AGA: EUFMD/RG/80

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

Vienna, Austria, 17-19 June 1980

Food and Agriculture Organization of the United Nations

Rome, 1980

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INTRODUCTION

A Session of the Research Group of the European Commission for the Control of Foot-and-Mouth Disease was held at the Federal Institute for Animal Virus Diseases, Vienna, Austria from 17 to 19 June, 1980.

The meeting was chaired by Dr. J.G. van Bekkum, Netherlands, Chairman of the Research Group. The members of the Research Group present were: Dr. G. Kubin, Austria; Dr. M. Mussgay, Federal Republic of Germany, Dr. J. Leunen, Belgium, Dr. G. Panina, Italy, Dr. M. Eskildsen, Denmark and Dr. R.F. Sellers, United Kingdom.

The following observers attended: Dr. P.D. McKercher, Plum Island Animal Disease Center, U.S.A.; Dr. J. Fontaine and Dr. H. Favre, IFFA-Mérieux, Lyons; Dr. T.W.F. Pay and Dr. M.M. Rweyemamu, Wellcome FMD Laboratory, Pirbright; Dr. U. Kihm, Federal Vaccine Institute, Basel; Dr. R.P. Strobbe, Veterinary Research Institute, Uccle; Dr. J.C. Lei. State Veterinary Research Institute, Lindholm; Dr. K. Strohmaier, Federal Research Institute for Animal Virus Diseases, Tübingen; Dr. S.J. Barteling, Central Veterinary Institute, Lelystad; Dr. J.R. Crowther, Animal Virus Research Institute, Pirbright and Drs. Silber, Bücher and Al-Nuktah of the Federal Institute for Animal Virus Diseases, Vienna.

Dr. P. Stouraitis and Miss J. Raftery from the European Commission, FAO, provided the secretariat and the rapporteur for the Session was Dr. G.N. Mowat, Animal Virus Research Institute, Pirbright. Interpretation facilities were kindly made available by the Austrian Government.

Dr. Pindur, on behalf of the Federal Minister of Agriculture, welcoming the participants expressed his gratitude for the contribution which FAO and the members of the Research Group have made in the past to the control of FMD in Europe.

The following agenda was presented and adopted:-

1. Advances in the production methods for concentrated FMDV antigen including the evaluation, in vivo and in vitro, of antigens during long-term storage.

2. FAO Collaborative International Study for the Standardization of Laboratory Methods in FMD Research. Discussion of the provisional results of Phase IV and proposals for the introduction of international reference preparations for FMD work.

3. Progress in the application of the newer laboratory techniques such as Radio-immuno Assay and Enzyme-Labelled Immunosorbent Assay in FMD research.

4. Review of the international situation with regard to SVD, including current research .

5. Demonstration of the facilities, current work and disease security measures at the Austrian Institute.

6. Topios raised by the Executive Committee of the European Commission for the Control of FMD.

7. Other business.

8. Final Conclusions and Recommendations.

Item 1 - Advances in the production methods for concentrated FMDV antigen including the evaluation in vivo and in vitro of antigens during long-term storage

At the present time this topic is becoming of increasing interest with the possibilities of national and international vaccine banks being set up. The economies to be achieved by the long-term storage of highly concentrated and therefore small space occupying batches of vaccine antigens, are very attractive. An essential prerequisite of course is that effects, if any, of concentration and long-term storage at low temperatures shall be well understood before the system is adopted unequivocally on a large scale.

The presentations at the Session were in effect a continuation of Item 2 of the Agenda of the Research Group meeting at Lindholm last year and were again concerned with evaluating various methods of concentrations and presenting the results of tests after storage. A series of six papers on various aspects of the topic were given (see Appendix A, 1-6).

In the methods of concentration reported, four of the contributors were concerned with the use of precipitation procedures based on polyethylene glycol (PEG), one made use of the Albertson two-phase separation method and in the remaining report, an ultrafiltration unit was used for concentration purposes. All of these methods were capable of producing highly concentrated virus (concentration factors of up to 400x were reported) and with the PEG procedures, some degree of purification (up to 20x) was also obtained. As potential industrial processes the productivity of these systems was attractive in that some 70 - 90% of the product could usually be recovered. The results of the two-phase separation method showed it to be rather less efficient in that only 50% recovery was obtained.

Some of the evidence supporting the view that FMD virions can be preserved for long periods by storage at low temperatures came from studies based upon following the retention of infectivity and the subsequent conversion of such infective material into effective vaccines. However, the advantages of storing inactivated material were clearly appreciated since the reports also dealt with the results of inactivation of virus either before or after concentration. The successful inactivation of concentrated virus by ethyleneimine (E.I.) was reported but on the other hand, inconsistent or variable results were also obtained when acetylethyleneimine (A.E.I.) was used. The consensus of opinion was that a preliminary filtration step to remove cellular debris was desirable and that at present until more experience of the kinetics of untypical conditions of inactivation is gained, it would be advisable to inactivate virus suspensions before the concentration step. It was of interest to note that inactivated antigen which had been adsorbed to Kieselguhr for storage at low temperatures could be used for the immediate preparation of vaccine without elution of antigen. Kieselguhr may have some adjuvant properties and the vaccine prepared with this material did not produce undesirable tissue reactions in the small number of cattle in which it was tested.

Conditions of storage appeared to present no major problems and both mechanical freezers and liquid nitrogen containers have been used successfully. At the industrial scale, since 200-400 times concentration of antigen should be possible, the storage of material from large cultures should be quite feasible.

Much emphasis both in the papers given and the discussion that followed, was placed on the importance of monitoring the quality of antigen during the processes of inactivation and concentration and also after its recovery from low temperature storage. Preliminary studies showed that concentration of fully infective virus followed by storage for various periods up to seven years, had no significant effect on the infectivity or structural integrity of the virus as demonstrated by measurements of the 146s content of stored material.

At a more detailed level of analysis, the Plum Island laboratory reported that the polyacrylamide gel electrophoreses pattern of the polypeptides obtained from antigens concentrated by hollow fibre filtration showed evidence of cleavage of the VP_3 (immunising) component. This was noted with both type A and C strains of virus. It was thought to

be due to proteolytic enzymes originating from low level bacterial contamination of the ultra filtration process. However, tests of immunogenicity of these altered antigens in guinea pigs, swine and cattle indicated that there had been no major loss of immunising power. These observations are rather in contrast to what might have been expected as the result of earlier work with trypsin treated virus.

Perhaps the most convincing evidence that low temperature storage of highly concentrated antigens would be of practical value was shown by the fact that all the results of the tests reported of stored materials as vaccines in either small laboratory animals or domestic livestock, indicated, without exception, that there had been no significant loss of immunising power of stored antigens.

Clearly there is still much to be done to establish the optimum conditions for the processing and storage of a wider range of vaccine strains and to complement this with more data of the performance of these materials as immunising agents; however, at present, this aspect of foot-and-mouth disease research appears to be very promising for the future.

Item 2 - FAO Collaborative International Study for the Standardization of Laboratory Methods in FMD Research. Discussion of the provisional results of Phase IV and proposals for the introduction of international reference preparations for FMD work.

Discussion at the Research Group's Session at Lindholm last year centred on the considerable inter-laboratory variation in the results of the earlier phases of this collaborative study. Despite this, however, it was generally agreed that the study was of considerable value since some measure of standardization of assay methods was desirable and some of the methods investigated showed some promise of further improvement.

It was, therefore, decided to continue with a further investigation (Phase IV) of two methods - one basically a biological method, i.e. an assay of infectivity, the other, a biophysical method for the estimation of viral mass. In the case of the former method it was agreed to pursue the concept of standardization even further than previously by the distribution of not only virus and cells, but also all the other components necessary for the method, i.e. tissue culture media, Petri dishes etc. together with a set of instructions which participants were asked to follow. With regard to the method for estimation of viral mass, it was thought not possible to go further towards greater standardization due to the wide range of equipment and methods used in the various laboratories. However, from the limited experience of Phase III, the extent of inter-laboratory variation was comparatively small and it was thought worth repeating the investigation.

For Phase IV, 16 laboratories accepted the invitation to participate. Thirteen sets of replies have so far been recorded.

In setting up this round of investigations some interesting problems arose in the distribution of the materials. Temperature requirements varied from -70°C for tissue culture cells to 4°C for media and providing these conditions in one box for up to three days (to cover travel time and delays) resulted in the somewhat complicated package eventually distributed. With the exception of two laboratories which received broken bottles of bicarbonate solution the materials arrived in good condition with something of the original 5 kg. block of dry ice still intact. Maintaining the cells at -70°C to ensure viability for the required time, was the most difficult task and this was the main cause of the delay in starting the work.

Reagents

The virus materials distributed were prepared from the O_1 strain OBFS 1860. For the infectivity assay the material was an 18 hour harvest prepared with BHK21 monolayer cells. As before, the plaque purified strain was used. Virus 2 was a 1/100 dilution of Virus 1 and both preparations were presented in 50% glycerol. The material for weight of virus

estimation was prepared from virus grown in BHK suspension cells (clone AC9) and inactivated with AEI. It was concentrated by hollow fibre filtration and defatted with trichlorethylene. Part of this preparation was used to make 12S antigen and Virus 4 was a 1 in 3 dilution of Virus 3 in this 12S suspension.

RESULTS

The results received so far are given in Appendix B, Tables 1 to 4. In assessing the results of the infectivity titrations in Phase IV in relation to those of the earlier phases of the study, the mean of each laboratory's results has been used to construct a range of means of all results and from this the standard deviation has been derived (Table 1). In comparison to the earlier phases, with the omission of two slightly untypical results, it would appear that the efforts to provide more standardized conditions for the assays have proved successful. The standard deviation values for Phase IV being markedly lower than those from the earlier work (Table 2).

In general, the 100-fold difference in titre between Virus 1 and Virus 2 has been fairly closely obtained by most laboratories.

In presenting the results of estimates of virus weight, the same method as for infectivity had been adopted. Table 3 gives the results from Phase IV and Table 4 compares them with those obtained from similar work in Phase III. In general terms the latest results showed an improvement over the earlier work.

In the discussion that followed, the dilution factor of the original Virus 3 to produce Virus 4 was questioned since the results obtained pointed to a 1:4 and not the 1:3 dilution which was in fact made. This led to queries about the linearity of response of the measuring systems with antigens at various concentrations. Doubts were expressed that linear responses were obtained when highly concentrated preparations were diluted and measured. Since none of the laboratories use identical equipment or methods for this work, it was difficult to resolve this problem but there was general agreement to the suggestion that efforts should be made in a further Phase of the Study to calibrate the methods and equipment of the various laboratories participating by means of a distributed highly stable, precise standard such as a solution of bovine serum albumin or possibly ferritin.

During the discussion the question of writing up the earlier work for publication was raised. Dr. Mowat explained to the Session that there had been unavoidable delays but that he and Dr. Garland now hoped to proceed rapidly with the preparation of a manuscript for at least the first three phases of the Study. It was agreed that the Journal of Biological Standardisation would be an appropriate publication to which this manuscript might be offered.

In closing this item of the agenda, the Chairman, Dr. van Bekkum, spoke of the value of international collaboration projects such as this one and again stressed the importance of publishing the results. The results of such studies would be of interest not only to those involved in FMD research, but also to the wider scientific community involved in biological research.

Item 3 - Progress in the application of the newer laboratory techniques such as the Radio-Immuno Assay and Enzyme Labelled Immunosorbent Assay in FMD research.

In keeping with the latest advances in methodology in other areas of microbiological research, the newer immunologically based assay systems have been adapted to the needs of FMD research.

The two methods of greatest interest have been the Enzyme Labelled Immunosorbent Assay (ELISA) and the Radio-immuno Assay (RIA). A series of four papers were presented (Appendix C 1-4) in which the relative merits of the ELISA and RIA systems were compared,

and the results of investigations into antigen specificity, antigen structure and the sub-type specificity of antibodies in the sera of vaccinated cattle were given as illustrations of the application of these methods to research topics.

The RIA, although somewhat more sensitive than the ELISA, has the disadvantage that it requires facilities for handling and counting radio activity. The ELISA system is a relatively simple procedure requiring no very specialised equipment in the first instance although, if large numbers of tests are undertaken, the system is amenable to automation.

The attraction of these two assay systems is their versatility in that they can be used for a wide range of investigations of both FMD antigens and antibodies. Both can be used for the rapid diagnosis and typing of virus and in comparison to the routine complement fixation tests, are of the order of 10-100 times more sensitive. Again, due to the high specificity and sensitivity of these tests for FMD antigens they can be used for both quantification of virus and for qualitative tests comparing one strain with another by means of competition for antibody.

As indicator systems for the detection of antibody they also have much to offer both for the screening of large numbers of sera for epidemiological purposes and for a quantitative assessment of the response of livestock to vaccination. In regard to the latter, however, it has to be noted that total antibody is measured and the relationships between that and protection have yet to be more fully investigated. Reports presented to the Session of the applications of the ELISA system have shown that it is capable of detecting very small differences between antigens. Working with anti-sera prepared against the structural proteins of the virion, it has been shown that virus adsorbed directly to the plastic wells of microtitre plates may undergo configurational changes which result in the exposure of the internal proteins VP_2 and VP_3. The resulting increase in cross-reactivity between serotypes may be unacceptable for certain investigations but modifications of the test procedure such as the 'sandwich' method, will almost certainly overcome this problem.

The 'sandwich' method is the most sensitive of the ELISA techniques available and was reported to be capable of detecting as little as 5 ng of protein. This method has been successfully applied to detecting the protein fragments of virus following trypsin and BrCN treatment. From these results and in conjunction with an analysis of the amino acids involved, a sequence of the peptides in the trypsin digestible coat protein VP Thr. has been derived.

The ELISA system has also been used to determine the type of specificity of immuno-globulins in the sera of vaccinated cattle. An interesting variation of the test procedure was used, in that antigen was chemically bound by means of cyanogen bromide to small paper discs, and the ELISA procedure was carried out in disposable glass haemolysis tubes. The advantages of the disc method was said to be that the antigen is firmly bound, that precise amounts of antigen can be used on each disc and also it is probable that prepared discs can be stored for lengthy periods. In tests with sera from vaccinated cattle and using pig antibovine IgG conjugate (alkaline phosphatase) as the indicator, it was shown that type specificity of FMD antibody in these sera could be clearly identified. Further, in tests comparing the sub-type identity of three type O strains, it was possible to show that O_1 Lombardy and O_1 Lausanne have a closer identity than O_1 Oltu.

The immunologically based test systems appear to offer many advantages. There is still a lot to be done in exploring their range of applications and in comparing the results with those from the older conventional test systems, however, it is clear that these newer methods will be applied more and more to FMD research in the future.

Item 4 - <u>Review of the international situation with regard to SVD, including research</u>

The present situation in the United Kingdom in relation to swine vesicular disease was reviewed by Dr. Sellers. The numbers of outbreaks for 1978, 1979 and 1980 were nil,

43 and 37 to date, respectively. Recent surveys on pigs sent for slaughter from herds fed swill, have shown that the figures for numbers of outbreaks may not reflect the true situation. Serum surveys now suggest that pigs may have had inapparent infections in 1978 and that the outbreaks in 1979 and 1980 may have been due to the continued presence of virus and not to a new introduction. At present there are no indications of major serological differences between virus isolates. The problem is still very much related to one type of pig husbandry, viz. swill feeding. Outbreaks have been only on premises where waste food is used. Also if pigs are housed on soft flooring, i.e. hay or straw, or, as frequently found, on mud, any lesions forming are not obvious and clinical disease is not observed. Spread of infection may be by direct contact on farms or at markets, but the two major factors in the continuance of this disease are (a) the use of swill feeding systems in which virus is not being completely inactivated by the heat treatment and (b) by motor vehicles which have transported inapparently infected pigs not being completely disinfected afterwards. The implementation of legislation governing these aspects of disease control has proved difficult.

The situation in Continental Europe

There was one outbreak of SVD in Austria in 1979 and since then no further outbreaks. Belgium is free at the present time and serum surveys show no evidence for the existence of virus in the country. There is no information available concerning the situation in France at present. The last outbreak in Switzerland occurred in 1973 and a recent small serum survey showed no evidence of infection. Denmark continues to remain free of S.V.D. and several serum surveys over the last seven years confirm this view. There were some outbreaks in North Italy in 1979. These were on large industrial type piggeries and were attributed to transmission of infection from contaminated vehicles. The virus isolated was only of low virulence, generalised infection being difficult to produce in laboratory animals. Sera prepared against this strain showed no differences when compared with the earlier isolates Italy 1972 and U.K. 1972. Further outbreaks have occurred in Italy during the first half of 1980. The last occurrence of the disease in the Federal Republic of Germany was in 1978 when there were two outbreaks. Greece had its first outbreak in 1979 when the disease was recognised in swill fed pigs near Athens. Compulsory slaughter of infected pigs is now required but there is no legislation relating to the use of swill. Holland had two outbreaks on neighbouring farms in 1973 and has been free since then. A subsequent survey of some 3,200 sera collected from 28 slaughter houses yielded two positive samples. The farms involved proved to be negative and the positive reactions are thought to be due to antibodies against other pig enteroviruses.

Pathogenesis of Swine Vesicular Disease

A paper on the influence of S.V.D. infection upon pregnancy was introduced by Dr. Al-Nuktah (see Appendix D). This recent investigation was made subsequent to a report of abortion in four sows in a group of 11 after an outbreak of S.V.D. in Vorarlberg, Austria in 1972. Tests involving six pregnant sows in contact with donor pigs inoculated with the WN strain, showed that infection with S.V.D. virus did not lead to abortion or had any detectable embryopathic effect. Serological tests on the piglets showed that there was no transplacental transmission of the virus. This is in accordance with the earlier findings of other workers.

Item 6 - Topics raised by the Executive Committee
Selection of strains for vaccines

The selection of strains for vaccines to be stockpiled as part of an International Vaccine Bank or for use in prophylactic vaccination programmes, continues to be of prime importance. In this context the field situation in Europe was reviewed and consideration given to which vaccines currently in use are likely to give adequate protection.

The introduction of new strains is most likely to come from the importation of meat

from South America. At present, the type O strains (O_1), active in South America and Europe, appear to have only minor serological differences and the prospects for cross protection with vaccines in use are good. Type C virus is widely distributed in Latin America and is predominantly of the C_3 subtype. European vaccines are prepared with C_1 strains and although, in view of the South American situation, C_3 would be a better choice, due to the relative similarity of C_3 and C_1 strains, vaccines prepared with the latter will probably give adequate protection.

At present in the South American meat exporting countries, the majority of type A strains belong to a group which are not all serologically identical but are characterised by the A_{24} subtype.

Europe has only had small numbers of outbreaks due to type A virus in recent years but the salient feature in all cases has been that the strains recovered were closer to A_{24} and different from A_5 and A_{22} (see Appendix E, Table 1). European authorities must therefore consider whether it would now be expedient to change the A_5, A_7 or A_{10} strains presently used for vaccine production, for one more appropriate to present day conditions. In the U.K. strategic vaccine reserve the A component has already been changed from A_5 to A_{24} and it is also of note that the report of the FAO Ad Hoc Consultation in December 1979 in Rome on the setting up of an International Vaccine Bank, contained the recommendation that for Phase I, relating to those countries not applying vaccination routinely, the A vaccine to be stored should be prepared from an A_{24} strain. In view of the recent field situation, whether or not A_{24} can still be regarded as non-indigenous to Europe is difficult to decide. In coming to a decision on the need to change vaccine strains or not, the following points have to be kept in mind:-

(1) A_5 vaccines will give some protection, especially after revaccination, against A_{24} strains but the amount of data is limited.

(2) Some of the recent outbreaks in Europe have been due to escape of virus from vaccine plants and also to vaccines containing incompletely inactivated virus (see Appendix E, Table 2). Therefore, during the initial period when changing from one vaccine strain to another, there may be risks to the livestock population at large.

(3) There may be some need for protection against A_5 in the future and at present there is no data on the ability of A_{24} vaccines to protect against this subtype.

Clearly there is need for further investigations into all of these aspects before recommendations can be made as to whether a change of vaccine strains is required. In addition, the question of the most appropriate strain to represent the A_{24} group would have to be considered since the members of the group are not identical and any strain chosen for vaccine use should give the widest available serological coverage.

The United States authorities in setting up a Strategic Vaccine Reserve have purchased A_5 antigen and will shortly be running comparative trials of A_5, A_{24} and A_{27} vaccines (the latter is a South American strain thought to be similar to A_5). It would therefore be best to wait until the results of these trials are available together with any other investigations from the European laboratories, before making further pronouncements on the situation.

Item 7 - Other business

A. Three papers presented in this section were concerned with FMD strain identity and associated serology.

The first paper gave the results of a study comparing two Austrian field strains O Zurndorf 1965 and O Gross Schweinbarth 1973 with two vaccine strains OBFS 1860 and O Svizzera (Lausanne) by means of neutralization tests with either guinea pig or bovine antisera. The OBFS 1860 and O Svizzera strains were shown to be closer in identity to

each other than either of the Austrian strains. The former strain gave the best overall coverage although the O Gross Schweinbarth was also useful in this respect and it was concluded that these two strains would be most appropriate for use as vaccines in Austria.

The second contribution reviewed the type C strains recovered from outbreaks in Europe and showed that since 1974 all of them have been classified as subtype C_1 by means of complement fixation tests. The latest strain from the current outbreaks in Spain was shown in a series of one way tests to be closest to C_1 Vosges 1960 but also to have some identity with the C_3 Philippines 1976 strain.

In the third paper the effects of animal species, use of individual animal or pooled sera, sera from primo-vaccinates or hyperimmune animals, anti-cellular antibody and antigen quantity or quality on the strain specificity of antisera used for vaccine strain selection were reported. The results suggested that when the antigen presentation was similar, 'r' values obtained by two-dimensional microneutralisation tests were consistent although individual serum titres varied from animal to animal and with the dose of the antigen. It had been reported in the literature that there was a decreased specificity in the sera of animals after hyperimmunisation but the results obtained indicated that the sera of cattle after revaccination were more specific than sera from the same cattle vaccinated only once. There was some evidence that sera from different species (guinea pigs or cattle) gave similar 'r' values. Variations in the specificity of sera were observed when the antigen was altered by trypsin cleavage or was live foot-and-mouth disease virus. Results indicated that it was probably valid to use pools of sera rather than to test with many individual sera when determining antigenic relationships.

B. Draft document on the characterisation of cells

Dr. Panina introduced this topic and reported that the draft document for the International Association for Biological Standardisation on the characterisation of cells to be used for vaccine production was proving difficult to prepare. The subject was a complex one and there were dangers in attempting to define the properties of cells too closely since once these definitions had been accepted, vaccine producers might find them unduly constraining at some time in the future.

In the discussion that followed, concern was expressed that not only oncogenicity of cells should be in the terms of reference but that freedom from adventitious agents should also be included. This was important in relation to the wide distribution of some veterinary products. The opinion was also expressed that there would be an increasing trend among national authorities for the screening of substrates to be used in the preparation of biologicals for sale prior to the issue of a licence. It was therefore best while there was still adequate time, to consider and decide the conditions for vaccine production, rather than have to adopt undiscussed requirements at short notice.

The Chairman pointed out that the Research Group was not empowered to change the original terms of reference; however, the meeting could make recommendations to the I.A.B.S. that the range of requirements for cells be extended and he suggested that a small Working Group be set up to consider the topic further and to offer advice to I.A.B.S. on the drafting of a suitable document.

C. Arrangements for the next session of the Research Group

The Chairman announced that Professor Mussgay had very kindly invited the Research Group to hold its next meeting at the Federal Research Institute for Animal Virus Diseases, Tübingen, Federal Republic of Germany, and it was hoped that the meeting could be held in late September, 1981.

The following provisional agenda for the Session was proposed:-

1. Further investigations of the production and evaluation of concentrated foot-and-mouth disease vaccine antigens for long-term storage.

2. Elution of viral antigens from vaccines and their application to the retrospective safety testing of fully formulated products.

3. Current developments in the application of ELISA methods to foot-and-mouth disease research.

4. Monoclonal antibodies - developments and progress in the application of this new technique to foot-and-mouth disease research.

5. FAO International Collaborative Study for the standardization of laboratory methods in FMD research. Discussion of the results of Phase V.

6. Results of investigations on type A strains which might be considered appropriate for the production of vaccines relevant to the current European field situation.

7. Topics raised by the Executive Committee of European Commission for the Control of Foot-and-Mouth Disease.

8. Demonstration of the facilities and some of the current research at the Federal Institute.

9. Any other business

10. Final conclusions and recommendations

In discussing the arrangements for the next meeting Dr. van Bekkum emphasised the advantages of distributing the texts of papers to be given well in advance. He requested that contributors should send copies of their papers to Professor Mussgay at least four weeks before the meeting, who would, in turn, ensure that they were distributed to all those intending to be present.

At the closing Session Dr. van Bekkum thanked the speakers and contributors to the discussions, the FAO Secretariat, Dr. P. Stouraitis and Miss J. Raftery, the rapporteur Dr. G.N. Mowat, and the interpreters.

In closing the Session, Dr. van Bekkum paid especial tribute to Professor Kubin and his staff for the excellent arrangements which they had made to receive members of the Research Group in Vienna and for their most generous hospitality. In all the Session had been a very pleasant and successful event.

Item 8 - Final Conclusions and Recommendations

Item 1: Advances in the production of concentrated antigen

The reports presented have shown that a range of different methods may be successfully used for the concentration of viral antigen. These included precipitation with polyethylene glycol, 2-phase polymer separation and ultrafiltration. The level of concentration achieved and the degree of purification varied according to the method employed. Preparations in which the antigen was concentrated by a factor of up to 400 times were reported and efficiency of product recovery was in the region of 70% - 90%.

One of the most important steps in the procedure is inactivation. Various inactivants were used either before or after concentration. At present inactivation before concentration appears to be preferable since problems of incomplete inactivation with concentrated antigen

were reported. It was also thought that a filtration step prior to inactivation might be advantageous but further investigations of this part of the process are needed.

Antigen preparations were stored either in mechanical freezers or in liquid nitrogen containers. In comparison to control preparations, stored materials showed no loss of infectivity or, when stored as inactivated antigen, no loss of immunogenicity. This was true for some preparations stored for up to seven years. In one study, polyacrylamide gel electrophoresis of stored concentrated antigens showed that the polypeptide responsible for immunogenicity had, for undetermined reasons, been cleaved. These materials were as immunogenic as comparable control preparations. These findings emphasize the need to continue to investigate the properties of stored concentrated antigens both at the biochemical level and by immunisation experiments in animals.

It is recommended that

(1) further research be devoted to developing and improving methods for the concentration and purification of FMD antigens for use in vaccines;

(2) concentrated antigen should be stored as inactivated material, since at present inactivation of concentrated live virus has given variable results and there is the additional advantage of increased safety in storing inactivated material;

(3) studies on the immunising properties of a more extensive range of concentrated antigens after storage at ultra low temperatures, be made;

(4) potency tests of vaccines made from concentrated antigens should be made in the animal species in which the vaccine will normally be used. Expiry dates of such vaccines should be defined;

(5) the use of stocks of concentrated antigen is considered of special interest for countries that have no systematic vaccination programme for cattle, and for those wishing to hold stocks against certain types or subtypes of the virus or for the protection of pigs in emergency situations.

Item 2 - The Results of Phase IV of the Collaborative International Study on Laboratory Methods in FMD Research

The investigations jointly undertaken in Phase IV consisted of two parts:

1. Assays of virus infectivity by the agar-cell suspension method, and
2. Estimations of viral mass by bio-physical methods.

The data presented have shown that with the infectivity assays the variation in results from different laboratories has significantly decreased following the distribution of materials for the performance of the test in addition to the virus samples and also a detailed description of the method. The conclusion to be drawn is that the results demonstrate the benefit of standardizing as many elements of the test as possible. This is amply confirmed when the results are compared to those of Phases I and II.

A similar trend was also shown in the results obtained from the estimates of viral mass. Compared with the results of Phase III the extent of variation was considerably reduced as was shown when the variation in each series was expressed as the standard deviation of the range of means of results from all the laboratories participating. It was noted that although the true difference in concentration of viral mass between the virus preparations that were compared was known to be 3-fold the difference observed in many of the participating laboratories was 4-fold or greater. This raised the question as to whether the laboratory systems in use were capable of giving a linear response over the range of antigen concentrations in the materials as distributed. Nevertheless this method was thought to have considerable promise as a means of measuring the amount of

antigen to be incorporated into vaccines in the future.

