin vaccine containing O_1 Campos antigen and were revaccinated with the same vaccine 21 days later. After 35 days the cattle were divided in 2 groups of 16 and IDL inoculated with strain O_1 Campos and O RS-Brasil/80, respectively.

The group vaccinated with O_1 Campos was fully protected against the homologous virus but in the group challenged with O RS-Brasil/80 ten of the 16 cattle developed lesions on one or more feet. It is clear that the spread of O RS-Brasil/80 and related strains should be watched carefully.

The A strains used in South America are shown in table 1. The A_{24} Cruzeiro, isolated in 1955 in Brazil, has been the reference strain for South America and is widely used for vaccine production. The A_{24}-Arg/68 is used for vaccine production in Argentina. This strain was the representative of the field strains in that country until 1977. The present field strains are similar to A-Arg/79 and this strain is now being incorporated in the vaccine production. A Venceslau was the predominant field strain during 1976-1979 in most of Brazil, with the exception of Rio Grande do Sul. Presently, this strain is used in the Brazilian vaccines.

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In table 5 a few more strains are included. The A_{27} Cundinamarca strain is representative for the strains acting in Colombia since 1967. This strain still is rather closely related to A_5 Westerwald/58. Its predecessor, A Colombia/67, was practically identical to A_5 Westerwald/58.

The strain A_{32} Venezuela/70 is used in Venezuela for the production of modified live virus vaccine.

Strain A Bagé was the representative strain in Rio Grande do Sul and Uruguay during 1976-1979. Presently, the strain A Brasil/79 is the predominant strain in all of Brazil.

It is important to note in table 5 that the strains A Bagé, A-Arg/79 and A Brasil/79 are closely related. It can be assumed that the A strains presently active in Brazil, Uruguay and Argentina all are descendants from A Bagé. A RS-Brasil/81 was identified during an outbreak in 1981 in the state of Rio Grande do Sul. Serologically this strain is quite different from any of the previous mentioned A strains. The incidence of this strain is now decreasing and again the majority of the isolates are more closely related to A Brasil/79.

Table 6 lists the results of the neutralization tests. Between the strains A_{24} Cruzeiro, A_{24} Argentina and A_{27} Colombia/76 there is a significant level of cross neutralization. The same is true for strains A Venceslau, A Argentina/79 and A Brasil/79. Table 7 summarizes the results of a protection test done at the official vaccine control unit (LARA/RS) of Brazil in cattle vaccinated with a commercial hydroxide-saponin vaccine containing antigen A Venceslau challenge with A RS-Brasil/81. It can be observed that there is a significant difference between the homologous and the heterologous response.

Table 8 lists the results of a cross protection test in cattle of A_{24} Argentina/68 and A Argentina/79 done by the official control laboratory (SELAB) in Argentina. With heterologous challenge both vaccines would have been rejected with a pass level of 3 PD_{50}.

Table 1. Strains used in South America for vaccine production

Country	Strains		
	O	A	C
Argentina	O_1 Caseros O_1 Campos*	A_{24} Arg/68 A Arg/79	C_3 Resende
Brazil	O_1 Campos	A Venceslau A Brasil/79	C_3 Indaial
Colombia	O_1 Campos* O Magdalena	A_{24} Cruzeiro A_{27} Cundinamarca	-
Ecuador	O_1 Urubamba	A_{24} Cruzeiro	-
Paraguay	O_1 Campos	A_{24} Cruzeiro	C_3 Resende
Peru	O_1 Urubamba	A_{24} Cruzeiro	C_3 Resende
Uruguay	O_1 Campos	A_{24} Cruzeiro	C_3 Resende* C_2 Pando
Venezuela	O_1 Campos** O_1 Campos	A_{32} Venezuela/70** A_{24} Cruzeiro	

*Not used in official vaccine control

**Modified live virus vaccine. / In Venezuela O_1 Cura is used for vaccine control.

Table 2. CF relationships (r_1/r_2) of FMDV O strains of epidemiological importance in South America since 1952

Virus strain	Serum					
	O_1 Cp	O_1 Cs	O_1 Ur	O_1 Cu	O Mg	O RS
O_1 Campos-Brazil/58	1.00	0.96	0.67	0.81	0.44	0.37
O_1 Caseros-Arg./67	0.87	1.00	0.73	0.92	0.67	0.35
O_1 Urubamba-Peru/63	0.77	0.88	1.00	0.78	0.48	0.26
O_1 Cura-Venez./71	0.80	0.88	0.65	1.00	0.56	0.29
O Magdalena-Col./78	0.51	0.45	0.54	0.37	1.00	0.28
O RS-Brazil/80	0.34	0.27	0.24	0.31	0.27	1.00

