VESICULAR STOMATITIS AND FOOT-AND-MOUTH DISEASE: ANALYSIS OF MIXED INFECTION IN CATTLE

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The occurrence in the same locality and at the same time of the viruses of both vesicular stomatitis and foot-and-mouth disease is a possibility disquieting to those who are responsible for the control of vesicular diseases of cattle and swine. This is particularly liable to occur in countries in which vesicular stomatitis is enzootic and in which relatively little attention is paid to this condition. In these circumstances it is feared that the existence of foot-and-mouth disease may be masked or obscured by the milder vesicular stomatitis until such time as it has become widespread in the animal population.

The possibility of concurrent infection with the two viruses has been suggested (Galloway & Elford, 1935), but the problem has not been studied in cattle before, and evidence cannot, therefore, be adduced on the probable behaviour of a mixture of the viruses during passage by natural transmission in a population of susceptible cattle. Our present knowledge, supported by the evidence to be given here, suggests that a mixed infection is possible if the requisite field conditions exist. It is less probable that a mixed infection could continue to exist during the spread of the disease without the stronger component becoming dominant, resulting in the extinction of the weaker virus. The occurrence of mixtures consisting predominantly of the virus of vesicular stomatitis would be favoured if the susceptibility of the cattle population to foot-and-mouth disease were modified by vaccination, especially if the immunity to foot-and-mouth disease were waning and the virus of foot-and-mouth disease were gaining a hold among the animals. The possibility of such a set of circumstances emphasizes the need for information on the reliability of methods for the detection of the virus of foot-and-mouth disease in the hypothetical early stage of the mixed outbreak when the virus of vesicular stomatitis should be present in great excess.

The differentiation of the viruses of vesicular stomatitis and foot-and-mouth disease has been discussed by Galloway (1936), and a method has been described for using the complement-fixation test (Brooksby, 1948). Some of the methods discussed are of uncertain value when the detection of a small amount of one virus in a mixture is required. In the resolution of mixtures, Galloway & Elford (1935) took advantage of the pathogenicity of the virus of vesicular stomatitis for the chick embryo in order readily to detect small amounts of this virus. More important, however, from the disease control aspect, is the possibility of detection of a small amount of the virus of foot-and-mouth disease which causes the more dangerous disease. For the detection of small amounts of the virus of foot-and mouth disease (10 M.I.D. or less), Galloway & Elford were successful in applying a filtration method, in which 'Gradocol' membranes of selected porosity were used to retain the larger virus and allow the virus of foot-and-mouth disease to pass.

In these experiments, the initial materials were mixtures of filtrates of guinea-pig adapted strains of the two viruses. The use of a similar filtration technique for materials of bovine or porcine origin is made difficult by the problem of species adaptation of the field virus, so that it is necessary to use cattle or swine for testing the filtrates. It is also possible that the quantity of the virus of foot-and-mouth disease in the original material might be insufficient to allow for the preparation of suitably clarified suspensions for filtration while retaining infectivity. For this reason the method reported in this paper is to be preferred, since a crude suspension of the original material can be used. The complement-fixation test can be dismissed on quantitative grounds as high initial titres of virus are necessary for success.

Shahan, Frank & Mott (1946) have referred to the conflicting evidence on the susceptibility of the sheep to vesicular stomatitis. Their own evidence, based on the inoculation of sixty-eight sheep, would suggest that this species is refractory, but too little is known of the susceptibility of sheep to varying quantities and different strains of the virus of foot-and-mouth disease for it to be used for differentiating these two viruses.

The experiments to be described here were therefore undertaken to study the possible use of cross-immunity tests in this field. Complement-fixation was used as an ancillary method.

EXPERIMENTS

The vesicular stomatitis viruses were the stock strains in use at Pirbright, Ind.C of the Indiana type and NJ.M of the New Jersey type (Brooksby, 1949). The strain of the virus of foot-and-mouth disease was the Pirbright strain 39 of Vallée O type. It had received seventy-nine passages in cattle at this Institute. The technique of complement fixation used was that described by the author (Brooksby, 1948), in which the degree of fixation is referred to the log dose of complement necessary for 50 % haemolysis. The figures given in Tables 1 and 2 represent the differences between the amount of complement necessary for 50 % haemolysis in the presence of the fixing system and the amount necessary in the control systems.