It was concluded that Phase IV had in fact been successful in that it had demonstrated that attention to detail could result in better agreement in results from different laboratories and it was agreed that the collaborative study should be continued.

It was therefore recommended that Phase V should be concerned with further study of the measurement of viral mass:

1. Bearing in mind the wide range of equipment and methods used to measure the weight of virus in different laboratories, it was agreed that an early essential step was to calibrate all these methods by means of a distributed standard. It was suggested that a suitable stable material capable of precise formulation might be provided by a suspension of ferritin and that following preliminary investigations to confirm this (to be carried out between the Lelystad and AVRI laboratories), the latter would send out to all participants a range of preparations for this purpose.

2. It was also recommended that an investigation of the linearity of the measuring systems with virus material should be pursued with the type O virus used in Phase IV. To this end AVRI will prepare a series of virus suspensions in which the 146S is diluted in known steps in homologous 12S material and these preparations will be distributed for assessment.

3. Further, it was recommended that the investigations be extended to include another virus type to observe whether the inter-laboratory variation would be of the same order as that found in the type O virus. It was recommended that type C inactivated antigen be used for this purpose and it was agreed that the AVRI laboratory would prepare a series of materials in the manner of that provided for the type O virus and would distribute them as before.

Item 3 - Application of radio immunoassay and enzyme labelled assay to FMD virus research

Radioimmuno assays (RIA) and enzyme linked immunosorbent assays (ELISA) have been successfully exploited for FMD virus studies by several research laboratories. A general conclusion may be made that the RIA is of limited use due to the faoilities needed for the handling and counting of radioactivity. The ELISA is therefore recommended as a sensitive and convenient method for further development; although the RIA will continue to be used by research laboratories to investigate some aspects of FMD virus. The ELISA should be workable by a wide number of laboratories with differing facilities.

Both FMD viruses and antibodies against viruses have been found suitable for use in the solid phase systems mainly using passive adsorption onto various plastic microtitre plates, although one laboratory has successfully attached FMD inactivated virus to paper discs by chemical methods - which might be exploited in future to obtain more stable pre-coated antigen plates.

The general methods used for the ELISA tests reported were similar to those described in the bulk of the literature concerning virus/antibody systems. No great difficulties were reported, although research is needed to investigate further any effects on virus interaction with antibody after it has been adsorbed to plastic.

Two types of development are recommended:-

1. Use of the ELISA test with its inherent sensitivity and convenience of solid phase microplate systems, to investigate the nature of the FMD virion, e.g. the antigenic characterization of 12S subunits and peptide fragments, and the comparison of virion structure at various stages during vaccine manufacture.

The use of the ELISA test as a basic research tool should continue to be promoted and may be of most use when monoclonal antibodies against FMD viruses are available.

2. Use of ELISA tests to replace or qualify existing immunoassays where the latter prove inconvenient or irreproducible e.g. to measure 146S directly from tissue culture fluids or measure antibodies against FMDV from a range of animal sera. These types of assays should be adapted for wide application. Research into the suitability of using stored inactivated antigen or antibody coated microplates should now be made with the aim of eventually supplying 'standardized' reagents to smaller laboratories.

Particular emphasis on the following research areas using ELISA techniques should be made:

1. application to the measurement of anti-FMD virus antibodies with the aim of correlating the relationship of ELISA results with SNT protection studies by screening large numbers of animal sera;

2. development of rapid diagnostic methods for antigen discrimination;

3. development and standardization of methods to measure the 146S virus from culture samples e.g. during vaccine manufacture;

4. the establishment of simple methods for the serological comparison of viruses from the field, and at various stages during the manufacture of vaccine-

It is also recommended that some liaison be set up between laboratories interested in, or who are using, ELISA techniques in FMD research so that current results may be exchanged or help given.

Item 6 - <u>Selection of Vaccine Strains</u>

In relation to the choice of vaccine strains considered to be appropriate for use in European countries, it was concluded that there was no need to change the O and C strains currently in use. Existing vaccine strains would probably give adequate serological cover. The situation was less clear with the type A strains. Prophylactic vaccination in Europe is carried out with vaccines prepared with A_5, A_7 or A_{10} strains. The small number of recent outbreaks in Europe due to type A virus have been caused by strains which appear to have a closer affinity to A_{24} than the vaccine strains in use. A_5 virus has not been recovered from the field during the last five years and the main threat to European livestock could come from the importation of meat products from South America where, at present, the majority of the type A strains in the field form a group characterised by the A_{24} subtype.

Before making recommendations on the need or otherwise to change the type A strains to be used for vaccine production, further investigations are required into the serological relationships of the various strains involved and in particular into the levels of protection that A_5 and A_{24} vaccines will give in cross protection tests. Trials investigating this point are shortly to be made with A_5, A_{24} and A_{27} vaccines at the Plum Island Laboratory.

It was therefore recommended that:-

1. The results of these trials plus those from any further investigations in the European laboratories, should be awaited before any pronouncements on the need to change vaccine strains are made.

2. This topic should be placed on the agenda for further discussion at the next Session of the Research Group.

Item 7: Standardization of cells for FMD vaccine production

At the International Symposium on Foot-and-Mouth Disease organised by the International Association of Biological Standardization (Lyons, France 5-8 October 1976) Drs. Nardelli and Panina were asked to prepare a draft document on testing of cells to be used in the production of inactivated FMD vaccines. Subsequently this preliminary draft was circulated for comments.

The Research Group of the European Commission for the Control of FMD recognised the importance of cell testing and considered that the draft was of interest. It recommends that after Dr. Panina has received comments and amendments from interested parties, a Working Group is set up to correlate the information for onward transmission to I.A.B.S. It is suggested that the Working Group consist of Dr. G.F. Panina, Dr. R.F. Sellers, Dr. J. Fontaine and Dr. T.W.F. Pay.

Appendix A-1

Preliminary results of *in vivo* and *in vitro* tests on concentrated and purified FMD viruses

by

A. Rey and C. Roulet *

The industrial methods of concentrating FMD viruses to be used in the preparation of cattle vaccines have gained some importance, especially regarding cell culture viruses.

Indeed, both the producer and the user can find numerous advantages in these methods. For the former, concentrated viruses can be stored in small volumes, which facilitates the production management and makes it possible to adapt the production work to the demand. The latter has the benefit of a vaccine prepared from an especially purified virus, which gives him full guarantees as to the perfect tolerance of the product.

All the currently used processes are based on virus precipitation, or its selective adsorption, by a ethylene-oxide high polymer. It is only in the case of selective adsorption, on a polymer-cation complex that the concentration is accompanied by a high degree of purification. The repeated process then results in a highly purified product. Desalting after adsorption is usually carried out using a virus-polymer-cation complex destructive agent.

The results obtained in both *in vivo* and *in vitro* tests carried out on industrial viruses subjected to such treatment are reported in this paper.

Materials

- The viruses used in this study belong to the French vaccinal strains, types O, A and C: O_1 Lausanne Switzerland 1965; A_5 Allier France 1960; C_1 Vosges France 1960. They are multiplied on a cell line adapted to the culture in suspension (IFFA-3 cells), in 1 400-litre fermentors (Ref. 1).
- The virus concentrates are obtained from the above-mentioned viruses after several concentration - purification cycles.
- The vaccines are aluminium hydroxide and saponin adjuvanted vaccines prepared according to the Institute's classical formula.

Methods

1) Concentration of the Virus Suspensions:

Polyox WSR is added to the virus suspension, kept at +4°C, in order to obtain final 4% proportion (w/v). After stirring, the solution is centrifuged at continuous flow in order to recover the polymer on which the virus is fixed. This sediment is then dissolved again in a buffer containing an amount of complexing agent enabling the desalting of the virus. The process can be repeated a second time.

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2) Inactivation

The 'concentrated - purified' viruses and their non-concentrated controls are inactivated at pH 8.02 by ethyleneimine at a concentration of 0.05 per thousand, whilst being stirred. (Ref. 2)

3) Conservation of the Inactivated Products

The non-concentrated control viruses are stored at +4°C following inactivation. The concentrated and inactivated preparations are stored at -70°C in glass containers. They are thawed rapidly before being used.

4) Preparation of the Vaccines

- Control Vaccines

  The control vaccines are prepared according to the industrial method from the inactivated control viruses. They are adjuvanted with saponin and adsorbed onto aluminium hydroxide.

- Purified Virus Vaccines

  The concentrated suspension is diluted after rapid thawing in an isotonic buffer at pH 7.9, in order to obtain the same amount of antigen per dose as in the control vaccine. Then, aluminium hydroxide and saponin are added at the usual concentrations.

5) In Vitro Testing Methods

a) Virus

The virus concentration, infectivity and antigenicity are determined by the usual techniques.

- Complement fixation using continuous flow Technicon apparatus (Ref. 3).
- Infectivity Assay by titration on sheep kidney secondary cells in microplates
- Determination of the 140 S Particles Concentration after ultracentrifugation on 45% - 15% saccharose gradient (Ref. 4)

b) Total Proteins

The proteins are precipitated by perchloric acid at a 10% final concentration and dosed according to the Lowry Method, after solution in 0.1 N soda (Ref. 5). Bovine albumin is used as a reference.

c) Electrophoresis in Polyacrylamide Gel

Electrophoresis in polyacrylamide gell allows a qualitative evaluation of the purification degree of the concentrated virus suspensions to be made. After precipitation by perchloric acid, the virus proteins are resuspended in a dissociation buffer (urea 8M - SDS 2% - Mercaptoethanol 5% - glycerol 10% - Tris 60mM) at the rate of 200 µl of buffer for 70 ug of proteins. The electrophoresis is then carried out according to the Laemmli modified discontinuous buffer system (resolution gel at 12.5% in acrylamide, containing 3M urea).

6) In Vivo Testing Methods

a) Safety

The safety is tested on cell culture at the inactivated antigen level, then in susceptible cattle at the vaccine stage.

b) Potency

Each vaccine has been tested in cattle by the Bovine Potency method, using a full dose and a quarter of a dose (Ref. 6). The challenges are carried out 21 days after vaccination.

Results - Discussion

The industrial viruses and the corresponding vaccines were subjected to *in vitro* and *in vivo* tests at the successive production and concentration stages.

1. 'In Vitro' Tests

a) Purification

The virus preparations were analysed at each step of the concentration-purification process. Tables 1, 2 and 3 give the results for each valency. The viruses were concentrated about 100 to 400 times. The proteins are concentrated 5 to 8 times. The purification rate is thus about 20 and more, but it greatly varies from one type of virus to another.

The highest purification rates are obtained in the A valency, whereas they are lower and about the same in the O and C valencies.

It is found that the complement fixation and the virion concentration results are closely related regardless of the concentration-purification stage. This is due to the use of hyperimmune sera. On the contrary, the infectivity does not increase in proportion to the other parameters.

The concentrated-purified viruses and their non-treated controls were analysed by electrophoresis in polyacrylamide gel, as well as the same viruses purified by ultracentrifugation (Figure 1).

The initial suspension contains a multitude of protein having very variable molecular weights. The bands corresponding to viral proteins are only slightly clear. After concentration, electrophoresis shows only a small number of bands. The viral proteins (molecular weight from 26 to 30 000) are then present in larger quantity. Most of the residual contaminating proteins have a higher molecular weight of 40 to 80 000 Daltons.

b) Conservation (Table 4)

The concentrated virus suspensions conserve well at low temperatures and, in a suitable buffer, retain their integrity during the freezing-thawing successive stages.

2. 'In Vivo' Tests (Table 5)

The vaccines prepared from concentrated-purified viruses have the same potency as the control vaccine, and sometimes even higher. The differences observed are not really significant but the result is interesting in that it demonstrates that it is possible to concentrate and to purify foot-and-mouth disease virus in large amounts and that after re-dilution it is still immunogenic.

Other study work is at present under way to verify if such a vaccine behaves in the same way as a classical vaccine after long-term storage.

Conclusion

The industrial methods of concentrating foot-and-mouth disease virus have been applied and published for some time.

The tests carried out on the products obtained at the successive stages of purification as well as after low temperature storage, show that these viruses retain their antigenic and immunogenic properties. The vaccines prepared with such viruses have a satisfactory potency.

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IN VITRO TESTS ON THE CONCENTRATED PURIFIED VIRUSES

Type O_1 Lausanne - TABLE -1-

	virus	CF	TCID	weight of virus : µg/ml	protéins (µg/ml)
initial virus	Batch 1	197	6,5	2,8	295
	Batch 2	157	6, 5	1, 5	246, 25
	Mean	177	6, 5	2, 15	271
concentrated virus 1st stage	Batch 1	4 668	6,5	71,7	1 300
	batch1 bis			91, 4	1 860
	batch 2				
	batch 2 bis				
	Mean	4 668	6, 5	81, 55	1 580
concentrated virus 2nd stage	Batch 1	56 000	7,1	543,9	2 360
	batch 1 bis	35 200		443, 3	2 540
	batch 2			217, 1	2 801
	batch 2 bis			168	2 528
	Mean	45 600	7, 1	343	2 557

IN VITRO TESTS ON THE CONCENTRATED PURIFIED VIRUSES
Type A_5 Allier 1960 - TABLE -2-

	virus	CF/0,8 ml	TCID /ml	weight of virus : µg/ml	protéins µg/ml
initial virus	batch1			1,17	290
	Batch 2	97	7,1	0,47	300
	batch3	272	7,1	1	240
	batch4	110	6,7	0,3	187
	Mean	159	7	0,77	254
concentrated virus 1st stage	batch1			67,7	898
	batch2			17	
	Batch 2 BIS			37,6	
	batch3	3 470	5,5	42,9	480
	batch3 bis	4 635		68,95	1 160
	batch4	1 084	6,3	10,2	780
	Mean	3 063	5,9	40,7	830
concentrated virus 2nd stage	batch1			574,2	1 035
	batch2	8 800	7,3	72,4	800
	Batch 2 BIS	11 670	7,1	172,8	820
	batch3	26 600	6,9	560	1 240
	batch3 bis			94,9	1 500
	Batch 4	4 180	7,1	40,1	1 143
	Mean	12 812	7,1	252,4	1 090

IN VITRO TESTS ON THE CONCENTRATED PURIFIED VIRUSES
Type C_1 Vosges 1960 - TABLE -3-

	virus	CF /0.8 ml	TCID/ ml	weight of virus : µg/ml	protéins µg/ml
initial virus	batch1			1,3	234,9
	batch2	168	7,3	1,1	340
	batch3	63	7,3	0,69	168
	batch4	163	7,7	2,43	175
	batch5			1,18	95
	Batch6	145	7,5	1,58	119
	batch7	100		1	340
	batch8	137	7,7	1,1	210
	Mean	106	7,5	1	210
concentrated virus 1st stage	batch 1			40,7	653
	batch 2			35,8	980
	batch 3			13	726,8
	batch 4				
	batch 5				
	Batch 6			50,49	506,6
	batch 7	3 470		28,8	1 025
	batch 7 bis	3 920		48,6	889
	batch 8	4 900	⩽ 5,9	47,7	1 387
	batch 8 bis	4 200	6,1	25,2	606
	Mean	4 122	6	36,28	847
concentrated virus 2nd stage	batch 1	11 764		186,8	632,5
	batch 2	12 272		74,1	552
	batch 3			108,9	712
	batch 4	13 087		116,02	857
	batch 5	12 810		110,6	710
	Batch 6	12 737		104,2	480
	batch 7	12 416		136	1 240
	batch 7 bis	8 445		98,9	1 120
	batch 8	8 936	7,7	75,6	1 283
	batch 8 bis	9 301	7,3	86,2	1 238
	Mean	11 307	7,5	109,75	882

Nature of the buffer		To	T 1 month	T 12 month
		← Tris EDTA	(0, 2 M. 0, 15 M) →	
CF/0, 8 ml	O control	201		
	O concentrated	55 090	52 400	
	A control	125		under way
	A concentrated	10 835	9 000	
	C control	184		
	C concentrated	13 084	12 737	
140 S ug/ml	O control	1, 8	1, 3	
	O concentrated	536	516	
	A control	0, 47	0, 2	
	A concentrated	203, 1	172	
	C control	1, 2	1, 3	
	C concentrated	92, 6	104, 2	

CONSERVATION OF THE CONCENTRATED AND EI-INACTIVATED VIRUS AT -70°C - TABLE -4-

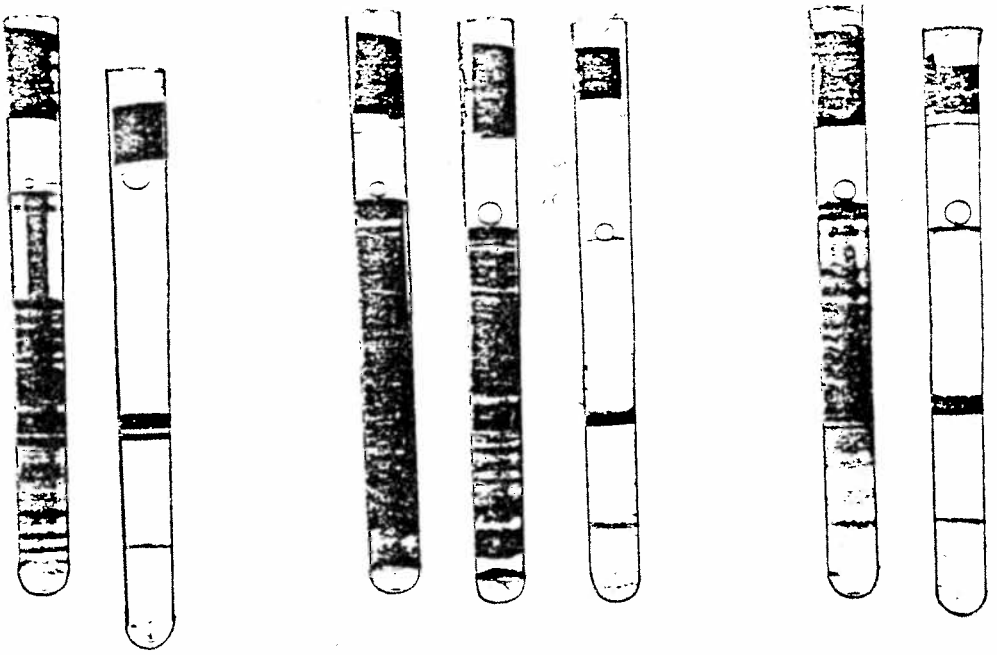
The controls are stored at +4°C.

virus of serotype	Antigens	CF	140 S	TCID	S.N antibodies		Number of animals protected	challenged
					dose	1/4 dose	dose	1/4 dose
■ O	non-concentrated control	201	1, 8	6, 5			4/5	2/5
	concentrated	55 090	516	7, 1			5/5	1/5
● A	non-concentrated control	125	0, 3	7, 1			5/5	3/5
	concentrated	9 000	172, 8	7, 1			5/5	5/5
● C	non-concentrated control	184	1, 2	7, 5			5/5	4/5
	concentrated	12 737	104, 2	7, 5			5/5	4/5

● 3 ml of virus are used to prepare the vaccines in the A and C valencies

■ 5 ml of virus are used in the O valency

- TABLE -5-



ELECTROPHORESE IN POLYACRYLAMIDE GEL OF THE CONCENTRATED AND PURIFIED VIRUSES AND THEIR CONTROLS.

- i : initial virus
- c : "concentrated-purified" virus
- p : pure virus

FIGURE -1-

Appendix A-2

Long Term Storage of FMD Virus for Vaccine Preparation: Concentration of Inactivated Virus by a Two-Phase System: Vaccine Potency Test on Cattle

by

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

Introduction

The purpose of the present work was to develop a procedure for production of highly concentrated foot-and-mouth disease (FMD) antigen.

This antigen is intended to be used for extemporaneous preparation of vaccine in emergency situations. The stock of classical strains antigen could allow to focus the full normal vaccine production capacity on a possible different field strain. To be practicable, the concentrated antigen procedure should be easily extrapolatable from experimental to industrial level and be of reasonable cost.

The procedure we experimented with involved formol inactivation of cellulose filtered virus, concentration in a two-phase system, storage at -70°C for four months and the preparation of vaccine immediately before use.

Materials and Methods

Virus suspensions

The virus was grown in Frenkel-type cultures. Equal volumes of O, A and C virus suspensions were combined, clarified by centrifugation and filtered on 1121-Schleicher and Schull filter pads. This constituted the original virus suspension.

Formol solution (Merck-4003), diluted 1:20 in PBS, was added to a final concentration of 0.75:1000 and the mixture was kept at 26°C for five days.

The inactivation of the virus was checked on baby mice after a 10x concentration by centrifugation. After completion of this test, part of the suspension was taken for the concentration procedure and part was used to prepare a control vaccine.

Concentration of virus suspensions

A PEG-Dextran two-phase system was used to concentrate the virus (1, 2, 3). A Dextran Sodium Sulphate 2000 (Pharmacia) solution 10 percent ww in water and a Polyethylene Glycol 6000 (Fluka) solution 50 percent ww in water were prepared and autoclaved at 110°C for 30 minutes. Solutions were mixed in the following order: virus suspension (84.4 percent ww); Dextran (2.0 percent ww); PEG (12.8 percent ww). Sodium chloride (0.87 percent ww) was also added.

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The system was prepared in a glass column which was siliconized to reduce the adsorption on the wall, and placed in the coldroom for about 18 h. The supernatans were pumped out down to about 7 percent of the original volume and discarded. The remainder was stirred and collected. The suspension was centrifuged at 4000 rpm for 20 minutes: upper phase, interphase and lower phase then appeared very distinctly. The upper phase was carefully taken off with a syringe. An attempt to take out the lower phase resulted in the loss of part of the interphase because of the very high viscosity of the phases. Interphase and lower phase were thus mixed: a sample was taken for titrations, the remainder was stored at -70°C.

Vaccine preparation

After storage at -70°C for four months, the tube containing the concentrated antigen was placed in a waterbath at 20°C. The antigen was suspended in PBS in a volume of 1:3 of the original virus suspension. Tube and pipet were rinsed several times to reduce loss due to adsorption. Aluminium hydroxyde gel was added to a final concentration of 20 percent of the resuspended antigen.

Vaccine testing

The vaccine was tested the next day. Irish cattle were divided into three groups of six animals. The vaccine dose was fixed at 10 ml. Vaccine dilutions 1:4 and 1:16 were prepared in PBS a few hours before the injection. O-type virus was used for challenge at three weeks after vaccination.

Results

Virus inactivation

The state of inactivation was verified on baby mice at the end of the inactivation step. No deaths occurred during the 96 h. observation period.

Virus concentration

In preliminary experiments, electron microscopy had shown that virions concentrated largely at the interphase. In this experiment the separations of interphase and lower phase were abandoned to avoid losses. Titration by CF-test was unsuccessful because of a strong anticomplementary activity which persisted even after resuspending pelleted virions. Previous experiments with physical methods had shown that about 60 percent of the virions collected in 1:100 of the original volume.

The quantity of virions per dose of vaccine was considered to be equivalent to that of vaccines customarily prepared at the Institute, considering the volume ratios, the volume of the sample taken for titration and the reduction of the virions content due to the concentration procedure.

Vaccine potency test

The experimental control vaccine appeared contaminated with bacteria four months after preparation and was therefore useless. The challenge of animals vaccinated with the concentrated antigen vaccine was made three weeks after vaccination: 25000 ID50 were injected intradermolingually, distributed in two points. The results of a one year old monovalent O vaccine, tested at the same time, are given as a reference in Table 1.

Seroneutralization test

The seroneultraization titres for animals vaccinated with one dose or with vaccine dilutions are presented in Table 1.

Conclusion

The inactivation of the original cellulose filtered virus suspension was complete, as shown by the baby mice test.

The PEG/Dextran two-phase system allowed a high concentration ratio with a yield of about 50 percent of the virus, after precautions were taken to limit loss by adsorption.

The immunogenicity of the virions was preserved as shown by the potency test on cattle, after storage of the antigen for four months. The relatively high loss of virus and the extra cost of the concentration procedure limit the applicability of this type of vaccine preparation to emergency situations.

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Table 1 : Results

Protection test			
Stored virus vaccine : dose 10 ml		Reference vaccine : dose 5 ml	
Vaccine dil.	Protected/total	Vaccine dil.	Protected/total
1:1	6/6	1:1	4/5
1:4	5/6	1:4	5/5
1:16	1/6	1:16	4/4
DP50=8		DP50=16	
Seroneutralisation test			
Stored virus vaccine : dose 10 ml		Reference vaccine : dose 5 ml	
Vaccine dil.	SN-titer	Vaccine dil.	SN-titer
1:1	162	1:1	162
1:4	108	1:4	119
1:16	42	1:16	90

Appendix A-3

Further experience with the use of concentrated foot-and-mouth disease virus stored at ultra-low temperatures

by

S.J. Barteling and P. W. de Leeuw *

On previous occasions we reported on foot-and-mouth disease (FMD) vaccines based on antigens produced in growing BHK-suspended cell cultures in the presence of polyethyleneglycol (PEG) treated serum (Ref.1, 2). The virus was precipitated with PEG, the precipitate collected in filter-aid, and the filter-cake stored at -70°C or over liquid nitrogen before further processing; a procedure similar to that described by Ley (Ref. 4). More recently the virus was concentrated 200-fold in a closed system by two successive cycles of precipitation, filtration, and elution ("PFE procedure") and stored in potassium phosphate (PB) buffer at -70°C (Ref. 2). Using vaccines based on similarly prepared and stored antigens satisfactory results were obtained both in cattle and in pigs (Ref. 1, 2).

The purpose of the present paper is to communicate some recent results obtained in our laboratory with respect to the use of frozen FMD virus antigens.

1. Preservation of FMD virus immunogenicity at low temperatures

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Appendix A-3

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by

S.J. Barteling and P. W. de Leeuw *

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Appendix-4

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by

J.C. Lei*

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The plates were incubated at 37°C for 4 days and the endpoints were determined on the basis of cytopathogenic changes. The titer of neutralizing antibody was expressed as the reciprocal of the serum dilution neutralizing 100 $TCID_{50}$-units of virus. For comparative reasons, antibody titers were determined as well against FMD virus propagated in BHK cells as against FMD virus propagated in small scale cultures of surviving bovine tongue epithelium (Frenkel cultures).

Results and discussion

Table I summarizes some of the findings illustrating the storage stability of PEG-precipitated FMD virus type C at -80°C for seven years. In the evaluation of the results it has to be noticed that the assays have been carried out with an interval of 7 years and that variations in the test systems over this span of time can not be excluded. With this reservation, the data of table I point to a high stability of infectious particles as judged by titration of infectivity and a good preservation of the immunogen as judged by the immunological response of the resulting vaccine in guinea pigs. The two-fold difference observed in titer of CF activity of the PEG-concentrates is most likely to reflect variations in the rather simple routine method used in titration of CF activity.

Unfortunately, the quantitation of 140 S particles was not yet introduced at the start of the experiments and no figures strictly comparable to the value of 6.6 ug/ml of 140 S material found in 1979 are thus available. The only indication as to stability of virions (140 S particles) may be provided by measurement of 140 S particles in batches of FMD virus type C grown in BHK 21 suspension cells i.e. the same system as used in the preparation of the virus under investigation. A total of 58 such batches produced from 1975 to 1979 yielded a mean value of 2.6 µg/ml 140 S particles with a standard deviation of 0.7 µg/ml. Taking into account that the 7% PEG-concentrates represent three-fold concentrations of the original virus harvest and assuming that a quantitative recovery of 140 S particles was achieved, then a content of 2,2 µg/ml 140 S particles in the original virus harvest can be estimated. Comparing this value to the above mean value suggests a survival in the region of 80%.

To provide additional evidence for the immunogenic effect of the vaccine, the potency in terms of PD_{50}-value in guinea pigs was determined. The vaccine was diluted in four-fold steps with dummy vaccine and 0.33 ml of vaccine dilution was injected sub-cutaneously into each of 10 animals. Four weeks after vaccination the animals were challenged. The result of the challenge was treated according to KÄrber (Ref. 4) and yielded a PD_{50}-value of 0.024 ml. This result is similar to that of a production scale PEG-vaccine prepared from material of the same serotype stored over liquid nitrogen for one year (2). With the latter vaccine a content of 6.7 µg/ml 140 S particles in the antigen preparation resulted in a PD_{50}-value of 0.016 ml. This seems to indicate that the immunogenic property of the non-fragmented virion (140 S particle) is not altered by prolonged storage at low temperature.

