

## SIGNIFICANCE OF TESTS FOR NON-INFECTIVITY OF FOOT-AND-MOUTH DISEASE VACCINES

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There are circumstances in which vaccination is an essential part of any programme for controlling the spread of foot-and-mouth disease. The methods of preparation of vaccine in current use involve the modification or inactivation of the virus by physical and/or chemical treatment and aim at producing a final product which is non-infective but of good immunizing value. Since the object of a test for non-infectivity is to detect the presence of any active virus in a vaccine, the most sensitive test available should be used. The general design must first be considered of any test for detection of a phenomenon of probably low frequency, and then the best means must be sought of applying such a design to the particular problem under consideration, i.e. the detection of foot-and-mouth disease virus by the inoculation of a susceptible host.

The presence of active virus may be proved by the result of one test, but no finite number of tests will ever prove the absence of active virus. If no active virus is detected in a test of a number of samples of vaccine it is, nevertheless, possible to calculate the greatest amount of virus that might be present and still be likely to give negative results in a test of that number of samples. This is a calculation of the upper limit of the probability of the occurrence of active virus. The greater the number of samples examined and found negative, the lesser will be the likelihood of any postulated percentage of infective samples and the greater will be the reliability of the test for non-infectivity.

By the binomial distribution, if  $p$  is the probability that a sample is positive and  $P$  is the chance of failure to detect a positive in  $n$  samples, then

$$(1 - p)^n = P$$

or 
$$p = 1 - q, \quad \text{when} \quad \log q = \frac{\log P}{n}.$$

The highest percentage of samples likely to be infective for various numbers of negative observations can thus be calculated for different levels of  $P$  (Table 1). The lower limit of probability of the occurrence of active virus may, of course, be zero if the inactivation treatment in the preparation of the vaccine has been complete, but the impossibility of proving the absence of a phenomenon restricts consideration to the upper limit.

Table 1 shows that a small number of negative observations may be consistent with a comparatively high frequency of infective samples, and that about one hundred observations have to be made before the possible percentage of infective samples begins to tail off. Up to about 100, any increase in the number of observations made greatly decreases the value of  $p$ , whereas this decrease becomes very

Table 1. *Highest percentage of samples likely to be infective for various numbers of observations, all being negative*

No. of observations all negative	Highest percentage of samples likely to be infective when $P$ , the chance of failing to detect a positive sample, equals		
	0.05	0.01	0.001
2	77.6	90.0	96.8
4	52.7	68.4	82.2
8	31.2	43.8	57.8
10	25.9	36.9	49.9
20	13.9	20.6	29.2
40	7.2	10.9	15.9
60	4.9	7.4	10.9
80	3.7	5.6	8.3
100	3.0	4.5	6.7
120	2.5	3.8	5.6
250	1.2	1.8	2.7
500	0.6	0.9	1.4
1000	0.3	0.5	0.7

much less for any increase in the number of observations beyond this point. The adequacy of a test for non-infectivity is usually limited by the number of samples that it is practicable to test, but what is the upper limit to the possible presence of active virus that can be accepted? How many observations must be made in order to be reasonably sure of detecting any active virus in excess of this amount? In other words, what levels of  $p$  and  $P$  can be accepted as being adequate and what is the corresponding value of  $n$ ? Table 2 provides similar information to Table 1, but it is arranged differently in an attempt to make it easier to appreciate the significance of these points.

Table 2. *Number of observations,  $n$ , required to attain various levels of  $p$  and  $P$  when  $(1-p)^n = P$*

$P \backslash p$	0.05	0.025	0.01	0.001	0.0001
0.1	29	36	44	66	88
0.075	39	48	60	89	119
0.05	59	72	90	135	180
0.04	74	91	113	170	226
0.03	99	122	152	227	303
0.02	149	183	229	343	457
0.01	299	368	459	689	918
0.001	3026	3726	4652	6977	9303

In Table 2,  $p$  is the probability of an event occurring and is represented by various percentages of infective samples. Different values of  $P$ , the chance of failing to detect a positive sample are shown. For  $P=0.05$ , or 1 in 20, we should expect failure to detect a positive sample once in 20 trials; for  $P=0.025$ , once in 40 trials; for  $P=0.01$ , once in 100 trials; for  $P=0.001$ , once in 1000 trials. The values of  $n$

are the numbers of observations necessary to attain the corresponding levels of  $p$  and  $P$ . For example, if we tested 100 samples and found them all negative we would expect to be correct 19 times out of 20 in assuming that the percentage of infective samples would not exceed 3.0. Values falling beyond the 0.05 and 0.01 levels are sometimes described as being unlikely and highly unlikely respectively.

