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The infectivity assay of foot-and-mouth disease virus in pigs

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The ability to assay the infectivity of a virus preparation for a particular host is an important requisite in the study of that virus–host relationship. It can provide an estimate of the degree of adaptation or attenuation of a virus strain for the host and of resistance, either natural or acquired, possessed by an individual or a group of animals.

The method of titration of foot-and-mouth disease virus (FMDV) in cattle by inoculation of the tongue (Henderson, 1949) has formed the basis for many of the standard procedures used in FMDV research involving cattle. Comparatively little work, however, has been recorded on the development of suitable methods of titration of FMDV in pigs. Brooksby (1950) inoculated individual animals with single dilutions of virus intradermally in the tongue and snout in his studies of virus strains showing natural adaptation to swine. Graves & Cunliffe (1960) carried out some comparative work on the sensitivity of pigs to different routes of inoculation and concluded that the inoculation of the coronary band region of the foot was the most sensitive for the detection of virus. They developed a titration scheme using the simultaneous inoculation of several dilutions of virus into individual animals and showed that their results were reproducible within good statistical limits. Lucam, Dhennin, Dhennin & Fedida (1962) subsequently compared titration end-points using coronary-band and snout inoculation and confirmed the high sensitivity of the coronary-band region. Burrows (Communication to the Research Group of the Standing Technical Committee of the European Commission for the Control of Foot-and-Mouth-Disease, June 1963, summarized in F.A.O. document 15766/E) reported that the bulb of the heel of the foot was a convenient and highly sensitive site of inoculation for titration, innocuity testing and challenge of immunity procedures. Some details of the use of this method for these purposes have been recorded (Girard *et al.* 1963; Burrows, 1964*a*, *b*).

It is desirable, when proposing an assay method, to attempt to determine the precision of the method and also to compare the results with those given by established titration procedures. This paper presents the results of a number of comparative titrations of some FMDV strains in cattle, mice and pigs, with particular reference to the results obtained in pigs by the bulb of the heel inoculation method.

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MATERIALS AND METHODS

Experimental animals

Details of the supply, housing and maintenance of cattle under experiment have been given by Henderson (1952). Similar details in relation to pigs have been recorded by Burrows (1964*a*).

Virus strains

Strains virulent for cattle

(*a*) A–119 Pirbright stock cattle strain used at the 26th cattle passage (*C* 26); (*b*) A–Kemron (Komarov & Goldsmit, 1958), *C* 2 (Pirbright); (*c*) O–Israel 1/63 World Reference Laboratory sample (WRLS), *C* 3; (*d*) C–997 British field sample (1953), *C* 6; (*e*) Sat 1–SA. 13/61 (WRLS), *C* 3; (*f*) SAT 1–Sudan 2/61 (WRLS), *C* 3; (*g*) SAT 1–Turkey 323/62 (WRLS), *C* 3; (*h*) SAT 3–SA. 57/59 (WRLS), C 5.

Seitz EK filtrates were prepared from 1/25 (w/v) suspensions of bovine tongue vesicle epithelium harvested 18–24 hr. after tongue inoculation. The suspensions were prepared in a mixture of equal volumes of M/25 phosphate buffer (pH 7·6) and Hartley's digest broth. Filtrates were held at 4° C. and usually titrated on the day of preparation. If titration was to be delayed for more than 24 hr., an equal volume of glycerol was added to the filtrate and the preparation stored at −20° C. Survival under these conditions was satisfactory.

Strains modified for cattle

A number of modified strains derived by passage in adult mice (*mo* strains) or in embryonated eggs (*E* strains) were examined. Detailed results of titrations are recorded for the following strains: A–119 *mo*, A–Kemron *E*, O–M 11 *mo*, SAT 1–Israel *E* and SAT 3–SA. 57/59 *E*. The *mo* strains were used as glycerinated filtrates of infected mouse carcases (Skinner, 1960). The A–Kemron *E* and the SAT 1–Israel *E* strains were supplied by the Wellcome Laboratory, Pirbright, as concentrated tissue culture preparations. In addition, the results of bulb-of-the-heel titration of several other modified strains derived from the C–997 and SAT 3–SA. 57/59 virus strains were used to prepare Table 6.