The vaccine was finally evaluated by assay of neutralizing antibody in cattle. Four animals were vaccinated and blood samples collected 4 weeks after vaccination. Titers of neutralizing antibody to virus of the same cellular origin as the vaccine antigen i.e. BHK 21 cells were estimated to be 32-64, 64, 8 and 16, respectively. The corresponding titers to virus of a different origin i.e. Frenkel culture were found to be slightly higher with values of 64-128, 64-128, 8-16 and 32, respectively. Thus, the performance of the vaccine in cattle appears to be acceptable.

In conclusion, this investigation shows that crude PEG-precipitated FMD virus can be preserved satisfactorily at low temperature for a period of time considerably longer than the generally accepted shelf-life of a fully formulated aluminium hydroxide gel vaccine.

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TABLE I

Storage stability at -80°C of crude PEG-precipitated FMD virus

BHK suspension cell virus, serotype C. Period of storage: 7 years

Virus preparation	Date of assay	Titer of infectivity	CF-titer	140 S µg/ml	Immunogenicity of vaccine	
					Antigen dose given 1:1	1:8
Original virus harvest	March 1972	7.1	8	n.d.	6/10[a)]	6/10
7% PEG-concentrate	March 1972	7.1	8-16	n.d.	10/10	5/10
7% PEG-concentrate	May 1979	7.1	4-8	6.6	19/20	15/20

Titers of infectivity are given in $\log_{10}$ ID_{50}/0.1 ml.

CF-titers are reciprocals of antigen dilution causing complete binding of 4 units of complement in the presence of a surplus of guinea pig hyperimmune serum.

n.d. - not done.

a) Number of immune guinea pigs to total number of guinea pigs within group.

Appendix A-5

Introductory study on the storage of inactivated, concentrated Foot-and-Mouth Disease antigen for emergency vaccinations

by

J.C. Lei *

Long term storage of foot-and-mouth disease (FMD) antigen for emergency purposes may take place by storing a crude, infectious concentrate or by storing a more or less purified, inactivated product (Ref. 1). In Denmark, the most serious objection to the practice of storing crude, infectious antigen concerns the aspect of safety. For this reason the following investigation on inactivation of FMD virus prior to storage of the concentrated product was initiated. This report illustrates that the techniques of concentrating polyethylene glycol (PEG)-precipitated FMD antigen by filtration with Kieselguhr (KG) (Ref. 2) may be used with advantage also in stock-piling inactivated antigen.

Experimental

The serotypes and strains of virus used in this study have been described previously (Ref. 2).

Viral harvest from BHK suspension cells (Ref. 3) treated with 0.5% (v/v) chloroform ($CHCl_3$) at 26°C for 3 hours and clarified by filtration with KG (Ref. 4) served as star material. This product is identical to the clarified virus suspension used in routine preparation of FMD vaccine in Denmark.

Inactivation of the $CHCl_3$-treated, clarified virus suspension was effected by treatment with 1 mM ethylenimine (EI) at 26°C for 42 hours. EI was added as a 0.1 M solution generated from 2-bromo-ethylamine hydrobromide and NaOH (Ref. 5). The inactivation was terminated by chilling the magnetically stirred reaction vessel with ice-water, and 6% (w/v) of PEG 6000 (Hoechst, Germany) was added. Stirring was continued for 1 hour, after which 0.28% (w/v) of KG (Standard Super Cel from Johns-Manville, USA) was thoroughly mixed into the system prior to filtration on filter paper in a Buchner funnel. The resulting filter cake was put into a screw-capped flask, flushed with nitrogen and frozen at -80°C for eleven days.

A vaccine was prepared from the stored material by stirring the frozen filter cake in one third of the original virus volume of modified Earles medium at room temperature for 1 hour. Preparation of the medium has been given in a previous report (Ref. 2). An equal volume of aluminium hydroxide gel (Alhydrogel 1.3% Al_2O_3), Superfos, Denmark) containing glycine buffer to yield a pH of 8.5 was then added. Finally, 0.25% (v/v) $CHCl_3$ and 10 ppm. of silicone antifoaming agent was introduced and the mixture was allowed to equilibrate by stirring overnight in the cold room.

Parallel to inactivation with EI, a sample of the $CHCl_3$- treated, clarified virus suspension was converted to standard vaccine by mixing with 30% (v/v) Alhydrogel followed by inactivation with 0.05% formalin at 26°C and pH 8.7 for 48 hours. The standard vaccine was concentrated by removal of supernatant liquid to give the same amount of original virus suspension per unit volume of final vaccine as the first vaccine.

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Samples were taken at the following steps during processing: 1. Before inactivation (the starting material) 2. After inactivation with EI, and 3. After resuspension of stored antigen for 1 hour at room temperature.

Innocuity testing of the sample after inactivation with EI was performed by inoculation of primary calf kidney cell cultures with material dialysed overnight in the cold against 500 volumes of Hanks salt solution with vitamins and lactalbumin hydrolysate. In the quantitation of 140 S particles, the corresponding material used was not subjected to dialysis. Prior to ultracentrifugation, sample no.3 was clarified by high-speed centrifugation to remove KG and other particulate matter.

Titration of infectivity and complement-fixing (DF) activity was carried out as described previously (Ref. 6). A complete description of the 140 S particle assay has been given elsewhere (Ref. 7).

The immunogenic effect of the two vaccines was tested comparatively in guinea pigs. Ten animals were each vaccinated with 0.33 ml of vaccine containing antigen from 0.5 ml of original virus suspension and 10 animals each received 0.33ml of a 1:9 dilution of vaccine in dummy vaccine. The vaccines and vaccine dilutions were given subcutaneously. Three weeks after vaccination all vaccinated animals together with 10 non-vaccinated guinea pigs were challenged by inoculation in one plantar pad with homologous guinea pig adapted cattle virus (titer of about 10^3 guinea pig ID_{50}/ml). Symptoms of generalization of the disease were recorded thrice with final inspection taking place 7 days after challenge with virus.

Results and discussion

Table 1 shows the analytical data obtained during processing of viral antigen. In addition to quantitation of 140 S particles, titration of infectivity and DF activity of the starting material was carried out. However, since the virus was subjected to inactivation with the concurrent loss of titer of infectivity as a parameter and since previous experience with CF activity as a reliable measure of recovery during processing of FMD immunogen with PEG was discouraging (Ref. 6), the final analysis of the new procedure was based on quantitation of antigen in terms of 140 S particles. With the present knowledge as to precision of the latter method it can be concluded from table I that inactivation with EI did not result in a measurable disintegration of virus particles of the three serotypes examined. That the inactivation with EI was carried out to completion was confirmed by the circumstance that no residual infectious particles could be demonstrated in the innocuity testing of the inactivated samples.

During inactivation, pH was found to rise from an initial value of 7.9-8.0 to 8.0-8.2 and a slight turbidity, probably due to denaturation of protein, was observed to develop. The efficiency of the subsequent precipitation with PEG was demonstrated by the absence of 140 S material in the filtrate from filtration with KG. The final volume of KG with deposited antigen was found to represent slightly more than a 100-fold concentration of the original viral harvest. This valuable improvement of the 50-fold concentration hitherto obtained in processing of crude viral harvest with PEG and KG (Ref. 2) was achieved by the removal of cellular debris prior to inactivation. Hereby a reduction in the amount of KG necessary to give a smooth filtration of the end product was made possible. It is to be expected from previous experiences with scaling-up of KG-filtration that the present 100-fold concentration may be increased even further.

The period of storage at -80°C was limited to eleven days since it was assumed that the process of freezing and thawing would be much more critical to the overall stability of the material than the actual holding at low temperature.

The last column of table I gives the percentages of recovery of 140 S particles after storage. In the case of antigen sample no. 350279 the calculation was based on 140 S particles in the starting material because the corresponding figure for the inactivated antigen preparation

was lacking. The percentages indicate good recovery of all three serotypes. It is of interest to note that the recovery with the present procedure, ranging from 73-90%, was lower than the range of 101-129% recovery previously found with correspondingly processed crude viral harvest (Ref. 2). This difference may reflect better preservation of 140 S material in the crude infectious product during the storage and/or rescue of 140 S particles by freezing and thawing of virus-containing cell debris. In favour of this explanation in an earlier series of experiments in which crude viral harvests of serotype O, precipitated with PEG, isolated by centrifugation and resuspended directly without freezing, storage and thawing gave an average recovery of about 80% (6,p. 1185). However, it can not be completely ruled out that the difference may originate from adsorption of 140 S particles to KG in the more purified inactivated material, but so far it has not been possible to demonstrate this experimentally.

In the present study no attempt to remove KG from the inactivated antigen prior to final formulation of vaccine was undertaken. Although removal of KG as judged by the recovery of 140 S particles (the samples used in the assay were freed of KG by centrifugation) presents no problem, the direct formulation of vaccine with KG will allow shipment of vaccine with minimal delay in an emergency. With the present formulation of the Kieselguhr-Alhydrogel (KAl)-vaccine, one cattle dose contains approximately 21 mg of KG and about 50 mg of alhydrogel. In order to test the KAl-vaccine for adverse local reactions, 3 calves were vaccinated subcutaneously with one dose volume (5 ml) of vaccine on one side of the neck and 2 dose volumes on the other side. The corresponding standard vaccine given to three other calves as described above served as reference material. During the first week after vaccination the KAl-vaccine was observed to induce a slightly more pronounced reaction at the injection sites than the standard vaccine did. Two weeks after vaccination, the difference between the two vaccines was negligible. In no case did the KAl-vaccine induce unacceptable reactions at the injection sites, and the general appearance of the animals did not seem to be affected. Although the KAl-vaccine is intended for emergency vaccinations only, this matter must be examined more carefully before approval of the direct formulation recipe. The use of KG as an adjuvant in immunization of guinea pigs with FMD antigen has been reported by Espinet (Ref. 8).

The results of the comparative testing of KAl-vaccine and standard vaccine for immunogenic activity in guinea pigs are presented in table II. Due to the introductory nature of this study, titration of the vaccines to yield PD_{50}-values was replaced by testing of the vaccines in undiluted form and in dilution 1:9. As can be seen from table II the KAl-vaccines gave immunological responses similar to those of the corresponding standard vaccines. This indicates a good recovery of immunogen in the overall storage procedure and supports the results obtained by quantitation of 140 S particles.

In conclusion, the present investigation shows that the techniques of concentrating PEG-precipitated crude FMD virus harvests by filtration with KG prior to storage at low temperature (REf. 2) may also be applied to virus preparations which have been clarified by treatment with $CHCl_3$ (Ref. 4) and inactivated with EI. Hereby the risk of storing large amounts of infectious material can be eliminated. As a further advantage the removal of cell debris before inactivation yields a considerable reduction in volume of the concentrated material and thus an improvement in the economy of stockpiling FMD antigen for emergency vaccinations. However, these advantages have to be weighed against the disadvantage met in all procedures based on inactivation prior to concentration i.e. the requirement of process equipment outside the virus-contaminated zone. Finally, the presented data indicate that the processing and storage of inactivated FMD antigen may be evaluated by quantitation of 140 S particles to compensate for the loss of infectivity titer as parameter.

Acknowledgements

The excellent technical assistance of Lene H. Hansen and Kit Frederiksen is highly appreciated. The author likewise expresses his gratitude to the Department of Serology for execution of the guinea pig experiments and testing of the KAl-vaccine in calves.

TABLE I

Assay of FMD virus particles during inactivation with EI
and estimation of percentage recovery after storage and resuspension

Virus sample no.	Sero-type	Starting material: Infectivity titer	Starting material: CF titer	Starting material: 140 S ug/ml	After inactivation 140 S ug/ml	After resuspension 140 S ug/ml	Recovery in % after storage
340179	A	6.3	2	1.4	1.7	4.3	84
350279	A	6.5	4	1.4	n.d	3.5	83
360179	O	6.9	8	2.6	2.4	6.3	88
370179	O	6.5	4	n.d	2.4	6.5	90
380179	C	6.9	4	3.4	3.4	7.4	73
390179	C	6.9	4	2.7	7.1	7.1	88

n.d - not done

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

CF-titers given are reciprocals of antigen dilution causing complete binding of 4 units of complement in the presence of a surplus of guinea pig hyperimmune serum.

TABLE II

Comparative testing of standard vaccine and KAI-vaccine for immunogenic activity in guinea pigs

Vaccine no.	Sero-type	Type of vaccine	Antigen dose given 1:1	1:9
340179	A	standard	3/10 a)	0/10
		KAl	3/10	0/10
350279	A	standard	4/10	0/10
		KAl	2/10	0/10
360179	O	standard	9/10	8/10
		KAl	7/10	7/10
370179	O	standard	10/10	8/10
		KAl	10/10	10/10
380179	C	standard	9/10	4/10
		KAl	7/10	3/10
390179	C	standard	9/10	6/10
		KAl	7/10	5/10

a) number of immune guinea pigs/total number of guinea pigs in group

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Appendix A-6

Concentrated Foot-and-Mouth Disease Virus Antigen: Its Use as a Vaccine

by

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H.L. Bachrach and G. Hardy**

INTRODUCTION

In FMD-free countries the need for vaccine is limited to emergency situations. Under such circumstances, the traditional method of holding FMD-prepared vaccine at 4°C for its storage life of approximately 18 to 24 months becomes economically unfeasible. Alternatives to vaccine storage presently being examined are: (1) the storage of characterized seed virus stocks in liquid nitrogen, (2) the storage of non-inactivated concentrated antigen and (3) the storage of an inactivated concentrated antigen. Each of these methods has advantages and disadvantages. The storage of seed virus allows immediate growth of virus for vaccine production without the disadvantage of adaptation required with a field virus (a virus freshly isolated from an outbreak). The latter two methods allow for long-term storage of a considerable vaccine potential in limited space. However, concentrated infectious virus must be reconstituted and inactivated before being compounded into a vaccine. The third method, the storage of a concentrated inactivated antigen, will be discussed here.

METHODS AND MATERIALS

Virus Preparations Foot-and-mouth disease virus types A_{24}, A_{27}, C_3, O_1 and A_{12} were grown in BHK monolayer roller bottle cultures as described (Polatnick and Bachrach, 1977). Harvested culture fluids with 1.0% chloroform added, were agitated and then clarified by high speed continuous flow centrifugation. In some preparations, 0.05% sodium azide was added immediately after harvesting culture fluids.

Plaque-forming unit and CF titers were established for each virus preparation, and the physical-chemical quality of purified virions was determined (see below).

Inactivation The virus was inactivated with 0.1 M. ethylenimine (EI) (Bahnemann, 1975) and held at 4°C until a sufficient volume was accumulated for concentration (200 liters being the minimal practical volume for the present system).

Concentration Concentration was accomplished by ultrafiltration using a Pellicon (R) ultrafiltration apparatus (Millipore Corp., Bedford, Mass.) as described (Moore and Morgan, 1979).

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Innocuity Assay of Concentrated Antigen Samples were assayed for residual virus infectivity by inoculation of BHK-21 cultures and plaque assays in pig kidney cell line (MVPK) cultures (Dinka, et al, 1977); and by intradermolingual inoculation of cattle as described by Henderson (1952).

Physical-Chemical Quality Control This procedure (described in detail elsewhere, Bachrach 1970, 1979) provides information on the concentration (mg/ml), stability and intactness of the 140 S virion and the intactness of its immunogenic protein VP_3. It works well for FMDV grown in roller-bottle BHK-21 cell cultures that produce 10^9 PFU (i.e., ≥ 3 ug/ml) of virus. Lesser concentrations of virus require additional preparatory steps. Virus is clarified (10,000 rpm, 10 min) and given a one-step purification; 42 ml of infectious or BEI-inactivated virus (original lots or concentrates resuspended to 42 ml in buffer) is centrifuged (Spinco 25.2 rotor, 25,000 rpm, 200 min) into 14.5 ml of a field-formed CsCl density gradient to its isodensity 1.43 g/ml. After dialyzing the banded virus against 0.02 M potassium phosphate pH 7.5, absorbance-temperature (A-T) and polyacrylamide gell electrophoretic (PAGE on 0.6 cm dia. SDS-8M urea gels) profiles are run to provide the physical-chemical data mentioned above. Staining intensities of the major protein bands VP_1, VP_2, VP_3 (or VP_3-cleavage products) and VP_4 are scanned photometrically at 600 nm and converted to protein concentrations (vs. Folin-Ciocalteau standard curves) and thence to virus concentration (FMD virus is 69% protein). The adsorbance at 259 nm of the initial plateau of the A-T profile gives a nearly independent (based mainly on RNA absorbance) value for virus concentration using the FMDV extinction coefficient $E^{1\%}_{259} = 76$. (Bachrach et al. 1964).

To screen for intactness of VP_3 only, virus was purified on cesium chloride gradients directly or after a single polyethylene/glycol precipitation (Wagner et al, 1970, Cowan et al, 1974). The purified virus was precipitated with 10% trichloro-acetic acid, washed with acetone, dried under nitrogen, dissolved in sample preparation buffer, electrophoresed on SDS-8 M urea slab PAGE (Moore and Cowan, 1978), stained and examined visually for content of VP_3 and/or VP_3-cleavage products.

Complement Fixation Assays Complement-fixation assays were performed on the virus before inactivation, after inactivation and after concentration using antisera specific for 140 S particle (Cowan and Trautman, 1967).

Storage The concentrated antigen was dispensed into 1 liter teflon bottles and stored in the gaseous phase of liquid nitrogen. Smaller bottles were stored at the same time to allow periodic sampling. When used, the frozen concentrate was thawed rapidly at 37°C and diluted with cell culture fluid to the desired concentration that had been calculated from CF data to a 140 S (intact virus) dose of 5 and 10 ug/ml.

Potency Tests Inactivated concentrated antigens were compounded into vaccines containing equal volumes of antigen and adjuvant (aluminum hydroxide gel with or without saponin or incomplete Freunds adjuvant). The vaccines were tested in guinea pigs, cattle and swine. Guinea pigs were also inoculated with the aqueous form of the vaccine only. Guinea pigs in groups of ten were inoculated subcutaneously with a 2 ml dose of the respective vaccine. Twenty-eight to 30 days post vaccination, serums from the guinea pigs were pooled and assayed for virus neutralization antibody in suckling mice (Table 1). Initially, two steers were used to determine the response of the different adjuvanted vaccines at the 5 to 10 ug level. Blood samples were collected at 7, 14, 21 and 28 days post vaccination and the serums assayed for virus neutralizing antibody. The immunity of the steers was challenged at 28 DPV by exposing them to FMDV-infected steers. These exposed steers were held for a period of 14 days, temperatures were taken daily, and they were examined at 5, 10 and 14 DPV for signs of FMD (Table 2). The United States Department of Agriculture requires that each serial lot (1,000,000 doses) of FMD vaccine purchased from a commercial producer be tested for potency using 18 FMD-susceptible cattle. Twelve cattle were inoculated subcutaneously with a 2 ml dose of vaccine diluted from frozen concentrated antigen to the required microgram dosage and adjuvanted with $Al(OH)_3$ plus saponin. Twenty-eight DPV, 3 non-vaccinated control cattle were injected intradermolingually with 10^4 bovine ID_{50} of the vaccine strain of FMD virus and placed with the 12 vaccinated cattle and 3 non-vaccinated contact control animals.

The 18 animals were observed carefully for clinical signs for 14 days and then thoroughly examined at necropsy for lesions of FMD. The test requires that at least two of the three non-vaccinated contact control cattle develop generalized signs of FMD with both oral and pedal lesions, and the third contact control must develop lesions in at least one site. If two or less of the vaccinated cattle develop lesions of FMD, the vaccine is satisfactory. The vaccine is rejected if 4 or more of the vaccinated animals fail to withstand challenge (i.e., become clinically infected). If there are 3/12 failures (75%) the serial can be tested in an additional 12 animals. If the cumulative total is 4/24 failures or less (83.3% protection) in the 24 vaccinated animals, the vaccine is accepted.

Results

Two hundred liters of inactivated antigen is the smallest volume which can be satisfactorily concentrated with our arrangement of this filtration equipment. Concentration of 100-fold was accomplished at a fluid removal rate of 1-2 liters per minute. The average infectivity of the virus lots prior to inactivation was 2.70×10^8 PFU/ml, with a C.F. titer of 1:60 before and after inactivation (data not shown).

The percentage recovery ranging from 70 to 100% was determined by comparison of C.F. titer times volume of both harvest pool and concentrated antigen. The physical chemical assays were considerably more conservative in the estimate of mass recovery.

Infectivity and complement fixation titers appear to vary independently from lot to lot (i.e., C.F.titer cannot be extrapolated to infectivity titer). A particular 200 liter pool showed almost complete cleavage of VP_3 after 160 liters of non-cleaved (VP_3) material had been pooled with 40 liters of material in which VP^3 was 40% cleaved (Fig.1). Also shown in the figure are representative samples of cleaved and intact VP_3 for FMDV types A_{12} and C_3.

Innocuity assays of the concentrated pools were negative both in tissue culture and animal tests. Concentrated antigen when reconstituted and compounded into vaccines elicited similar antibody responses in guinea pigs regardless of whether they were composed of virus with VP_3 cleaved, uncleaved or a mixture of the two (Table I).

In the potency test in steers, the pooled antigen at a 10 ug dose adjuvanted with $Al(OH)_3$ and saponin stimulated an excellent antibody response and complete protection from infection, when the immunity of the 12 test animals was challenged at 28 days postinoculation (Table 3).

DISCUSSION

Initial results (Moore and Morgan, 1979) showed that concentrated A_{24} antigen which had been stored a few days in the gaseous phase of liquid nitrogen produced neutralizing antibodies in guinea pigs similar to that produced by fresh unfrozen antigen. In our present study we have examined the antigens for FMD types A_{24}, O_1 and C_3 after storage in the gaseous phase of liquid nitrogen from 1 to 116 days. When these stored antigens were reconstituted and formulated into a vaccine, they produced antibody responses in guinea pigs similar to vaccines prepared from the fresh antigen (Table 4).

Presently, different lots of antigen are kept separated until their content of physical-chemical intact or cleaved virus proteins is detemined. Initially, however, pools were made from the different lots of antigen prior to such determinations and thus some of the results discussed in this presentation involve vaccines made from pools of antigen with both cleaved and uncleaved VP_3.

One might expect, based on the findings of Wild and Brown (1967) in which trypsin-treated FMDV, type O_1 was found to be less infective and less antigenic, that the cleaved A_{24} virus would be less immunogenic than the uncleaved. However our results indicate that cleavage of VP_3 does not markedly decrease the immunogenicity of the vaccine preparation.

Vaccines prepared from A_{12} FMD intact virus, trypsin-cleaved virus and naturally cleaved virus produced similar responses when inoculated into swine (Table 5). As indicated in this table, when the immunity of these swine was challenged by contact exposure to infected non-vaccinated swine, all three groups were equally protected.

Similar antibody responses and immunogenicity in guinea pigs and cattle appear to result with C_3 FMD antigen containing cleaved VP_3. The present results, however, do not necessarily justify the use of VP_3 cleaved virus, because antigen stability, dose-response relationships and duration of immunity may be found to be adversely affected. Also, the immunogenicity of different serological FMD types and subtypes might be affected differently with cleavage of the VP_3 polypeptide. For example, VP_3-cleaved type A_{12} FMDV loses 3-4 logs of infectivity (Bachrach, 1977), whereas VP_3 cleavage of type C virus with trypsin has little or no effect on its infectivity (Rowlands et al 1971, Bachrach, 1977).

Cleavage of VP_3 by chymotrypsin, trypsin or a mixture, to three different size classes of polypeptide fragments and changes or lack of changes in infectivity and antigenicity associated with the different products have been reported (Moore and Cowan, 1978). The protease present in some vaccine antigen batches was not identified. Despite cleavage of VP_3, the effect on immunogenicity of the vaccines containing cleaved proteins cannot be predicted. It is our opinion that the VP_3 cleavage that occurs from time to time in some lots of antigen is caused by an enzyme or enzymes from bacterial contamination. Our monolayer system is not a closed system and despite routine precautions, some contamination is occasionally evident. Chloroform and/or sodium azide (1/.05%) is added immediately after harvest and physical-chemical assays are conducted after clarification to determine whether the VP_3 polypeptide has been cleaved. Our immediate concern is to determine the actual effect of the cleavage on the immunogenic properties of the antigen. With the FMDV types A_{12}, A_{24} and C_3, there does not appear to be any great difference in immunogenicity of vaccines compounded with cleaved or uncleaved antigens. However, one would prefer a method of production that would ensure an antigen without cleavage. At this time we have not had an opportunity to test concentrated antigen stored for periods longer than 6 months. Responses obtained within that time frame appear adequate.

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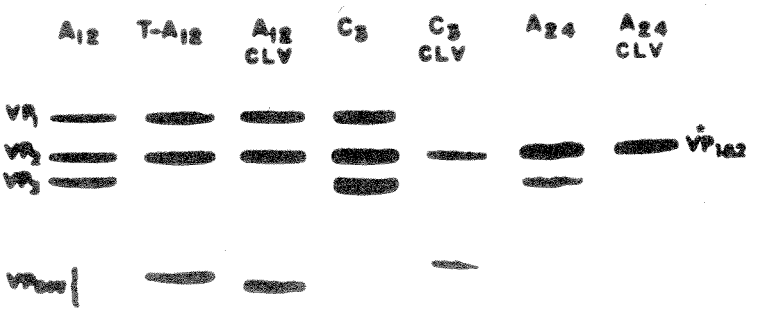

Fig. 1: SDS-Urea polyacrylamide slab gel of virus purified from vaccine concentrates. Foot-and-mouth disease type A_{12} lines demonstrate cleavage produced by trypsin and a protease found in some harvest fluids; types C_3 and A_{24} lines demonstrate intact and enzyme cleaved VP_3 in various harvest lots.

($T\text{-}A_{12}$ = trypsinized FMDV A_{12}; CLV = naturally cleaved FMDV; VP_3 CLV = cleavage products of VP_3).

*$VP_{1\ \&\ 2}$ co-electrophorese with FMDV A_{24}.

Table 1 Neutralizing Antibody Titers of 28 Day Serums from Guinea Pigs Inoculated with Ethylenimene-Inactivated Foot-and-Mouth Disease Virus Antigen A_{24} Grown in Baby Hamster Kidney Cell Culture and Concentrated by Ultrafiltration

ADJ	ANTIGEN xx		ANTIBODY
	CLEAVED	UNCLEAVED	TITER
OIL	+	+	3.8 *
AL(OH)3	+	+	3.1
AQUEOUS	+	+	2.7
OIL		+	4.2
OIL	+		4.2
OIL	+	+	3.7

*Mouse Log_{10} PD_{50} neutralizing titer pool of 10 serums from 10 guinea pigs each vaccinated with 10 ug of antigen.
xx antigen-cleaved and/or uncleaved pooled.

Table 2 Neutralizing Antibody Titers of Serums from Steers Vaccinated with 10 ug of Ethylenimine-Inactivated A_{24} Foot-and-Mouth Disease Virus Antigen Adjuvanted with with Oil, AL $(OH)_3$ or AL $(OH)_3$ + Saponin

BOV. ET.	ADJUVANT	DAYS POST VACCINATION 7	14	21	28	CHALL. IMM.
108	OIL	1.9 *	2.5	1.7	3.0	**NVL
109	OIL	.5	2.6	2.0	1.6	+++
103	AL(OH)3	1.0	1.4	1.7	1.9	NVL
106	AL(OH)3	1.4	2.5	2.3	1.6	NVL
104	AL(OH)3+S	1.4	2.2	2.5	2.4	NVL
110	AL(OH)3+S	.6	2.5	2.1	2.0	NVL
Controls						
161						++++
163						++++

*Mouse Log_{10} PD_{50} neutralizing titer.

**NVL - no visible lesions of FMD.

++++ - Generalized foot-and-mouth disease.

Table 3 Potency Test in Steers of 10 ug in 2 ml of Ethylenimine-Inactivated A_{24} Foot-and-Mouth Disease Virus Antigen Adjuvanted with AL $(OH)_3$ with Saponin

	DAYS POST VACCINATION				CHALL.
BOV. ET.	7	14	21	28	IMM.
140	1.6 *	2.5	2.8	3.4	NVL**
150	1.4	2.2	2.3	2.8	NVL
147	2.2	2.3	2.9	3.0	NVL
148	0.2	1.7	2.1	2.8	NVL
138	1.2	1.8	2.4	2.5	NVL
149	1.4	2.9	2.7	3.1	NVL
137	2.6	2.8	3.5	3.2	NVL
152	2.6	3.5	3.4	3.5	NVL
141	2.4	2.8	2.7	2.8	NVL
154	1.4	2.4	2.1	2.7	NVL
151	1.3	3.0	2.9	2.7	NVL
139	2.9	3.9	3.3	3.7	NVL
controls					
1-6					***Gen.

