ANALYSIS OF THE DOSE/RESPONSE RELATIONSHIP IN FOOT-AND-MOUTH DISEASE VACCINATION OF PIGS

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SUMMARY

Based on first-order models for vaccine dose/immunological response relationships, a series of foot-and-mouth disease (FMD) vaccine assays in pigs were analyzed in terms of protection against challenge, levels of circulating antibodies contemporaneous to challenge, and presence of VIA antibodies at 15 days post challenge. According to the models, the first two dose/response relationships would show positive slopes, whereas the last one would be negative. Statistical analysis confirmed the expected results, showing that it is possible to immunize pigs against FMD using good-quality vaccines, and that protection against challenge, presence of VIA antibodies and levels of specific circulating antibodies are functions of the dose of immunogen applied. These relationships indicate that vaccine potency can be estimated by means of the common biological assays or others that involve direct challenge. The antibody levels, although they show a significant correlation with the immune status of pigs, do not present sufficient validity to replace direct challenge in estimating potency.

INTRODUCTION

If the biological assays for estimating the potency of foot-and-mouth disease (FMD) vaccines in pigs follow the general features of biological assays, a direct relationship should exist between the vaccine dose (stimulus) and the pigs' immune response (biological reagents). The response is presented as a variable artificially classed as "protected" and "unprotected"; the interpretation varies according to the type of FMD virus challenge, which comprises the instrument for immunity measurement. If the challenge is by inoculation of a certain quantity of virus—generally in the coronary band or in the heel bulb of a hind hoof—the appearance of any specific lesions elsewhere is considered as indicating an "unprotected" status (infection or generalization). When the challenge is by exposure through contact with pigs previously infected by inoculation, any animal not exhibiting FMD lesions is considered protected or resistant. An alternative method of estimating vaccine potency would be by determining the specific circulating antibody levels by means of serum neutralization techniques.

The results of pig vaccinations have ranged from very poor, like those obtained with some aluminum hydroxide adsorbed vaccines—saponin coadjuvanted or not—(*5, 21*) to very good, produced with mineral oil-adjuvanted vaccines (*3, 22, 25, 26*) or DEAE-Dextran (*30*), or with large doses of aluminum-hydroxide saponin vaccine (*10*). Some reports state that there is no correlation between the pigs' immune status and their serum antibody levels when challenged (*9, 18, 29, 30*). Conversely, Lucam *et al.* (*21*) state that the lesions exhibited by vaccinated pigs in places other than the point of inoculation would be due to reinfection or reinoculation through the skin as a consequence of handling during observation; these authors call them "primary reinoculation lesions" and do not consider them as either secondary or generalization lesions. Based on the discrepancy between antibody levels and immunity in convalescent pigs inoculated with the homologous virus, Gomes (*16*) suggests that determination of the circulating antibody levels could replace the direct challenge test as a means of vaccine potency estimation. On the other hand, other experiments (*2, 3, 10, 25, 26*) indicate that the vaccinated pigs' immunological response is a function of the quantity and quality of the immunogen, and that

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there is a correlation between antibody levels and protection. Other authors even suggest that oil-adjuvanted vaccines containing an appreciable quantity of immunogen should provide better and longer-lasting immunization than through infection with FMD virus (*4, 23*).

In 1966, Cowan and Graves (*8*) reported the existence of a virus-infection-associated (VIA) antigen that induced the formation of specific antibodies in infected animals. McVicar and Sutmöller (*24*) emphasized the potential usefulness of VIA antibodies research for serological epidemiology. Alonso Fernandez *et al.* (*1*) used the detection of these antibodies to estimate cattle exposure to FMD. Gomes *et al.* (*17*) reported that vaccinated pigs that were protected against challenge by contact showed a lower percentage of VIA antibodies than unprotected pigs. However, the type of relationship between vaccination and presence of pisitive VIA sera (VIA+) immediately after challenge has not been studied.

This work presents the statistical analysis of several experimental results of pig vaccination against FMD (*2, 3, 5, 10, 17, 20, 25, 26*), following the theoretical model outlined in Figure 1.

