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Enzyme-linked immunosorbent assay (ELISA) for the detection of antibodies against foot-and-mouth disease virus

III. Evaluation of antibodies after infection and vaccination

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SUMMARY

Investigations using a liquid-phase blocking sandwich enzyme-linked immunosorbent assay (ELISA) for the measurement of antibodies against foot-and-mouth disease virus (FMDV) in sera from sheep and from cattle are reported, and results compared with those obtained by virus neutralization (VN) tests.

Serum antibody titres in sheep after primary vaccination and in cattle challenged with a natural aerosol after vaccination were similar by ELISA and VN. However, the antibody levels detected in sera of cattle during early infection and of vaccinated cattle after intradermolingual challenge were clearly greater by ELISA than by VN.

The ELISA titres in cattle sera following synthetic peptide vaccination indicated some relationship to protection and were clearly different from those recorded by VN. On the other hand, the antibody levels following conventional vaccination showed that ELISA and VN titres in cattle sera were related to protection. Although there was a good agreement between the ELISA antibody titre and protection for the four vaccines used, by VN the titre which afforded protection varied depending on the vaccine used.

The ELISA was considered therefore to be more reliable than the VN and may prove useful for evaluating the immunological response of animals following infection and following vaccination.

INTRODUCTION

The virus neutralization (VN) test has been used for many years to measure antibodies against foot-and-mouth disease virus (FMDV). The VN titres recorded after vaccination have been shown to correlate with protection against FMDV challenge in cattle (Martin & Chapman, 1961; Sutmoller & Vieira, 1980; Pay *et al.* 1983), and in pigs (Bauer, Lorenz & Wittmann, 1975; Black *et al.* 1984). Whereas the VN test measures those antibodies which neutralize the infectivity of the virion *in vitro*, the enzyme-linked immunosorbent assay (ELISA) probably measures all classes of antibodies, including those generated against incomplete and non-infectious virus. Results from the two assays, therefore, may not be

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directly comparable in the assessment of immunity against foot-and-mouth disease (FMD), particularly since the mechanisms of *in vivo* protection are not fully understood.

A liquid-phase blocking sandwich ELISA, described by Hamblin, Barnett & Hedger (1986) and Hamblin, Barnett & Crowther (1986), was developed for the detection of antibodies against FMDV. The ELISA titres showed a good correlation with those recorded by VN tests. The authors suggested that this ELISA could replace the traditional VN for assessing antibodies in sera from convalescent and vaccinated animals and for the routine screening of sera from animals intended for export.

In this study, sera were collected from naturally infected cattle, from vaccinated cattle which were challenged experimentally or naturally, and from vaccinated sheep. These sera were examined using the liquid-phase blocking sandwich ELISA and VN. Results recorded by each assay were compared, to determine if the natural and acquired immunological reactions could be discriminated and whether protection could be predicted from the acquired immunological response.

MATERIALS AND METHODS

Viruses

Foot-and-mouth disease virus strain O_1 BFS 1860, A_{22} IRAQ 24/64, A_{24} CRUZEIRO and C OBERBAYERN were propagated in BHK-21 monolayer tissue culture cells. Cultures were harvested between 18 and 24 h after inoculation, when 100% cytopathic effect was observed. Supernatant fluids were clarified at 2000 rev./min for 10 min stored at 4 °C.

Rabbit and guinea-pig antiserum

Type-specific rabbit antisera were prepared by two subcutaneous inoculations of inactivated 146S FMDV in Freund's complete adjuvant (FCA) (Have & Jensen, 1983). Animals were exsanguinated after the second inoculation. Sera was dispensed into aliquots and stored at −20 °C.

Type-specific guinea-pig antisera were prepared by single inoculation of inactivated 146S FMDV in FCA as described by Ferris & Donaldson (1984). Guinea-pigs were exsanguinated after 28 days. Collected sera were pooled, dispensed into aliquots and stored at −20 °C.

Virus neutralization test

Virus neutralization tests were performed in flat-bottomed tissue-culture grade microtitre plates using methods previously described by Golding *et al.* (1976). Controls in each test included two virus titrations, from which the actual amount of virus used in the test was calculated, and two quadruplicate twofold dilution series of convalescent cattle reference antisera. Titres were expressed as the reciprocal of the last dilution of serum in the serum/virus mixtures which neutralized an estimated 100 $TCID_{50}$ of virus at the 50% end-point according to the method of Kärber (1931).