***Gen. - Generalized FMD - lesions tongue, mouth, nose and feet.

**NVL - No visible lesions of FMD.

*Mouse Log_{10} PD_{50} neutralizing titer.

Table 4 Potency Tests in Guinea Pigs of Ethylenimine-Inactivated Foot-and-Mouth Disease Virus Antigens A_{24}, O_1 and C_3 Produced in Baby Hamster Kidney Cell Culture and Concentrated by Ultrafiltration and stored in the gaseous phase of liquid nitrogen.

FMDV TYPE	DAYS STORAGE	VACC. AQUEOUS	$AL(OH)_3$	OIL
A_{24}	O	1.4 *	2.3	N.D
A_{24}	1	1.7	2.6	3.0
A_{24}	116	2.1	2.0	3.0
O_1	56	1.7	1.9	3.5
C_3	52	2.0	2.6	3.4

* Mouse LOG_{10} PD_{50} Neutralizing Titer.

Table 5 Potency Test in Swine of 10 ug in 2 ml of Ethylenimine-Inactivated Foot-and-Mouth Disease Virus Antigen A_{12}(Intact Virion) A_{12}(Naturally Cleaved VP_3) A_{12}(Trypsin-Cleaved) VP_3 Adjuvanted With Oil

SWINE	ANTIGEN	DAYS POST VACCINATION			CHALL
		28	38	58	IMMUNITY
208	whole virus	3.6*	3.3	3.8	NVL**
215	whole virus	2.6	2.7	2.2	NVL
228	whole virus	3.1	2.9	2.8	NVL
214	naturally cleaved	2.7	3.0	2.8	NVL
221	naturally cleaved	3.1	3.2	3.2	NVL
230	naturally cleaved	2.8	2.6	2.8	NVL
216	trypsin cleaved	2.7	3.0	2.6	NVL
222	trypsin cleaved	2.4	2.9	3.4	NVL
226	trypsin cleaved	2.5	2.6	2.4	NVL

* Mouse Log_{10} PD_{50} neutralizing titer
** NVL- no visible lesions of FMD.

Appendix B

FAO International Collaborative Study for the Standardisation of laboratory methods for the assay of virus and antibody in Foot-and-Mouth Disease vaccine preparation and control

Preliminary Report of the results of Phase IV *

Table 1: Assay of virus infectivity by the agar cell suspension plaque method - standardised materials.

Lab.No.	Virus No. 1					$\bar{x}$	Virus No. 2					$\bar{x}$
1	8.0	8.0	7.89			7.96	5.89	5.61	5.86			5.79
2	7.31	7.32	7.30	6.95	7.26	7.23	5.03	5.22	5.03	4.56	5.16	5.0
3	8.18	8.16	8.38			8.24	5.88	5.89	6.04			5.94
4	8.21	8.27	8.32			8.27	5.97	5.86	6.03			5.95
5	8.21	8.11				8.16	5.58	5.50				5.54
6	8.23	8.19	8.21			8.21	6.0	5.99	5.88			5.96
8	7.93	7.32	8.30			7.85	5.44	4.95	6.03			5.47
10	8.01	7.86	7.83			7.90	5.52	5.56	5.42			5.50
13	8.93	9.13	9.15			9.07	6.54	6.59	6.61			6.58
14	8.26	8.35	8.31			8.31	6.02	5.92	5.70			5.88
19	8.44	8.52	8.23			8.39	6.18	6.26	6.06			6.17
	n = 11						11					
	Range of means 7.23 - 9.07 .						5.0 - 6.58					
	$\bar{x}$ 8.14						5.79					
	Stand. dev. 0.45						0.41					
Lab. No.												
9	(own materials)	7.9	7.9	7.86		7.88	5.34	5.39	5.39			5.37

* AVRI, Pirbright

Table 2: Agar - Cell Suspension Plaque Assays

Comparison of the results of Phase IV with earlier work

Phase I: BHK Suspension or Monolayer cells with various materials and methods

Number of participants (n) = 7 Range of means 5.04 - 7.76

$\bar{x}$ 6.88 Stand. dev. of means 1.01

Phase II: (a) Various BHK cell sources with different materials and methods

n = 10 Range of means 5.33 - 7.94

$\bar{x}$ 7.15 Stand.dev. of means 0.87

(b) Standard Suspension cells and agar ex Lelystad

n = 10 Range of means 6.75 - 8.12

$\bar{x}$ 7.35 Stand. dev. of means 0.47

Phase IV: Standardised BHK Cells (Monolayer) materials and method

Virus 1	n = 11;	range of $\bar{x}$ = 7.23 - 9.07;	$\bar{x}$ = 8.14;	Stand.dev. 0.45
Virus 1 (omitting Labs. 13 & 2.)	n = 9;	range of $\bar{x}$ = 7.85 - 8.39;	$\bar{x}$ = 8.14;	Stand.dev. 0.19
Virus 2	n = 11;	range of $\bar{x}$ = 5.0 - 6.58;	$\bar{x}$ = 5.79;	Stand.dev. 0.41
Virus 2 (omitting Labs. 13 & 2)	n = 9;	range of $\bar{x}$ = 5.47 - 6.17;	$\bar{x}$ = 5.80;	Stand.dev. 0.24

Table 3: Weight of virus estimation (µg 146s/ml)

Lab. No.	Virus No. 3	$\bar{x}$	Virus No.4	$\bar{x}$
1	11.0 10.0 9.5	10.16	3.1 3.4 2.9	3.13
2	12.1 12.25 13.5 13.25 11.5	12.52	5.3 3.9 3.7 3.8 3.5	4.04
3	12.3 13.5 12.3 11.9	12.50	3.2 3.0 3.06 3.02	3.07
5	17.5 16.5 15.3 18.0 17.4	16.94	4.0 3.5 4.3	3.93
6	18.25 18.67 18.04 18.40	18.34	4.0 3.80 3.85 3.64	3.82
8	17.2 15.0 16.4	16.2	4.2 4.4 4.1	4.23
9	13.5 12.4 13.3	13.07	3.17 4.49 3.96	3.87
10	15.30 14.72 14.97 14.62	14.90	3.47 3.69 3.35 3.73	3.56
13	10.93 9.92 10.44 9.49 9.67 10.05	10.12	2.28 2.00 2.19 2.21 2.11 2.35	2.19
14	10.1 10.5 10.9 11.1 11.1 11.3 11.6 11.9 12.4	11.21	2.17 2.27 2.48 2.71 2.69 2.49 2.35 2.46 2.64	2.47

n =	10	10
Range of means	10.12 - 18.34	2.19 - 4.23
$\bar{x}$	13.60	3.43
Stand. dev. of means	2.87	0.69

Table 4: Weight of virus estimation - Comparison with results from Phase III

Phase III				
Virus A	n = 9;	range of $\bar{x}$'s = 3.34 - 44.0;	$\bar{\bar{x}}$ = 9.64;	Stand. dev. = 13.22
Virus A (omitting Lab.No. 1)	n = 8;	range of $\bar{x}$'s = 3.34 - 13.1;	$\bar{\bar{x}}$ = 5.35;	Stand. dev. = 3.19
Virus B	n = 9;	range of $\bar{x}$'s = 1.1 - 26.0;	$\bar{\bar{x}}$ = 4.33;	Stand. dev. = 8.14
Virus B (omitting Lab. No.1)	n = 8;	range of $\bar{x}$'s = 1.1 - 2.5;	$\bar{\bar{x}}$ = 1.62;	Stand.dev. = 0.51
Virus C	n = 9;	range of $\bar{x}$'s = 1.65 - 15.1;	$\bar{\bar{x}}$ = 4.90;	Stand.dev. = 5.76
Virus C (omitting Labs. Nos. 1 and 9	n = 7;	range of $\bar{x}$'s = 1.65 - 2.5;	$\bar{\bar{x}}$ = 2.00;	Stand.dev. = 0.32
Virus D	n = 9;	range of $\bar{x}$'s = 0.79 - 26;	$\bar{\bar{x}}$ = 5.62;	Stand.dev. = 7.72
Virus D (omitting Lab.No.1)	n = 8;	range of $\bar{x}$'s = 0.79 - 4.7;	$\bar{\bar{x}}$ = 3.07;	Stand.dev. = 1.17
Phase IV				
Virus 3	n = 10;	range of $\bar{x}$'s = 10.12 - 18.34;	$\bar{\bar{x}}$ = 13.60;	Stand. dev. = 2.87
Virus 4	n = 10;	range of $\bar{x}$'s = 2.19 - 4.23;	$\bar{\bar{x}}$ = 3.43;	Stand. dev. = 0.69

Appendix C-1

Application of Radioimmunoassay and Enzyme Linked Immunosorbent Assay to Foot-and-Mouth Disease Virus Research

by

J.R. Crowther *

Problems associated with the study of foot-and-mouth disease (FMD) antigen and antibody have been investigated using the radioimmunoassay (RIA) and enzyme linked immunosorbent assay (ELISA) at the Animal Virus Research Institute, Pirbright.

The following summarizes the main points made in the paper given at Vienna.

The areas of research outlined were:

1. Rapid Diagnosis

Double-sandwich ELISA used successfully for rapid and unequivocal typing results on 'original' epithelium tissue. Superior to CF test in sensitivity and is unaffected by anti-complementary activity. Results of CF and ELISA studies were described.

2. Differential Diagnosis

FMD virus diagnosis in Britain and some parts of Europe can be complicated since swine vesicular disease causes the same symptoms in pigs. Rapid differential diagnosis of these diseases is essential and ministerial wishes for a "pen-side" test are being followed. Sandwich-ELISA techniques are being used towards this end.

3. Qualitative Examination of Antigen

Antigenic variation occurs intratypically and may be sufficient to overcome low levels of specific antibody in vaccinated animals. The routine method of subtyping using the complement fixation test will be compared to the competition - ELISA and competition - RIA methods developed. The latter tests have the advantages that (a) the whole virion antigens are compared and (b) 'crude' tissue culture samples can be compared.

Comparison of viruses throughout vaccine manufacture indicates whether any antigenic changes have occurred before vaccine formulation, e.g. action of proteolytic enzymes. These changes can dramatically affect potency of the vaccine.

4. Quantification of Whole Virions

The ELISA and RIA - sadwich tests have proved successful in assessing whole virus antigen and are superior to other quantitative tests in that:

a) A large number of samples can be processed without concentration.

b) Whole virion can be measured from tissue culture preparations without need for separation from subunit and virus-induced proteins.

c) The process can be automated for continuous assessment of virus during vaccin manufacture.

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Results to substantiate these conclusions were shown.

5. Antibody Detection and Quantification

The serum neutralization test (SNT) is widely used to detect antibodies to FMD virus. This is laborious, not rapid, and involves the use of tissue culture with its inherent variation. The indirect - ELISA has been used successfully to detect antibodies to FMD virus from a variety of sera, particularly from cattle.

Results to show the sensitivity and correlation of these tests were discussed.

The solid-phase aspect of the ELISA offers a clear advantage over other techniques in the easy separation of specific classes of immunoglobulin. This was illustrated by showing results of the examination of anti-viral IgG_1, IgG_2, IgA and IgM from cattle sera after infection or vaccination.

Enzyme labelled and ^{125}-iodine-labelled protein A has been used with great success at the AVRI and its application as a tracer was discussed.

Since the full text of the paper submitted was too long for publication, the following references concerning the various aspects of research outlined above, might be consulted.

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(Submitted for publication) 27th June, 1980

Appendix C-2

Use of antisera against intact FMDV and against its structural proteins for demonstration of cross reactivity in ELISA, RIA and MNT

by

R.H. Meloen and J. Briaire*

Summary

Of two recently introduced laboratory techniques for FMDV research, the ELISA and RIA, the ELISA has definite advantages.

Our results with the ELISA and antisera against the structural proteins of FMDV indicate that complete virus when adsorbed to the wells of microtitre plates might, to some extent, expose internal proteins indicating a configurational change. This results for instance in increased cross reactivity between serotypes as compared to the RIA and the MNT and makes the ELISA technique less suitable for some of its proposed applications. The need arises therefore to keep the virus unchanged upon absorption to the microtitre plates. Some attempts to obtain this were however not successful.

Introduction

In recent years both the radioimmunoassay (RIA) and the enzyme-linked immunosorbent assay (ELISA) have been applied in FMDV research. The RIA has been used to differentiate between virus strains and sub-types; the ELISA for measuring antibody titres and virus content in suspensions, its use for virus typing and subtyping has been proposed. (Crowther 1977, Abu Elzein & Crowther 1978, 1979, Crowther and Abu Elzein 1979). The ELISA appears to be at least as sensitive as the RIA and is easier to handle, is more rapid and has a much higher sample capacity. It does not need radio active compounds. The ELISA therefore has definite advantages over the RIA.

In our laboratory, the RIA and ELISA have been used to study the structure of intact virus (140S) and its degradation product (12S) with the aid of antisera raised against the isolated viral proteins. (Meloen & Briaire, in prep.). It is known that the configurational change which accompanies the disentegration from 140S to 75S and/or 12S particles results in the exposure of internal antigens. We showed that this change exposes internal proteins - VP_2 and VP_3. Both VP_2 and VP_3 exhibited antigens which were cross reacting with antisera against the virus VP_2 proteins VP_3 of different serotypes. In case of VP_2 no type specific antigens were exposed at all. This explains why configurational changed FMDV gives rise to increased cross reactivity. (Meloen & Briaire in prep.).

These findings suggest that for most applications of the ELISA in FMDV work (serodifferentiation, quantification of anti-140S activity, etc.) the adsorbed virus should maintain its native configuration. We investigated the effect of adsorption of the virus to the wells of the microtitre plates with the aid of antisera raised against VP_1, VP_2, VP_3 and intact 140S.

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Materials and Methods

Viruses: FMDV of types A_{10}, O1-BFS and C Dettmold were produced in BHK suspension culture and concentrated and purified by precipitation with PEG and isopycnic banding in CsCl - respectively - 12S particles were prepared by acid treatment of the purified virus.

Antisera: Antisera against the isolated viral structural proteins were prepared as described (Meloen and Briaire, in prep.). In short, viral structural proteins of O_1 virus were separated on a 10% polyacrylamide gel slab using the discontinuous Tris-glycine buffer system of Laemmli (1970) in the presence of 0.1% SDS. The proteins of A_{10} virus were separated on the same gel system but in the presence of 8 M urea. The stained protein bands were cut out, dried under nitrogen gas and suspended in an appropriate volume of 0.05 M phosphate buffer, pH 7.4 containing 0.2 M NaCl and 0.1% SDS. This suspension was left overnight at 50°C and then emulsified with complete or incomplete Freund adjuvant. Antisera were raised in duplicate in rabbits, using an injection of 150 ug of virus protein emulsified in complete Freund's adjuvant followed four and eight weeks later by injections of the same quantity of antigen in incomplete Freund's adjuvant. Antisera against complete virus (140S) and 12S antigen were obtained using three doses of respectively AEI-inactivated and acid degraded 140S material in doses of 1 and 10 ug.

Micro neutralization test: Neutralizing activity was determined in a microtitre system using secondary pig kidney cells (De Leeuw et al. 1979). The sera were tested in two-fold dilutions against about 100 TCID. End points were determined according to Reed and Muench and are expressed as the negative $_{10}$logarithm of the serum dilution endpoint. Sera were considered positive if their titres were 0.45 or more.

Enzyme-linked immunosorbent assay (ELISA): The method described by Abu Elzein & Crowther (1978) was used, with the exception that the plates were coated with antigen in a 0.05 M phosphate buffer, pH 7.4, containing 0.2 MNaCl instead of in a carbonate buffer, pH9.6. The antisera were serially diluted in the coated plates. After incubation and washing, the plates were incubated with a 1/1000 diluted goat antirabbit peroxidase conjugate. After washing the colour was developed with 5-amino salicylic acid as substrate. The extinctions were read with a Multiscan apparatus (Titertek R) at a wave length of 450 nm. The endpoint was taken as the negative log of the serum dilution giving twice as much extinction as the background.

Radio-immuno assay (RIA): Essentially the micromethod described by Crowther (1977) was used. S^{35} - methionine labelled virus was incubated with serial dilutions of the specific rabbit antiserum in microtitre plates. After addition of goat-anti-rabbit gamma-globulin the precipitates were spun down and the radioactivity left in the supernatants was counted. The endpoint was taken as the negative log of the serum dilution precipitating 70% of the labelled virus.

Results and Discussion

Antisera against the structural proteins were prepared as shown in figure 1. Only anti-VP_1 and anti-140S serum had neutralizing activity, in contrast to anti-VP_2 and anti-VP_3 serum (Table 1). In the RIA (Table 2) labelled 140S particles reacted only with anti-VP_1 but not with anti-VP_2 and VP_3. When in this case 12S particles were used anti-VP_2 and anti-VP_3 serum of type O and anti-VP_2 of type A reacted as well.

When the serum were tested in the ELISA (Table 3) using plates coated with 140S particles definite but not very strong reactions occurred with anti-VP_2 and VP_3 sera. If 12S was used all antisera reacted including those against the internal proteins. The difference observed between the results of ELISA on the one hand and the MNT and the RIA on the other, probably indicate a conformational change of 140S. Because such a change is expected to be accompanied by the appearance of reactivity between serotypes we measured the cross reactions with the anti-140S sera too. Table 4 shows that cross reactivity was neither observed in the MNT nor in the RIA when 140S antigen is used, however, when in the

RIA 12S is used the expected cross reactions do occur.

In contrast to the other two methods if 140S virus particles were applied in the ELISA cross reactivity is observed, again indicating that some kind of conformational change has occurred upon adsorption of the virus to the microtitre plates. With 12S of type O and A cross reactivity increases. Because the value of the ELISA might be diminished due to a change in the specificity of the antigen we made some attempts to develop a method of keeping the virus in its native state. The use of flexible polyvinyl-chloride microtitre plates (Cooke Engineering) instead of the usually applied polystyrene plates did not improve results. Table 5 shows the results were the opposite of what we wanted. On PVC plates 140S changes much more than on polystyrene plates: both reactions of antisera against the internal proteins and the cross reactivity are increased. Coating of the antigen to the microtiter wells at pH 9.4 again was no improvement at least for type O virus. (Table 5). Also the sera were less reactive when the antigen is coated at a higher pH. Purification of the virus on a sucrose gradient instead of a CsCl gradient also gave no improvement nor did fixation of the virus with 1.0, 0.1; 0.01 and 0.001% formaldehyde or 0.01, 0.005 and 0.001% glutaraldehyde prior to adsorption. Thus according to our data a conformational change of the 140S particle occurs when attached to the wells of microtiter plates. This change may, at least theoretically, make some of the applications of the ELISA debatable, but we do not know whether it is large enough to have a substantial effect on all applications, which perhaps explains why this change has not been observed by others (Abu Elzein & Crowther 1978, Crowther and Abu Elzein 1979). Other explanations might be differences in anti-12S activities in the sera used, in the virus strains or experimental conditions. Our data only apply when the antigen is coated directly to the wells.

To solve this problem further study is required.

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Text figure 1: Structural proteins of FMDV type A_{10} separated on poly-acrylamide gel.

SDS - PAGE

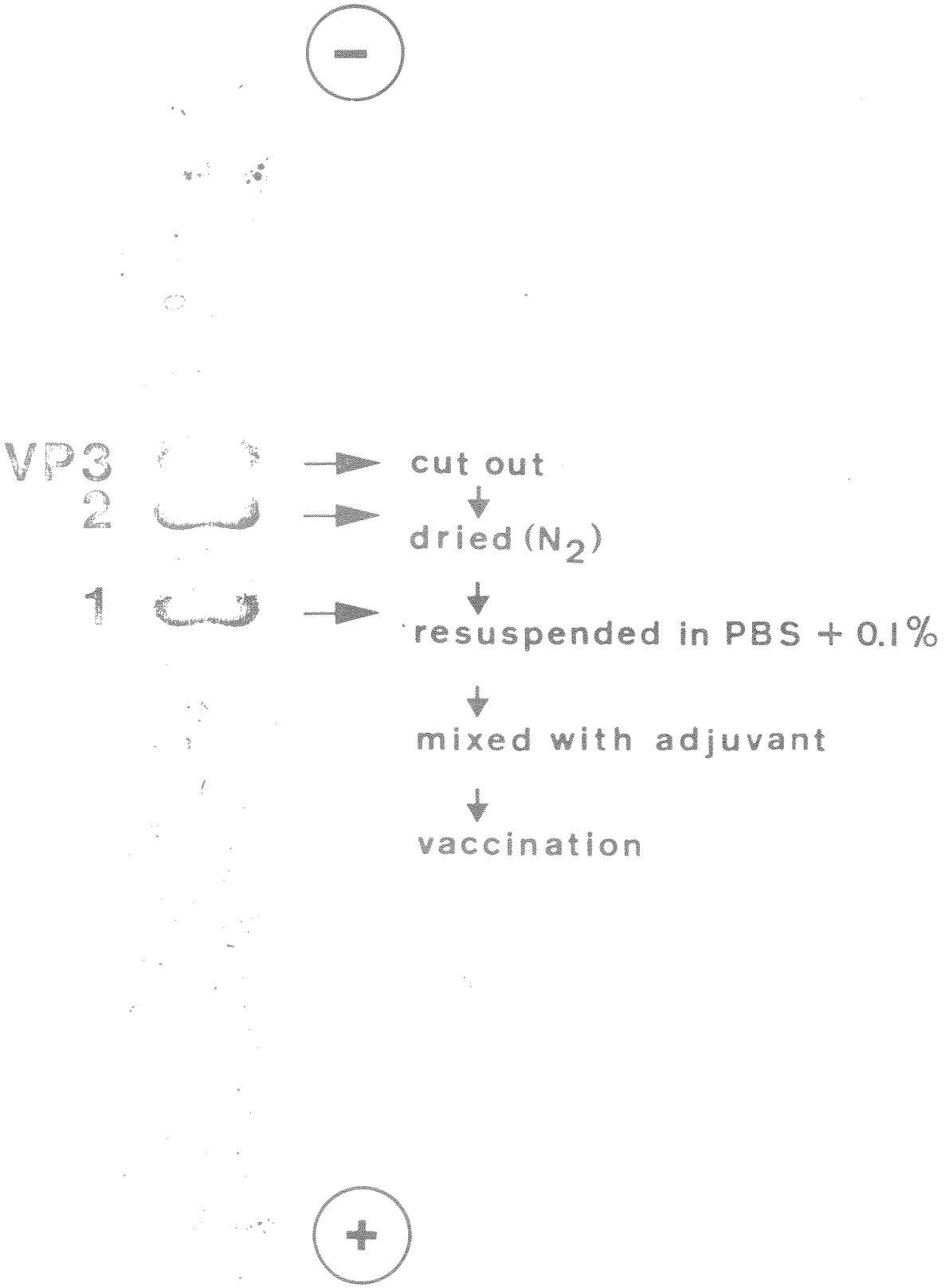
−
VP3
2
1
cut out
dried (N_2)
resuspended in PBS + 0.1%
mixed with adjuvant
vaccination
+

Table 1: Neutralizing activity of antisera prepared against the structural proteins of FMDV.

		MNT	
		A_{10} virus	O_1 virus
type A sera	anti-VP_1	2.0*	-
	VP_2	-	-
	VP_3	-	-
	anti-140S	2.0	-
type O sera	anti-VP_1	1.6	2.4
	VP_2	-	-
	VP_3	-	-
	anti-140S	-	2.4

Table 2: Activity of antisera prepared against the structural proteins of FMDV measured in the RIA with 140S and 12S.

		RIA with 140S		RIA with 12S	
		A_{10} virus	O_1 virus	A_{10} virus	O_1 virus
type A sera	anti-VP_1	4.0*	-	3.0	2.9
	VP_2	-	-	2.2	2.2
	VP_3	-	-	-	1.6
	anti-140S	3.6	-	3.2	2.7
type O sera	anti-VP_1	2.4	3.5	2.6	2.9
	VP_2	-	-	2.2	2.7
	VP_3	-	-	2.5	1.6
	anti-140S	-	4.0	2.5	3.4

x) log serum dilution precipitating 30% of the labelled antigen; - means less than 1.6.

Table 4 (contd.)

	ELISA		
	140S A	O	C
anti-140S A	4.7	4.1	4.4
O	3.8	5.1	4.2
C	4.1	4.4	4.7
	12S A	O	C
anti-140S A	5.0	4.5	4.5
O	4.5	5.1	4.5
C	4.9	5.2	5.8

Table 5: Comparison of polystyrene and polyvinylchloride microtiter plates.

		140S A	
		polystyrene	PVC
type A sera	anti-VP_1	3.4	3.4
	VP_2	-	2.5
	VP_3	-	2.5

		140S O	
		polystyrene	PVC
type O sera	anti-VP_1	3.7	4.0
	VP_2	2.2	3.7
	VP_3	2.2	4.0

Table 3: Activity of antisera prepared against the structural proteins of FMDV measure in the ELISA with 140S and 12S coated plates.

		140S A	12S A
type A sera	anti-VP_1	4.8^{+}	4.9
	VP_2	3.7^{x}	4.6
	VP_3	3.7^{x}	4.1
	anti-140S	4.7	5.0

		140S O	12S O
type O sera	anti-VP_1	4.7	5.2
	VP_2	3.7^{x}	3.7
	VP_3	4.2^{x}	4.2
	anti-140S	5.1	5.1

		140S C	12S C
type C sera	anti-VP_1	5.1	5.2
	VP_2	3.7	3.8
	VP_3	3.8^{x}	4.2
	anti-140S	5.7	5.8

+) log serum dilution giving an extinction twice the background

x) weak reaction

Table 4: Cross reactions in the MNT, RIA and ELISA

	MNT	
	A	O
anti-140S type A	2.0	-
O	-	2.7

	RIA	
	140S A	140S O
anti-140S type A	3.4	-
O	-	4.2
	12S A	12S O
anti-140S type A	3.2	2.5
O	2.5	3.2

Table 5 (contd.)

		140S C	
		polystyrene	PVC
type C sera	anti-VP_1	3.1	3.7
	VP_2	1.9	2.5
	VP_3	-	3.1

	polystyrene			PVC		
	140S A	140S O	140S C	140S A	140S O	140S C
anti-140S A	3.1	2.8	3.1	3.7	3.1	3.4
O	1.6	3.7	3.1	3.1	4.0	3.4
C	-	1.6	4.3	3.4	3.7	4.6

Table 6: Coating at different pH's of 140S type O

		pH 7.4	pH 9.6
anti-O sera	anti-VP_1	4.0	2.8
	VP_2	3.7	2.6
	VP_3	3.4	2.6
	anti-140S	4.2	2.9

Appendix C-3

Utilization of immunoenzymatic methods to reveal the presence of FMD type and sub-type antibodies in the sera of vaccinated cattle

by

R. Piroird and M. Lombard *

The immunoenzymatic methods have only recently appeared but they have developed with surprising rapidity. Any laboratory, even with limited means, can use these very sensitive methods.

The application of these new techniques to FMD has only been made, so far by Crowther and Abu-Elzein at Pirbright, for cattle-antibody research work and the comparison of virus strains.

We applied the immunoenzymatic methods in heterogeneous phase to the demonstration of cattle FMD antibodies and to the determination of their specificity. We retained the ELISA method (Enzyme Linked Immunosorbent Assay) which possesses an original characteristic: the utilization of filter paper discs as heterogeneous phase, supporting the antigen. This method is the transposition of the RAST (Radio-Allergo-Sorbent-Test) used in radioimmunology to the immunoenzymatic field.

In our opinion, this method has many advantages over the ELISA technique in microplates. Firstly, the fixation of the antigen on the support through chemical binding (Cyanogen Bromide) : it is, therefore, especially strong. Moreover, as we shall see later on, it can be measured and anticipated. Finally, it can be realised in advance, enabling ready-to-use reagents to be prepared.

The main purpose of our study is to demonstrate the presence of FMD antibodies in cattle and subsequently to identify the viruses found in biological fluids by the so-called "double antibody" sandwich ELISA method.

This paper reports our trials relating to cattle antibody detection. In the first two parts, the technique of reactive disc preparation and testing by isotope followed by the ELISA technique which was applied, will be explained. In the last part, the results will be presented and discussed.