Consideration of the conventional level of significance,  $P = 0.05$ , may assist in reaching a decision as to what levels of  $p$  and  $P$  are adequate. It would hardly be acceptable to be less exacting, but with the risk involved, even  $P = 0.05$  is too great a chance of failure to detect the presence of active virus, and 5% of samples infective is a rather high percentage to accept as a possibility. If no other factors had to be considered, the 0.01 level for  $p$  and  $P$  would appear to be more adequate, as it would then be highly unlikely that more than 1% of samples were infective, but this demands a total of 459 observations which, in most biological work, it would be quite impracticable to provide. As a compromise, 100 observations would seem a more reasonable figure to choose, and, if all the observations were negative, the percentage of infective samples would be unlikely ( $P = 0.05$ ) to exceed 3.0 and highly unlikely ( $P = 0.01$ ) to exceed 4.5 (see Table 1).

The discussion, so far, is applicable to any test for the detection of anything of low frequency, but further discussion must be related to the particular test in question, as various factors may make it easier or more difficult to reach an adequate standard of reliability. Some biological products, for example, can be prepared and tested *in vitro* using a concentrated material that may then be greatly diluted before issue, whereas others necessitate the inoculation of an expensive susceptible animal for the provision of each observation.

In a test for non-infectivity it is obvious that the most sensitive means must be used for detection of active virus as, if any is present, the amount is likely to be small. Very small quantities of foot-and-mouth disease virus can only be detected by inoculation of a susceptible host and, in general, the most sensitive host is the one to which the virus strain is adapted. Foot-and-mouth disease vaccines are prepared, almost without exception, from strains of cattle adaptation, and the most sensitive animals for use in the test for non-infectivity are, consequently, susceptible cattle. The possibility of using unweaned mice for tests of the non-infectivity of vaccines cannot, however, be excluded in view of the observations of Skinner (1951) that they may be as sensitive as cattle when judged by the results of comparative titrations of bovine virus suspensions. It is not yet possible to give an assessment of their value in the tests for the non-infectivity of vaccines. A number of factors must be considered in choosing the route of inoculation of the cattle in a test for non-infectivity. The significance of some of these factors has been studied experimentally, particularly in a comparison of intradermal tongue inoculation and subcutaneous inoculation.

#### METHODS

Most of the methods employed in the present experiments have been described in the previous paper (Henderson, 1952). Reference is made to certain virus strains being of 'S.A.T. 1', 'S.A.T. 2' or 'S.A.T. 3' type. These are three new immuno-

logical virus types identified while examining strains recovered from outbreaks of foot-and-mouth disease in Africa (Galloway, 1950; Galloway, Brooksby & Henderson, unpublished work).

#### RESULTS

##### *The relative sensitivity of the method of inoculation.*

A comparison in cattle of intradermal tongue inoculation with subcutaneous, intracutaneous and intravenous inoculation showed tongue inoculation to be the most sensitive (Henderson, 1952). With some strains there was no significant difference in the amount of virus required to infect by the subcutaneous route, and with such a strain there might be an advantage in using this route because of the larger volume that could be inoculated. With other strains, however, when perhaps as much as 250,000 times more virus was required to infect by the subcutaneous route than by the tongue route, it would be quite useless attempting to use the subcutaneous route for the detection of minimal quantities of virus.

##### *Number of observations that can be provided and volume of material that can be tested by the intradermal tongue route and by the subcutaneous route*

With intradermal inoculation of the tongue it is possible to have as many as twenty separate sites on one tongue inoculated with about 0.1 ml., thus one animal can provide twenty observations. If other routes are used, however, each animal can provide only one observation, but the volume inoculated can greatly exceed that used by the tongue route and, presumably, the chance of including widely dispersed particles of active virus would be increased. For example, one observation from the subcutaneous inoculation of 100 ml. would be based on 1000 times the volume of vaccine providing each intradermal tongue inoculation.