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Liquid-phase blocking sandwich ELISA

The ELISA was performed in flexible, polyvinyl chloride microtitre plates (Dynatech) using the methods described by Hamblin, Barnett & Hedger (1986). Briefly the method involved the incubation of constant pre-titrated virus with a dilution series of test serum in liquid-phase. Any virus which was not totally blocked by antibodies in the test serum was assayed using a trapping ELISA. Each virus was used at a dilution estimated to give an absorbance reading of 1·5 by ELISA (Hamblin, Barnett & Hedger, 1986). Virus controls and quadruplicate twofold dilutions of homologous reference antiserum from convalescent cattle were included on the first and last plate of each test. End-point titres were expressed as the reciprocal $\log_{10}$ of the final dilution of serum in the serum/virus mixtures giving 50% of the absorbance recorded in the virus control. Based on previous data (Hamblin, Barnett & Crowther, 1986), ELISA titres less than $\log_{10}$ 1·6 were considered negative.

Cattle infection

Three crossbred Friesian calves were infected with an artificial aerosol of FMDV strain O_1 BFS 1860 generated using a May spinning-top apparatus as described by Donaldson *et al.* (1987) and then housed in separate loose boxes in a high-security isolation unit. Calves numbered 1 and 2 received an estimated dose of $\log_{10}$ 5.2 $TCID_{50}$ FMDV. Animal number 3 received an estimated dose of $\log_{10}$ 4·4 $TCID_{50}$ FMDV. Serum samples were collected daily from each animal between day 0 and 16 after infection and stored at −20 °C before being assayed by ELISA and VN.

Primary and secondary vaccination

Two crossbred Border Leicester sheep were inoculated subcutaneously on the side of the neck with 1·0 ml of monovalent saponin-alhydrogel 0_1 FMD vaccine. Each animal was bled before vaccination (day 0) and then daily until day 12. Both animals were re-vaccinated on day 37. Daily blood sampling was continued until day 45. Sera were stored at −20 °C before being assayed by ELISA and VN.

FMD vaccination and natural aerosol challenge

Nine crossbred Hereford calves which had been inoculated with the recommended dose of saponin-alhydrogel O_1 FMD vaccine were bled on days 0, 7, 14 and 21. The calves were challenged on day 22 for 1 h by indirect contact exposure to two pigs with early acute FMD. During challenge each of the calves received an estimated dose of $\log_{10}$ 3·4 $TDID_{50}$ FMDV. The calves were isolated from the pigs and bled daily until day 14 and on days 22 and 29 after the challenge. Two non-vaccinated cross bred Hereford calves were also exposed to infection on day 22.

Synthetic peptide vaccination

Three groups of calves, numbered 1–9, were vaccinated with varying doses of a chemically synthesized peptide (DiMarchi *et al.* 1986) based on the virus coat protein (VP1) sequence of FMDV strain O_1 KAUFBEUREN. Each group received a subcutaneous inoculation of 3 ml peptide in FCA. Blood samples were

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collected from all animals on days 0, 7, 14, 21, 26 and 32. Calves numbered 1–3 were re-vaccinated on day 32 with 3 ml peptide in Freund's incomplete adjuvant and further blood samples taken on days 39, 46 and 53. Animals numbered 4–9 and 1–3 were challenged intradermolingually on days 32 and 53 respectively with 10^5 cattle infective doses (ID_{50}) of virulent virus. The calves were examined for 7 days after challenge for evidence of generalized FMD.

FMD vaccine potency tests

Four monovalent vaccines (O_1 LAUSANNE, A_{22} IRAQ 24/64, A_{24} CRUZEIRO and C OBERBAYERN) prepared from antigens stored in the International Vaccine Bank, Institute for Animal Disease Research, Pirbright, were each tested in 24 crossbred Hereford cattle. The cattle were randomly divided into three groups of eight animals. A blood sample was collected (day 0) before each group was inoculated subcutaneously on the side of the neck with 3 ml of either a 1 in 2, a 1 in 10 or a 1 in 50 dilution of vaccine. Serum samples were collected from each animal at 7, 14 and 21 days after vaccination. The cattle were challenged on day 21 by intradermolingual inoculation into ten sites (0·1 ml per site) of homologous virus containing 10^5 cattle ID_{50} per ml and examined daily for 19 days for evidence of virus generalization. In most cases blood samples were also collected from the animals following challenge.