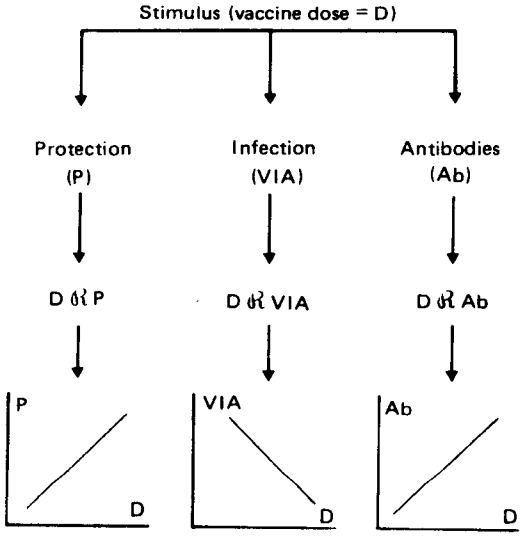

FIGURE 1. *Theoretical model of dose/response relationships produced by FMD vaccination in pigs.*

The model implies: (a) a direct relationship between vaccine dose and protection against challenge; (b) an indirect relationship between vaccine dose and infection after challenge, and; (c) a direct relationship between vaccine dose and levels of circulating antibodies.

MATERIALS AND METHODS

The main substratum of this analysis is the content of Tables 3 and 4 of the work by Gomes *et al.* (*17*). The methodology for calculating the vaccines' potency and estimating the parameters of the dose/effect regression is as indicated by Finney (*14, 15*) for the probit analysis. The transformations used were: x = log vaccine dose expressed in complement fixing units (CFU) (*3, 17*[2]) in virus nanograms (*25, 26*) or in milliliters (*10*); Y = probit percentage of protected or VIA+. For the dose/antibody level study, with the data presented by Gomes *et al.* (*17*), the following was used: x = log CFU and Y = log neutralizing dose 50% (ND_{50}) of the individual sera determined through the microtiter neutralization test described by Ferreira (*13*). The methodology proposed by Ostle (*27*) was utilized for the analysis of variance of the dose/antibody level regression.

RESULTS

Dose/protection relationship

A preliminary analysis showed that the potency of the two vaccines used in the experiment by Gomes *et al.* (*17*) did not differ significantly (p = 0.1028 ...) and that the coefficients of regression were practically parallel (p = 0.1687...), which justified the pooling of the results corresponding to each dose (Table 1).

Figure 2 illustrates the dose/response regression of the pooled results. The variance analysis for the parallelism of the individual slopes yielded a sum of squares χ^2 = 2.077 which, with one degree of freedom (df), is not significant; likewise, the sum of squares (χ^2 = 6.604 with 3 df) due to the heterogeneity between the experimental values

[2]Calculation of the CFU dose was based on the data in Table 1, Reference *17*.

and those forecast according to the regressions was not significant either. The results fitted well to the corresponding model proposed in Figure 1.

TABLE 1. *Distribution of protection according to antigen dose in pigs challenged by contact at 30 days post-vaccination*[a]

Dose (CFU)[b]	Vaccine 1	Vaccine 2	Total (1+2)
30.0	8/8[c]	7/7	15/15
10.0	7/8	5/8	12/16
3.3	2/8	7/8	9/16
1.1	1/6	2/6	3/12
PD_{50} (CFU)	4.13	1.54	2.91
Lower limit	2.41	0.37	1.70
Upper limit	7.08	6.44	4.98
a	3.43	4.78	4.19
b	2.55	1.16	1.75
χ^2 (2 d.f.)	1.787	4.816	1.293

[a]Taken from Gomes *et al.* (17).
[b]Complement fixing units.
[c]Protection/total.

The results of the analyses of the other assays are given in Table 2; the slope variance comparison and heterogeneity are shown in Table 3. It can be observed that although the values of the slopes vary from 1.096 to 3.194, the differences are not significant and yield $\chi^2 = 8.757$ with 7 df. All the slopes differed significantly from zero ($p < 0.05$).

Dose/infection relationship

As a criterion of infection the positivity of sera to the VIA antigen at 15 days post exposure was taken. The negativity of the sera was considered as indicating either the absence of infection or a local, transient infection incapable of inducing the formation of detectable quantities of antibodies. Table 4 shows the distribution of VIA+ and VIA− sera according to the immunogen dose, the vaccine dose that permitted the presence of 50% VIA+ and the regression parameters corresponding to each vaccine and to the per-dose pooled results.