##### *Incubation period following inoculation of active virus by the intradermal tongue route and by the subcutaneous route*

The intradermal inoculation of the tongue with a series of dilutions in a titration of a virus suspension invariably shows that lesions are slower in developing at those sites receiving least virus. For example, sites receiving the limiting infective dilution may show no reaction until about 30 hr. after inoculation compared with the 18–24 hr. required for the development of lesions at sites receiving 100–1000 times more virus. With inoculations consisting only of minimal infective doses, where the complication in a titration of the development of secondary lesions can be excluded, it is exceptional for a positive reaction to appear later than 48 hr. after inoculation.

After subcutaneous inoculation, the incubation period is always a matter of days and not hours. In titration experiments with six strains of virus in which the subcutaneous route was used (Henderson, 1952), the mean incubation period for a total of 41 reactors was  $5.9 \pm 2.6$  days, with the longest incubation period in those animals which had received least virus.

The importance of considering the incubation period is that a test for non-

infectivity consists of the inoculation of possibly a trace of active virus along with virus inactivated but of good antigenic potency. A reaction will usually be produced by the intradermal tongue inoculation of an infective dose of virus in less time than an immune response can be detected. Considerable protection can be demonstrated by the fifth day after the subcutaneous inoculation of vaccine (unpublished work). This might limit the chance of detecting a trace of active virus in a vaccine inoculated subcutaneously. These expectations are confirmed by the result of the following experiment.

*Detection of active virus added to an inactivated virus vaccine*

An incubated blood vaccine, Vac/CV/Bec 1 111, was prepared from strain Bec 1 (S.A.T. 1 type) for use in another set of experiments, as a result of which it was found that no active virus could be detected in the vaccine by the intradermal tongue inoculation of ten cattle at ten sites each and the inactivated virus was shown to be antigenic in that of forty cattle vaccinated with 100 ml. doses subcutaneously, twenty-nine were completely protected against the development of secondary lesions when later inoculated on the tongue with active virus.

A filtrate of a suspension of freshly collected strain Bec 1 bovine tongue epithelium was prepared and titrated by the intradermal tongue route in two cattle; the 50 % positive end-point dilution was  $10^{-5.1}$ . Since the object of this experiment was to determine whether a small quantity of active virus could be detected in the presence of a large amount of inactivated virus vaccine, it was decided to add sufficient active virus to the vaccine to make 5 % the proportion of infective samples of 0.1 ml. volume. The required amount of virus filtrate for addition to the vaccine was calculated as follows: by the Poisson distribution, if  $m$  is the mean number of infectious units per sample, the proportion of samples containing no infectious units is  $e^{-m}$ ; thus the mean number of infectious units corresponding to any percentage of infective samples can be calculated (see Fisher, 1941, § 17). The mean number of infectious units corresponding to 5 % infective samples is 0.05 per sample. If, therefore, there are 5 % infective samples in amounts of 0.1 ml., there will be a mean count of 500 infectious units per litre. It has usually been considered sufficiently accurate with these titrations of foot-and-mouth disease virus to take the reciprocal of the 50 % end-point dilution as an estimate of the number of infectious units per sample of the undiluted filtrate, so 10 ml. of the  $10^{-4.4}$  dilution of the filtrate, end-point  $10^{-5.1}$ , was added to 1 l. of the vaccine. In fact, however, when the percentage of negative samples is 50, i.e.  $e^{-m} = 0.5$ , then  $m = 0.7$ . Instead, therefore, of describing the vaccine/virus mixture as containing 500 infectious units per litre, it would be more correct to say it contained 350 units per litre and the expected percentage of infective 0.1 ml. samples would be 3.4 and not 5.

Eight cattle were inoculated subcutaneously with 100 ml. of the vaccine/virus mixture, i.e. 100 ml. vaccine + 35 infectious units of virus.

Four cattle were inoculated subcutaneously with 10 ml. of the  $10^{-5.4}$  dilution of the filtrate, i.e. 35 infectious units of virus with no vaccine.

Four cattle were inoculated intradermally on the tongue with not more than 0.1 ml. of the vaccine/virus mixture at each of twenty-one sites.

All these cattle were housed separately in loose boxes, and the maximum practicable precautions were taken to prevent the spread of infection. Those inoculated on the tongue were examined daily for the first 3 days, but the boxes containing the subcutaneously inoculated cattle were not entered until the 10th day except in the case of an obvious reactor. The results are shown in Table 3.