RESULTS

The antibody titres recorded by ELISA and VN in calf sera collected between days 0 and 14 following experimental infection are shown in Fig. 1. The low titres recorded by ELISA and VN between days 0 and 5 were similar and were considered to be non-specific. Positive antibody titres could be detected in sera from all calves after day 5 by ELISA and after days 8–9 by VN. From day 5 onwards the ELISA titres were considerably greater than those recorded by VN (approximately twenty-fold by day 14).

Fig. 2 shows the mean antibody titres recorded by ELISA and VN in the sera of two sheep following primary and secondary vaccination against O_1 BFS 1860 FMDV. The antibody titres recorded by both assays were similar following primary vaccination and until 5 days after secondary vaccination. Thereafter the antibody slopes by ELISA and VN diverged. Positive antibody titres could be detected by both assays 5–6 days after the first vaccination.

The mean antibody titres in the sera of nine vaccinated calves which were challenged with a natural aerosol of homologous type O_1 FMDV are shown in Fig. 3. No clinical disease was observed in any of the animals and there was no rise in serum titres following challenge. The two non-vaccinated control calves developed clinical disease, and ELISA and VN titres showed that both animals had sero-converted five days after infection. The VN antibodies reached plateau height by day 7 and were maintained until day 21 after exposure to FMDV. The ELISA antibody titres peaked on day 12, when they were approximately twentyfold greater than the VN titres, and then declined.

Fig. 4 shows the mean antibody titres recorded by ELISA and VN in the sera of three groups of eight cattle which had received varying dilutions of vaccine followed by intradermolingual challenge with FMDV strain C OBERBAYERN.

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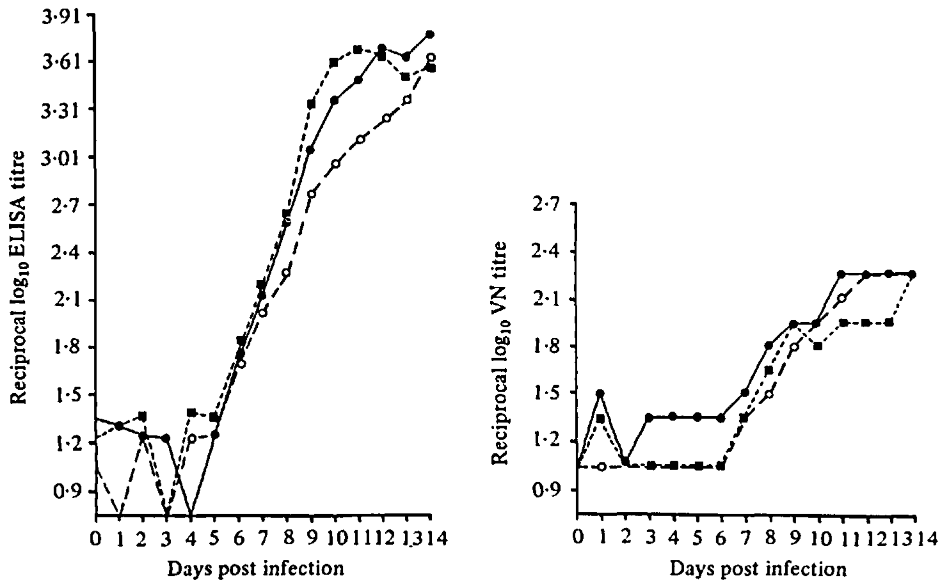

Fig. 1. Antibodies detected by ELISA and VN in the sera of three calves infected with an artificial aerosol of FMDV strain O_1 BFS 1860. ●——●, Calf 1; ■------■, 2 and ○---○, 3.