PREPARATION OF REACTIVE DISCS

Materials

- Pre-cut paper discs (6 mm diameter) supplied by Schleicher and Schüll 1/
- Cyanogen Bromide, Sigma 2/
- Intertechnique liquid scintillation spectrometer, type SL 30, with logarithmic amplification and tape-punching teleprinter. This spectrometer was equipped with Gammavials Sohibo (ND) ref. 6724 i., for gamma readings.
- Pharmacia (ND) gel filtration equipment

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1/ Schleicher and Schüll - Dassel B.R. Deutschland

2/ Sigma C 6388

Virus:

The viruses consist of viral suspensions inactivated by glycidaldehyde, purified and tested after BACHRACH. They were of the O type-sub-types O_1 Lombardy 1946, O_1 Lausanne 1965 and O_1 Oltu 1978, the A type-sub-type A_5 Allier 1950 and the C type-sub-type C_1 Vosges 1960. All these viruses were labelled with Iodin 125, supplied by the CEA 1/ (213 millicuries/ml).

Methods

1 - Activation of the reactive paper and fixation of the FMD viruses

The activation of the discs is derived from the AXEN and ERNBACH method (Ref. 2) modified by BRIGHTON (Ref. 3). These discs were first activated with bromide cyanogen then after washing, they were put in contact with the virus. About 100 discs (0,2 g) are necessary for 3 ml of virus suspension containing 300 µg per ml in sucrose. The discs fixed a large amount of antigen after slow stirring overnight at room temperature. Their conservation at +4°C is satisfactory. They can also be frozen.

2 - Verification of the amount of virus fixed onto the discs

The fixation of radio-active virus onto the discs and its persistence, can be monitored with Iodin 25 (a gamma ray generator). The iodin-labelling method used in this case, has been described by Greewood and al. (Ref. 4).

The method should be fast in order to avoid excessive oxidation of the viral proteins. The non-fixed iodin is separated from the virus-fixed iodin by gel filtration using A50. The labelled viruses are found in the dead part of the column.

The radio-active virus made it possible to demonstrate a dose-effect relationship between the virus concentration in the suspension and the amount of virus chemically bound to the discs. (See Results). In the same way, the discs' reactivity was evaluated under varying time, pH and temperature conditions.

Results

1 - Labelling of FMD viruses with Iodin 125

The efficiency rate of the labelling was comprised between 20% and 35%. 2/ Recording n. 1 and the annexed table show the radioactivity of the fractions collected after gel filtration with the labelled virus being collected the first. The iodin 125 distribution has been studied by LAPORTE, J. who showed that 95% of the iodin was fixed on VP_1 (Ref. 5) and 5% on VP_3. The fact that VP_4 and VP_2 are not labelled, is explained by their internal position. The conservation of the protein VP_1 and therefore the integrity of the virion under the ELISA reaction conditions is demonstrated by the fact that the discs' radio-activity remains constant during the work and the serological reactions.

2 - Influence of the virus concentration on the fixation onto the discs

The knowledge of the weight of virus per ml of virus suspension and of the global radio-activity before the fixation makes it possible to calculate the weight of virus fixed onto each disc by measuring the individual radio-activity of the discs.

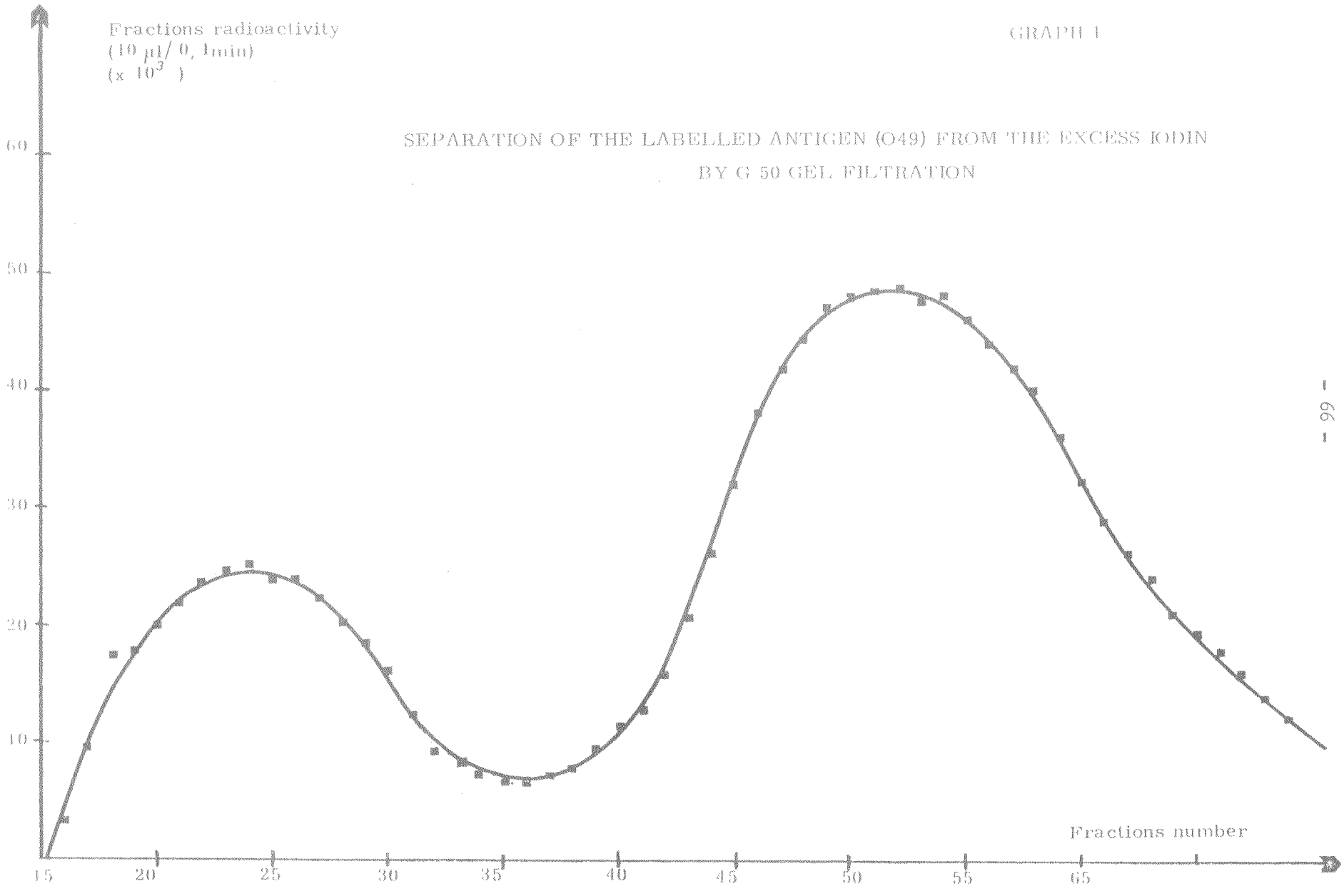
1/ CEA: Commissariat à l'Energie Atomique

2/ Efficiency rate (%) $= \frac{\text{Radio-activity of the 1st peak (virus)}}{\text{Radio-activity of the 1st (virus) and 2nd peak (free iodin)}}$

Recording n° 1 : - Radioactivity of the fractions collected after G 50 Sephadex gel filtration

fraction number	1/10 min measuring time	Radioactivity	10 µl of each fraction are measured		
4	.10	1335	1	0	
5	.10	1280	4	0	
6	.10	1232	5	0	
7	.10	1211	3	0	
8	.10	1462	0	0	PBS buffer of the column
9	.10	1464	1	0	
10	.10	1749	3	0	
11	.10	8652	2	0	
12	.10	27045	7	0	
13	.10	23131	4	0	
14	.10	32891	4	0	Tube 12 to 24 : Total radioactivity
15	.10	34246	8	0	
16	.10	30834	4	0	
17	.10	33524	12	0	405 097
18	.10	30642	7	0	
19	.10	37720	2	0	
20	.10	33724	7	0	Labelled antigen
21	.10	33927	7	0	
22	.10	34333	4	0	
23	.10	30281	4	0	
24	.10	22997	6	0	
25	.10	14550	1	0	
26	.10	14604	5	0	
27	.10	15803	6	0	
28	.10	18983	3	0	
29	.10	22623	4	0	
30	.10	28220	6	0	
31	.10	31937	5	0	
32	.10	32271	8	0	
33	.10	38380	2	0	
34	.10	43123	6	0	
35	.10	38772	8	0	Tube 29 to 49 : Total radioactivity
36	.10	39615	7	0	
37	.10	46574	2	0	749 742
38	.10	41110	10	0	
39	.10	50876	6	0	
40	.10	44935	6	0	
41	.10	46267	9	0	
42	.10	45826	8	0	
43	.10	33941	5	0	
44	.10	35733	10	0	
45	.10	32309	7	0	
46	.10	28482	3	0	Iodin in excess
47	.10	25988	3	0	
48	.10	21105	2	0	
49	.10	21655	8	0	
50	.10	19551	6	0	
51	.10	17490	5	0	
52	.10	15746	3	0	Efficiency of the labelling
53	.10	14625	7	0	
54	.10	13197	4	0	
55	.10	11925	5	0	$R° = \frac{405\ 097}{1\ 154\ 839} = 35,1\ \%$
56	.10	11133	7	0	
57	.10	9900	1	0	
58	.10	9194	5	0	
59	.10	8010	5	0	
60	.10	7553	4	0	
61	.10	6553	2	0	
62	.10	6560	3	0	
63	.10	6314	3	0	
64	.10	6005	3	0	

GRAPH 1


Fractions radioactivity
(10 µl/ 0, 1min)
(x 10³)
SEPARATION OF THE LABELLED ANTIGEN (O49) FROM THE EXCESS IODIN
BY G 50 GEL FILTRATION
60
50
40
30
20
10
Fractions number
15
20
25
30
35
40
45
50
55
65
I 66 I
I 99 I

The number of FMD virions* fixed onto each side of the disc can even be calculated, using Avogadro's number.

Table 2 and figures 2 and 2 bis show that the chemical binding of the viruses to the reactive discs follows a linear pattern in direct relation with the initial virus concentration.

3 - Influence of temperature and the incubation period

Table 3 shows clearly that a maximum fixation is obtained for a period of 24 hours at room temperature.

Time in hours / discs radio-activity	Radio-activity of 1 disc at +4°Cppm	Radio-activity of 1 disc at +22°Cppm	Radio-activity of 1 disc at +37°Cppm
1	133 606	124 296	101 447
2	153 032	220 075	166 622
4	138 138	221 544	200 954
6	135 874	255 179	241 422
24	145 217	345 622	301 410

TABLE 3 : Influence of temperature and the incubation period

Conclusion

The chemical fixation of purified viruses inactivated by glycidaldehyde can be achieved. It can be verified and quantified using a radio-active marker.

The conservation of the discs ready to use is good at a temperature of +4°C. This is proved by the results obtained with discs kept for a few weeks although we have as yet no experience regarding conservation over a period of several months.

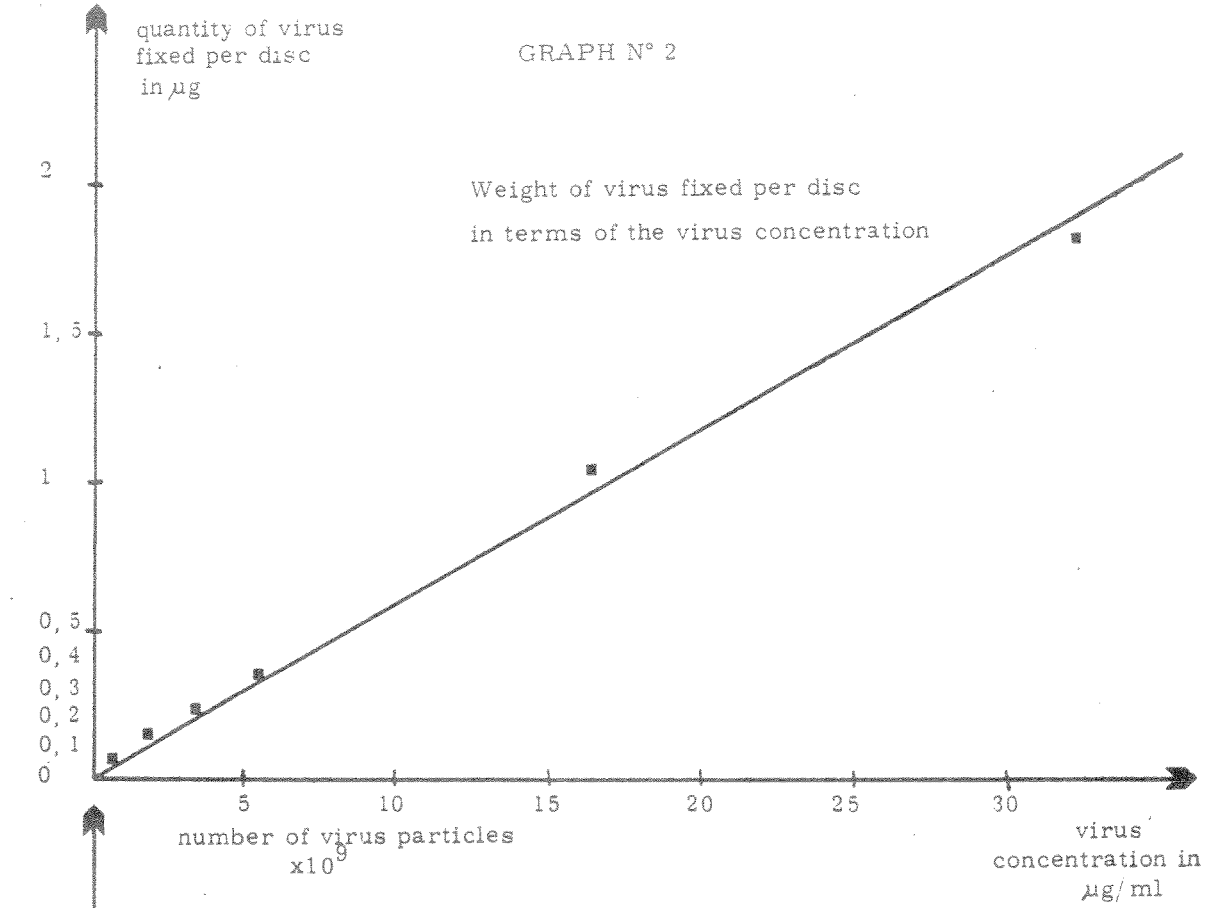
As these "FMD antigen" reactive discs are prepared from inactivated viruses, they can be used in non-protected laboratories, which is not the case for the seroneutralisation method.

---

* $N_{virions} = \frac{\text{Virus mass expressed in grams x Avogadro's number}}{\text{Virion molecular mass}}$

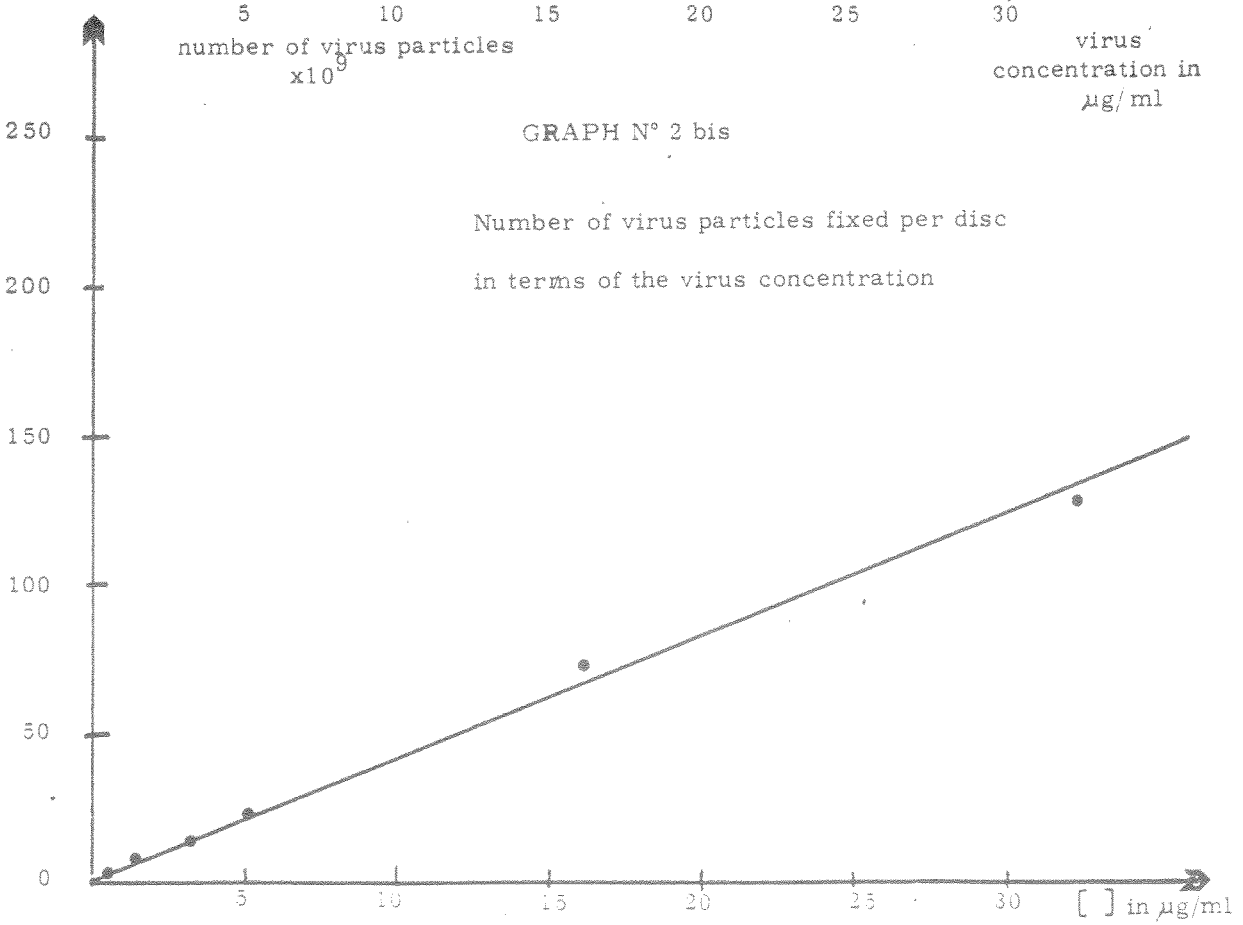
	RADIOACTIVITY in ppm in million of pulses per minute (10^6 ppm)							
	1 ml	1 ml	1 disque	1 disque	1 ml	1 ml		
Antigen dilution	virus suspension before contact	virus suspension after contact	Disc - unwashed - undried	Disc - washed - dried	washing fluid 1	washing fluid 2	Amount of virus fixed per disc (ug)	Number of virions fixed per disc (milliards)
pur 160 µg/ml	25, 78	11, 90	1, 03	1, 07	2, 48	0, 41	5, 63	396
1/3 53 µg/ml	6, 49	5, 50	0, 76	0, 68	0, 24	0, 035	3, 58	252
1/5 32 µg/ml	5, 02	3 41	0, 45	0, 34	0, 21	0, 042	1, 79	126
1/10 16 µg/ml	2, 27	1, 43	0, 25	0, 19	0, 07	0, 016	1, 00	70
1/30 5, 3 µg/ml	0, 75	0, 38	0, 065	0, 058	0 036	0, 011	0, 30	21
1/50 3, 2 µg/ml	0, 54	0, 26	0, 032	0, 036	0, 027	0, 009	0, 19	13
1/100 1, 6 µg/ml	0, 26	0, 13	0, 023	0, 021	0, 016	0, 004	0, 11	8
1/300 0, 53 µg/ml	0, 11	0, 05	0, 005	0, 008	0, 008	0, 004	0, 04	3

Table 2 : Influence of the virus concentration



GRAPH N° 2

Weight of virus fixed per disc in terms of the virus concentration



GRAPH N° 2 bis

Number of virus particles fixed per disc in terms of the virus concentration

ELISA METHOD APPLIED TO THE DETECTION OF FMD ANTIBODIES IN CATTLE

The ELISA method makes it possible to detect type and even sub-type specific antibodies in bovine sera using reactive discs and to obtain information as useful as that supplied by seroneutralisation.

Materials

- The ELISA reaction is conducted in disposable glass haemolysis tubes. The substrate is read with a continuous flow photometer equipped with an interferential filter (λ = 405 nm).
- The reactive discs used as antigen were prepared with A_5Allier, C_1Vosges, O_1, O_1Lausanne, O_1Lombardy and O_1Oltu viruses.
- The bovine sera were taken from animals twice vaccinated at a 15-day interval (Biological) Standardisation Requirements Sera: anti O_1Lausanne, anti A_5Allier). The anti-bovine IgG conjugate was a pig serum distributed by Institute Virion Ltd. The enzyme-substrate system selected was alkaline phosphatase and paranitrophenylphosphate Sigma (ND).

Method

The discs must be treated with polyamines or foal serum before being engaged in the ELISA reaction in order to neutralize the still active chemical bonds.

1 - Incubation of the "reagent-antigen" disc with the serum to be tested

The serum is diluted in tubes at 1:64, 1:128.....up to 1:1024. One reactive disc and one ml of serum dilution are incubated for 4 hours at room temperature. When this time of specific immunoglobulin fixation is over, the discs are washed in the tube and dried on filter paper.

2 - Incubation with the conjugate

The discs are put in contact with 0,2 ml of previously titrated anti-bovine IgG conjugate in a clean tube and incubated overnight at room temperature. The discs are then washed and dried and transferred into another clean haemolysis tube.

3 - Incubation with a substrate

1 ml of freshly prepared pNPP solution is added to each tube. The enzymatic reaction causes a staining to appear after 45 minutes at +37°C.

4 - Photometer reading out

The reading out is made on the stained medium by aspiration and analysis of the adsorption at 405 nm (adsorption wavelength for Paranitrophynol). The usual reaction controls must be prepared: substrate, conjugate and bovine negative serum controls.

In brief, the time required to determine the specificity of the FMD antibodies in a bovine serum is:

- 4 hours for the fixation of the antibodies onto the reagent
- 1 night to fix the anti-antibody conjugate
- 45 minutes to reveal the enzymatic reaction

This is much less than the time necessary for a seroneutralization test.

RESULTS

The stock of reactive discs that we had prepared enabled use to carry out, with the anti-bovine IgG conjugate, research work on the type and sub-type specificity of the immunoglobulins in the sera of cattle having received a booster vaccination (about 12 trials).

1 - Demonstration of type specificity

Three types of antigen discs (O_1 Lausanne, A_5 Allier, C_1 Vosges) and two bovine sera (anti O_1 Lausanne and anti A_5 Allier) were used. The results obtained in time work are gathered in tables 5 and 6.

Anti O_1 Lausanne bovine serum - 1078 - Vaccine 8175

antigen \ serum dilution		1/64	1/128	1/256	1/512	1/1024	T serum	T conjugate
O_1 Lausanne	O.D.	0,49	0,45	0,35	0,29	0,26	0,10	0,13
	$O.D._{c}$*	0,36	0,32	0,22	0,16	0,13		
A_5 Allier	O.D.	0,25	0,23	0,23	0,20	0,18	0,10	0,14
	$O.D._{c}$*	0,11	0,09	0,09	0,06	0,04		
C_1 Vosges	O.D.	0,27	0,21	0,175	0,17	0,14	0,10	0,13
	$O.D._{c}$*	0,14	0,08	0,045	0,04	0,01		

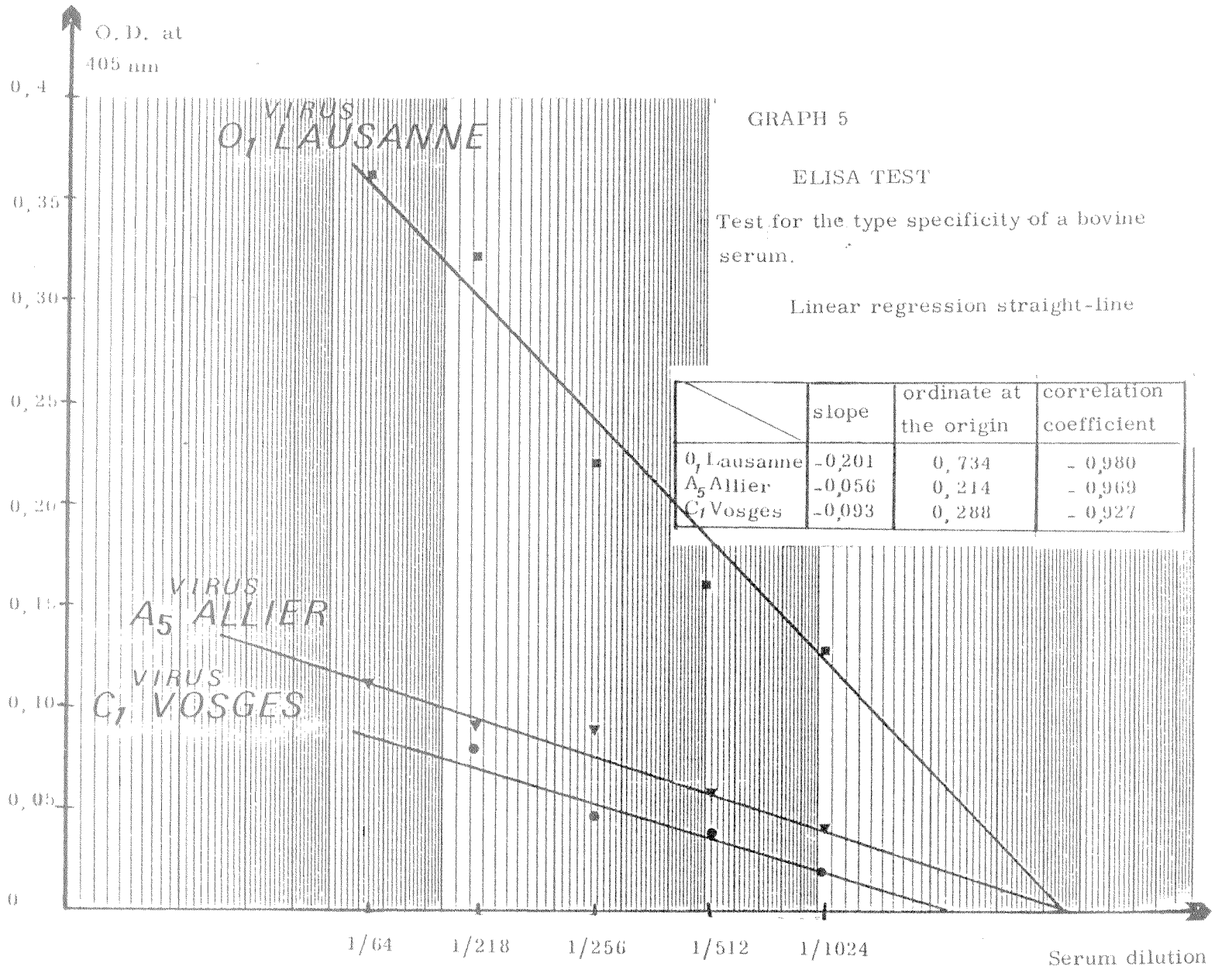
Table 5 - ELISA TEST - O_1 Lausanne bovine anti serum against type O_1 Lausanne, A_5 Allier and C_1 Vosges discs.

The reaction is maximum for the couple O_1Lausanne-Antigen - O_1Lausanne-antiserum is put in contact with O_1 Lausanne, A_5 Allier and C_1 Vosges antigen discs.

The serum also reacts with A_5 Allier and C_1 Vosges antigen discs, but the staining intensity at the end to the ELISA is three times lower than that obtained in the homologous system.

Plotting the O.D. at 405 nm in terms of the serum dilution gives a series of negative slope straight lines. (Graph 5) We calculated the parameters of the linear regression straight line of type $y = mx + b$ (where m is the slope and b the ordinate at the origin). The linear correlation is excellent for the three pairs under study: it is known on the graphs.

* $O.D._{c}$: collected optical density



GRAPH 5

ELISA TEST

Test for the type specificity of a bovine serum.