Table 3. *Detection of active virus added to an inactivated virus vaccine*

Group	Animal no.	Result
100 ml. vaccine + 35 infectious units of virus by subcutaneous inoculation	C/T 21	Negative
	22	Negative
	23	Negative
	24	Negative
	25	Negative
	26	Negative
	27	Reacted + 5, T, Ls, 4F
	28	Reacted + 6, T, L, 4F
35 infectious units of virus by subcutaneous inoculation	29	Reacted + 5, T, L, 4F
	30	Reacted + 9, T, 4F
	31	Reacted + 3, T, L, 4F
	32	Reacted + 6, T, L, 4F
Vaccine/virus mixture at 21 sites on the tongue	33	2 sites positive, + 1
	34	1 site positive, + 2
	35	Negative
	36	1 site positive, + 2

+ 5 denotes the 5th day after inoculation and T, Ls, 4F denotes lesions on the tongue, lips and four feet.

The result of the intradermal tongue inoculation of the vaccine/virus mixture in which four sites were positive out of 84, i.e. 4.76 %, compared with the expected 3.4 %, shows clearly that the presence of the inactivated but antigenic virus played no part in preventing the trace of active virus producing a reaction with its appropriate frequency on tongue inoculation, presumably because of the shortness of the incubation period and, in any case, the total quantity of the vaccine inoculated into each animal was only about 2 ml. When the mixture was inoculated subcutaneously, however, in one thousand times the quantity it is apparent that the mass of antigen hindered this infective dose of active virus in producing the disease in that only two out of eight cattle reacted compared with the complete reaction of the group of four given the same dose of virus subcutaneously without any vaccine. As these four cattle reacted to 35 tongue infectious units, it is probable that the amount necessary with this virus strain, Bec 1, to cause 50 % of positives by the subcutaneous route is  $\leq 35$  tongue units. The amount of virus necessary to produce 50 % of positives by the tongue route is 0.7 tongue unit. Therefore, the ratio of the amounts necessary to produce infection by the two routes (subcutaneous/tongue)  $\leq 50$  (see Table 5 of the previous paper (Henderson, 1952) for comparison with some other strains). It will be appreciated that it is only with such strains in which this ratio is small that the point illustrated in the above experiment could be

demonstrated. A strain in which the subcutaneous infective dose was many times the intradermal tongue infective dose would not have produced any reactors to the small amount of active virus inoculated subcutaneously.

*Simultaneous intradermal tongue inoculation of active virus and subcutaneous inoculation of vaccine*

The wide variation that has been demonstrated between strains in their ability to infect if inoculated subcutaneously compared with intradermally into the tongue suggests that perhaps the most logical way in which to attempt to detect the presence of a trace of active virus of a strain in which this characteristic was unknown might be to inoculate the material at multiple sites on the tongue and also to give a large dose subcutaneously. The most sensitive method of inoculation would then be used for multiple observations and, if the strain infected readily by the subcutaneous route, the much larger volume that can be given by that route would aid the chance of including an infective dose.

One disadvantage is that, as shown by the result of the experiment just described, if a vaccine is being tested the amount of antigen in the dose inoculated subcutaneously might mask the presence of active virus. It was, nevertheless, thought to be worth while to determine whether the simultaneous inoculation of an immunizing dose of vaccine subcutaneously would hinder the detection of active virus inoculated intradermally into the tongue.

A filtrate of a suspension of strain RV 7 (S.A.T. 3 type) bovine tongue epithelium was prepared and titrated by the intradermal tongue route in two cattle. The 50 % end-point dilution was  $10^{-4.6}$ , and  $10^{-5.3}$  was estimated as being the 15 % positive dilution. This dilution was inoculated intradermally on the tongue at twenty sites on each of eight cattle. Four of these cattle also received a subcutaneous inoculation of 100 ml. of a strain RV 7 incubated blood vaccine, Vac/CV/RV 7 120, having a 50 % protection dose of between 80 and 100 ml. The results of the intradermal inoculations are shown in Table 4, from which it can be seen that the subcutaneous dose of vaccine had no apparent influence on the sensitivity of the

Table 4. *Simultaneous tongue inoculation of virus and subcutaneous inoculation of vaccine*

Group	Animal no.	Reactions to tongue inoculation at 20 sites of the $10^{-5.3}$ dilution of a virus filtrate of titre (50 % end-point) $10^{-4.6}$		
		Positive	Negative	Group total
Virus on the tongue	C/BA 17	8	12	19 positive 61 negative 23.75 % positive
	18	2	18	
	19	4	16	
	20	5	15	
Virus on the tongue, vaccine subcutaneously	21	6	14	22 positive 58 negative 27.5 % positive
	22	5	15	
	23	2	18	
	24	9	11	

tongue route for the detection of active virus. All these cattle subsequently developed secondary lesions on all four feet, and at no time was any difference detected between the reactions in the two groups.