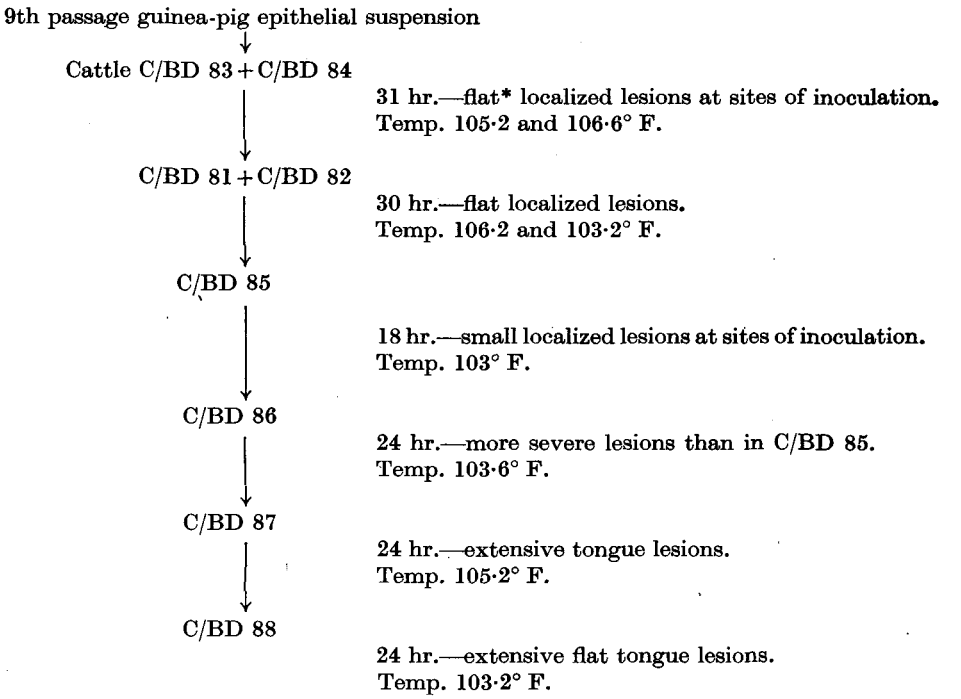
(1) *Preliminary passage of vesicular stomatitis strains of virus*

Strain Ind.C. An attempt to revive this strain from dried bovine epithelium which had been stored for 4 years having failed, a series of passages was made beginning from material of the 9th passage of the strain in guinea-pigs. The material passaged was bovine lingual epithelium obtained from the lesions and ground with sand in 0·85 % sodium chloride solution buffered with M/200 phosphate. The details of this serial passage were as shown in Diagram 1.

Only two animals in this series of six passages showed any sign of secondary lesions. C/BD 83 at 5 days after inoculation had a temperature of 103·4° F. and on the next day there were redness and marked pain on pressure in the interdigital spaces of both fore-feet. No vesicular lesions could be detected then or later. C/BD 88 developed a small lesion on the lower lip on the second day after inocula-

tion. A suspension of epithelium collected from a vesicular lesion in C/BD 88 (6th passage of strain Ind.C) was titrated in two cattle, C/BD 91 and C/BD 92 by the method described by Henderson (1949). A filtrate (Gradocol A.P.D. 0·71μ) of a 1/10 epithelial suspension had a titre of $10^{-2.1}$.

Diagram 1



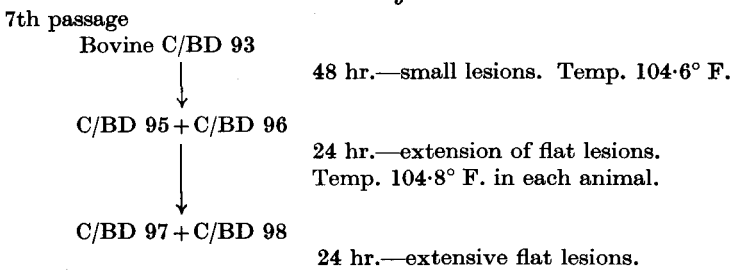
* 'Flat' lesions are described in contrast to the severe vesicles frequently seen in foot-and-mouth disease. The surface epithelium in the early lesion in vesicular stomatitis is frequently not perceptibly raised, though of a whiter colour than the normal epithelium. It is not readily separated from the underlying tissue.