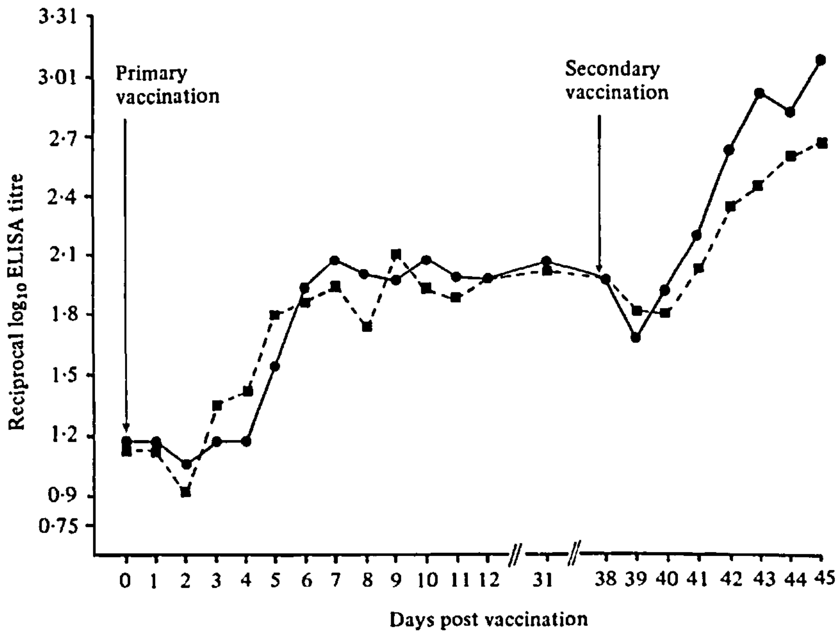

Fig. 2. Mean antibody titres recorded in the sera of two sheep following primary and secondary vaccination against FMDV strain O_1 BFS 1860. ●——●, ELISA titres; and ■——■, VN titres.

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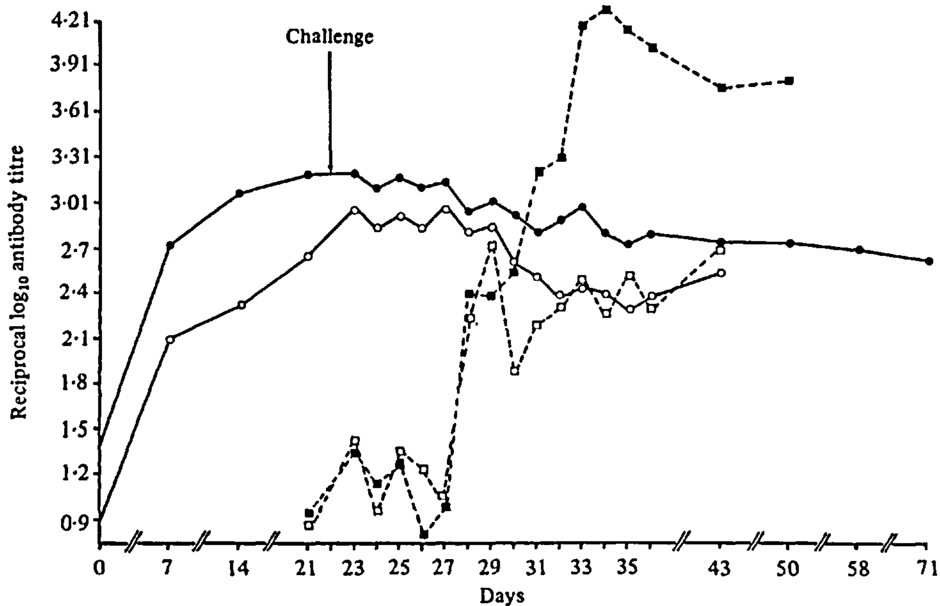

Fig. 3. The mean antibody titres in the sera of nine vaccinated and two non-vaccinated calves which were challenged on day 22 with a natural aerosol of FMDV strain O_1 BFS 1860. ●——● and ○——○ are the mean ELISA and VN titres, respectively, for the vaccinated calves. ■----■ and □----□ are the mean ELISA and VN titres for non-vaccinated control animals.