*Determination of the susceptibility of cattle used in a test for non-infectivity*

The importance has already been stressed of being able to obtain cattle in this country with a 'clean' history whose initial susceptibility to foot-and-mouth disease virus is assured. Even with such cattle, however, there is considerable variation in individual susceptibility, as is particularly evident when they are inoculated on the tongue in titrations of the virus (see Henderson, 1949). The standard deviation in different animals of the end-points of such titrations by multiple tongue inoculation of each animal is  $\pm 0.46$  on the logarithm scale to the base 10 of the dilutions, and one of the principal components contributing to the magnitude of this deviation is the variation in the individual susceptibility of the tongue tissue. The only measure of other than gross differences in the susceptibility of the tongue is the response of the animal compared with that of its fellows in the titration of a virus suspension by multiple inoculation of the tongue. Any animal providing an end-point lower by more than two standard deviations from the mean end-point should be regarded as having tongue tissue of less than normal susceptibility and certainly insufficiently susceptible for the detection of minimal quantities of virus by this route of inoculation. The relevance of this to the discussion of the test for non-infectivity is that although 2 ml. of the best of the types of foot-and-mouth disease vaccines in current use may protect cattle against the development of secondary lesions following intradermal inoculation of the tongue with virus, the intradermal tongue inoculation of  $20 \times 0.1$  ml. of such a vaccine does not usually produce any detectable resistance to primary infection of the tongue by inoculation, and the susceptibility of this tissue can subsequently be tested. If, however, a vaccine is tested for non-infectivity by subcutaneous inoculation and advantage is taken of being able to test a much larger dose than is possible by the tongue route, then the non-reactor will be immunized and no estimate of its initial susceptibility relevant to subcutaneous inoculation can be made.

In practice, the susceptibility of the non-reactors is compared by titration with that of a group of normal cattle, the end-point provided by the latter being taken as the standard. It must be emphasized that the quoted standard deviation applies to the cattle used at this Institute, and it would not necessarily be the same for other cattle. The following examples illustrate this method of determining susceptibility.

(i) Nine cattle were inoculated on the tongue at ten sites each in a test for non-infectivity of a strain M1 (Vallée A type), aluminium hydroxide vaccine. No reactions were observed at any of the inoculated sites. Six weeks later a strain M1 filtrate was prepared and titrated by the inoculation of four dilutions at five sites each on the tongues of the nine non-reactors and on two normal cattle. The result is shown in Table 5. The end-point provided by the two normal cattle,  $10^{-4.3}$ , is taken as the standard, and twice the standard deviation,  $\pm 0.46$ , for this method



of titration is used to define the limits of the normal range. Thus, any animal providing an end-point lower than or equal to  $10^{-3.38}$  is regarded as being of less than normal susceptibility, and its contribution should be excluded in the assessment of the reliability of the test for non-infectivity. This applies to animal no. C/AO 22. Instead of the test being based on ninety negative observations from nine cattle, it is only justifiable to cite eighty observations from eight cattle. The lapse of 6 weeks between the test for non-infectivity and the test of susceptibility was unusually long. These tests are part of a larger programme of work and are done when it is expedient. Normally, the period between them is about 3 weeks.

Table 5. *Test of susceptibility of non-reactors in a test for non-infectivity of a strain M 1 vaccine. Reactions to virus filtrate dilutions inoculated on the tongue*

Animal no.	Dilutions				50 % end-point
	$10^{-3}$	$10^{-4}$	$10^{-5}$	$10^{-6}$	
	Non-reactors				
C/AO 22	-----	-----	-----	-----	
23	+++++	-----+	-----+	-----	$10^{-3.7}$
24	+++++	+++++	---++	-----	$10^{-4.8}$
25	+++++	++---	-+---	+++--	$10^{-4.5}$
26	+++++	+-+--	-----	-----	$10^{-4.2}$
27	+++++	---++	-----	-----	$10^{-3.8}$
28	+++++	+-+--	-----	-----	$10^{-3.8}$
29	+++-+	---+-	-----	-----	$10^{-3.5}$
30	++++-	+++++	-----	-----	$10^{-4.4}$
	New cattle				
C/AO 32	+++++	++-++	-----	-----	$10^{-4.3}$
C/AP 76	+++++	++-++	-----	-----	