Strain NJ.M. This strain was revived from dried bovine vesicular epithelium stored for 4 years at 4° C. Two cattle in one loose box were inoculated, one with a suspension of the dried material and one with a suspension of a sample of the same epithelium which had been stored frozen. The animal (C/BD 93) which received the suspension of dried material reacted 48 hr. after inoculation with lesions on the tongue on one portion of the area inoculated. The other animal (C/BD 94) failed to react to the initial inoculation of frozen material, but was found, 7 days after the appearance of lesions in C/BD 93, to have a ruptured lesion on the tongue and another on the upper lip. This reaction was presumably due to contact infection from C/BD 93. There was no thermal reaction in C/BD 94, although C/BD 93 had had a temperature of 104·6° F. on the appearance of the initial tongue lesion and of 104·8° F. on the appearance of a lip lesion 3 days later.

The details of serial passage of this strain are shown in Diagram 2. Secondary lesions, on the lower lip, were observed in C/BD 95 and C/BD 96, 6 days and 1 day, respectively, after the appearance of primary lesions.

An 0·57 μ Gradocol membrane filtrate of a 1/25 suspension of bovine epithelium from C/BD 95 and C/BD 96 (8th passage of strain NJ.M) was titrated in the same way as strain Ind.C in two cattle C/BD 99 and C/BE 0. The titre of the NJ.M filtrate was $10^{-2\cdot3}$.

Diagram 2



(2) *Verification of identity of strains by cross-immunity tests*

Although complement-fixation tests on material from each series of passages had confirmed the presence of virus of the correct immunological type, a cross-immunity test was carried out with each of the two strains. This was considered a desirable check for the freedom of each type from traces of the other which could not be detected by complement fixation. Information was also required on the degree of the immunity of recovered animals before proceeding to the experiments involving the virus of foot-and-mouth disease.

Strain Ind.C. A 1/25 suspension of the bovine epithelium from C/BD 87 (5th passage of the strain) was filtered through a Gradocol membrane of A.P.D. 0·71 μ. This filtrate was titrated, in the same manner as earlier preparations, in two cattle, C/BE 85 and C/BE 86, and the titre was found to be $10^{-3\cdot5}$. At the same time, this undiluted filtrate was inoculated intradermally into the tongues of the following four cattle:

C/BD 91 and C/BD 92 (cattle which had reacted to infection with strain Ind.C 30 days previously).

C/BD 97 and C/BD 98 (cattle which had reacted to infection with strain NJ.M 22 days previously).

Animals C/BD 91 and C/BD 92 did not react to this inoculation.

Animals C/BD 97 and C/BD 98 developed lesions at the site of inoculation 1 and 2 days after inoculation respectively. There were no secondary lesions when the animals were destroyed, 4 days after infection.

Strain NJ.M. A 1/25 suspension of bovine epithelium from animals C/BD 97 and C/BD 98 (9th passage of strain NJ.M) was prepared and filtered through a Gradocol membrane of A.P.D. 0·71 μ. This filtrate, titrated in two cattle C/BE 87 and C/BE 88 had a titre of 10^{-3}. The undiluted filtrate was inoculated intradermally into the tongues of the following four cattle:

C/BD 83 and C/BD 84 (cattle which reacted to Ind.C infection 51 days previously).

C/BD 95 and C/BD 96 (cattle which reacted to NJ.M infection 30 days previously).

Animals C/BD 95 and C/BD 96 did not react to this inoculation.

Animals C/BD 83 and C/BD 84 developed flat lesions at the sites of inoculation on the tongue 48 hr. after inoculation. No secondary lesions were observed.

The results of these two cross-immunity experiments confirm the purity of the two strains examined, and at the same time demonstrate the validity of the method of carrying out the tests using the intradermal inoculation of the tongue as the route for the second, or challenging, inoculation. It would be of interest to investigate the duration of immunity to this method of challenge. In the absence of this information it would appear to be justifiable to use animals in the first 2 months after recovery from initial infection. The development of the local lesions following inoculation of the virus of the heterologous type does not appear to be inhibited, although all animals used had recovered for 22 days or more from the initial infection.

(3) *Cross-immunity studies with mixtures of the viruses of vesicular stomatitis and foot-and-mouth disease*

First experiment

The components of the mixture were first prepared and titrated as follows:

Vesicular stomatitis strain NJ.M. A 1/10 suspension of epithelium from C/BD 97 and C/BD 98 (9th passage of strain NJ.M stored for 5 weeks) was prepared and titrated in two cattle. The titre of this suspension was $10^{-3\cdot4}$.

Foot-and-mouth disease strain 39 (*Vallée O type*). A 1/10 suspension of epithelium from C/BB 6 (79th passage of strain 39 stored for 8 months) was prepared and titrated in two cattle. The titre of this suspension was 10^{-4}.