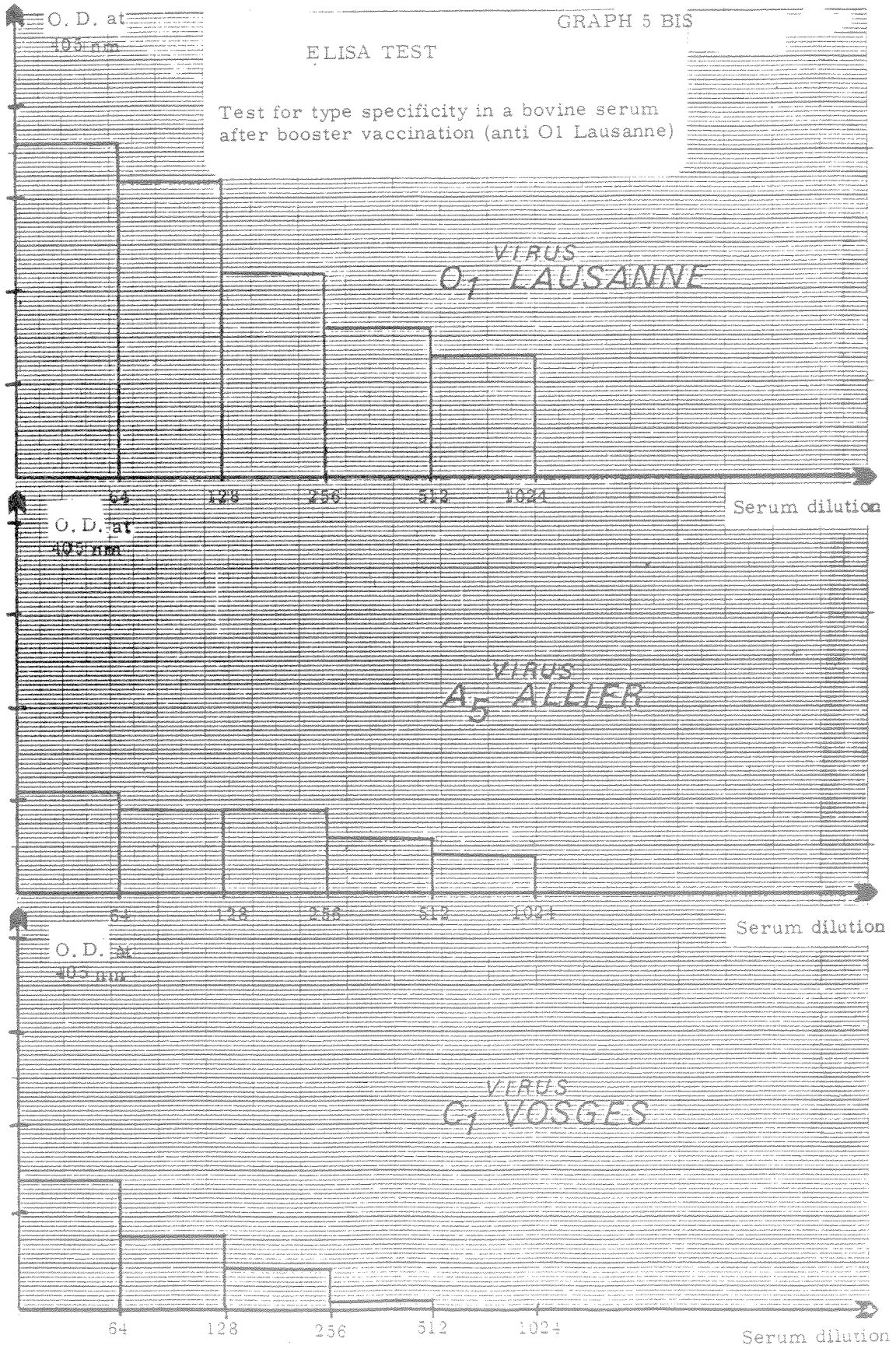
Linear regression straight-line

	slope	ordinate at the origin	correlation coefficient
O_1 Lausanne	-0,201	0, 734	- 0,980
A_5 Allier	-0,056	0, 214	- 0,969
C_1 Vosges	-0,093	0, 288	- 0,927

GRAPH 5 BIS

ELISA TEST

Test for type specificity in a bovine serum after booster vaccination (anti O1 Lausanne)



antigen \ serum dilution		1/64	1/128	1/256	1/512	1/1024	T serum	T conjugate
O_1 Lausanne (1)	O.D.	0,49	0,42	0,38	0,30	0,22	0,10	0,14
	O.D. $_c$	0,35	0,28	0,24	0,16	0,08		
O_1 Lausanne (2)	O.D.	0,29	0,27	0,26	0,20	0,19	0,10	0,19
	O.D. $_c$	0,10	0,08	0,07	0,01	0		

- Negative serum control at the dilution 1:64 = OD = 0,03

(1) O_1 Lausanne bovine anti-serum - 1078 - Vaccine 8175

(2) A_5 Allier bovine anti-serum - 1061 - Vaccine 8205

Table 6 - ELISA - O_1 Lausanne and A_5 Allier bovine anti-sera against A_5 Allier discs.

In this second ELISA, we engaged O_1 Lausanne antigen discs against 2 different bovine sera (one anti O_1 Lausanne and one anti A_5 Allier). Table and Graph 6. Here again, the results are significant since the O_1 Lausanne discs react three times more with the O_1 Lausanne anti-serum than with the A_5 Allier anti-serum.

2 - Test for sub-type specificity

The same O_1 Lausanne bovine anti-serum was tested against O_1 Lausanne, O_1 Lombardie 1946 and O_1 Oltu 1978 inactivated antigen discs. The results are gathered in Table 7.

antigen \ serum dilution		1/64	1/128	1/256	1/512	1/1024	T serum	T conjugate
O_1 Lausanne 1965	O.D.	0,49	0,42	0,38	0,30	0,22	0,10	0,14
	O.D. $_c$	0,35	0,28	0,24	0,16	0,08		
O_1 Lombardie 1946	O.D.	0,40	0,36	0,29	0,22	0,15	0,10	0,14
	O.D. $_c$	0,26	0,22	0,15	0,08	0,01		
O_1 Oltu 1978	O.D.	0,33	0,27	0,25	0,22	0,19	0,10	0,15
	O.D. $_c$	0,18	0,12	0,10	0,07	0,04		

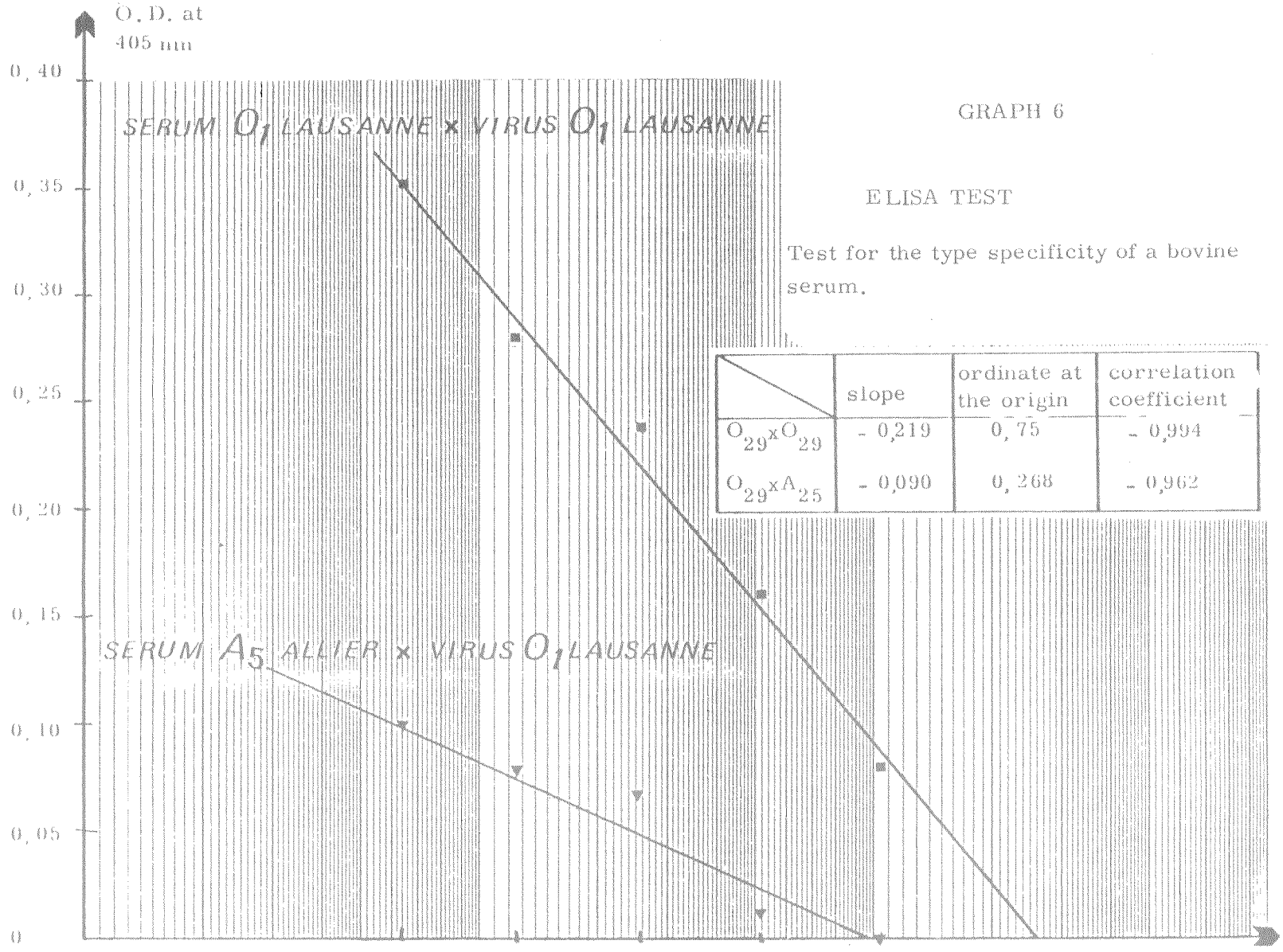
- Negative serum control: at the dilution 1:64 with the three types of discs, the O.D. is about 0,03

Table 7 - Anti O_1 Lausanne bovine serum - 1078 - Vaccine 8175

GRAPH 6

ELISA TEST

Test for the type specificity of a bovine serum.

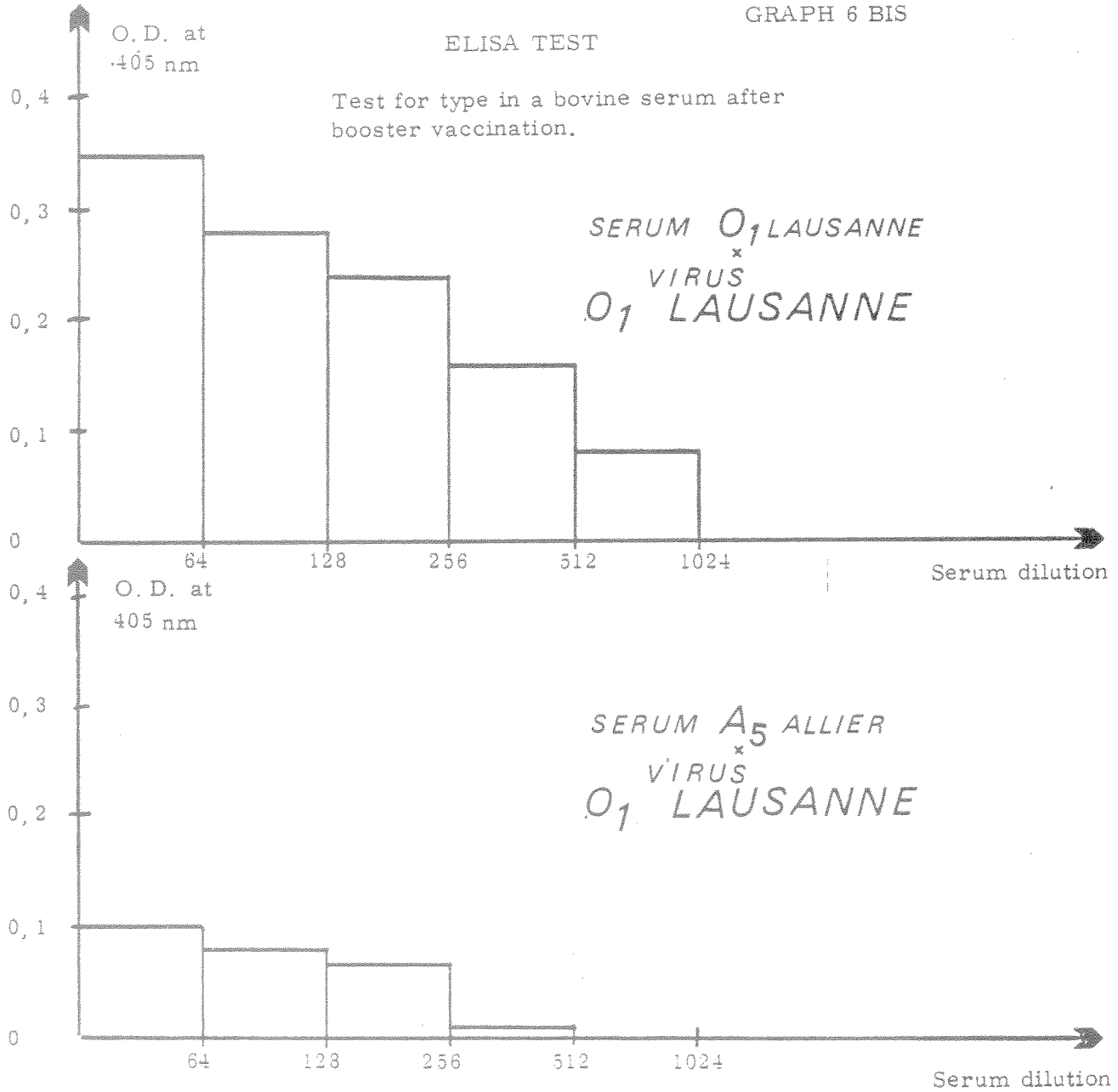


	slope	ordinate at the origin	correlation coefficient
O_{29} x O_{29}	- 0,219	0,75	- 0,994
O_{29} x A_{25}	- 0,090	0,268	- 0,962

GRAPH 6 BIS

ELISA TEST

Test for type in a bovine serum after booster vaccination.

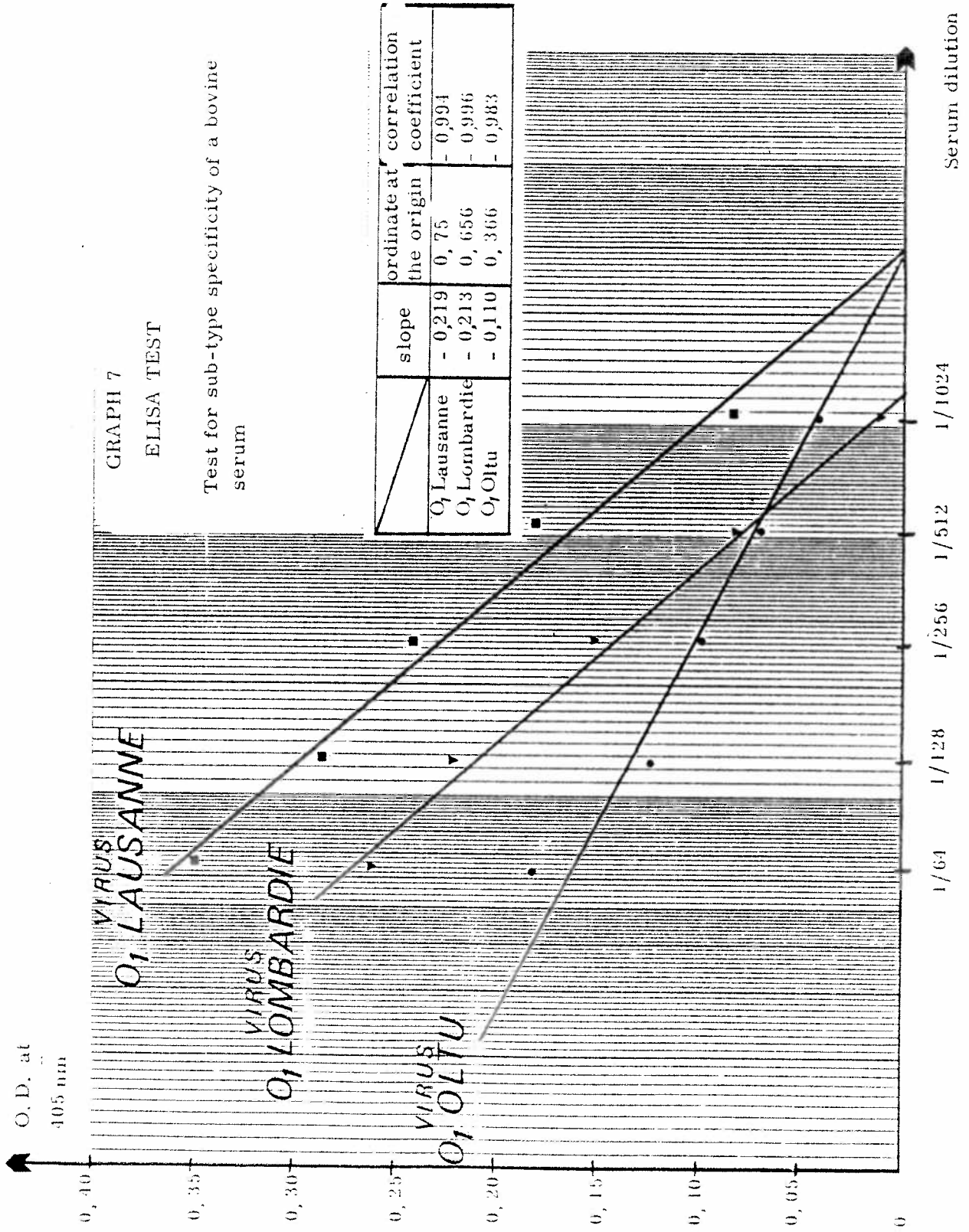


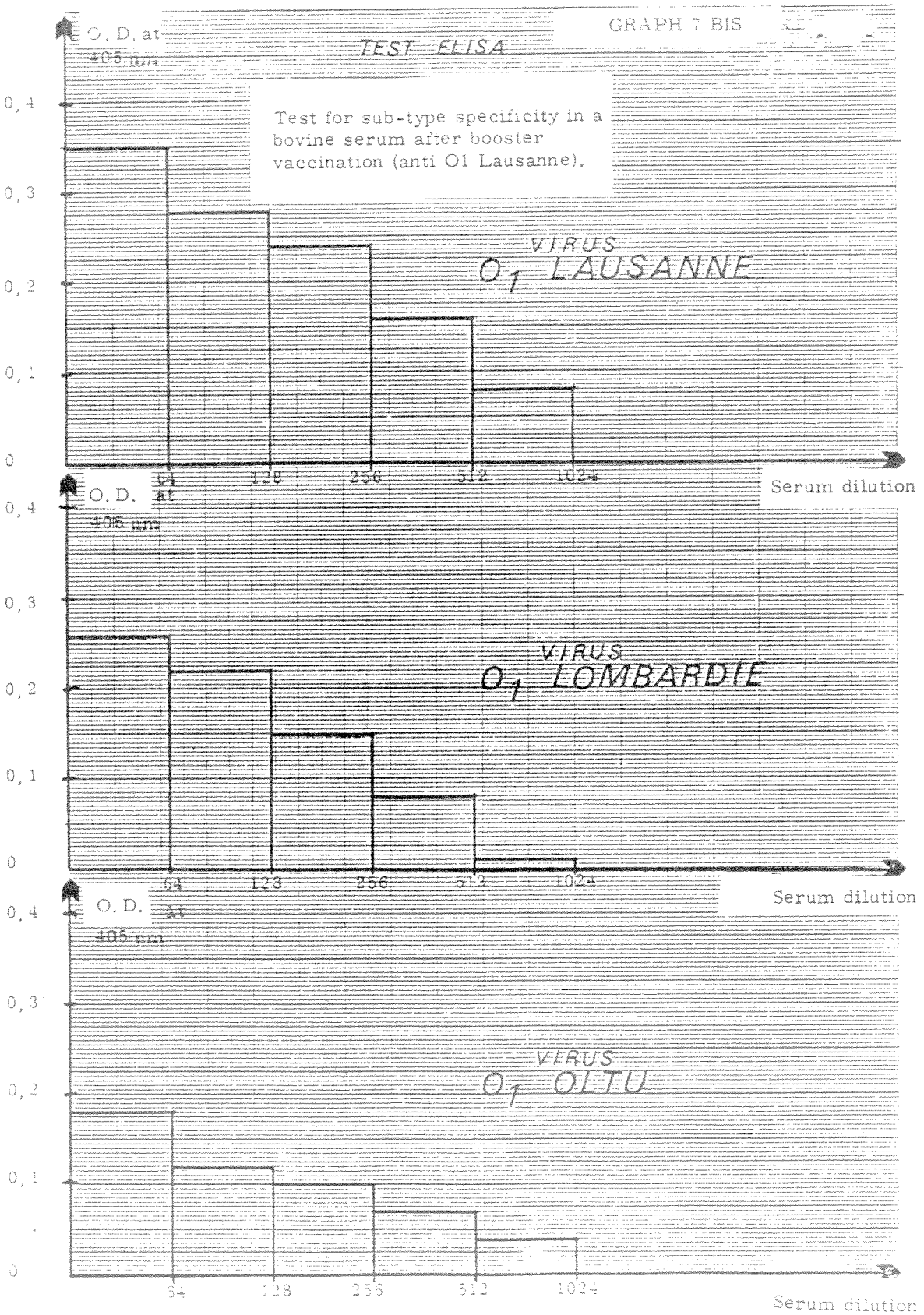
GRAPH 7

ELISA TEST

Test for sub-type specificity of a bovine serum

	slope	ordinate at the origin	correlation coefficient
O_1 Lausanne	- 0,219	0, 75	- 0,994
O_1 Lombardie	- 0,213	0, 656	- 0,996
O_1 Oltu	- 0,110	0, 366	- 0,983




GRAPH 7 BIS
TEST ELISA
Test for sub-type specificity in a bovine serum after booster vaccination (anti O1 Lausanne).
O. D. at 492 nm
0,4
0,3
0,2
0,1
0
VIRUS
O1 LAUSANNE
64
128
256
512
1024
Serum dilution
O. D. at 405 nm
VIRUS
O1 LOMBARDIE
O. D. at 406 nm
VIRUS
O1 OLTU

DISCUSSION

The ELISA method that we have adapted to FMD serology, makes it possible to determine the type specificity of the immunoglobulins in the sera of FMD vaccinated cattle.

The sub-type specificity can also be determined but a larger stock of reactive discs would be necessary in this case in view of the larger number of different FMD virus sub-types. It is also necessary that these discs have fixed a similar amount of virus particles before being engaged in this type of immunoenzymatic test (antigen standardisation).

It is interesting to compare results obtained in our system including only 3 sub-types and using inactivated antigens with the results published about the serological relationships between the virus strains that we have studied.

The classical methods of determining the serological relationships call for either guinea-pigs hyperimmune sera (complement fixation) or primo-vaccinated or twice vaccinated bovine sera (seroneutralisation tests), engaged against cultured viruses. These classical methods have made it possible to demonstrate that O_1 Lombardy and O_1 Oltu are both just as distant from the O_1 Lausanne virus strain.*

Using inactivated antigens and an immunoenzymatic method, we have shown that the O_1 Lombardy virus strain for the O_1 FMD sub-type, is closer to O_1 Lausanne than is the O_1 Oltu antigen.

From a quantitative point of view, such a difference in the appreciation of the methods is very small. From a qualitative point of view, a difference can be explained by the fact that the systems of analysis are different, (inactivated or virulent antigens, antibody populations of different nature and specificity).

In this respect, it should be pointed out that a relative homogeneity of results is obtained despite the heterogeneity of the means of sub-types. The antigenic characteristics of the three virus strains are equally well detected by immunoenzymology, complement fixation and seroneutralisation. Using bovine serum gives to immunoenzymology the advantage shared with seroneutralisation of assaying the sera of susceptible animals for whom the vaccine is prepared. The ELISA has another advantage, residing in the use of high serum dilutions avoiding interference with non-specific antibodies and increasing the discriminating power of the method.

Crowther and Abu El Zein are so far the only authors to have reported on the application of the ELISA to FMD. They are of the opinion that the ELISA method possesses advantages over other serological tests including radioimmunology. Our brief experience in this field leads us to share this point of view.

We would further insist on the easiness of implementation which makes it possible to obtain, within 24 hours and without any special work, the equivalent of a seroneutralisation with a reactive disc which we have reason to believe can be re-used, as regeneration is possible.

* M. Lombard: XXIII Session de la Commission européenne de lutte contre la fièvre aphteuse Rome, mars 1979; Réunion du Groupe de recherche, Lindholm, Danemark, juin 1979.

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Appendix C-4

Application of the Enzyme Labelled Immunosorbent Assay (ELISA) for the Study of the Structure of Foot-and-Mouth Disease Virus (FMDV)

by

K. Strohmaier, R. Franze and K.H. Adam *

The object of the experiments in our laboratory is the detection of the physical-chemical background of the biological behaviour of the FMDV (Ref. 1, 2). For this purpose we try to determine the amino acid sequence of that portion of proteins which carries the immunising component of the FMDV.

For the investigation of several peptides concerning the estimation of the pattern of them in the protein and location of the active center we used three methods:

1) the ELISA for the detection of the antigenic activity;
2) the plaque neutralization test (PNT) for the detection of the neutralizing capacity and;
3) the terminal amino acid determination for the chemical characterization.

This report is restricted to experiences gathered with the ELISA and some results obtained.

At the beginning of our investigations we found that the classical methods to detect antigenic determinants such as the complement fixation and the agar gel precipitation were not sensitive enough to detect activities in the amounts available.

We therefore compared first the practicality of the radio immune assay (RIA) and the ELISA for our demands. The employment of the RIA requires a γ - counter. The ELISA can be carried out by using a simple photometer as it is available in most laboratories. For large quantities of samples, however, an automatic device appears more appropriate. We used the "Titertek Multiskan" (Flow Laboratories, Irvine, U.K.), which measures the 96 wells of a plate within approximately one minute and prints the values of the optical density of each well.

The higher sensitivity of the RIA for positive samples is partially diminished by in general a higher background in cases of weak samples.

Whereas the ^{125}J labelled antibodies for the RIA are only stable for limited periods of time the enzyme conjugate is almost imperishable if stored properly. On the other hand there are factors which influence the enzyme reaction such as temperature, time, type and amount of substrate and others. It is, however, no problem to conduct the test under constant conditions.

The determining factor, however, in taking the ELISA for the studies was the more complicated and expensive nature of the RIA as a test with ^{125}J for routine check of many samples.

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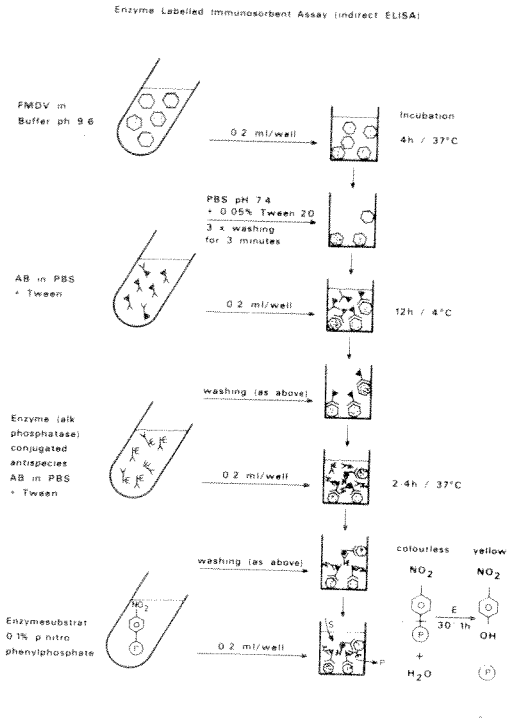


Fig. 1

In general we used the ELISA technique originally developed by Engvall and Perlmann (Ref. 3) adapted to the FMDV system by Crowther and Aleu-El Zein (Ref. 4). Its principle is demonstrated in Fig. 1: The virus is attached to the wells of the microplate during an incubation period at pH 9.6. Loose virus left is then removed by washing the wells with Tween 20. Next mouse serum is added and after a second incubation period the wells are washed again (3 times). Then enzyme conjugated goat anti-mouse IgG is placed into the wells. The substrate is added after another period of incubation followed by the washing process.

We do not stop the reaction by adding sodium hydroxide because the color reaction in the microplate can be measured within one minute and repeated if necessary.