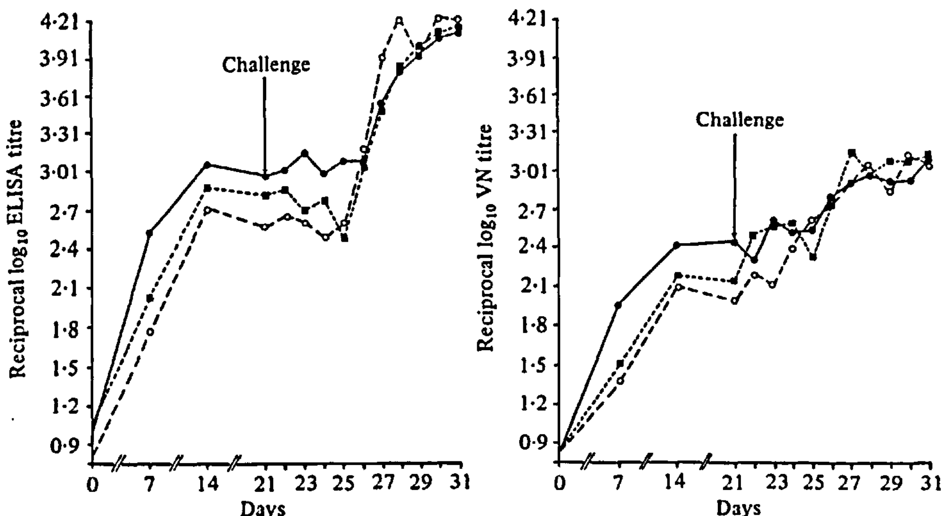

Fig. 4. The mean ELISA and VN antibody titres in the sera of three groups of eight cattle which been vaccinated against and challenged with homologous FMDV strain C OBERBAYERN. Group 1 ●——● had received vaccine diluted 1 in 2, group 2 ■------■ vaccine diluted 1 in 10 and group 3 (○---○) vaccine diluted 1 in 50.

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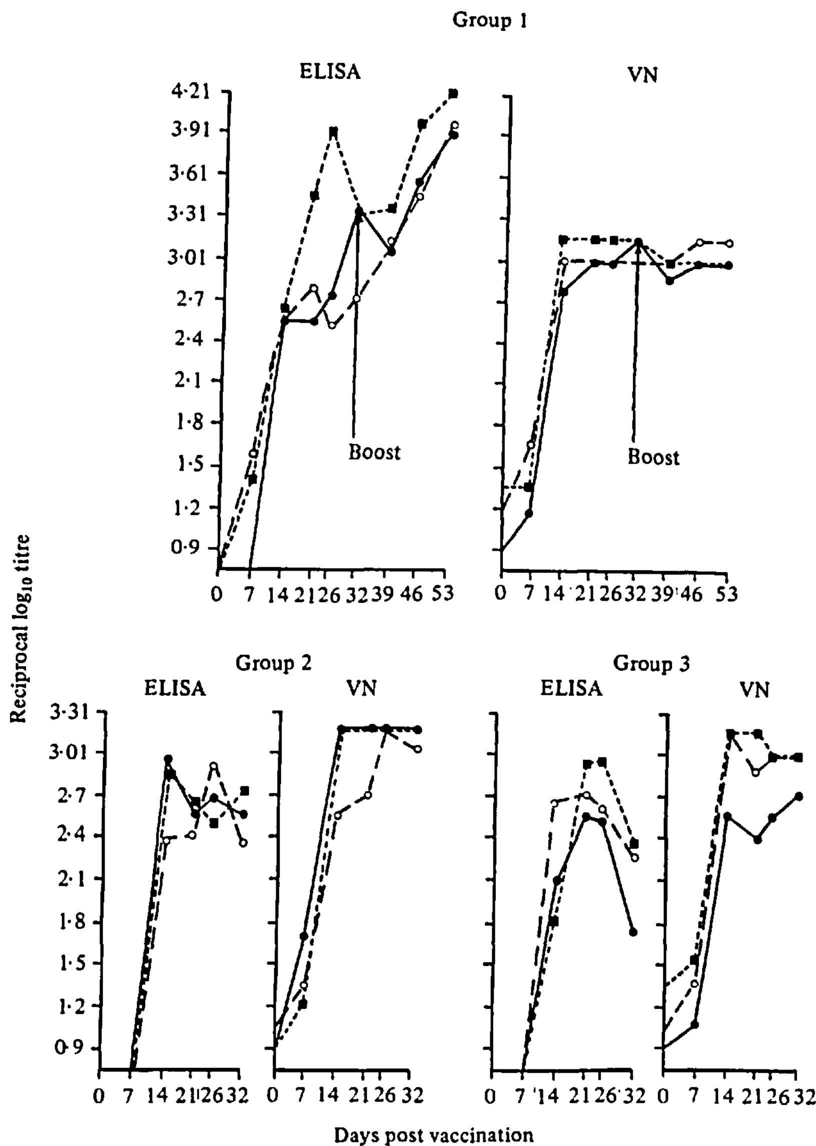