Figure 3 illustrates the regression corresponding to the cumulative results and their respective limits for 95% reliability. This line fits well into the corresponding model in Figure 1. The variance analysis showed that the departure from linearity was not significant ($p > 0.3$), whereas the coefficient of regression $b = -1.4$ differed significantly from zero ($p < 0.025$).

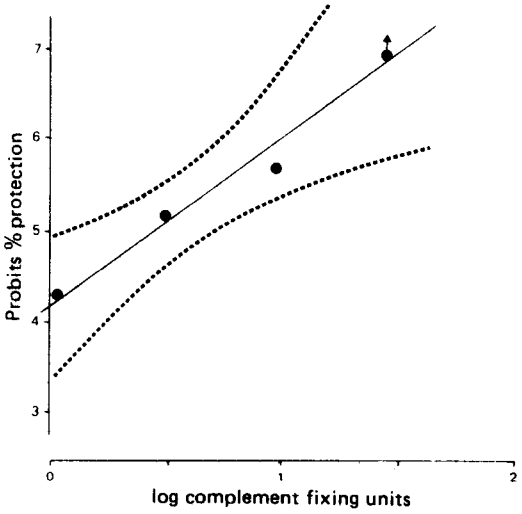

FIGURE 2. *Antigen dose/protection relationship in pigs at 30 days post vaccination. Cumulative results per dose of Vaccines 1 and 2 (17).*
$\hat{Y} = 4.19 + 1.75x$, χ^2 (2 d.f.) = 1.293.

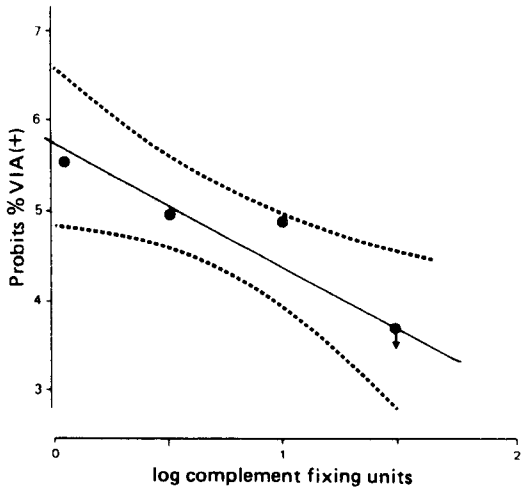

FIGURE 3. *Antigen dose/VIA(+) relationship at 15 days post challenge in pigs. Cumulative results per dose of Vaccines 1 and 2 (17).*
$\hat{Y} = 5.80 - 1.40x$, χ^2 (2 d.f.) = 4.802.

TABLE 2. *Comparison and analysis of dose/response regression slopes in eight potency tests of FMD vaccines in pigs*

Virus	Vaccine	ED50%	Units	Regression	χ^2	d.f.	Ref.
C[a]	DEAE-D	10.98	CFU[b]	$\hat{Y} = 3.516 + 1.426x$	0.804	2	(3)
A[a]	O–PE	209.41	ngr	$\hat{Y} = 0.643 + 2.431x$	0.697	1	(25)
O_1	O–DE	4.13	CFU[c]	$\hat{Y} = 3.431 + 2.547x$	1.787	2	(17)
O_1	O–DE	1.54	CFU[c]	$\hat{Y} = 4.781 + 1.165x$	4.817	2	(17)
A_{22}	O–DE	13.49	ngr	$\hat{Y} = 1.390 + 3.194x$	0.058	3	(26)
Asia 1	O–DE	5.36	ngr	$\hat{Y} = 3.456 + 2.119x$	1.404	3	(26)
SAT 2	O–DE	6.96	ngr	$\hat{Y} = 4.076 + 1.096x$	6.846	3	(26)
C[a]	AH	2.39	ml	$\hat{Y} = 4.139 + 2.274x$	1.278	2	(10)

[a]Subtype not indicated.
[b]Complement fixing units; AVRI technique, Pirbright, United Kingdom.
[c]Complement fixing units; PAFMDC technique.
DEAE-D = Diethylaminoethyl-Dextran; O-PE = oil, primary emulsion; O-DE = oil, double emulsion; AH = aluminum hydroxide; ngr = nanograms.