(ii) Eight cattle were inoculated on the tongue at fifteen sites each in a test for non-infectivity of a strain Rho 1 (S.A.T. 2 type) incubated blood vaccine. All were negative. In connexion with the main experiment for which this vaccine was required it was necessary to have a group of susceptible cattle each receiving 10,000 ID<sub>50</sub> of virus at multiple sites on the tongue (ID<sub>50</sub> = the 50 % infective dose). Accordingly, a strain Rho 1 filtrate was prepared and titrated by tongue inoculation of two cattle. The 50 % end-point was  $10^{-5.1}$  and the  $10^{-1.1}$  dilution was prepared to provide 10,000 ID<sub>50</sub>. Eighteen days after the test for non-infectivity the eight non-reactors and eight new cattle were inoculated on the back of the tongue at five sites with the 10,000 ID<sub>50</sub> dose and at five sites on the front of the tongue with approximately the 50 % end-point dilution. The result is shown in Table 6. Seven of the eight non-reactors were obviously of normal susceptibility, and although C/AW 30 might have been shown to be sufficiently susceptible if a full titration had been done, its contribution was excluded in an assessment of the significance of the test for non-infectivity.

*Consideration of how limitations of method can best be suited to requirements of design*

The following advantages and disadvantages can be summarized between the intradermal tongue route and the subcutaneous route for inoculation of cattle in the test for non-infectivity.

The advantages of tongue inoculation are: (i) this method is the most sensitive for the detection of infectivity; (ii) at least twenty observations can be provided by one animal; (iii) the presence of non-infective but antigenic material in the inoculum does not hinder the detection of a trace of active virus; (iv) an estimate of the initial susceptibility of the tongue tissue of non-reactors can usually be obtained. The only disadvantage of tongue inoculation is the smallness of the dose that can be inoculated at each site.

Table 6. *Test of susceptibility of non-reactors in a test for non-infectivity of a strain Rho 1 vaccine. Reactions to virus filtrate dilutions inoculated on the tongue*

Animal no.	Dilutions	
	10 <sup>-1</sup>	10 <sup>-5</sup>
Non-reactors		
C/AW 28	++++	- - + - +
29	++++	+ - - + -
30	++++	- - - - -
31	++++	- + - - +
32	++++	+ + + - -
33	++++	- - - - +
34	++++	- + + - +
35	++++	- + + - +
New cattle		
C/AX 14	++++	+ - - + -
15	++++	+ - + - +
16	++++	- + + + -
17	++++	- + - - -
22	++++	- - - - -
24	++++	- - + - -
26	++++	- + - - -
28	++++	- + + + -

The only advantage of subcutaneous inoculation is that a large sample of the vaccine can be tested on one animal.

The disadvantages of subcutaneous inoculation are: (i) with some strains very large amounts of virus are required for infection by this route; (ii) only one observation is provided by each animal; (iii) the presence in the inoculum of non-infective but antigenic material may mask the presence of active virus; (iv) non-reactors to an immunizing dose of vaccine cannot subsequently be tested for a measure of their initial susceptibility relevant to this route of inoculation.

Sound design of the test for non-infectivity demands that many samples of the vaccine be tested by the most sensitive means for the presence of active virus. The multiple intradermal tongue inoculation of a number of cattle and the subsequent test of their susceptibility would appear to fulfil these requirements, and this method has been adopted at this Institute for the routine testing of vaccines for non-infectivity.

A vaccine is not normally tested for non-infectivity until the inactivation treatment has been completed and when the probability of virus surviving is small.

Occasions on which active virus has been detected have, therefore, been few, but the following are two examples which, besides illustrating the method used, emphasize the necessity of making large numbers of observations.

*Test for non-infectivity of two incubated blood vaccines prepared from strain ASJ (Vallée O type)*

(i) *Vaccine Vac/CV/OASJ 81.* A test for non-infectivity of this vaccine was made after the infective blood had been incubated for 4 days at 37° C. Sixteen cattle, C/42-57 T, were inoculated intradermally on the tongue with 0.1 ml. at each of six sites. Two cattle had reacted by 24 hr., each having a lesion at one site only. Thus two sites were positive and ninety-four were negative.