An account of the behaviour in pigs of some of these modified strains has been recorded (Burrows, 1964*a*, *b*).

Virus assay

Cattle

Tongue inoculation (Henderson, 1949). Two cattle were inoculated intradermally in the tongue with four tenfold dilutions, using five sites on each tongue for each dilution.

Pigs

Bulb-of-the-heel inoculation (*heel*) (Burrows, 1964*a*). Four pigs were inoculated at two sites (inner and outer main digit) on each foot with a series of tenfold dilutions. This allowed the estimation of individual and group 50% positive end-point dilutions. With certain groups of pigs the dilutions were distributed in a

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factorial arrangement to allow additional calculation of group end-points based on particular feet.

Coronary-band inoculation (Graves & Cunliffe, 1960). Four pigs were inoculated with a series of tenfold dilutions following a pattern similar to that used for heel titration.

Tongue inoculation. Two or four pigs were inoculated intradermally in the tongue with four tenfold dilutions, using three sites on each tongue for each dilution.

Mice

Intraperitoneal inoculation (Skinner, 1951). Three to five litters of randomized 5- to 7-day-old 'P' strain mice were inoculated with a fivefold dilution series, using two subjects per litter for each dilution (Subak-Sharpe, 1961).

End-point determinations

The majority of cattle and all pigs were examined for lesions 18, 24, 30, 42 and 48 hr. after inoculation and daily thereafter. Mice were examined twice daily for 6 days. The 50% end-point dilution estimations were computed according to the method of Reed & Muench (1938) and were expressed as the number of 50% infective doses (ID 50) per ml. of the virus filtrate.

Table 1. *Time taken for vesicles to develop in relation to the concentration of virus inoculated into the heel*

Virus	Number of ID 50* inoculated			Secondary lesions
	4	5–40	> 40	
A–119	30†	28	23	42
A–Kemron	29	28	23	46
O–Israel 1/63	30	26	O.T.R.	41
C–997	26	26	24	42
SAT 1–Sudan 2/61	28	24	18	41
SAT 1–SA. 13/61	26	26	24	47
SAT 3–SA. 57/59	30	24	24	43

* Estimated from group end-point 30 hr. after inoculation.
† Mean time in hr. after inoculation.
O.T.R., Outside titration range.

RESULTS

The development of lesions following heel inoculation

The time of appearance of vesicles after heel inoculation was found to be dependent to some degree on the strain of virus and the concentration of virus inoculated and also on the individual animal. Table 1 records the mean times, in hours, required for vesicles to develop in relation to virus concentration for seven cattle strains of virus. Lesions were recorded once they had attained the diameter of 0·5 cm. The rate of vesicle growth was such that, with some strains of virus, they had involved some 2 cm.^{2} or more of the inoculation areas within 6 hr. of first appearance. Trials using two inoculation sites on each main digit were discontinued

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because the rapid extension of a vesicle at one site frequently involved the second site before it had time to develop a visible reaction. Secondary lesions were recognizable in the majority of animals 40–48 hr. after inoculation and were first seen on the heel and coronary regions of the main digits and the coronary regions of the accessory digits. Vesicles on the tongue, lips and snout were usually apparent 72 hr. after inoculation and with certain strains of virus lesions were also observed on the skin covering the anterior surfaces of the limbs and on the teats and prepuce.

As a result of these observations, the final reading for end-point determinations was standardized at 30 hr. after inoculation of cattle strains of virus.

Lesions produced by virus strains modified for cattle were slower in development and limiting infective doses required 2 or 3 days to become apparent (Burrows, 1964*a*). Secondary lesions usually appeared on the 3rd or 4th day after inoculation.

Table 2. *Variation in experimental* 50 % *end-points observed in titration of cattle strains of virus using the 'heel' method*

Virus	Pig number 1	2	3	4	*V*
A–119	3·8	4·8	4·8	4·3	0·2291
A–119	3·3	5·3	5·8	4·3	1·2292
A–119	5·3	4·8	6·3	6·3	0·5624
A–Kemron	4·5	5·5	4·5	6·0	0·5624
O–Israel 1/63	5·5	5·0	4·5	6·5	0·7292
C–997	6·0	6·0	5·5	5·0	0·2291
SAT 1–Sudan 2/61	4·7	6·0	6·0	6·5	0·5993
SAT 1–SA. 13/61	4·8	6·8	5·8	6·3	0·7292
SAT 1–Turkey 323/62	4·6	5·6	5·6	4·6	0·3333
SAT 3–SA. 57/59	5·0	4·5	5·5	5·5	0·2291

Homogeneity of variance $\chi^2 = 5{\cdot}62$ (9), $> 0{\cdot}5$, $< 0{\cdot}9$; $\bar{V}$ 0·5432.