Fig. 5. Antibodies detected by ELISA and VN in sera from calves following vaccination with varying doses of a synthetic peptide prepared against FMDV strain O_1 KAUFBEUREN. Group 1 received 1 mg peptide in FCA on day 0 and 0·2 mg peptide in Freund's incomplete adjuvant on day 32. Groups 2 and 3 received 5 mg and 1 mg peptide in FCA respectively.

No difference in ELISA and VN titre could be detected in sera collected on days 0, 7, 14 and 21 and for 4 days after challenge. Thereafter ELISA titres were up to twelvefold greater than the VN titres. Clinical FMD did not occur after intradermolingual challenge, although a rise in the ELISA and VN titre could be measured in all the animals. The localized replication of virus at the site of inoculation always resulted in a secondary antibody response, which could be differentiated by ELISA.

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Table 1. *The relationship between the antibody titres recorded by ELISA and VN 21 days after vaccination and protection against challenge with FMDV*

Animal Number	Vaccine	O_1 LAUSANNE VN	O_1 LAUSANNE ELISA	A_{22} IRAQ 24/64 VN	A_{22} IRAQ 24/64 ELISA	A_{24} CRUZEIRO VN	A_{24} CRUZEIRO ELISA	C OBERBAYERN VN	C OBERBAYERN ELISA
1	1/2	2·85	3·16 P	2·55	2·65 P	2·55	2·70 P	1·95	2·87 P
2		2·70	3·13 P	2·70	2·70 P	2·25	2·26 P	2·70	3·07 P
3		2·70	3·24 P	2·70	2·80 P	2·10	2·29 P	2·40	2·89 P
4		2·70	2·98 P	3·16	3·70 P	2·25	2·51 P	2·10	2·65 P
5		2·70	3·08 P	2·85	2·55 P	2·55	2·51 P	2·70	2·98 P
6		3·16	3·16 P	2·70	2·82 P	1·95	2·29 P	2·55	3·12 P
7		1·95	2·50 P	3·01	2·55 P	1·65	2·05 P	2·25	3·03 P
8		3·01	3·06 P	3·01	3·34 P	1·50	2·04 P	2·40	3·08 P
9	1/10	1·80	2·30 P	2·25	2·83 P	2·25	2·20 P	2·25	3·04 P
10		2·55	2·51 P	2·70	2·85 P	1·95	2·04 P	1·95	2·79 P
11		3·16	2·89 P	2·40	2·62 P	1·20	1·95 F	2·10	2·72 P
12		2·40	2·42 P	2·25	2·70 P	1·95	2·04 P	2·25	2·77 P
13		1·80	2·04 P	1·50	2·04 P	1·65	2·03 P	2·25	2·76 P
14		2·55	2·80 P	2·10	2·16 P	1·05	1·52 F	2·25	2·77 P
15		2·70	2·44 P	1·65	1·67 F	1·20	1·83 F	2·25	3·01 P
16		1·95	1·99 F	2·40	2·54 P	2·40	2·54 P	1·65	2·55 P
17	1/50	1·80	1·95 F	1·95	2·12 P	0·75	1·30 F	2·10	2·34 P
18		2·70	2·44 P	1·80	2·08 P	0·90	1·35 F	2·10	2·79 P
19		2·25	2·10 P	1·35	1·64 F	0·75	1·64 F	2·10	2·72 P
20		2·25	2·00 F	2·10	2·55 P	0·75	1·35 F	2·25	2·68 P
21		1·65	2·04 F	1·95	2·08 P	1·50	1·87 F	1·80	2·38 P
22		2·55	3·01 P	1·95	2·42 P	1·35	1·93 F	1·95	2·72 P
23		1·65	1·99 F	2·10	2·18 P	1·35	1·35 F	1·95	2·25 P
24		1·35	1·64 F	1·95	2·02 P	1·05	1·84 F	1·95	2·25 P

P denotes protected and F denotes not protected.