(ii) *Vaccine Vac/CV/OASJ 87.* Nine litres of this vaccine had been prepared using three 3 l. bottles. After 4 days' incubation at 37° C. a 10 ml. sample was withdrawn from each bottle and the contents of the bottles then pooled. This pooled vaccine was tested for non-infectivity by intradermal tongue inoculation of thirteen cattle, C/88-99 W and C/O X, at ten sites each. All 130 sites were negative. Four days later the individual samples from each of the three bottles were tested on twelve of these non-reactors, C/89-99 W and C/O X, four cattle per bottle. On the second day C/97 W, inoculated with the bottle III sample, developed lesions at two out of ten sites. Two interpretations of this result are possible. Either the contents of bottle III were more infective than those of the others and on pooling became diluted below the infective level, or the quantity of surviving virus was evenly distributed but was so low that the frequency of infective samples was 2 out of 250 (13 × 10 in the first test and 12 × 10 in the second test).

*Amount of virus that may be undetected in various doses of vaccine*

Table 7 gives the mean number of infectious units, *m*, corresponding to various percentages of infective samples assuming that the numbers of samples containing 0, 1, 2, . . . infectious units are distributed in the Poisson series in which case  $e^{-m}$  is the proportion of non-infective samples. This is of some importance when the size of the sample of vaccine being tested by the intradermal tongue route is limited to 0.1 ml., whereas the dose of vaccine given in immunization may vary from, say, 2 to 100 ml. If, therefore, 100 negative observations are made in

Table 7. *Relationship of mean number of infectious units of virus per sample for various percentages of infective samples*

Percentage of infective samples	Mean number of infectious units of virus
10.0	0.1053
7.5	0.0779
5.0	0.0516
4.0	0.0412
3.0	0.0296
2.0	0.0198
1.0	0.0099
0.1	0.0010

the non-infectivity test there still may be 3.0 % of 0.1 ml. samples infective (Table 1,  $P=0.05$ ), corresponding to 0.0296 infectious units per sample (Table 7); thus a 100 ml. dose of vaccine might contain as many as 29.6 tongue infectious units of virus. Table 8 shows for  $P=0.05$  the highest likely mean number of infectious units that might be undetected in various doses of vaccine.

Table 8. *Amount of virus that may be undetected in various doses of vaccine when the volume of the samples tested is 0.1 ml. and  $P$ , the chance of failing to detect a positive sample, equals 0.05*

No. of observations in non-infectivity test, all being negative	Highest likely mean number of tongue infectious units of virus per 0.1 ml.	Highest likely mean number of tongue infectious units of virus that might be undetected in a vaccinating dose of			
		2 ml.	10 ml.	30 ml.	100 ml.
10	0.2994	5.99	29.94	89.82	299.40
40	0.0751	1.50	7.51	22.53	75.10
80	0.0373	0.75	3.73	11.19	37.30
100	0.0296	0.59	2.96	8.88	29.60
120	0.0247	0.49	2.47	7.41	24.70
250	0.0119	0.24	1.19	3.57	11.90
500	0.0060	0.12	0.60	1.80	6.00
1000	0.0029	0.06	0.29	0.87	2.90

#### DISCUSSION

An attempt has been made to set out the various factors involved in testing inactivated foot-and-mouth disease virus vaccines for the presence of any surviving active virus. In common with the preparation of most inactivated virus vaccines, the process of inactivation is necessarily delicate so that non-infectivity is achieved without loss of antigenicity. If, in this case, the virus could be subjected to the normal methods of sterilization the same doubt would not be felt about the thoroughness of the treatment.

A theoretical consideration of the design of the test for non-infectivity suggests that not less than 100 samples of the vaccine should be tested with negative results before there is reasonable assurance that the vaccine is safe to use. Theoretically the volume of the sample for test should, without doubt, be at least that of the dose to be used in vaccination. With most foot-and-mouth disease vaccines such a dose could only be given by some route other than into the susceptible tissue of the tongue, thus necessitating having 100 cattle for the provision of 100 observations.

It does not seem possible to be able to meet all the requirements of sound design by any one method. The requisite number of observations can readily be obtained by multiple intradermal tongue inoculation of a few cattle, but the volume of the sample tested is small and, whereas the required volume can easily be tested by subcutaneous inoculation, the number of observations that it is practicable to make is usually very limited for reasons of expense and restrictions of accommodation.

If it were known that the test animals were fully susceptible and that the virus strain being used could infect as readily by the subcutaneous route as by the

tongue route, then  $100 \times 0.1$  ml. samples could be tested as adequately by making one subcutaneous inoculation of 10 ml. Unfortunately, it has been shown that, even when cattle with a 'clean' history are available, a small proportion are markedly less susceptible than the average and no strain of virus has been found to infect as readily subcutaneously as by tongue inoculation although, with some, the difference observed was not significant.