Table 3. Mean cross neutralization titers of FMDV O type strains

Virus strain	Serum					
	O_1 Ca	O_1 Cs	O_1 Ur	O_1 Cu	O Mg	O RS
O_1 Campos	2.5	2.6	2.46	2.25	1.65	$\leq$1.35
O_1 Caseros	3.12	3.15	3.00	2.76	1.89	1.86
O_1 Urubamba	2.85	3.18	3.34	2.67	2.01	1.92
O_1 Cura	2.63	2.73	2.64	2.42	1.8	$\leq$1.35
O Magdalena	1.98	1.97	$\leq$1.50	$\leq$1.5	2.43	$\leq$1.35
O Rio Grande so Sul	$\leq$1.44	$\leq$1.47	$\leq$1.35	1.63	$\leq$1.41	3.12

Table 4. Protection of cattle against strains O_1 Campos and O RS-Brazil/80, vaccinated 2 x with O_1 Campos aluminum hydroxide-saponin vaccine at 21 days interval and challenged 35 days after the last vaccination

Challenge virus	No protected /total	Mean MPI*
O_1 Campos	16/16	3.84
O RS-Brazil/80	10/16	2.99

*Mean mouse protection index of all cattle.

Test done in LARA/RS, Brazil

Table 5. CF relationships ($r_1/4_2$) of FMDV A strains of epidemiological importance in South America since 1952

Virus strain	Serum									
	A_5 West.	A_{24} Cruz.	A_{24} Arg.	A_{27} Cund.	A_{32} Ven.	A Ven.	A Bag.	A Arg.	A Br.	A Br/81
A_5 Westerwald/58	1.00	0.51	0.53	0.54	0.23	0.24	0.26	0.60	0.55	0.07
A_{24} Cruzeiro-Br/55	0.29	1.00	0.48	0.27	0.15	0.08	0.14	0.49	0.32	0.05
A_{24} Argentina/68	0.46	0.61	1.00	0.45	0.27	0.24	0.32	0.76	0.61	0.14
A_{27} Cundinamarca-Col/76	0.50	0.37	0.48	1.00	0.14	0.20	0.18	0.52	0.37	0.05
A_{32} Venzuela/70	0.30	0.19	0.39	0.16	1.00	0.14	0.27	0.52	0.68	0.28
A Venceslau-Br/76	0.13	0.22	0.30	0.15	0.15	1.00	0.44	0.60	0.37	0.05
A Bagé-Br/76	0.18	0.28	0.40	0.25	0.23	0.37	1.00	0.92	0.95	0.06
A Argentina/79	0.12	0.21	0.28	0.16	0.15	0.30	0.51	1.00	0.61	0.06
A RS-Brazil/79	0.16	0.24	0.30	0.18	0.23	0.44	0.58	0.89	1.00	0.05
A RS-Brazil/81	0.09	0.05	0.12	0.05	0.16	0.06	0.10	0.15	0.21	1.00

Table 6. Mean cross neutralization titers of FMDV A type strains

Virus strains	Serum					
	A_{24} Cruzeiro	A_{24} Arg/68	A Cund.	A Venc.	A Arg/79	A Br/79
A_{24} Cruzeiro-Br/55	2.36	2.28	1.71	<1.35	<1.53	<1.35
A_{24} Argentina/68	2.42	2.97	2.07	<1.43	1.83	<1.50
A_{27} Cundinamarca-Col/76	2.05	2.52	2.85	<1.50	<1.43	<1.35
A Venceslau-Br/76	<1.59	2.16	<1.43	2.94	2.40	2.34
A Argentina/79	<1.53	1.98	<1.35	2.13	2.31	2.22
A RS-Brasil/79	<1.50	1.96	<1.43	2.25	2.40	2.22

Table 7. Protection against strains A Venceslau and A RS-Brazil in cattle 30 days after vaccination A Venceslau vaccine

Challenge virus	No protected/total	Mean MPI*
A Venceslau	9/9	4.75
A RS-Brazil/81	5/9	1.44

*Mean MPI of all cattle.

Test done in LARA/RS, Brazil

Table 8. Cross protection test of cattle vaccinated with two Argentine A strains

Vaccine Dilution	Vaccine: A_{24} Argentina/68 — Virus: A Arg/68	Vaccine: A_{24} Argentina/68 — Virus: A Arg/79	Vaccine: A Argentina/79 — Virus: A Arg/79	Vaccine: A Argentina/79 — Virus: A Arg/68
1/1	5/5	4/5	5/5	4/5
1/3	5/5	1/5	5/5	2/5
1/9	4/5	1/5	1/5	0/5
BPD_{50}	12.5	2.2	6.5	2.2

Test done in SELAB, Buenos Aires, Argentina

Appendix C-3

Comparative Serological Study of two Foot-and-Mouth Disease Virus Strains which appeared in March and April 1981 O Austria* and O Israel*

BY

M. Lombard**

Abstract

In March 1981, there was an outbreak of foot-and-mouth disease (type O) in Austria. During the following month, further outbreaks of the same type were noted in Israel.

Despite the distance separating the two outbreaks it is interesting to note that the comparative serological study carried out with nine different sera gave similar results for the two strains in question.