The ELISA and VN titres recorded for calves vaccinated with different doses of synthetic peptide are shown in Fig. 5. In most cases VN titres reached plateau height by day 14 and remained between $\log_{10}$ 2·7 and 3·31 until challenge. Animals numbered 1–3 from group 1, which received a second vaccination, and numbers 4 and 5 from group 2, which received a single vaccination of 5 mg peptide, resisted challenge with virulent FMDV type O_1. The ELISA titres varied depending on the dose of peptide administered, but at the time of challenge animals with antibody titres equal to or greater than $\log_{10}$ 2·55 were protected, and animals with antibody titres less than or equal to $\log_{10}$ 2·0 were not protected.

Table 1 shows the ELISA and VN titres in the sera of four groups of 24 cattle 21 days after conventional vaccination with the four monovalent vaccines. The clinical signs seen in the cattle during the 10 days following intradermolingual challenge were recorded. Animals were considered protected when no sign of generalized FMD was observed. The highest and lowest ELISA titre recorded with sera from animals which failed or were protected was $\log_{10}$ 2·04 and 2·02 respectively, whereas by VN the titre was $\log_{10}$ 2·25 and 1·5 respectively.

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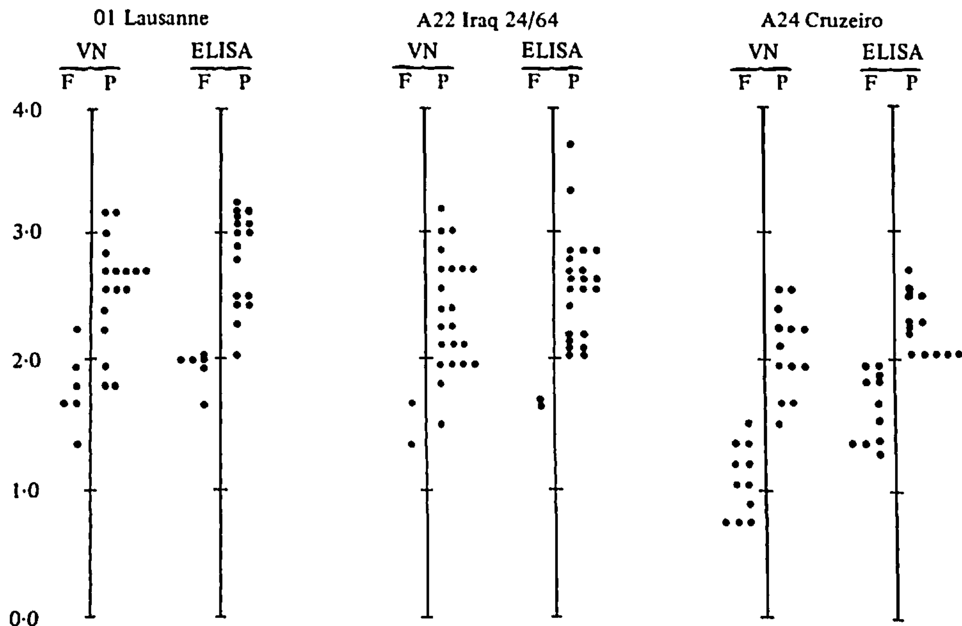

Fig. 6. The relationship between ELISA and VN antibody titres (reciprocal $\log_{10}$) and protection following monovalent FMD vaccination against O_1 LAUSANNE, A_{22} IRAQ 24/64 and A_{24} CRUZEIRO. P and F signify protected and non-protected respectively.

The relationship between ELISA and VN antibody titres and protection 21 days after conventional vaccination is shown in Fig. 6. Serum from one protected and one non-protected animal which had been vaccinated against FMDV type O_1 recorded an ELISA titre of $\log_{10}$ 2·04. Overlapping VN titres were recorded in 21-day sera of seven, two and two of the immune/non-immune cattle vaccinated against O_1, A_{22}, and A_{24} FMDV respectively.

DISCUSSION

Many countries vaccinate against FMD, and the first consideration when selecting a vaccine is whether it will protect against the strains of virus circulating in the country or in neighbouring countries. Serological data is valuable when assessing a response to vaccination and for epidemiological surveillance of vaccinated and non-vaccinated animals, particularly when vaccine breakdown is suspected.