The variation of end-points determined by heel titration

An estimate of the total variation in the assay system can be obtained by calculating the variance of the individual 50 % end-points. The end-points recorded in ten titrations of cattle virus strains and eight titrations of modified virus strains are displayed in Tables 2 and 3. Differences in individual end-points within a titration group ranged from 0·5 to 2·5 $\log_{10}$ units with a mean of 1·34. The variances calculated for different titration groups ranged from 0·06 to 1·23. The results of Bartlett tests for homogeneity of the variances justify the calculation of average variances for (i) the cattle virus titration group (0·543), (ii) the modified strain virus groups (0·356) and (iii) the total data (0·460).

These variance values include components contributed by random sampling error and differences in susceptibility between digits and between animals. The experimental design does not allow assessment of the magnitude of these various components by an analysis of variance procedure. However, some general observations as to the major source of variation can be inferred from data collected from these and other experiments.

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Differences between animals (breed and weight)

The pigs used in the majority of these experiments were commercial crossbreds (Wessex/Large White/Landrace) and ranged in weight from 45 to 70 lb. They were usually purchased in groups of at least ten and it is unlikely that all animals in a group were litter-mates. A single trial was carried out to see if there was any indication of extreme variation in the susceptibility of pigs of different breeds. The results of titration in purebred Large White (A), Landrace (B) and Wessex (C) showed no significant differences in end-points (Table 3, groups A–119 *moC*, A, B and C).

Table 3. *Variation in experimental* 50 % *end-points observed in titration of modified virus strains using the 'heel' method*

Virus	Pig number 1	2	3	4	*V*
A–119 *moC* A	2·0	3·5	3·5	2·5	0·5624
A–119 *moC* B	2·4	3·5	2·4	2·4	0·3058
A–119 *moC* C	3·0	2·0	4·0	3·5	0·7259
A–Kemron *E*	6·3	6·8	5·8	6·8	0·2291
SAT 1–Israel *E*	5·5	6·5	5·5	5·5	0·2500
SAT 3–SA. 57/59					
E 19	2·0	2·7	3·5	2·5	0·3891
E 30	2·5	2·5	2·5	2·0	0·0623
E 40	3·0	2·0	2·5	3·3	0·3266

Homogeneity of variance $\chi^2 = 5{\cdot}58$ (7), $> 0{\cdot}5$, $< 0{\cdot}9$; $\bar{V}$ 0·3564.

The weight of pigs has been shown to be of importance in the development of foot lesions following intramuscular inoculation of certain modified strains of virus (Burrows, 1964*a*). As pigs of slightly different weights were used in some titrations, this could have been a source of variation. However, titration of the O–M 11 *mo* virus strain in pigs of greatly different weight did not indicate that this factor was of importance with this particular virus strain; similar end-points were obtained with the two groups of animals (group 1–11 pigs, mean weight 69 lb., range 52–84 lb., mean end-point 3·7; group 2–16 pigs, mean weight 210 lb., range 140–284 lb., mean end-point 3·8).

Difference between digits

It is assumed that this error is probably not very great, i.e. that the eight main digits are of uniform susceptibility to virus, that identical inoculations are made at each of the sites and that similar volumes of inoculum are retained in each case. In respect of the latter two assumptions, the degree of wear and the thickness of the epithelium covering the bulb of the heel are variables of importance. These are fairly uniform within each pig but not necessarily uniform between different pigs. The validity of the assumption that the different feet are of equal susceptibility could be tested by using a large number of pigs and inoculating each foot with a small amount of virus; experiments of this nature were not attempted. Tests for

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major differences in susceptibility were carried out by distributing virus dilutions in such a fasion as to allow the calculation of 50 % end-points on the responses of individual feet (group end-points) and also in each individual of the group. Table 4 gives the results of six titrations carried out in this manner. The variation in end-points estimated from the reaction of different feet (average variance 0·598) was of similar magnitude to that calculated from individual animal end-points (average variance 0·535) and no marked difference in sensitivity was noted.