TABLE 3. *Analysis of the regression χ^2 components of eight potency tests of FMD vaccines in pigs*

Component	d.f.	χ^2	Significance
Heterogeneity	11	17.691	0.098033...
Parallelism	7	8.757	0.270576...
Total	18	26.448	0.089947...

TABLE 4. *Distribution of VIA(+) and VIA(–) pigs according to antigen dose[a]*

Dose (CFU)[b]	Vaccine 1	Vaccine 2	Total (1+2)
30.0	0/8[c]	0/7	0/15
10.0	3/7	4/8	7/15
3.3	5/8	3/8	8/16
1.1	3/4	4/6	7/10
PD_{50} (CFU)	4.6	2.9	3.7
Lower limit	9.7	8.9	7.1
Upper limit	3.1	3.4	4.3
a	6.16	5.53	5.80
b	– 1.74	– 1.15	– 1.40
χ^2 (2 d.f.)	1.872	3.444	4.802

[a]Taken from Gomes *et al.* (17).
[b]Complement fixing units.
[c]No. VIA(+)/No. total.

Table 5 indicates the distribution of VIA+ and VIA– pigs according to their immune status. The coefficient of concordance $\phi = -0.70$ is significant at $p < 0.001$, indicating a strong negative correlation between protection and presence of antibodies to VIA antigen. This additional data confirms that the results fit the corresponding theoretical model (Figure 1).

TABLE 5. *Distribution of VIA(+) and VIA(–) pigs according to protection[a]*

	Protection		
VIA	Yes	No	Total
(+)	6	16	22
(–)	32	2	34
Total	38	18	56

$\phi = -0.70$, $\chi = 24.38$ ($p < 0.001$).
Protection % = 67.9.
VIA(–) % = 60.7.
$t = 1.26$ ($p > 0.1$).

[a]Taken from Gomes *et al.* (17).

Dose/antibody level relationship

The dose (log CFU)/antibody levels correlation was analyzed for each particular vaccine. For

Gomes *et al.* original values (Ref. *17*, Tables 3 and 4) the log CFU/ND_{50} regressions slopes of Vaccines 1 and 2 differed significantly between themselves $|b_1 - b_2| = 0.882$, $t = 9.91$ ($p < 0.001$), although both are positive and different from zero. The large proportion of indeterminate values (equal to or greater than) corresponding to the highest doses of Vaccine 2 produced a marked departure from the linear model: $F_{(2,25)} = 6.06$ ($p < 0.001$) whereas the $ND_{50} \leq 1.2$ produced by the lower dose of Vaccine 1 did not produce a significant lack of fit ($p > 0.6$). After sorting out all the indeterminate values, which meant disregarding all Vaccine 1 lower dose values and all Vaccine 2 higher dose values, the slopes for both vaccines turned out to be almost parallel $|b_1 - b_2| = 0.211$, $t = 0.17$ and their departure from linearity ceased to be significant.

Table 6 lists the expurgated means of indeterminate values and the analysis of the pooled data. It can be seen that the ratio of variance for the lack of fit to linearity is not significant. Figure 4 shows the linear regressions for each vaccine calculated after expurgating the indeterminate values (indicated by arrows).

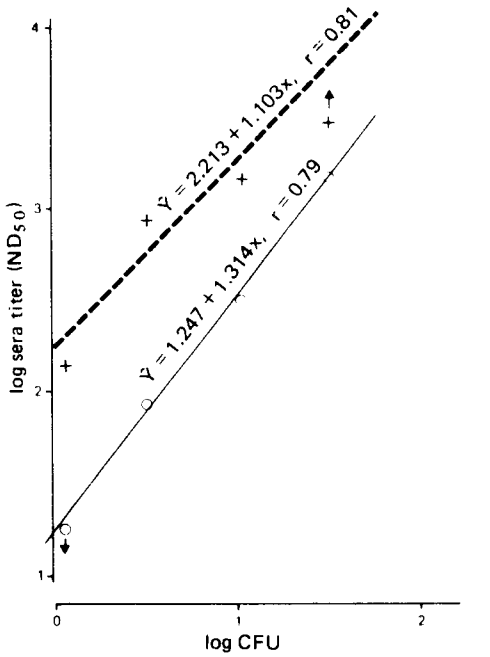

FIGURE 4. *Antigen dose/antibody levels regression in pigs at 30 days post-vaccination.* —— = *Vaccine 1,* – – = *Vaccine 2. Indeterminate values are indicated by arrows.*