A mixture was prepared by pipetting 1 ml. of a 10^{-2} dilution of the strain 39 suspension into 9 ml. of the undiluted strain NJ.M suspension. The amount of the strain 39 suspension was estimated on the basis of a reading of the titration before all lesions had developed, and therefore the titre was underestimated so that more of the foot-and-mouth disease suspension was used than had been intended. In terms of I.D. 50 (the difference between the reciprocal of the 50 % end-point of the suspension and the dilution used) the mixture contained, per dose inoculated at each separate site on the bovine tongue, 10 I.D.50 of strain 39 (foot-and-mouth disease) and 2500 I.D.50 of strain NJ.M (vesicular stomatitis).

The mixture was then inoculated into the tongues of the following cattle, by the intradermal route, about ten sites being inoculated on each tongue:

C/BF 3 and C/BF 4—normal cattle.

C/BD 99 and C/BE 0—cattle which had reacted to inoculation with strain NJ.M (V.S.) 33 days previously.

C/BD 91 and C/BD 92—these cattle had reacted to inoculation with strain Ind.C (V.S.) 48 days previously and had resisted reinfection with the same strain 32 days later.

The subsequent history of these animals is given in Table 1, together with the result of complement-fixation tests on samples of epithelium collected at the times indicated from the primary lesions on the tongues. It may be noted that the

Table 1

Animal no.	Day 1: 22 hr.	Day 1: 28 hr.	Day 2	Day 3	Day 5	Complement fixation: Log dose complement fixed with epithelium from primary lesion and serum: O (F. & M.)	NJ.M (V.S.)	Ind.C. (V.S.)
C/BF 3 (normal)	104·4° F. Full vesicular lesions covering all sites of inoculation. Epithelium collected. Animal destroyed	—	—	—	—	0·79	0·68	0·12
C/BF 4 (normal)	102° F. No lesions yet visible	102·4° F. Flat* lesions at sites of inoculation. Epithelium collected	101·4° F. Small lesions on right fore-feet and left hind-feet	—	—	0·91	0·62	0·10
C/BD 99 (NJ.M immune)	100° F. Small tongue lesion spreading from a vesicle about 1 cm. diameter	101·2° F. Slight extension of lesion. Many sites still negative	102° F. No further lesions	103·4° F. Vesicular lesion on dental pad	101° F. Lesions all four feet. Animal destroyed	0·88	0·12	0·09
C/BE 0 (NJ.M immune)	100° F. All sites negative	101·8 F. Small flat lesions at several sites of inoculation	101° F. No further lesions. Epithelium collected	102·6° F. Lesions on right hind-foot	100·6° F. Lesions all four feet. Animal destroyed	0·77	0·12	0·10
C/BD 91 (Ind.C immune)	101° F. Flat lesions covering all sites of inoculation	—	100·4° F. No further lesions. Epithelium collected	102° F. Lesions on both fore-feet	100·2° F. Lesions all four feet. Animal destroyed	0·63	0·59	0·13
C/BD 92 (Ind.C immune)	104·6° F. Vesicular lesions covering all sites of inoculation. Epithelium collected	—	—	—	—	0·94	0·58	0·10

* 'Flat' lesions are described in contrast to the severe vesicles frequently seen in foot-and-mouth disease. The surface epithelium in the early lesion in vesicular stomatitis is frequently not perceptibly raised, though of a whiter colour than the normal epithelium. It is not readily separated from the underlying tissue.

O(F. & M.) = Vallée O type of virus of foot-and-mouth disease; NJ.M(V.S.) and Ind.C(V.S.) = New Jersey and Indiana types of vesicular stomatitis virus.

poorest primary lesions were those in the cattle immune to strain NJ.M (C/BD 99 and C/BE 0), although the difference between these and the lesions in the 'normal' animal C/BF 4 was not great. All four animals which were allowed to survive for more than 28 hr. after inoculation developed secondary lesions on the feet and, in view of the infrequent occurrence of foot lesions in vesicular stomatitis infection, this would confirm the presence of the virus of foot-and-mouth disease. The evidence supplied by the complement-fixation test is most illuminating. All six cattle gave evidence of infection with the virus of foot-and-mouth disease, Vallée O type, and in the four not immune to the New Jersey type of vesicular stomatitis, concurrent infection with strain NJ.M. In these virus samples from the NJ.M immune animals only the virus of foot-and-mouth disease of the Vallée O type could be detected by complement-fixation tests. These results suggest that use of the cattle immune to the two types of vesicular stomatitis readily brings to light the small proportion (1/250th) of the virus of foot-and-mouth disease in the original mixture.