Table 4. *Experimental* 50 % *end-points calculated in individual animals and on the feet of groups of animals*

	Individual animal end-points*					
				A–119 *moC*		
	SAT 1 SA. 13/61	SAT 1 Sudan 2/61	SAT 1 Israel *E*	A	B	C
Pig 1	4·6	4·7	5·5	2·0	2·4	3·0
2	6·8	6·0	6·5	3·5	3·5	2·0
3	5·8	6·0	5·5	3·5	2·4	4·0
4	6·3	6·5	5·5	2·5	2·4	3·5
Group $\bar{x}$	5·93	5·8	5·75	2·88	2·63	3·13
	$\bar{V}$, 0·535					
	Group end-points calculated on one foot of each animal					
Right fore	4·7	5·6	6·0	2·5	2·8	3·0
Left fore	5·5	4·7	5·5	3·0	1·8	4·5
Right hind	6·5	6·0	6·5	2·5	3·5	2·5
Left hind	5·5	7·0	5·0	3·3	3·6	2·5
Group $\bar{x}$	5·55	5·83	5·75	2·83	2·93	3·13
			$\bar{V}$, 0·598			
Group overall†	5·60	6·00	5·85	2·87	2·66	3·29

* These results also appear in Tables 2 and 3.
† Group end-point estimated in the standard way from the whole group.

Table 5. *Distribution of foot lesions on the main digits of pigs following intramuscular inoculation of seven modified strains of virus*

	Fore feet		Hind feet	
Site of lesion	Outer digit	Inner digit	Outer digit	Inner digit
Coronary-band area	60*	45	62	36
Bulb of the heel	54	47	58	38

* No of lesions found in fifty-four reactors.

Previous experience of the response of pigs to intramuscular inoculation of modified virus strains had indicated that the outer (IV) digit was more likely to develop secondary lesions than the inner digit (III). Table 5 details the distribution of foot lesions recorded on the main digits of fifty-four animals which developed lesions following intramuscular inoculation. The difference in the frequency of

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occurrence of lesions on the outer and inner heels is significant at the 1% level (χ^2 6·8). A similar difference was also observed in the distribution of lesions involving the coronary band area.

The results of replicate inoculation of the heels of the titration animals were analysed in similar fashion (Table 6). No significant difference in the frequency of occurrence of lesions on the two heels could be shown in those animals used for titration of cattle strains of virus, but a slight but significant difference was observed in animals used for titration of virus strains modified for cattle.

Table 6. *Responses of outer and inner heels to replicate inoculation—titration animals*

Virus	No. of strains	No. of pigs	Outer heel	Inner heel	χ^2	*P*
Virulent for cattle	8	40	93/160*	83/160	1·26	0·5–0·1†
Modified for cattle	15	99	228/372	181/372	11·99	0·001

* No. of positive sites/no. of sites inoculated.
† Not significant.

Table 7. *The frequency of positive reactions to heel inoculation in relation to the concentration of virus inoculated*

	Virus strains virulent for cattle			Virus strains modified for cattle		
Approximate number of ID50 inoculated	No. of sites	No. of positive sites		No. of sites	No. of positive sites	
		Observed	Expected		Observed	Expected
10	116	113	116	94	87	94
3	40	33	35	42	31	37
1	40	18	20	22	11	11
0·3	40	7	8	40	9	7
0·1	84	5	⩽ 8	58	6	⩽ 5
	320	χ^2	1·63	256	χ^2	2·26

Additional evidence for the assumption that the 'between digit' component is negligible was obtained from the titration animals by observing the frequency of reaction of sites inoculated with progressive dilutions of virus and testing this for agreement with expectation from a Poisson distribution (employing the conversion 1 ID50 = 0·693 infectious units). The approximate number of ID50 inoculated at each site was calculated for each animal from the end-point observed in that animal. Table 7 lists the data for both the cattle virus and the modified strain virus groups. Chi-square values were not significant but a larger value was determined for the modified strain group of animals and there was a trend towards a flatter dosage response curve.