TABLE 6. *Analysis of regression antigen dose/antibody levels in pigs at 30 DPV against FMD*

Dose (CFU)	Vaccines 1	2
30.0	3.20 ± 0.24	>3.50
10.0	2.54 ± 0.55	3.20 ± 0.12
3.3	1.95 ± 0.35	2.96 ± 0.34
1.1	<1.2	2.17 ± 0.33
a =	1.247	2.213
b =	1.314	1.103

$|b_1 - b_2| = 0.211$, $t = 0.17$ (not significant)

Analysis of variance

Source of variation	d.f.	Sum of squares	Mean of squares	R.V.
Total	41	17.3848	–	–
Regression	1	5.0805	5.0805	15.87[a]
Fit of results	2	0.1381	0.0691	0.22[b]
Error	38	12.1662	0.3202	–

[a] $p < .001$. [b] Not significant.

R.V. = Ratio of variance.

The correlation between antibody titers and protection to exposure was also studied. That correlation ($r_{bp} = 0.53$, $t = 4.73$ with 57 df) is highly significant although, as seen in Table 7, the distribution of protection is somewhat irregular.

TABLE 7. *Distribution of protection according to antibody levels in pigs at 30 DPV against FMD*[a]

–log ND_{50}	Protection Yes	No	Total
<1.2	0	7	7
1.2 – 1.7	3	1	4
1.8 – 2.3	5	4	9
2.4 – 2.9	5	5	10
3.0 +	26	3	29
Total	39	20	59

$r_{(bp)} = 0.53$, $t_{(57)} = 4.73$ ($p < .001$).

[a] Taken from Gomes *et al.* (*17*).

Table 8 illustrates the sensitivity and specificity of several ND_{50} screening values for classifying the pigs as either "protected" or "unprotected". ND_{50} = 2.6 is the value showing the best equilibrium, corresponding to a sensitivity of 79.5% and a specificity of 75.0%.

TABLE 8. *Sensitivity and specificity of some values of serum neutralization to indicate protected pigs*

	Percentages	
Value	Sensitivity	Specificity
1.8	92.3	40.0
2.3	81.6	60.0
2.6	79.5	75.0
3.0	66.7	85.0

DISCUSSION

Statistical analysis of the results presented by Gomes *et al.* (*17*) shows that there are significant relationships among the variables considered, and that protection, infection and antibody levels fit well to the theoretical models shown in Figure 1. Those analyses and others conducted with data found in other publications lead to several observations:

1. Protection in vaccinated pigs, regardless of the method of challenge employed (contact or inoculation), appears as a variable dependent on the immunogen dose administered; this dependency shows that it is possible to conduct FMD vaccine control tests following experimental designs based on direct virus challenge (50% protective dose or others). Moreover, the response appears to be independent of the type of vaccine coadjuvant whenever the design complies with minimum requirements for the analysis of the biological assays such as the utilization of doses yielding results close to 100% and others near 0%. The 8 published results that were analyzed (Table 2) included the following vaccines: aluminum hydroxide-saponin (*10*), diethylaminoethyl dextran (*3*), primary emulsion oil-adjuvanted (*25*) and double emulsion (*26*) oil-adjuvanted vaccines—all with challenge by inoculation—and double-emulsion vaccines (*17*) challenged through contact. The assumption of Lucam *et al.* (*21*) that many "primary reinoculations" lesions would appear in the pig would be baseless and such lesions, regardless of their origin, should be interpreted as being due to vaccines of low efficacy for pigs, as the authors themselves suggest. Burrows (*7*) also found that the vaccines he tested did not yield good results and also observed that although most of the generalization lesions appeared in the vaccinated pigs on the second and third days after challenge, new lesions could be observed up to the fourth and fifth days post challenge. These new lesions were usually minor. This description coincides with that reported by Lucam *et al.* (*21*).