The result of the experiment in which a mixture of vaccine and virus inoculated subcutaneously caused reactions in only two out of eight cattle, compared with four out of four animals reacting to the same amount of virus alone, indicates an important limitation to the use of the subcutaneous route in attempts to detect small quantities of active virus that may be present in otherwise non-infective but antigenic material.

The possibility of being able to show that non-reacting test animals would have been capable of detecting minimal quantities of active virus is a great advantage of the intradermal tongue route of inoculation, and is a strong recommendation for the use of this route in the test for non-infectivity. If further research produces foot-and-mouth disease vaccines of better immunizing quality than those in current use, it might no longer be possible to determine the initial susceptibility of the cattle in this way, but the other advantages of the tongue route would remain unchanged.

The test for non-infectivity currently used at this Institute consists of the multiple intradermal tongue inoculation of not less than six cattle, thus providing an initial total of at least 120 observations. These cattle are later tested for susceptibility in comparison with the reaction of at least two new cattle, and the contribution is discarded of any animal failing to pass this test. This usually ensures having 100 negative observations if not 120. As even this number of observations may not detect an infective level of 3 % of samples positive, it is important to have some knowledge of the relative sensitivity of the cattle to inoculation with the virus strain in question by the route used in vaccination and to consider this in relation to the data given in Table 8.

Whatever the method of test, it must be remembered that the absence of active virus can never be proved, and reference to the non-infectivity of a vaccine should always include a statement of what test was performed so that its limitations are known.

#### SUMMARY

An appreciation of the chance of failing to detect small amounts of infectivity is the main consideration in the design of any test for non-infectivity. The absence of infectivity can never be proved, and many samples must be tested and found negative before there is reasonable assurance that a supposedly non-infective vaccine is safe to use.

The highest percentage,  $p$ , of samples likely to be infective for various numbers of negative observations,  $n$ , has been calculated from the expression  $(1 - p)^n = P$  for different levels of  $P$ , the chance of failure to detect a positive. For example, if 100 observations are made and found negative the percentage of infective samples

would be unlikely ( $P = 0.05$ ) to exceed 3.0 and highly unlikely ( $P = 0.01$ ) to exceed 4.5. It is suggested that at least this number of observations be made.

The particular problem of detecting foot-and-mouth disease virus by inoculation of a susceptible host is considered in relation to the theoretical requirements of a test of sound design.

The multiple intradermal tongue inoculation of a number of cattle is the method that best meets these requirements. It is shown that this method is the most sensitive for the detection of foot-and-mouth disease virus, at least twenty observations can be provided by one animal, the presence of non-infective but antigenic material in the inoculum does not hinder the detection of a trace of active virus, and an estimate of the initial susceptibility of the tongue tissue of non-reactors can usually be obtained.

Although a much larger volume can be tested by subcutaneous inoculation, disadvantages of this route are shown to be that with some strains very large amounts of virus are required for infection, only one observation is provided by each animal, the presence of non-infective but antigenic material in the inoculum may mask the presence of active virus, and non-reactors to an immunizing dose of vaccine cannot subsequently be tested for a measure of their initial susceptibility relevant to this route of inoculation.

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#### REFERENCES

- FISHER, R. A. (1941). *Statistical Methods for Research Workers*, 8th ed. Edinburgh: Oliver and Boyd.
- GALLOWAY, I. A. (1950). Observations on immunological and other characteristics of strains of the virus of foot-and-mouth disease with special reference to experimental methods, epizootiology and methods of control, including vaccination. Report submitted to Joint Meeting of the Food and Agriculture Organization of the United Nations and Office international des Epizooties, Paris, May, 1950.
- HENDERSON, W. M. (1949). The quantitative study of foot-and-mouth disease virus. *Rep. Ser. agric. Res. Coun., Lond.*, no. 8. H.M. Stationery Office.
- HENDERSON, W. M. (1952). A comparison of different routes of inoculation of cattle for detection of the virus of foot-and-mouth disease. *J. Hyg., Camb.*, **50**, 182.
- SKINNER, H. H. (1951). Propagation of strains of foot-and-mouth disease virus in unweaned white mice. *Proc. roy. Soc. Med.* **44**, 1041.

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