Residual variation

This appears to be the major source of variation in the titration system. Interbreed differences in susceptibility were not marked and differences in susceptibility between digits were not demonstrably large. Thus, the main source of

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variation would appear to be the result of other causes, i.e. the sum of the theoretical statistical minimum variance, differences in inoculum volume and differences in individual susceptibility. Clinical observation of the development and character of lesions following heel inoculation indicated that differences in individual susceptibility probably account for the major part of this residual variation.

The precision of end-points determined by heel titration

The average variance of 0·46 determined for the eighteen titrations gives a standard deviation of 0·68 for individual end-points. The 95 % confidence limits for a mean end-point can be determined for any number of animals, using the expression $(t_{17} \times \sqrt{0{\cdot}46})/\sqrt{n}$. Thus, the precision of an end-point based on four animals equals $\pm 0{\cdot}718$ and that based on nine animals $\pm 0{\cdot}479$.

Table 8. *Results of comparative titration of cattle strains of virus*

Virus	Mouse intra-peritoneal	Cattle tongue	Pig		
			Heel	Coronary	Tongue
A–119	7·5*	6·1	4·6	2·4	2·4
A–Kemron	6·5	5·8	5·2	1·3	–
O–Israel 1/63	7·2	6·7	5·3	3·0	–
C–997	7·0	5·6	5·6	2·7	3·9
SAT 1–SA. 13/61	7·9	7·2	5·6	–	–
SAT 1–Sudan 2/61	7·5	6·5	6·0	3·3	–
SAT 1–Turkey 323/62	7·8	6·6	5·1	2·6	4·1
SAT 3–SA. 57/59	7·9	6·4	4·9	4·0	4·0

* Log_{10} ID 50 per ml. of a 1/25 EK filtrate of bovine tongue epithelium.
–, Not tested.

Titration in pigs by other routes of inoculation

Coronary band inoculation

The development of primary lesions and the subsequent appearance of secondary lesions followed a pattern similar to that described for those following heel inoculation. It was found, however, that considerably more virus was required to produce visible lesions at the coronary-band site (Table 8). The variation in response of individual pigs was greater than that determined for heel inoculation. The average variance determined for eight titrations was 0·742 with 95 % confidence limits of 0·489–1·236 (the 95 % confidence limits for the average variance of end-points obtained by heel inoculation equalled 0·344–0·652). The standard deviation of the mean end-point determined in four animals ($\pm 0{\cdot}43$) was not substantially different from that of $\pm 0{\cdot}34$ recorded by Graves & Cunliffe (1960) in their original description of this titration procedure.

Tongue inoculation

Some difficulty was experienced in confining the inoculum to the thin epithelial layer and subsequent examination of the tongue for the presence of lesions could only be carried out satisfactorily with the pig under general anaesthesia. The results of four titrations are recorded in Table 8.

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Primary lesions produced by tongue inoculation were inconspicuous, showed little tendency to extend and had usually healed within 48 hr. With some strains of virus and in certain pigs neither primary nor secondary lesions developed.

Comparative titration in cattle, pigs and mice

The results of comparative titrations of cattle strains of virus are given in Table 8. With all strains, mice gave slightly higher infectivity end-points than cattle (mean difference 1·05 $\log_{10}$, range 0·5–1·5). Titration in pigs by the heel route gave infectivity end-points approximately tenfold lower than those recorded for cattle (mean difference 1·07, range 0·0–1·6). Titration in pigs by the coronary-band route gave infectivity end-points several thousandfold lower than those found for cattle (mean difference 3·54, range 2·8–4·1). Tongue titration in pigs gave end-points similar to those determined by coronary-band inoculation.

The results of comparative titration of virus strains modified for cattle have already been recorded (Burrows, 1964*a*).

DISCUSSION

The observation that lesions appeared only on the bulbs of the heels of pigs after intramuscular inoculation of certain modified strains of virus led to the investigation of the susceptibility of the area to inoculation. Titrations comparing the sensitivity of the heel area with that of the coronary band region have shown the heel to be consistently more sensitive to those strains of FMDV which have been tested.