Analysis of the dose/response regression coefficients in the results presented by Durand *et al.* (*10*), Anderson *et al.* (*3*), Morgan *et al.* (*25*) and Gomes *et al.* (*17*), under the assumption of a common slope b = 1.61 calculated from the total sums of weighted squares, showed that the individual slopes did not differ significantly from it, nor among themselves; the value of χ^2 = 8.351 with 7 df, found to verify the assumption of parallelism, is not significant. Given the small number of animals used in each assay, this result is not surprising. The variance of the slope $V(b) = 1/\Sigma nwx$, i.e., the reciprocal of the weighted sum of squares of x (log dose), depends on the number of replicates per dose (n) and the number of doses in the assay (k); because both figures are small ($n \leqslant 8$ and $k \leqslant 5$), the slope variances are large for each assay. It is possible that the slopes for each vaccine type, and even each virus type, can be characterized in assays with larger numbers of animals per dose.

Although the scope of this study is not to compare the potency of vaccines analyzed—an impossibility because of the diversity of units used to express a dose—a short comment is in order on the potency difference expressed in virus nanograms and found between the results reported by Morgan *et al.* (*25*) and by Mowat (*26*). The latter suggests that the lower potency shown by the Morgan *et al.* immunogen could be due to its being stored for more than 9 months. However, the difference in the quantity of virus used in the challenge may have had some influence; whereas Mowat challenged his pigs with 100 infective doses/pig, Morgan *et al.* used 40,000 infective doses/suckling

mouse. The ratio is about 1/400 and may have influenced the results because pigs show a clear dose/response relationship with respect to the quantity of virus inoculated, which permits the titration of virus in that species. The studies by Graves and Cunliffe (*18*), Burrows (*5, 6, 7*) and Thomas *et al.* (*28*) on susceptible pigs, and those by Fedida *et al.* (*12*) on vaccinated pigs, are conclusive in this respect. The latter authors present Tables of results whose analysis reveals significant dose/response slopes in both susceptible and vaccinated pigs ($p < 0.01$). The significance of these facts should be considered in the design of FMD vaccine potency tests in pigs, whether for experimental or official control purposes, in order to prevent adding uncontrollable variations to those inherent in the tests themselves.

2. The distribution of pigs with VIA+ sera at 15 days post challenge indicated a clear dependency on the vaccine dose. In that case, when the dose/response relationship fitted the forecast model, it indicated that the vaccine not only protected the animals against the appearance of clinical lesions, but also prevented the appearance of infections that were long-lasting and extensive enough to induce the production of detectable quantities of VIA antibodies. This fact is in agreement with the experimental results reported by de Leeuw *et al.* (*19, 20*), who found that individually isolated vaccinated pigs challenged by inoculation showed a correlation between the quantity of virus collected in the oral cavity and the level of antibodies induced by the vaccine and the score of lesions.

Analysis of the paired protection and VIA+ responses disclosed a coefficient of concordance $\phi = -0.70$ ($\chi^2 = 24.38$, $p < 0.001$), which indicated that both variables were inversely correlated. It is likewise noteworthy that the values corresponding to the effective doses for protection and VIA+ of the individual vaccines and of the pooled results are very similar, as are the respective slopes, in absolute values regardless of the sign (Tables 1 and 4).

3. As well as being correlated with the vaccine dose, the circulating antibody levels are also positively correlated with protection (Table 7), according to other authors (*3, 5, 7, 19, 26*). This fact was implicit in the corresponding model in Figure 1. In spite of that good correlation, the validity of serum neutralization for classifying pigs in protected and unprotected categories is not good enough because it lacks satisfactory sensitivity and specificity, at least with respect to the technique utilized in the study by Gomes *et al.* (*17*). Taking $ND_{50} \geqslant 2.3$ as the screening value, the sensitivity —the percentage of pigs classed as positive and actually protected—is 81.6%, while the specifificity —the percentage correctly classed as negative—is 60%; this means that some 40% of the pigs would have been classified as protected by serum neutralization whereas they actually had not been protected. If the screening value is raised to $ND_{50} \geqslant 2.6$, the sensitivity remains at 79.5%, whereas the specificity improves to about 75% (Table 8). These sensitivity and specificity percentages are comparable with those obtained from analyzing the data presented by Burrows (*5*) with the metabolic inhibition test and by de Leeuw *et al.* (*19*) with a plaque reduction assay. For Burrows' data, a screening value of $ND_{50} \geqslant 1.4$ yields 72.7% sensitivity and 93.5% specificity, while sensitivity drops to 40% and specificity reaches 100% for $ND_{50} \geqslant 1.7$. Analysis of the de Leeuw *et al.* (*19*) data shows good correlation between protection and antibody levels; that correlation is highly significant for the pigs challenged by inoculation ($r_{bp} = 0.69$, $t = 5.48$ with 33 df), while for animals challenged through contact it is only significant at the 5% level ($r_{bp} = 0.35$, $t = 2.15$, with 33 df). With respect to sensitivity and specificity, the percentages obtained in the inoculation assays are also better than the contact values: sensitivity 84% and 73% respectively, and specificity 88% and 58%, for $ND_{50} \geqslant 1.5$ screening values. The pooled inoculation and contact results are 80% sensitivity and 70% specificity.