Second experiment

In this experiment the quantity of the virus of foot-and-mouth disease in the mixture was reduced to a level at which it could be expected that not all samples inoculated would produce lesions of the disease. The sample of the virus of vesicular stomatitis was, however, of lower titre than in Exp. 1 so that the ratio of the virus of vesicular stomatitis to that of foot-and-mouth disease was not greatly altered. It was 300 : 1 compared with 250 : 1 in the first experiment. The data on the components of the mixture were as follows:

Vesicular stomatitis strain Ind.C. A 1/10 suspension of epithelium from C/BD 87 (5th passage of strain Ind.C stored 10 weeks) was prepared and titrated in two cattle. The titre of the suspension was $10^{-2.6}$.

Foot-and-mouth disease strain 39 (*Vallée O type*). A 1/10 suspension of epithelium from C/BB 6 (79th passage of strain 39 stored 8 months) was prepared and titrated in two cattle. The titre of this suspension was $10^{-5.1}$.

A mixture was prepared by pipetting 1 ml. of a 10^{-4} dilution of the strain 39 (foot-and-mouth disease) suspension into 9 ml. of the undiluted suspension of strain Ind.C (vesicular stomatitis). In terms of I.D. 50, the mixture contained per dose inoculated at each site on the bovine tongue, 1·3 I.D. 50 of strain 39 and 400 I.D. 50 of strain Ind. C.

The mixture was than inoculated intradermally into the tongues of the following cattle at ten sites on each tongue:

C/BF 39 and C/BF 40 (two normal cattle).
C/BD 81 and C/BD 82 (two cattle which developed lesions after inoculation with strain Ind.C (V.S.) 73 days previously).
C/BD 95 and C/BD 96 (two cattle which developed lesions after inoculation with Strain NJ.M (V.S.) 54 days previously).

The reactions of these animals and the result of complement-fixation tests on epithelium collected from the primary lesions are shown in Table 2. The data in

Table 2

Animal no.	Day 1		Day 2	Day 3	Day 4	Day 5	Day 6	Complement fixation: Log dose complement fixed with epithelium from primary lesion and serum		
	22 hr.	28 hr.						O (F. & M.)	NJ.M (V.S.)	Ind.C (V.S.)
C/BF 39 (normal)	101·8° F. Negative at all sites	102·6° F. Small lesion at one site of inoculation	101·8° F. Lesion now 2·5 cm. in diameter. Epithelium collected	101° F. No further lesions	104·8° F.	102° F. Lesions both fore-feet	Lesions all four feet	0	0·08	>0·8
C/BF 40 (normal)	102·6° F. Vesicular lesions at all sites	103·6° F. Epithelium collected	101·6° F. No further lesions	101·6° F. No further lesions	103·4° F.	102·8° F. Lesions all four feet	—	0·02	0·05	>0·8
C/BD 81 (Ind.C immune)	101·4° F. One site showing vesicular lesions	102·6° F. Slight extension from one site. Others negative. Epithelium collected	105·4° F. Two vesicular lesions on dental pad	101·6° F. Lesions on left fore-foot	102·2° F.	101° F. No further lesions	Lesions all four feet	>0·8	0	0
C/BD 82 (Ind.C immune)	101° F. Negative at *all sites*	102° F. Negative at *all sites*	101·2° F. Negative at *all sites*	100·6° F. Negative	103·4° F.	100·8° F. Lesions over all inoculated area of tongue, also ruptured lower lip	Lesion left fore-foot	No test—insufficient material		
C/BD 95 (NJ.M immune)	100·4° F. Irregular blanching at sites inoculated. No definite lesions	105·2° F. Flat extensive lesions at all sites. Epithelium collected	104° F. Further lesions on tongue	101·8° F. Lesions on dental pad and both fore feet	103·2° F.	102·6° F. Lesions all four feet	—	>0·8	0·04	>0·8
C/BD 96 (NJ.M immune)	107·2° F. Negative at all sites	105° F. Slightly vesicular lesions. Epithelium collected	101° F. No further lesions	104° F. Lesions left hind-foot	104° F.	103° F. Lesions dental pad and all four feet	—	0·05	0·05	>0·8

O(F. & M.) = Vallée type of virus of foot-and-mouth disease; NJ.M(V.S.) and Ind.C(V.S.) = New Jersey and Indiana types of vesicular stomatitis virus.

the table appear to justify the following conclusions relating to the individual animals.