This higher sensitivity might be accounted for by the more efficient exposure of the basal layers of the epithelium to virus. The heel procedure consists essentially of making a needle track in the depths of the epithelium without entering the sub-epithelial region. Inoculation requires a considerable amount of pressure and the bulk of the inoculum (0·1 ml.) escapes when the needle is withdrawn owing to the dense and elastic nature of the heel epithelium. The amount of inoculum retained is thought to be fairly constant and is likely to be determined by the thickness of the needle and the length of the inoculation track. Syringes capable of accurate measurement of volumes less than 0·1 ml. were not sufficiently robust or suitable for the heel inoculation procedure. All infectivity estimates were calculated on the basis of a 0·1 ml. inoculum although it was recognized that the true infectivity of a virus preparation for the pig heel would be underestimated. A similar situation has been demonstrated in the calculation of infectivity estimates based on tongue inoculation of cattle (Hyslop & Skinner, 1964–see below).

In comparison, inoculation of the coronary band consists of an oblique penetration of the epithelium of the area and the delivery of the inoculum deep to the hoof wall. Little resistance is experienced to inoculation and experiments have shown that much of the inoculum is dispersed in the subcutaneous region underlying the coronary band and in the region between the hoof wall and the laminar matrix.

The susceptibility of pigs to the cattle strains of virus used was not much less than that of cattle. In six of the eight trials, uninoculated control pigs were housed

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in contact with titration animals. In two trials (C–997 and O–Israel 1/63) some or all of these control animals did not develop clinical lesions. All these 'insusceptible' animals were later shown to have acquired a subclinical infection. Specific neutralizing antibodies were present 10 days after exposure and the animals did not develop secondary lesions when challenged in the heel with 100–1000 ID 50 (pig heel). Thus, failure to develop clinical lesions after a period of exposure to infection does not necessarily imply insusceptibility, an observation which has been noted on previous occasions at this Institute (Henderson & Brooksby, 1948; Burrows, 1964*b*) and by Nathans (1965).

Comparisons of infectivity titres in mice and cattle have usually been expressed in terms of end-point dilutions and usually these have been found to be similar for titration of cattle strains of virus (Skinner, Henderson & Brooksby, 1952; Heatley, Skinner & Subak-Sharpe, 1960). Conversion of these measurements to ID 50/ml. results in end-points approximately 0·5 $\log_{10}$ units higher being recorded by mice inoculated by the intraperitoneal route. The mean difference in end-points (ID 50/ml.) between mice and cattle determined in the present experiments has been somewhat greater; mice have given end-points approximately 1·0 $\log_{10}$ units higher than cattle. Four of the eight comparative titrations were carried out using aliquots from a master dilution series to obviate the minor errors inherent in preparing replicate series of dilutions up to 10^{-7}.

Hyslop & Skinner (1964) have speculated on the precise amount of the dose inoculated into the cattle tongue which contributes to the initiation of a local lesion. From preliminary observations they have indicated that the true infectivity for cattle of a virus suspension titrated in cattle by the standard method could well be 20-fold higher than that calculated on the inoculated dose per site of 0·1 ml. The results obtained in the series of titrations catalogued in Table 8 would support this view.

Essential requirements for the evaluation of the efficacy of FMD vaccines for pigs include the ability to titrate the challenge virus and a challenge procedure which will produce both primary and secondary lesions with regularity in the non-immune animal. If immunity is to be evaluated by a method akin to the Lucam K test (Lucam & Fedida, 1958), a titration technique which is capable of analysis by statistical methods is a prerequisite. Inoculation of the bulb of the heel fulfils all these requirements and has been used regularly during the past three years at this Institute for titration and for the challenge of animals vaccinated with both inactivated vaccines and modified virus strain vaccines.

SUMMARY

FMDV strains were titrated in pigs by inoculation of the bulb of the heel of the foot, the coronary band region of the foot, and the tongue. Heel inoculation gave end-points approximately 300-fold higher than did the other two methods.

Parallel titrations in cattle and mice gave infectivity estimates approximately 10-fold and 100-fold higher, respectively, than did heel titration of pigs.

The average variance of individual end-points determined for heel titration was

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0·46 ($\log_{10}$ units) and that determined for coronary-band titration was 0·74. The sources of variations in end-points given by heel titrations are discussed.

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