The antibody level/protection correlation, even though not valid enough to substitute direct challenge in FMD potency tests—because, in the final analysis, circulating antibodies are only a partial aspect of immunity (as pointed out by Cunliffe (*9*) and the presence of antibodies does not always mean immunity (*11*)—may nevertheless be an important tool in serological epidemiology once its classification errors and other limitations are more accurately known.

4. The dose/response relationships analyzed herein show that pigs fit the general conditions required for biological assays for estimating the potency of biologically active products. Therefore, a vaccine control method designed for that species should be based on direct challenge protection to satisfy minimum quality conditions.

REFERENCES

1. ALONSO FERNANDEZ, A.; AUGÉ DE MELLO, P.; GOMES, I.; ROSENBERG, F. The use of virus-infection-associated antigen (VIA) in the detection of cattle exposed to foot-and-mouth disease virus. *Bltn Centro Panamericano Fiebre Aftosa 17-18*: 17-22, 1975.
2. ANDERSON, E.C. Some observations on the serological response of pigs to emulsified foot-and-mouth disease vaccine. Europ. Comm. Control FMD Mtg. Res. Group. Stdg. Techn. Committee, Brescia, Italy, 1969.
3. ANDERSON, E.C.; MASTERS, R.C.; MOWAT, G.N. Immune response of pigs to inactivated foot-and-mouth disease vaccines. *Res. vet. Sci. 12*: 342-350, 1971.
4. BACHRACH, H.L.; McKERCHER, P.D. Immunology of foot-and-mouth disease in swine: Experimental inactivated-virus vaccines. *J. Am. Vet. med. Assn. 160*: 9-16, 1972.
5. BURROWS, R. The behavior of some modified strains of foot-and-mouth disease virus in pigs. II. Immunogenicity. *Bull. Off. int. Épizoot. 61*: 1277-1293, 1964.
6. BURROWS, R. The infectivity assay of foot-and-mouth disease virus in pigs. *J. Hyg.,* Camb., *64*: 419-429, 1966.
7. BURROWS, R. Notes on the results of experiments using inactivated foot-and-mouth disease virus vaccines in pigs. *EUFMD/Mtg. Stdg. Techn. Comm.,* Pirbright, Great Britain, 1966.
8. COWAN, K.M.; GRAVES, J.H. A third antigenic component associated with foot-and-mouth disease infection. *Virology 30*: 528-540, 1966.
9. CUNLIFFE, H.R. Antibody response in a group of swine after infection with foot-and-mouth disease virus. *Can. J. med. vet. Sci. 26*: 182-185, 1962.
10. DURAND, M.; GUILLOTEAU, B.; GIRAUD, M.; GUERCHE, M.; PESSON, M.; PRUNET, P. Étude d'agents alkylants pour l'inactivation des virus aphteux et la préparation d'un nouveau vaccin inactivé. *Bull. Off. int. Épizoot. 69*: 429-465, 1968.
11. FAZEKAS de St. GROTH, S. Neutralization of viruses. *Adv. Virus Res. 9*: 1-116, 1962.
12. FEDIDA, M.; COUDERT, M.; THOMAS, J.-P.; DANNACHER, G.; PEILLON, M.; LUCAM, F. Mesure de l'immunité anti-aphteuse post-vaccinal du porc par épreuve virulente. *Rcl. Méd. Vét. 147*: 309-325, 1972.
13. FERREIRA, M.E.V. Prueba de microneutralización para estudios de anticuerpos de la fiebre aftosa. (Microtiter neutralization test for the study of foot-and-mouth disease antibodies). *Bltn Centro Panamericano Fiebre Aftosa 21-22*: 17-20, 21-24, 1976.
14. FINNEY, D.J. Statistical methods in biological assays. Griffin & Co., London, 2nd ed., 1964.
15. FINNEY, D.J. Probit analysis. Cambridge University Press ed., 1971.
16. GOMES, I. Fiebre aftosa: reacción de cerdos convalecientes a la exposición de virus homólogos. (Foot-and-mouth disease: reaction of convalescent pigs to homologous virus exposure). *Bltn Centro Panamericano Fiebre Aftosa 26*: 15-18, 19-22, 1977.
17. GOMES, I.; AUGÉ DE MELLO, P.; ALONSO FERNANDEZ, A.; FREITAS COSTA, K. Vacuna antiaftosa con adyuvante oleoso para cerdos. III. Respuesta inmunitaria con vacunas emulsificadas por vibración ultrasónica o por agitación mecánica. (Foot-and-mouth disease oil adjuvanted vaccines for pigs. III. Immune response of vaccines emulsified by ultrasonic or mechanical equipment). *Bltn Centro Panamericano Fiebre Aftosa 35-36*: 19-25, 27-33, 1979.
18. GRAVES, J.H.; CUNLIFFE, H.R. The infectivity assay of foot-and-mouth disease virus in swine. *In*: 63th ann. Proc. U.S. Livestock Sanit. Assoc.: 340-345, 1959.
19. LEEUW, P.W. de; TIESSINK, W.A.; FRENKEL, S. Vaccination of pigs with formaldeyde inactivated aluminium hydroxide foot-and-mouth disease vaccines, potentiated with diethylaminoethyldextran (DEAE-D). *Zbl. Vet. Med. B 26*: 85-97, 1979.
20. LEEUW, P.W. de; TIESSINK, W.A.; van BEKKUM, J.G. The challenge of vaccinated pigs with foot-and-mouth disease virus. *Zbl. Vet. Med. B 26*: 98-109, 1979.
21. LUCAM, F.; DHENNIN, L.; DHENNIN, L.; FEDIDA, M. Essais de vaccination anti-aphteuse intradermique chez le porc; résultats obtenues au laboratoire. *Bull. Off. int. Épizoot. 57* (5-6): 924-936, 1962.