The kinetics of the color reaction is shown in Fig. 2. All three variants described in the literature were tested for their usefulness as to our studies (Fig. 3):

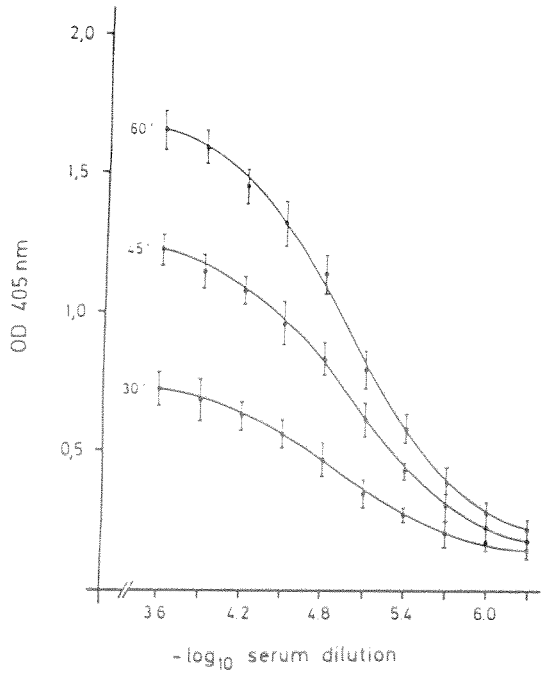


Fig. 2

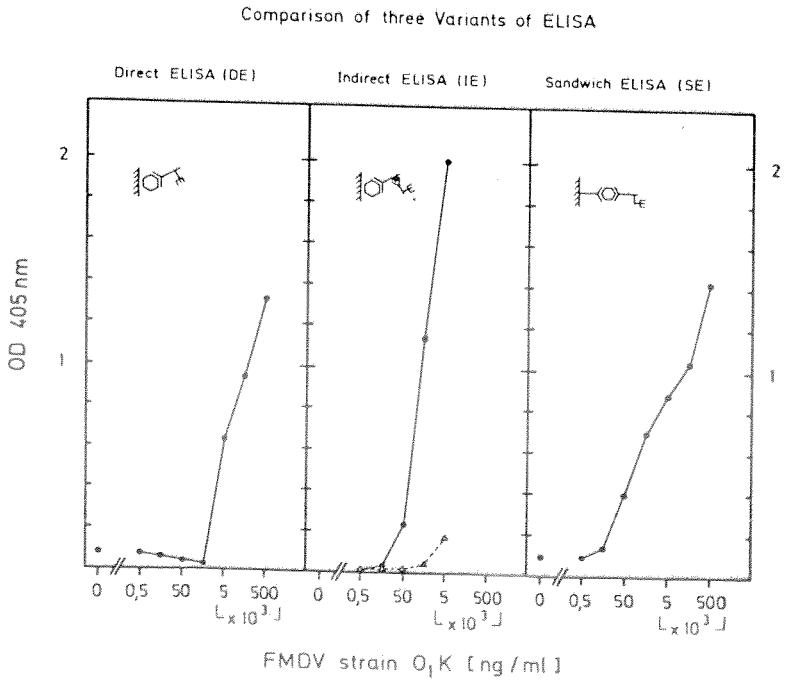


Fig. 3

(1) In the direct ELISA the antigen is attached to the microplate and the enzyme is conjugated to the specific antibody.

(2) In the indirect ELISA the antigen is also attached to the microplate but the enzyme is conjugated to an antispecies IgG and bound indirectly via a second antigen-apecific antibody to the antigen. The advantage of this method is the possibility to use the same enzyme conjugate for the detection of different antigens with corresponding antibodies of the same animal.

(3) The most sensitive variant is the sandwich ELISA where the specific antibodies are attached to the wells. The antigen is then added and during a further reaction the enzyme conjugated antibodies are bound by the antigen. A prerequisite for this test is a polyvalent antigen.

In our experiments we could determine the following sensitivities:

Direct ELISA:	500 ng/ml	FMDV
Indirect ELISA:	50 ng/ml	"
Sandwich ELISA:	5 ng/ml	"

As enzyme we used alkaline phosphatase and as substrate p-nitrophenyl phosphate. The enzyme was conjugated to the specific antibodies with glutar aldehyde. The yellow color reaction is visible by the naked eye and quantitatively measurable at a wave length of 405 nm.

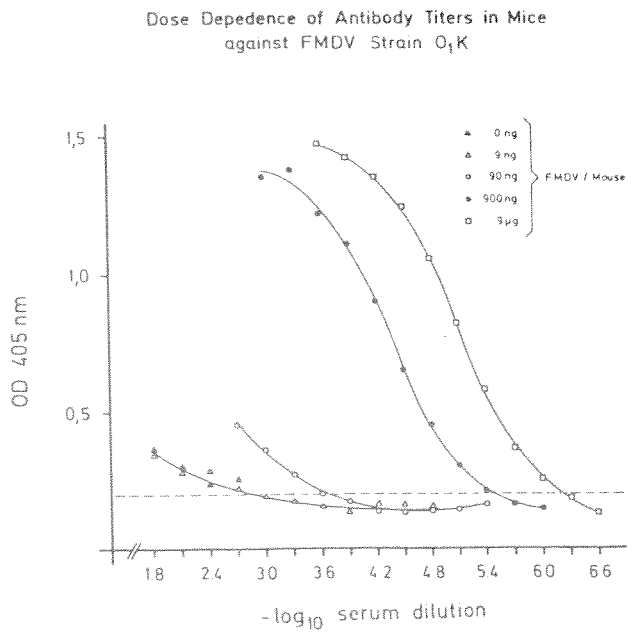


Fig. 4

In Fig. 4 a series of typical titrations is shown. Mice had been immunized with different doses of FMDV.

The dilution of a serum with the optical density of 0.2 was found to represent a useful value for the characterization of the serum activity.

The dependence of the result of the enzyme reaction on different parameters has been mentioned already. The correlations found agree with those of others described already (Ref. 3, 4).

Normally, we do not use the ELISA for the differentiation of various types of FMDV but mainly for the detection of antigenic determinants of virus fragments. Thereby we check the reaction of antibody containing sera as far as their activity is directed against the specific fragment or the whole virus.

In the following an example of such an experiment is presented. A suspension of purified FMDV strain O_1K was incubated after adding trypsin and the electrophoretically detectable fractions T_1 and T_2 isolated by chromatography. Moreover, the trypsin digestible coat protein VP_{Thr} was isolated by ionic exchange chromatography. With suspensions of the polypeptides mice were immunized and the sera checked by means of the ELISA and PNT against the whole virion. The results are given in Table 1:

Immunity against $VP_{Thr/T1}$, $VP_{Thr/T2}$, VP_{Thr} and FMDV-O_1K in Mice

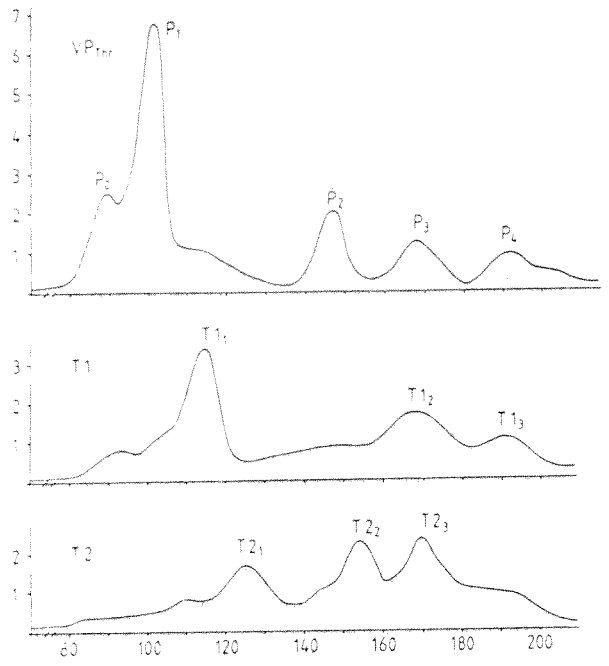
Immunization (10µg/Mouse)	Plaque-neutralizing Antibody Titer ($-\log_{10}$)	Antibody Titer against FMDV ELISA ($-\log_{10}$)
$VP_{Thr/T1}$ + CFA	< 0.3	5.1
$VP_{Thr/T2}$ + CFA	< 0.3	< 3.0
VP_{Thr} + CFA	2.1	5.5
FMDV-O_1K + CFA	2.7	6.3
.... CFA	< 0.3	—

Table 1

In the ELISA besides the protein VP_{Thr} also the fragment T_1 gave a reaction, but only the non-treated, non-split VP_{Thr} induced an antibody formation directed against the whole virus.

The same experiment was carried out with the BrCN fragments of the viral protein VP_{Thr}. It and the trypsin fragments T_1 and T_2 were split with BrCN and the peptides separated on a BioGel P 150 column. The elution diagram is shown in Fig. 5. The protein VP_{Thr} contains 4 methionins. This means, we expect to have 5 separate peptides. So far, we only found 4 because the first peak contained a mixture of non-split and incompletely split VP_{Thr}.

The reaction of the peptides in the PNT and ELISA is shown in Table 2:



Elution diagrams of BrCN peptides of VP_{Thr}, T1 and T2

Fig. 5

Only peptides P_1 and P_2 were able to induce neutralizing antibodies, whereas in the ELISA also P_3 would be recognized as a virus specific protein. Antibodies against P_4 do not seem to react with the whole virus. From all the peptides the molecular weights were calculated and the terminal amino acids estimated using the dansyl method and carboxy peptidase Y in connection with an amino acid analyzer. The results are listed in Table 3:

It is remarkable that both tryptic fragments have arginin at the C terminal but the whole protein ended with leucin, which means that a tiny peptide at the end of the protein was lost during the digestive process. It remained therefore undetected in the electrophoresis and the elution diagram.

Immunity against purified VP_{Thr}-BrCN-Peptides of VP_{Thr} in Mice

Immunization (10µg/Mouse + CFA)	Plaque-neutralizing Antibody Titer ($-\log_{10}$)	Antibody Titer against FMDV ELISA ($-\log_{10}$)
Fraction P 0	1.7	5.6
Fraction P 1	2.1	5.9
Fraction P 2	1.2	5.1
Fraction P 3	< 0.6	4.4
Fraction P 4	< 0.6	< 3.0
VP_{Thr}-BrCN Mixture	1.0	5.2
VP_{Thr}	1.5	5.1

Table 2

Molecular Weights and terminal Aminoacids of BrCN Peptides

	Mol. Weights	Aminoacids	
		N-terminal	C-terminal
Virusprotein VP_{Thr}	28 000	Thr	Leu
P_1	15 000	Val	Met*
P_2	5 000	Lys	Met
P_{3a}	3 250	Pro	Met
P_{3b}	4 100	Thr	Met
P_4	2 600	Asp	Met
Trypsinfragment T_1	15 500	Pro	Arg
$T1_1$	10 000	Val	Arg
$T1_2$	3 250	Pro(Thr)	Met
$T1_3$	2 600	Asp	Met
Trypsinfragment T_2	8 700	Gly	Arg
$T2_1$	7 500	Gly	Met
$T2_2$	4 500	Gly	Met
$T2_3$	3 800	Lys	Arg

* = Methionin was detected as homoserin after splitting by BrCN

During the splitting process of VP_{Thr} by BrCN also only methionin was detected as homoserin by the amino acid analyzer at the carboxyl end of the peptides. The fifth peptide - probably also a tiny one from the end of the protein - could also not be detected in the elution diagram.

The peptides P_3 and P_4 from VP_{Thr} and peptide $T1_2$ and $T1_3$ from the trypsin fragment T1 were identical as far as their distinguishing marks were concerned. The first one is the peptide from positions 10-36 of the known N terminal sequence of VP_{Thr} (Ref.2), (Fig.6).

The Asp-Pro binding of positions 9-10 will be split by acids.

The peptide P_1 has the same N terminal amino acid valin as $T1_1$ but the molecular weight is considerably higher. Obviously peptide $T1_1$ originates from the trypsin split P_1 leaving another portion in the fragment T_2.

Table 3

VP_{Thr}

```
 1                          5                          10
Thr - Thr - Ser?- Ala - Gly - Glu - Lys?- Ala - Asp - Pro -

11                         15                         20
Val - Thr - Thr - Thr - Val - Glu - Asn - Tyr - Gly - Gly -

21                         25                         30
Glu - Thr - Gln - Ile - Gln - Arg - Arg - Gln - His - Thr -

31                         35                         40
Asp - Val - Ser - Phe - Ile - Met - Asn - Arg - Phe - Val -
```

Fig. 6

Sequence of peptides in virus protein VP_{Thr}.
The lengths correspond approximately to the molecular weights

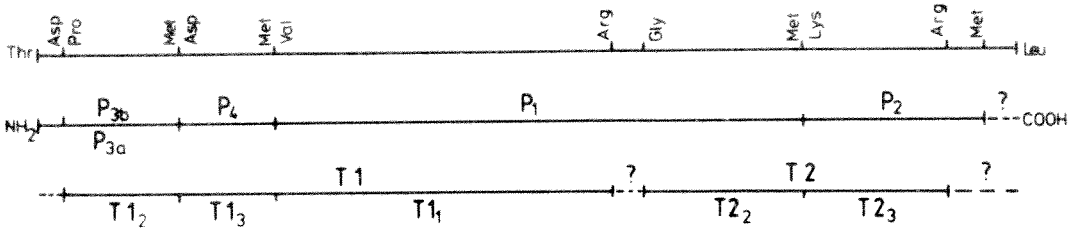


Fig. 7

Even if the results lack at present some details it is possible by combining all information to draw the following, in Fig. 7 summarized conclusions.

Since the neutralizing capacity of P_1 is destroyed by trypsin, the sequence of the amino acids in that area seems to be responsible for the neutralizing potency of the protein VP_{Thr} and perhaps the whole virion. Further experiments are in progress to confirm this assumption.

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Appendix D

Contribution to the Pathogenesis of SVD (Swine Vesicular Disease)

The influence of infection upon pregnancy

by

G. Kubin and M. Al-Nuktah (*)

On the occasion of an SVD-outbreak in Vorarlberg in 1972 we were informed that after a massive virus infection, four out of eleven pregnant sows aborted in the sixth or eighth week of gestation.

Due to isolation tests made on fetuses from pregnant sows which had been slaughtered seven to 14 days after the intracoronary or intravenous infection with the strain England 72, Burrows et al. became increasingly convinced that a vertical infection was unlikely and concluded that this form of transmission was of no importance in the epidemiology of SVD.

The question, whether an infection with the SVD virus strain isolated in Austria would behave differently and whether this strain would be able to cause abortions or fetal death, seemed, due to the observation made in practice, to call for a review.

Material and Methods

For the infection of the test pigs the virus strain WN 1972, isolated in Austria, was used.

Test pigs: Feeder hogs and pregnant sows of the breed "deutsches Edelschwein".

In order to simulate the form of infection corresponding to the field conditions, contact infection was chosen. As a source of infection one pig (1631) was infected intravenously and another pig (1627) intramuscularly. The two infected feeder hogs were kept in one pen together with six sows.

As it is already known from experience with other virus strains, such as Aujeszky virus, swine fever virus, Parvo virus, SMEDI virus, that an effect causing abortion or fetal death depends on the stage of gestation, we exposed our test animals to infection in various stages of pregnancy.

At the time of infection, two sows had been served 32 days earlier, one sow 63 days earlier, one 68 days earlier and two sows were pregnant 94 and 96 days, resp.

The rectal temperatures of all test animals were recorded daily, at certain periodic intervals the animals were subjected to a clinical examination, supplemented by the taking of faecal samples and swabs of nasal and pharyngeal mucus. At one week intervals blood samples were taken from the vena cava cranialis.

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The intravenously infected virus donor was killed seven days p.i. and pathologically/ anatomically examined as well as virologically tested for virus persistence.

Virus, virus detection, serum neutralization test

Virus:

For the infection the virus strain WN 72 was used as fourth tissue culture passage. In the neutralization test the virus was tested with specific antisera produced in guinea pigs, as well as with porcine enterovirus antisera 1-8 and Coxsackie antisera B 1 - B 6. Virus strain WN 72 could only be neutralized with the specific SVD antiserum as well as Coxsackie B 5 antiserum.

Enterovirus antisera 1 -8 as well as Coxsackie antisera B 1 - B 4 and B 6 did not show any indication of neutralization.

For the serologic test of the virus strain varying doses of virus and constant amounts of serum were used.

For virus isolation and neutralization tests mainly IB-RS 2 cells were used, in exceptional cases a permanent cell line SNH, developed in our laboratory, was applied, which in comparative tests proved to be as susceptible to SVD virus as IB-RS 2 cells.

The swabs and tissue samples were sometimes examined directly, but in most cases they were first of all frozen at -70°C and then thawed shortly before processing. The mucus swabs were extracted by means of Hanks-medium plus 1 p.c. serum and antibiotics and inoculated on tissue cultures. The tissue cultures were observed for 4 days, then at least 3 subcultures or cover slip cultures were examined by specific fluorescent antisera for the presence of SVD virus.

Neutralization test:

This test was carried out on IB-RS 2 cells, with constant virus concentration (100 KD 50) and varying serum dilutions. The reading of the cytopathogenic effect was carried out 3 - 4 days after setting up the test. Neutralization was effected partly as a tube test, partly as a microtest.

Colostrum and milk samples taken from the sows were serologically tested for antibodies by the initial adding of rennet ferment and then being shaken with chloroform and centrifuged. The whey thus obtained was used for the neutralization test.

Casuistics

The test pig (1631) which was i.v. infected for the contact infection, was already ill on the third day after infection, with typical symptoms and moderate fever, all four extremities clearly showing formation of vesicles. Changes on the lateral hoofs were especially striking. In the intramuscularly infected pig 1627, the vesicles developed one day later, first of all on one extremity. This pigs temperature rose up to 41,2°C and on the 5th day pig 1627 fell generally ill.

The pregnant sows showed on the 4th day after contact with the virus donors differently developed symptoms of SVD. Sow n. 4 had vesicular lesions on all extremities, sows 1, 3 and 5 had vesicles on one extremity, sows 4 and 6 did not show any clinically provable changes on the 4th day p.i. Gradually, the remaining sows fell ill with generalized formation of vesicles on the hoofs and on the snout with the exception of sow 6, in which 8 days after infection only a rudimentary vesicle formation on the snout could be identified. Temperature never rose in those sows above a limit value of 39,8°C.

Pig 1631, killed 7 days p.i., and pig 1627, killed 14 days p.i., were examined pathologically and virologically by means of tissue cultures and immunofluorescence. The following organs were examined: larynx, neck, lymph nodes, pharynx, tonsils, salivary gland, snout,

pancreas, spleen, esophagus, vesicles on hoofs, horn of hoofs, central nervous system, muscles, kidneys, lungs, urinary bladder, trachea, stomach, large and small intestines, lymph nodes of intestines, thymus, inguinal lymph nodes, liver and heart. From both pig 1631 and pig 1627 a cytopathogenic agent could be isolated from the remains of the vesicles on the extremities and from the sole horn and large intestines. Only the isolates from the vesicles turned out to be SVD virus under immunofluorescence and neutralization tests. The cytopathogenic agents isolated from the large intestines and the lymph nodes turned out under neutralization tests with specific antisera to be enterovirus strains 8 and 2.

Sow n. 1 gave birth to nine healthy piglets at the expected time, 18 days p.i. Immediately after birth the piglets were separated from their mother to prevent intake of colostrum. We took blood samples from 4 piglets and subsequently killed them, in order to examine organ samples of the animals for virus content. The organs removed comprised all parenchymatous organs, the central nervous system, tonsils, muscles, lymph nodes, intestines and testicles.

In addition, a colostrum sample was taken from this sow, which served for the serologic determination of the antibody titre.

21 days p.i. sow n. 2 gave birth to 12 healthy and one still-born but fully developed piglet. From three piglets, which had no colostrum intake, blood samples were taken; subsequently, one piglet was killed, and organ samples were taken just as in case of the piglets of sow 1. The individual organ samples of the still-born piglet were also subjected to a virological examination in tissue cultures. Likewise, from three piglets, which had had an opportunity of colostrum intake over a period of six hours, blood samples were taken and were subjected together with the colostrum to an antibody determination.

Sow n. 2 and eight surviving piglets were killed as late as 14 days after birth. The blood samples of the animals were tested for antibodies, the organ samples for virus content by the method described above.

Sow n. 4 gave birth to only 3 piglets 47 days p.i. 2 piglets were separated from their mother before colostrum intake, one piglet was left with the mother and could feed on colostrum for 10 hours. We took blood samples from the piglets and colostrum from the brood sow.

One piglet was killed shortly after birth and the other together with the brood sow 8 days after birth and then examined serologically and virologically in the usual way.

The pathologic/anatomic examination showed a purulent inflammation in the left horn of sow n.4, but there were no indications of a fetal resorption.

Sow n.3 gave birth with complications 44 days p.i. Three piglets were born spontaneously. Two still-born but fully developed piglets and a mummified fetus had to be manually extracted. After killing of the sow two living piglets were discovered in a uterine horn.

Two of the living piglets had taken in colostrum. The examination of the blood samples comprised the still-born piglets, piglets without colostrum intake and piglets after colostrum intake.

Virological examination was made of organ samples from all piglets including samples from the mummified fetus.

82 days p.i. sow n. 5 bore first of all two healthy piglets and subsequently one still-born piglet. After obstetric help it bore a further six live piglets. Blood samples of the still-born piglet and of one piglet without colostrum intake were serologically tested for antibodies. Moreover, organ samples were taken from the piglets and examined by means of a tissue culture for virus content. Immediately after birth a whey sample and two blood samples from piglets without any colostrum intake and from 2 piglets with colostrum intake were examined. Sow n. 6 unfortunately turned out not to be pregnant.

Results

The virological examination of the faecal samples and pharyngeal and nasal swabs, showed that until the sixth day cytopathogenic agents could be repeatedly isolated from the faeces of the pigs, which under further examination turned out not to be SVD virus. However, in the virus donors from the 3rd day onwards and in virutually all pigs from the 7th day onwards, SVD virus could be isolated from the faeces and be identified as such by immuno-fluorescence. In the case of sows 4, 5 and 6 SVD virus could still be detected in the faeces as late as on the 15 th day after contact infection. In nasal swabs we found SVD virus on the sixth day from sows 4 and 5, the test of the pharyngeal swabs showed a positive result in the case of 5 sows. From the organ and blood samples of the dead or killed piglets and from the organs of the slaughtered sows, it was in no case possible to isolate the SVD virus.

The development of antibodies clearly showed that all sows in contact with the virus donors had become infected with SVD virus (table 1-3). The infection led to the formation of high antibody levels, which further rose during the period of observation and persisted until the conclusion of the experiment.

The colostrum, taken immediately after birth, contained virus neutralizing antibodies, corresponding to the serum antibodies. Blood samples from dead piglets and equally from healthy piglets, which had had no opportunity for colostrum intake, contained without exception no antibodies, some hours after the intake of colostrum such piglets showed high antibody titres, their level depending on time and quantity of the colostrum intake. Already one week after birth a significant reduction of the passive antibodies could be detected.

Conclusion

It can be stated that the present tests did not give any indications that a contact infection with the SVD virus leads to abortion in pregnant sows or has an embryopathic effect.

The occasional death of fully developed piglets is probably due to uterine insufficiency and birth trauma. The negative serological findings in immune-competent, fully developed dead piglets also prove that there had not been any intra-uterine infection with SVD virus. The cause for the death of the mummified fetus in case of sow n.3 could not be clarified. No cytopathogenic agent could be isolated from this fetus.

The negative isolation tests and serological examinations of the piglets indicate that there is no transplacentary transmission of the SVD virus to the piglets. In accordance with the findings of Burrows our investigations have thus given no indication that an infection with the SVD virus strain WN, isolated in Austria, causes abortion or fetal death. The practical observations that during the course of an SVD epizootic, abortions repeatedly occurred in pregnant sows, could not be confirmed by our experiments.

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TABLE 1 Serumneutralization - Titre

		Infection	Birth									
		18.12.79	5. 1.80	17.12	24.12	31.12	5.1	7.1	14.1	21.1		
SOW 1	Serum			0	0	> 2048		> 256	512	> 2048		
	Whey						8192			64		
	Piglet						0					
	Piglet						0					
no	Piglet						0					
colostrum	Piglet						0					
	Piglet 1							> 2048	128	4		
After	Piglet 2							> 2048	64	16		
colostr.	Piglet 3							2048	128	64		
intake	Piglet 4							> 2048	256	128		
		18.12.79	7.1.80	17.12	24.12	31.12	5.1	7.1	14.1	21.1		
SOW 2	Serum			0	0			> 256	1024	> 2048		
	Whey							16384		128		
	Piglet							0				
no	Piglet							0				
colostrum	Piglet							0				
	Piglet							0				
	Piglet											
After	Piglet 1							> 16384		128		
colostr.	Piglet 2							> 16384		128		
intake	Piglet 3							> 16384		128		

TABLE 2 Serumneutralization - Titre

		Infection	Birth									
		18.12.79	11.2.80	17.12	24.12	31.12	5.1	7.1	14.1	21.1	4.2	12.2
SOW 3	Serum			0	0	> 2048		> 256	1024	> 2048	> 2048	2048
	Whey											16384
	Piglet +											0
	Piglet +											0
without colostrum	Piglet											0
	Piglet											0
	Piglet											0
	Piglet											0
	Piglet											0
After col. intake	Piglet											2048
	Piglet											2048
SOW 4		18.12.79	3.2.80	17.12	24.12	31.12	5.1	7.1	14.1	21.1	4.2	12.2
	Serum			0	2	2048		> 256	2048	> 2048	> 2048	
	Whey										16384	1024
no col.	Piglet										0	
no col.	Piglet										0	
After col. intake	Piglet										16384	

TABLE 3 Serumneutralization - Titre

		Infection	Birth													
		18.12.79	10.3.80	17.12	24.12	31.12	5.1	7.1	14.1	21.1	4.2	12.2	19.2	26.2 4.3	10.3	13.3
SOW 5	Serum			0	4	2048			512	1024	2048	1024	>8192	16384		16384
	Whey														16384	
	Piglet +														0	
no col.	Piglet														0	>4096
a. col. it.	Piglet														1024	4096
a. col. it.	Piglet														2048	2048
a. col. it.	Piglet															2048
a. col. it.	Piglet															> 4096
a. col. it.	Piglet															512
SOW 6		18.12.79	not pregnant	0	2	2048	>256		512	>2048	256	512	> 8192	1024		2048

no col. = without colostrum

a. col. it = after colostrum intake

Table 1

Type 'A' Foot-and-mouth disease in Europe 1969 - 1980

Appendix E

Type 'A' Europe	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980
W. Germany			A	A	A				Related to Mor 77, Alg77 Hol 77, A24. Diff. to A5 & A22			
E. Germany												
Austria												
Belgium			A A69Gre									
Bulgaria					A5							
Denmark	A	A										
Spain	A			A related A5,A24								
France												
Greece	A	A Gre 1969	A diff. Europ. strains.	A22	A22	A22		Closest A24(50%). Diff. A5 & A22	Related to Gre 76. Closest A24. Diff. A5, A22			
Holland									Related Mor 77, Alg 77, W.Germ * 77, A24,Arg25230 Diff.A5, A22			

*Hol 77 vs Arg 25230 - this result received from CPFA. Argentine 25230 not held in WRL

Type 'A' Europe	1969	1970	1971	1972	1973	1974	1975	1976	1977	1978	1979	1980
Hungary												
Italy	A		A		A		A		Closest to Venceslan Base. Diff. Europ. & M.E.	No results available	A	
Malta										Related to A5, A24. Diff. A22		
Poland												
Portugal												
Rumania												
Switzerland												
Czechoslo-vakia							A					
Turkey	A	A22	A22	A22	A22	A22	A	A22	A22	A22	A22	A22
U.S.S.R.	A	A22	A22	A22	A22	A22	A	A22	A22	A22		
Yugoslavia										Related to A5, A24. Diff. to A22		

TABLE 2 — Appendix E

EUROPE — New isolations of FMD virus from foci related either to disease introduced from outside the continent or obviously connected with vaccine use and manufacture

Year	Type	Country	Way of introduction	Post-vaccination cases	Virus escape from plants
1968	-A (A Valais) (A 26?)	Switzerland piggery in Valais followed by 20 foci	frozen meat from South America (garbage fed to pigs)		
	-A5	Denmark			2 foci near Lindholm
1969	-C Thorout " -C5 " (C Arg. 1969)	Belgium pigs and cattle involved	frozen meat from South America (suspected)		
	-A Spain 1969	Cadiz Huelva	foci coincided with importation of young cattle from South America		
	-A Greece	Crete and Macedonia	frozen meat imported - garbage fed to pigs (many foci)		
	-A5	Czechoslovakia (Bohemia) single focus			escape from vaccine plant suspected