Several authors have used the VN test to study the relationship between antibody titre and protection in FMD-vaccinated animals but results have not always been consistent. For example, Martin & Chapman (1961) Mackowiak *et al.* (1962) and Pay *et al* (1983) reported a correlation between VN titre and protection in cattle with each vaccine studied, but concluded that the antibody titre which corresponded to protection depended on the batch and strain of FMD vaccine used. On the other hand, Bauer, Lorenz & Wittmann (1975) reported that a correlation between antibody titre and protection depended on the strain of FMDV used in the vaccine, and concluded that an antibody titre alone does not

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provide a useful measure for potency testing in pigs. Black *et al.* (1984), however, reported a correlation between VN antibody titre and protection in vaccinated pigs and suggested that a group mean serum antibody titre may provide the basis for a revised oil-emulsion vaccine potency-testing procedure.

Despite these anomalies the VN test has been used extensively and is considered to be the best method available for the measurement of antibodies in sera, not only from vaccinated but also from infected animals. The test is generally considered to be sensitive and specific, but the wide variation in antibody titres recorded previously in replicate tests (Hamblin, Barnett & Hedger, 1986) suggests that the assay is not always reliable. This variation is probably due to differences in the sensitivity of the tissue culture cells, which ultimately affect the dose of virus used in the test. Therefore, tests are only considered valid if the actual virus dose and the reference serum titre are within predetermined limits. Consequently the test serum antibody titres also vary, making the interpretation of results difficult.

In a previous publication Hamblin, Barnett & Crowther (1986) showed that ELISA and VN titres in the sera of animals which had received a single vaccination against FMD or had recovered from clinical FMD were generally in agreement. In these studies, the ELISA titres in sera of sheep after primary vaccination were also comparable to those recorded by VN and therefore support the earlier findings. Although the anamnestic response which followed the second vaccination was detected by both assays, the slopes of the antibody curves by ELISA and VN appeared to be divergent after day 42. Unfortunately sera were not available after day 45. However, further studies to investigate the possible difference in the antibodies detected by ELISA and VN following secondary vaccination may be rewarding.

In contrast, the ELISA titres in sera collected from animals during early infection were considerably higher than those recorded by VN. These results suggested that, in addition to neutralizing antibodies, the ELISA was measuring other classes of antibodies which did not neutralize FMDV *in vitro*. In this study, no serum was available from the clinically infected animals after day 28 and therefore the persistence of these 'non-neutralizing' antibodies must remain speculative. However, the lack of any difference in titre by ELISA and VN in convalescent animals (Hamblin, Barnett & Crowther, 1986), together with the results presented here, suggests that some of the early antibodies detected by ELISA are short lived.

Whereas an anamnestic response was detected in vaccinated cattle after intradermolingual challenge, no secondary antibody response was measured in the vaccinated calves challenged with a natural aerosol of FMDV. This is probably a reflexion of the difference in the method of challenge used, in particular of the quantities of virus, the degree of tissue damage induced and the amount of virus replication. Although the difference in the antibody response following natural and artificial challenge was distinguished by both assays, these differences were more apparent by ELISA.

The interpretation of VN antibody titres in sera from animals which had been vaccinated with a synthetic peptide was difficult, both because the antibody levels in protected and non-protected animals were similar and because of the small number of animals involved. The antibody titres by ELISA, however, appeared to

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be different in sera from protected and non-protected calves. Although these data are limited, these assays probably measure different antibodies. The ELISA, therefore, may be more relevant to protection than the VN test. On the other hand, the data recorded by ELISA and VN following conventional vaccination were similar for the four vaccines tested. Individual ELISA titres, however, were more closely related to protection than were VN titres. Further assessment of the ELISA is being made to evaluate it fully as an alternative to the VN test for testing and monitoring vaccines.

In conclusion, the ELISA is considerably more precise than the VN test and is probably a more reliable measurement of the antibody status of infected and vaccinated animals. The results presented here also indicate that the ELISA measures antibodies which appear to be relevant to protection but which are not measured in VN tests. This may be useful in assessing differences in the antibodies present in sera from infected and vaccinated animals and for identifying early infection, particularly in sheep, where clinical FMD can be absent or mild. The nature of these antibodies and the factors influencing their detection in ELISA remain to be evaluated.

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