22. McKERCHER, P.D.; GAILIUNAS, P. Response of swine to inactivated foot-and-mouth disease vaccine. *Arch. ges. Virusforsch. 28*: 165-176, 1969.
23. McKERCHER, P.D.; GIORDANO, A.R. Foot-and-mouth disease in swine. I. The immune response of swine to chemically-treated and non-treated foot-and-mouth disease virus. *Arch. ges. Virusforsch. 20*: 39-53, 1967.
24. McVICAR, J.W.; SUTMÖLLER, P. Foot-and-mouth disease: The agar precipitation test for antibodies to virus-infection associated (VIA) antigen as a tool for epidemiologic surveys. *Am. J. Epid. 92*: 273-278, 1970.
25. MORGAN, D.O.; McKERCHER, P.D.; BACHRACH, H.L. Quantitation of antigenicity and immunogenicity of purified foot-and-mouth disease virus vaccine for swine and steers. *Appl. Microbiol. 20* (5): 770-774, 1970.
26. MOWAT, G.N. Quantities of purified antigens required to immunize swine against foot-and-mouth disease. *Bull. Off. int. Épizoot. 77*: 887-897, 1972.
27. OSTLE, B. Statistic in research. Iowa State University Press, 2nd ed., 1963.
28. THOMAS, J.P.; FEDIDA, M.; COUDERT, M.; DANNACHER, G.; PEILON, M.; LUCAM, F. Mise au point d'une méthode de titrage du virus aphteux sur le porc. *Bull. Acad. vét. 44*: 353-357, 1972.
29. WITTMANN, G.; BAUER, K.; MUSSGAY, M. Essais de vaccination de porcs avec des vaccins à base de virus aphteux inactivé. I. Essais avec du virus O inactivé par l'hydroxylamine, le formol, la chaleur et le pH. *Bull. Off. int. Épizoot. 71*: 351-379, 1969.
30. WITTMANN, G.; BAUER, K.; MUSSGAY, M. Experiments on vaccination of pigs with ethyl-ethyleneimine (EEI) diethylaminoethyl-Dextran (DEAE-D) foot-and-mouth disease vaccines. Influence of route of inoculation and dose of antigen on the duration of immunity. *Arch. ges. Virusforsch. 36*: 251-264, 1972.