(1) *C/BF* 39. The only virus detected by complement fixation with epithelium collected at 48 hr. was vesicular stomatitis, and the infection would therefore appear to be predominantly due to this virus. Nevertheless, the animal did later (at 5 days) develop severe lesions on the feet and must be considered to have become infected with the virus of foot-and-mouth disease.

(2) *C/BF* 40. The history of this animal is similar to that of C/BF 39, except that the initial lesions were more severe.

(3) *C/BD* 81. The immunity of this animal to the virus of vesicular stomatitis type Indiana is demonstrated by the result of the complement-fixation test in which only the virus of foot-and-mouth disease, Vallée O type, was detected.

(4) *C/BD* 82. The reaction of this animal, at 5 days after inoculation, would seem to have been due to contact infection from C/BD 81 with which it was confined in a loose box.

(5) *C/BD* 95 *and C/BD* 96. These two animals appear to have developed a mixed infection, differing only in that the multiplication of Vallée O type virus of foot-and-mouth disease was much less in C/BD 96 than in C/BD 95, as judged by the result of the complement-fixation test.

In the conditions of minimal infecting dosage with the virus of foot-and-mouth disease in this mixture, evidence of multiplication was found in five of the six animals.

DISCUSSION

The two experiments with mixtures of the two viruses support the view that the detection of small amounts of the virus of foot-and-mouth disease in the presence of excess of the virus of vesicular stomatitis can be effected simply and conclusively by the inoculation of the suspected mixture into cattle immune to vesicular stomatitis, one group of animals immune to each of the two immunological types being used. The limitation on recovery of the virus of foot-and-mouth disease would appear to be no greater than that imposed on its detection in normal cattle, in the unmixed suspension, since, when the small dosage of 1·3 I.D.50 was used, five animals inoculated reacted with lesions of foot-and-mouth disease, and the remaining animal in the experiment, a vesicular stomatitis immune, later became diseased as a result of contact infection. In other words, immunity to vesicular stomatitis does not prevent the detection of small amounts of the virus of foot-and-mouth disease.

The delicacy of the test for detecting a trace of foot-and-mouth disease virus in a given mixture will obviously be governed by economic and practical considerations, rather than by the statistical reasoning on how many cattle will be needed to detect a certain low concentration of the virus. The inevitable outcome of inoculation of the two mixtures described appeared to be the development of a mixed infection in the cattle susceptible to both viruses. This gives some support to the view that a hypothetical mixed infection in the field is unlikely to exist

through many natural passages without clinical manifestations of foot-and-mouth disease or the detection in complement-fixation tests of antigens of foot-and-mouth disease.

The strain of the virus of foot-and-mouth disease used in these experiments is not regarded as highly invasive, but no doubt strains of even lower invasiveness exist. In the event of mixtures occurring, involving such strains, the possibility of the virus of foot-and-mouth disease being supplanted by that of vesicular stomatitis under certain conditions, although less likely than the alternative, cannot be definitely excluded.

The importance of these experiments lies in their application to the problem outlined in the introduction to this paper. The conclusion must be that, if repeated tests on field samples of virus from areas where vesicular stomatitis is enzootic are carried out in cattle immune to this virus, it is very unlikely that the virus of foot-and-mouth disease will go for long undetected.

The method, however, does require a special organization to maintain cattle immune to strains of vesicular stomatitis. The maintenance of a supply of suitable animals might be a serious obstacle in some countries unless cattle could be obtained from an area where foot-and-mouth disease had not occurred and no vaccination had been practised against this disease. Nevertheless, for the reasons which have been stated earlier in this paper, the method described would appear to be the most satisfactory available at the present time for the early detection of traces of the virus of foot-and-mouth disease.

SUMMARY

1. It has been found possible to detect traces of the virus of foot-and-mouth disease in mixtures of this virus and that of vesicular stomatitis by cross-immunity tests in cattle. A quantity of the virus of foot-and-mouth disease as small as 1·3 I.D.50 was revealed by the inoculation of such mixtures into cattle immune to vesicular stomatitis.

2. It is suggested that such a method would be useful in the analysis of possible mixed infections in field outbreaks.

3. The usefulness of complement-fixation tests to confirm the resolution of mixtures has been demonstrated.

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