Year	Type	Country	Way of introduction	Post-vaccination cases	Virus escape from plants
	-C	Switzerland Neuchatel single focus	garbage fed to pigs; imported frozen meat strongly suspected		
1970	*C Greece 1970 identical with C Thorout =C5	Greece (Attica) started in garbage fed pigs	frozen meat imported from South		
	*O 1970	Greece (Lamia) one focus in cattle	coincided with importation of young cattle from eastern Mediterranean		
	*A (Dutch prod. strain)	Denmark (Jutland)		nine post-vaccination foci	
1971	-A (similar to A Gr. 1969)	Belgium one focus in garbage fed pigs	frozen meat imported from South America by an intern. agency		

Year	Type	Country	Way of introduction	Post-vaccination cases	Virus escape from plants
	*C (production strain)	Germ. Fed. Rep. (Baden-Würtenberg) 2 foci		post-vaccination cases	
	*A (similar to A Gr. '69)	Greece (Crete)	imported frozen meat (same situation as in 1969)		
1972	*A Santander (A24 - A26?)	Spain Port of Santander where disease started	coincided with importation of cattle from South America		
	*A22 (second invasion)	Greece, Thrace and Thessaloniki	meat and lambs introduced from Turkey		
	*C (prod.strain)	Hungary and neighbouring countries			escape from a Waldmann plant
	*A5 (prod.strain)	Germ. Fed. Rep. (sporadic foci)		post-vaccination cases	
	*O Peplos	Greece - north eastern Greece and Attica	garbage fed pigs in Attica; eastern origin		

Year	Type	Country	Way of introduction	Post-vaccination cases	Virus escape from plants
1973	ASIA$_1$	Turkish Thrace (Istanbul)	arrival of slaughter animals from eastern Anatolia		
1974	O (production strain)	Belgium 50 foci		post-vaccination mechanical failure in an inactivator	
	C (prod.strain)	Germ. Fed. Rep. 14 foci			disease started ne a vaccine plant
1975	A5 (prod.strain)	Czechoslovakia single focus			disease found near the vaccine plant of Terezin
	A Italy '75	Italy - six foci at Alessandria including garbage fed piggeries	frozen meat imported from South America and distributed in the area		
1976	C (prod. strain)	German Democratic Rep. 9 foci			disease started at Rostock and Greifswald i.e. near the vaccine plant
	C (prod.strain)	German Fed. Rep. one focus		post-vaccination cases	

Year	Type	Country	Way of introduction	Post-vaccination cases	Virus escape from plants
	A Aachen Dec. 1976	Fed. Rep. of Germany 4 foci	garbage fed pigs; containing frozen meat from South America		
1977	A Netherlands January '77	Netherlands (Limburg) one focus	extension of the German outbreak		
	A Sicily '77 June & Dec.	Italy Two foci in different places	garbage fed pigs and use of frozen meat from South America		
	A Greece '77	Greece three foci	garbage fed pigs and use of frozen meat from South America		
	A Morocco and Algeria	episcodic in both countries	suspected origin: imported meat and sheep from South America		
	C (prod. strain)	German Fed. Rep. 2 foci			disease found nea vaccine prod. pla
	O (prod. strain)	German Dem. Rep. one focus			disease at Greifswald i.e. near production plant (Riems)

Year	Type	Country	Way of introduction	Post-vaccination cases	Virus escape from plants
1978	A Italy 1978	Italy (no outbreaks)	virus isolated from home of South American origin - tested at entry		
	A Italy similar to Sicily '77	Italy one focus	supposed to be related to imported frozen meat		
	C (Prod.strain)	Switzerland one focus		post-vaccination cases	
1979	A similar to A Sicily '77	Northern Italy	supposed to be related to imported frozen meat (pigs involved)		
	C (prod.strain)	Spain Gerona and other provinces			escape of virus from a vaccine plant near Gerona suspected
	O Calvados	France Normandy	unknown origin		
1980	O (prod.strain)	Switzerland		post-vaccination cases	
	O (prod.strain)	Fed.Rep.Germany		post-vaccination cases	

NOTE: The isolations of virus from outbreaks related to infection spread from endemic areas within Europe was not considered in this table.

Appendix F-1

Relationship between two Austrian virus strains of Foot-and-Mouth Disease, type O, and the vaccine strains O_1BFS 1860 and O_1 Svizzera

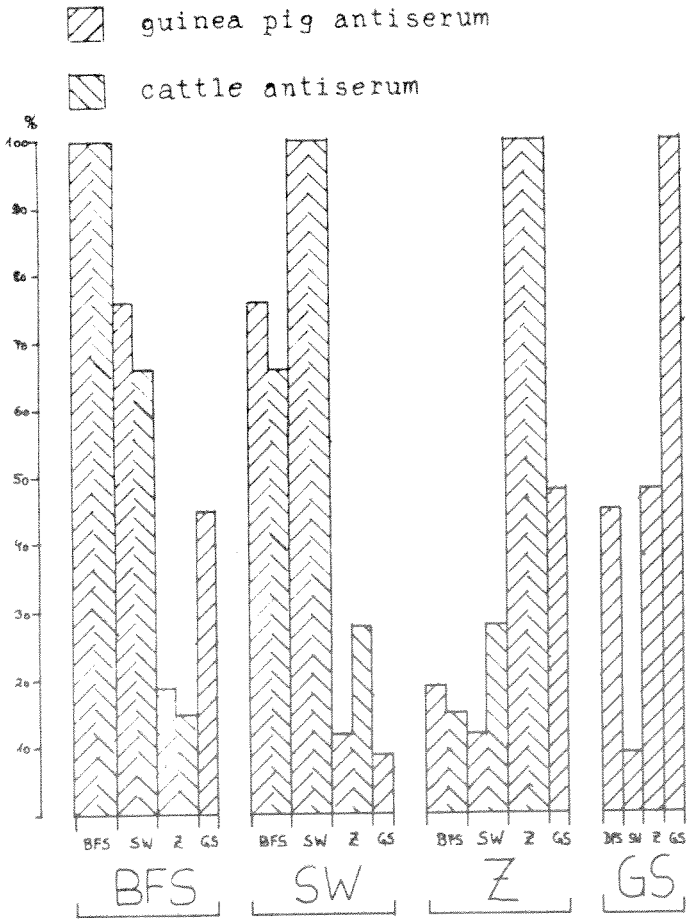
by

R. Silber*

From among the four virus strains compared O_1BFS 1860, O_1 Svizzera 1965 Lausanne, O Zurndorf 1965 and O Gross Schweinbarth 1973, O_1BFS and O Gross Schweinbarth seem to be the most suitable for the production of vaccine. With these the most effective protection against such FMD outbreaks of type O as occurred in Austria in the past, could be expected.

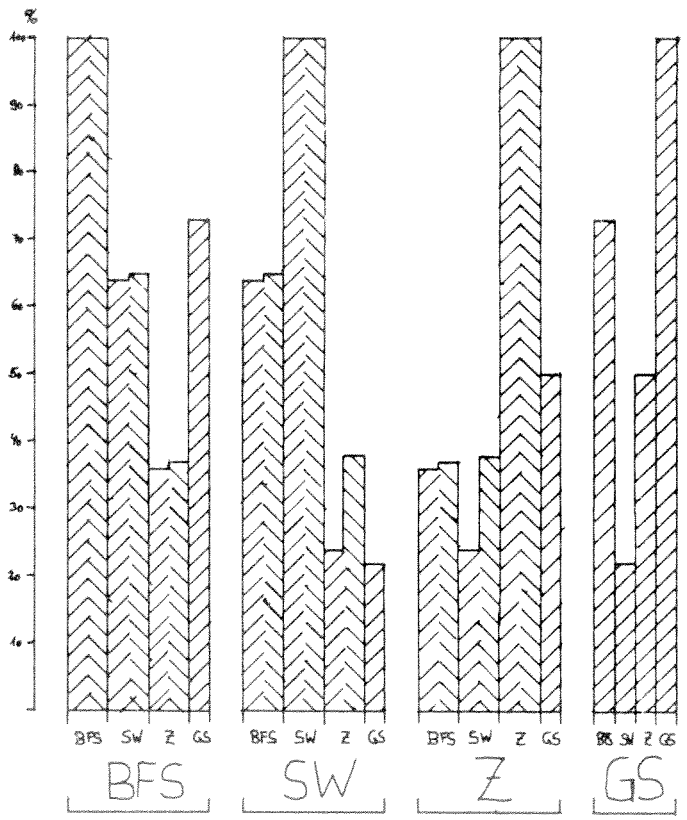
Graphs 1 and 2 show the R values of the four test arrangements according to the four virus strains.

Graph 1 tube method



* Federal Institute for the Control of Animal Virus Diseases, Vienna, Austria

Graph 2 micro method



Present results of the unilateral study for the virus C which appeared in Spain in 1979

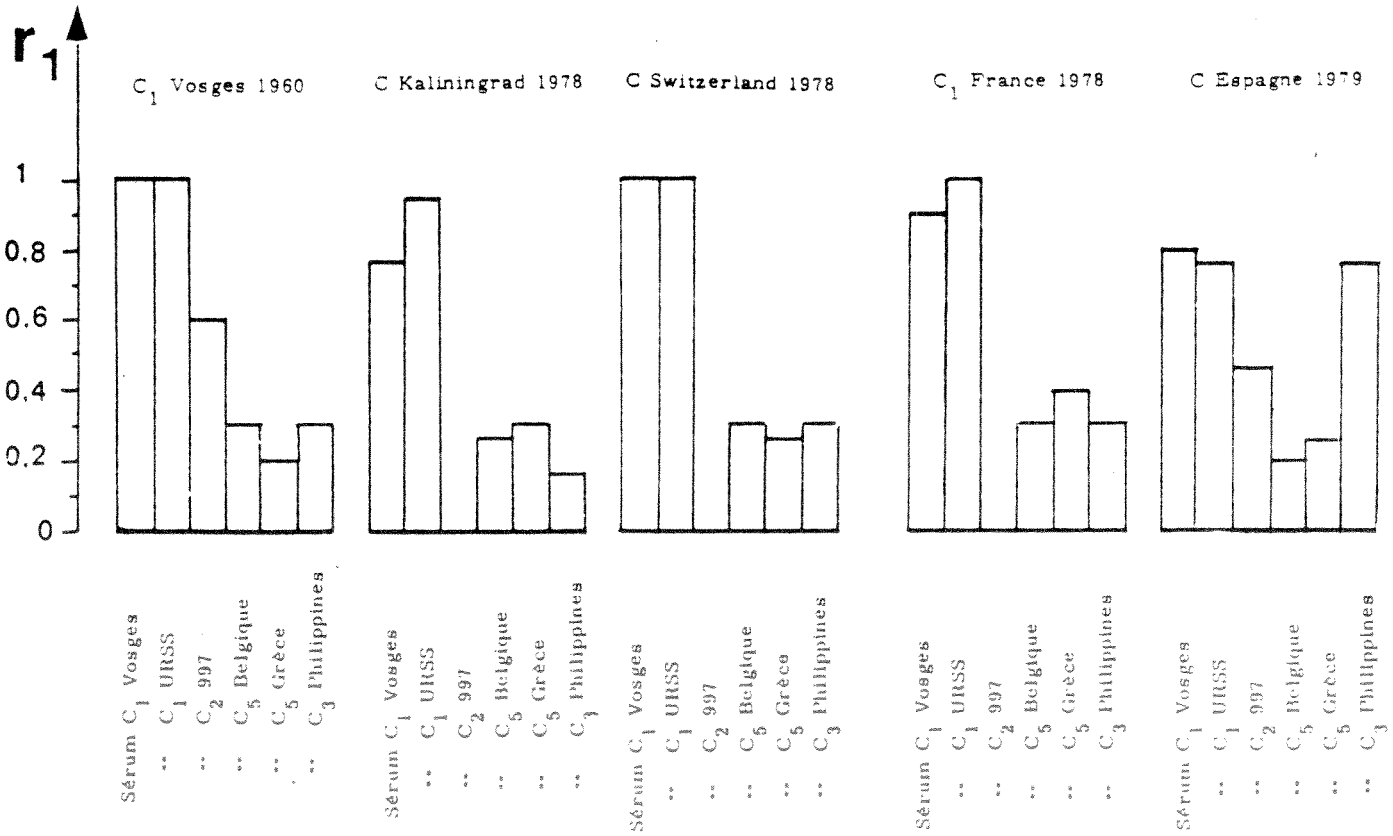
Comparison with viruses C recorded in Europe in 1978

by
M. Lombard*

Conclusion

The present complement fixation results show that the C Spain 1979 virus strain belongs to sub-type C_1 like the viruses C which appeared in Europe in 1978; however, it has a marked relationship with sub-type C_3.

All the viruses officially identified in Europe since the outbreak of sub-type C_5 in Greece in 1971 have been identified as belonging to sub-type C_1.



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SERUM \ VIRUS	C1 Vosges 1960	C1 URSS 1972	C2 997 1953	C5 Belgique 1969	C5 Grèce 1971	C1 France 1974	C1 Kaliningrad 1978	C1 Switzer. 1978	C1 France 1978	C Espagne 1979
Sérum C1 Vosges 1960	1	0, 80	0, 45	0, 20	0, 30	0, 65	0, 75	1, 0	0, 90	0. 80
Sérum C1 URSS 1972	1, 0	1	0, 40	0, 24	NT	0, 83	0, 92	1, 0	0, 95	0, 76
Sérum C2 997 1953	0, 60	NT	1	NT	NT	NT	NT	NT	NT	0, 45
Sérum C5 Belgique 1969	0, 30	0, 20	0, 12	1	0, 40	< 0, 15	0, 26	0, 30	0, 30	0, 20
Sérum C5 Grèce 1971	0, 20	0, 40	0, 23	0, 30	1	<0, 15	0, 30	0, 25	0, 40	0, 25
Sérum C3 Philippines 1976	0, 30	NT	0, 25	0, 30	NT	NT	0, 15	0, 31	0, 30	0, 76

TABLE OF THE SEROLOGICAL RELATIONSHIPS "r_1"

$$r_1 = \frac{\text{mean of 3 to 5 heterologous serum titrations}}{\text{mean of 10 to 20 homologous serum titrations}}$$

Appendix F-3

Further studies on the effect of antiserum quality on strain specificity in foot-and-mouth disease virus strain differentiation

by

M. M. Rweyemamu, Elizabeth Ouldridge, P. Hingley and Morwen Head *

INTRODUCTION

At the Uccle and Lindholm meetings of the Research Group it was recommended that tests based on the virus neutralization reaction were to be preferred over those based on the complement fixation reaction for the purpose of vaccine strain selection. The importance of using vaccine seed virus stocks for the preparation of reference antisera was stressed and it was also appreciated that in the neutralization tests the anti-12S reaction did not exert a significant influence on virus strain specificity. Although opinion was divided on the use of individual animal versus pooled sera, it was generally agreed that the testing of individual animal sera was to be preferred in the assessment of vaccine potency, whereas the use of pooled sera offered practical advantages in the rapid screening of the relationships between virus strains.

We have, therefore, examined the effects of animal species, use of individual animal or pooled sera, sera from primovaccinates or hyperimmune animals, anti-cellular antibody and antigen quantity or quality on the strain specificity of antisera. Results of our experiments are summarised below.

INDIVIDUAL ANIMAL TO ANIMAL VARIATION AND THE EFFECT OF POOLING SERA

Ten 18-24 months old sero-negative Hereford steers were vaccinated with monovalent O_1 Campos vaccine in Uruguay on premises known to be free from FMD. They were revaccinated 21 days later. Individual animal sera were collected on the 21st day post-vaccination and the 16th day post-re-vaccination. Pooled serum samples were prepared by mixing 1 ml aliquots from each of the 10 animals.

Strain specificity of the individual animal sera and pooled serum samples was tested in the two-dimensional microneutralization test (Rweyemamu, et al, 1978) using O_1 Campos virus as the homologous strain and O_1BFS 1860 and O Malaysi 1/78 as the heterologous strains. These strains were selected because of unequal relationships between O Malaysia 1/78 and subtype O_1 virus strains. Previous studies had shown the r value relationships of O Malaysia 1/78 against O_1BFS 1860 (bovine vaccinates) as 0.98; against O_1BFS 1860 (guinea pig) as 0.71; against O_1 Campos (rabbit) as 0.07; against O_1 Campos (guinea pig) as 0.17.

Table 1 shows r values obtained with individual and pools of sera both 21 days post-vaccination and 16 days post re-vaccination.

In order to compare differences arising between individual sera and pooled sera r values, the differences between homologous and heterologous log SN_{50} readings were calculated. One-way analysis of variance was used to determine a pooled variance estimate for differences

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between sera within a treatment (this estimate was 0.039 on 34 degrees of freedom).

Mean values of the differences in Log SN_{50} were calculated for the various treatments. These means were then compared with the differences from the pooled samples by t tests, using the pooled estimate of variance.

There were no significant differences (at P = 0.05) in pooled vs. mean individual r values for either comparison at either 21 days post-vaccination or 16 days post-re-vaccination. Further comparisons using multivariate analysis of variance showed a statistically significant increase in specificity after re-vaccination.

EFFECT OF ANIMAL SPECIES

Guinea pig, rabbit and bovine antisera were used in these studies. Guinea pig antisera had been prepared to AEI-inactivated, sucrose-density gradient purified 140S antigens (Rweyemamu, et al, 1979); convalescent rabbit antisera were raised by intravenous inoculation of live BHK-tissue culture virus and the rabbit was exsanguinated at 28 days post-inoculation; bovine antisera were derived either from convalescent cattle or from monovalently vaccinated cattle.

The r values obtained with guinea pig antisera were compared with those obtained using other species antisera when testing the same virus strains. The comparison was on the basis of whether or not we would have drawn differnt conclusions on vaccine strain selection using pooled antisera from different species. Values of $r < 0.1$ were regarded as 0.1 and those > 1.00 were assumed to be 1.00.

Table 2 summarises the comparison of strain specificities obtained. r values obtained with guinea pig antisera were therefore compared with 63 obtained using convalescent rabbit antisera, 11 using convalescent bovine antisera and 27 pooled antisera from vaccinated cattle. Virus strains from 6 of the 7 FMDV serotypes were represented.

Bovine vaccinate and guinea pig antisera gave similar specificities (r value ratios within the range 1/2 to 2-fold were observed). Divergencies in strain specificity were observed with convalescent rabbit and bovine antisera. These divergencies are probably more related to the difference in antigen preparations i.e. inactivated versus live virus than to animal species differences. Since uncloned virus preparations were used it might be expected that convalescent animal sera are more likely to have a more heterogenous antibody population than antisera raised against inactivated antigens.

QUALITY OF IMMUNIZING ANTIGEN

The immunogenic determinant and neutralizing antigen determinant for FMD virus is generally accepted to be located on the trypsin-sensitive polypeptide (VP1) and is most effectively expressed by the intact capsid. Trypsin treatment of the capsid results, with many strains, in the reduction of immunogenicity as expressed by the diminished ability to provoke neutralizing antibody and this is believed to be a function of the cleavage of VP1 by trypsin. Although so-called trypsin resistant variants have been observed no virus strain has been reported to possess an uncleavable VP1 polypeptide. Whether the neutralizing antibody to trypsin-treated virus is of the same antigenic specificity as that against native virion, to the best of our knowledge, does not seem to have been elucidated.

Trypsin -treated virus was obtained by treating a concentrate of AEI-inactivated O_1BFS 1860 virus with 1 mg/ml trypsin at 37°C for 30 minutes. This material and a similar quality of untreated virus were sedimented on sucrose density gradients. The 140S antigens so obtained were emulsified with incomplete Freund's adjuvant and inoculated intramuscularly into groups of 10 guinea pigs. The guinea pigs were exsanguinated 28 days later and sera from groups of similarly vaccinated animals were pooled. The r values against O_9K120/64 virus for each pool were determined in two-dimensional microneutralization tests. This comparison was made at least 12 times to provide adequate data for statistical analysis.

The r value obtained for the untreated serum was 0.22 whereas that for the trypsin-treated serum was 0.35. This small difference was shown to be significant at the 5% confidence limit by both a 't' test and by analysis of variance. This result suggests that the trypsin-treated virus may have a broader serological specificity than the intact virion. The extent of this change is being investigated further.

EFFECT OF ANTIGEN DOSE ON THE SPECIFICITY OF ANTISERA

Groups of ten guinea pigs were inoculated with doses varying between 5 and 30 µg of AEI-inactivated, sucrose density gradient purified 140S antigen of strain O_1BFS 1860, emulsified in Freund's incomplete adjuvant, as specified in Tables 3 and 4. Samples of antigens were fractionated by SDS-polyacrylamide gel electrophoresis to ascertain that the virus proteins were intact. Five guinea pigs from each group were exsanguinated at 28 days post-inoculation to provide primo-vaccinate sera. The remaining five were re-inoculated with similar doses of antigen to those they received before and were exsanguinated 14 days later. The sera from guinea pigs in the same group were pooled. The pools were tested for strain specificity in the complement fixation test according to Forman (1974) against O_1BFS 1860, O Caseros, O_9K120/64 antigens as well as against the heterotypic antigen, C Noville and BHK cell lysate. They were also tested in the two-dimensional microneutralization test (Rweyemamu, et al, 1978) against the same homologous and heterologous virus strains. The O_9 K120/64 strain was selected as representing the East African vaccine strain and had previously been shown to be representative of African strains until at least 1977 (unpublished data). The O Caseros, which is the Argentinian vaccine virus, was chosen for this study because although it is generally regarded as belonging to the O_1 subtype in South America, our studies have shown it markedly differs serologically from the other O_1 virus strains we have examined.

Table 3 summarises the results of the complement fixation tests. Except for the highest antigen dose studied (31 µg), re-vaccination resulted in greater heterologous and heterotypic responses than after a single vaccination. In addition, sera from re-vaccinated animals demonstrated a strong anti-BHK response. This may account for part of the apparent heterotypic reaction with C Noville but as anti-BHK titres were always lower than those against 140S C Noville antigen it would not account for all of it. Nonetheless, results like these cast doubt on the validity of the continued use of complement fixation tests in FMD virus strain differentiation even when so called purified 140S antigens are used as inocula and in the test.

Table 4 summarises data from the two dimensional microneutralization test. The heterotypic reaction observed was of an extremely low order which for all practical purposes was inconsequential. We have not attempted to assess its specificity. In contrast to the CFT data, there was no evidence from this study that serum titre as a function of either antigen dose or booster responses altered significantly the strain specificity in the two-dimensional microneutralisation test.

CONCLUSIONS

The results that we present here suggest that when the antigen presentation is similar, although individual serum titres may vary from animal to animal and with the dose of the antigen, r values obtained by two-dimensional microneutralization tests with these sera are consistent. Other workers have reported a decreased specificity in the sera of animals after hyperimmunization. Our results are in conflict with this and, in fact, showed that the sera of cattle after revaccination were more specific than the sera from the same cattle after a primary vaccination.

We also have some evidence that sera from different species (i.e. guinea pig and cattle) give similar r values. Variations in the specificity of sera were observed where the antigen was altered by trypsin cleavage or was live FMDV.

We have shown in this paper that it is probably valid to use pools of sera rather than to test with many individual sera when determining antigenic relationships. It is proposed that for virus strain differentiation, pools of well characterized reference antisera should be prepared to inactivated antigens from vaccine virus seed stocks. We envisage two types of antisera:

1) Pools of guinea pig antisera
2) Pools of monovalently vaccinated cattle sera collected after re-vaccination since primo-vaccinate antisera tend to be of too low titre to cover an adequate antigenic spectrum.

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TABLE 1

r Values Obtained Using Individual and Pools of Sera from Cattle Vaccinated with an O_1 Caseros Vaccine

Serum No.	vs O_1 BFS 1860		vs O Malaysia 1/78	
	21 dpv	16 dprv	21 dpv	16 dprv
1	0.87	0.23	0.06	0.06
2	1.00	0.13	0.15	0.02
3	0.52	0.65	0.19	0.07
4	0.65	0.35	0.29	0.09
5	0.81	1.02	0.19	0.07
6	0.66	-	0.22	-
7	0.65	0.51	0.17	0.14
8	0.55	0.56	0.17	0.09
9	1.05	0.58	0.08	0.05
10	0.98	1.20	0.26	0.08
Mean Individual* $\underline{r}$	0.72	0.48	0.16	0.06
Pooled Serum	0.65	0.91	0.23	0.05

* determined from mean log $\underline{r}$ values

dpv = days post-vaccination

dprv = days post-revaccination

TABLE 2

Comparative Specificities of Antisera from Different Species

(Ratio of r values from guinea pigs : r values from other species)

Serum Type Compared	Virus Serotype	No. of sera showing following levels of divergence				
		<0.25	0.25-0.5	0.5-2.0	2.01-4	>4
Convalescent Rabbit	O	0	0	13	2	0
	A	1	2	7	0	0
	Asia 1	0	0	8	0	0
	SAT 1	0	0	1	0	0
	SAT 2	1	8	19	0	1
	Total 63	2	10	48	2	1
	(%)	(3)	(16)	(76)	(3)	(1.6)
Convalescent Bovine	O	2	2	4	0	0
	A	0	0	1	1	0
	SAT 2	0	0	0	1	0
	Total 11	2	2	5	2	0
	(%)	(18)	(18)	(45)	(18)	
Bovine Vaccinates	O	0	0	8	0	0
	C	0	0	2	0	0
	Asia 1	0	0	2	0	0
	SAT 2	0	0	14	0	1
	Total 27	0	0	26	0	1
	(%)	(0)	(0)	(96)	(0)	(4)

TABLE 3

Effect of Antigen Dose on Strain Specificity

(Complement Fixation Responses)

140S Primary Dose (µg)	140S Secondary Dose (µg)	Log_{10} Complement Fixing Antibody titre to the following antigens (r values in bracket)				
		O_1BFS 1860 (Homologous)	O Caseros	O_9K120/64	C Noville	BHK Lysate
5	-	1.35	< 0.9 (< 0.35)	< 0.9 (< 0.35)	< 0.9 (< 0.35)	N.T.
8	-	2.25	0.9 (0.04)	1.65 (0.25)	0.9 (0.04)	< 0.9 (< 0.04)
8	10	2.7	1.65 (0.09)	2.25 (0.35)	1.8 (0.13)	1.36 (0.05)
18	-	1.95	< 0.9 (< 0.09)	1.35 (0.25)	< 0.9 (< 0.09)	1.05 (0.13)
18	14	3.15	2.85 (0.50)	3.15 (1.00)	2.7 (0.35)	2.33 (0.15)
24	-	2.55	1.5 (0.09)	1.95 (0.25)	< 0.9 (< 0.02)	0.9 (0.02)
24	24	2.85	2.25 (0.25)	2.7 (0.71)	2.55 (0.50)	2.18 (0.21)
31	-	2.25	1.95 (0.50)	1.65 (0.25)	1.65 (0.25)	1.05 (0.06)
31	30	3.15	2.55 (0.25)	3.15 (1.00)	2.55 (0.25)	1.96 (0.06)
31 (BHK absorbed serum)	30	2.4	1.65 (0.18)	2.1 (0.5)	1.5 (0.13)	< 0.9 (< 0.03)

N.T. = Not Tested

TABLE 4

Effect of Antigen Dose on Strain Specificity

(Virus Neutralization Responses)

Primary Dose (µg 140S)	Secondary Dose (µg 140S)	Mean Log_{10} SN 50 against following Viruses (r values in brackets)			
		O_1BFS 1860	O Caseros	O_9K120/64	C Noville
5	-	2.56	<1.2 (<0.04)	1.82 (0.18)	<1.2 (<0.04)
8	-	3.09	1.29 (0.016)	2.41 (0.21)	
8	10	3.92	1.73 (0.006)	3.42 (0.32)	
18	-	3.69	1.01 (0.002)	2.81 (0.13)	
18	14	4.32	1.69 (0.002)	3.80 (0.30)	
24	-	3.83	1.56 (0.005)	3.19 (0.23)	
24	24	4.71	1.82 (0.001)	3.78 (0.12)	
31	-	3.76	1.67 (0.008)	3.12 (0.23)	1.05 (0.002)
31	30	4.81	1.96 (0.001)	4.14 (0.21)	1.40 (0.0004)