Serologically speaking, they are very similar.

Serums \ Virus	Virus O Israel 1981	Virus O Austria 1981
Serum O_1 Lausanne Switzerland 1965	0,40	0,37
Serum O_1 Lombardy Italy 1946	0,49	0,40
Serum O_1 BFS 1860	0,37	0,35
Serum O_1 Manisa Turkey 1969	0,65	0,64
Serum O_1 Sharquia Egypt 1972	0,68	0,67
Serum O Thailand 1980	0,18	0,17
Serum O Austria 1973	0,44	0,47
Serum O_1 Calvados France 1979	0,37	0,35
Serum O_2 Brescia Italy 1947	0,34	0,35
Serological relationship (r_1) for O Israel 1981 - O Austria 1981 strains		

*Autriche : notes O.I.E. 1981 - nos 32 and 33
*Israel : bulletin O.I.E. no. 413, May 1981

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Appendix C-4

Titration of FMD antibodies in cattle sera

Comparative Study of two methods: Seroneutralisation on cells and ELISA

by

M. Lombard - R. Piroird*

The manufacture and use of ELISA reagents (discs of paper on which inactivated virus is placed) has been previously reported (Vienna, 1980). It appeared to be of interest to see whether the ELISA (quick method - 24 hours) could be as useful as the seroneutralisation method in the immunitary surveillance of cattle used in FMD vaccine testing. The results of titrations, in parallel, using the two above-mentioned methods are the beginning of a much longer series of experiments which are under way.

Discussion

The study of the results suggests that the ELISA titre values are only slightly different to those obtained by seroneutralisation in a few limited cases.

- Before vaccination and on the cow number of sera tested, the ELISA method detects, in one case, the presence of antibodies which have perhaps a cross-reactivity with the FMD virus without having neutralising properties;
- 21 days after vaccination or 21 days after booster, titres observed are only slightly different, the parallelism being evident in only certain cases;
- 3 months after vaccination, the ELISA method does not reveal a marked drop in the antibody level as is the case for seroneutralisation. It could be that in this case, the ELISA method is sensitive to the presence of non-neutralising antibodies.
- Lastly, 6 months after the booster (booster - 3 months after primo vaccination) the antibody level detected by ELISA is slighly higher, on average, than that observed with seroneutralisation.

Conclusion

With specific inactivated antigens which are chemically bound to paper discs, it is possible to recognize the specificity of FMD antibodies of the sera of vaccinated cattle. The choice of a method of calculating the ELISA serum titre expressed in log 10 enables comparisons with titres obtained by seroneutralisation to be made.

If the values found 21 days after vaccination and 21 days after booster are comparable in the two methods, the sensitivity of the ELISA technique enables populations of antibodies which are not detected by seroneutralisation to be revealed, either in a few animals prior to vaccination or in the majority 3 months post vaccination.

It is only by a more detailed study of the antibody populations concerned that the respective role they play in titres observed by seroneutralisation and ELISA will be able to be appreciated.

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Appendix C-5

Titration of FMD Antibodies in Cattle Sera
Comparative Study of Serum-neutralization on Mice and ELISA*

by

F. De Simone, E. Brocchi, G.F. Panina and S. Barei

The antibody level of sera collected from cattle used in FMD industrial vaccine testing (PD_{50}), has been comparatively studied by serum-neutralization and ELISA.

Materials and Methods

FMD virus - the following strains were used: A_5 Parma 1962, O_1 Switzerland 1965, C_1 Brescia 1964.

Sera were collected from cattle before vaccination (FMD antibody free) and 21 days post-vaccination with different doses of vaccine. All sera were heated at 56°C for 30 minutes.

Vaccine - aluminium hydroxide-saponin FMD vaccines produced in our Institute have been used. Dilutions for the PD_{50} test were performed in buffer without adjuvant.

Serum-neutralization was performed in baby mice testing twofold serum dilutions against 500 mice ID_{50}.

ELISA was performed according to Abu Elzein and Crowther (1978), using: infectious or inactivated 146S antigens; 1/50 dilution of sera; anti-bovine IgG, prepared in goats and labelled with alkaline phosphatase; optical density (=OD) recorded by Titertek Multiskan at 405 nm.

Conclusions

A good agreement between virus neutralizing antibodies and ELISA reacting antibodies was observed, either in sera of trivalent-vaccinated cattle or in sera of monovalent-vaccinated cattle 21 days post-vaccination.

The type-specificity of ELISA as regards FMD viruses was investigated by testing sera of monovalent-vaccinated cattle either with the homologous type of virus or with heterologous types.

According to the authors' experience, the erratic results sometimes observed in the ELISA can be highly reduced using inactivated 146S antigens instead of the infectious ones.

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* The original paper was presented at the 35th Meeting of the 'Società Italiana delle Scienze Veterinarie'. Gardone Riviera, 16-19 September 1981.