

***In vitro* comparison of foot-and-mouth disease virus subtype variants causing disease in vaccinated cattle**

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SUMMARY

Foot-and-mouth disease virus isolates of types O, A and SAT 2, from diseased animals in herds routinely vaccinated twice a year were compared antigenically with the vaccine strains in the complement-fixation, neutralization and radial immunodiffusion tests. It was found that strains which had readily infected vaccinated cattle had *R* values against the vaccine strain in the complement-fixation and radial immunodiffusion tests of 30 or less, while strains causing primary outbreaks with little spread had *R* values of 30–40. Threefold differences in humoral neutralizing antibody concentration between the field variant and the vaccine strain in sera from vaccinated animals were likely to be significant in terms of protection.

INTRODUCTION

The results of a taxonomic study of the range of antigenic subtype variants found in the enzootic areas of Kenya have been reported (Anderson, Anderson & Doughty, 1974*a*). Subtype variants are distinguished by the fact that immunization to one subtype does not confer as solid an immunity to another variant of the same immunological type as to the homologous strain (Traub & Mohlmann, 1946; Galloway, Henderson & Brooksby, 1948; Ubertini *et al.* 1964). It is desirable to be able to compare the degree of antigenic variation between vaccine strains and field strains as shown by *in vitro* methods with the ability of such field strains to cause disease in vaccinated animals. In this paper we relate the degree of antigenic variation determined by three *in vitro* methods to the severity of disease and ease of spread of FMD variants in herds regularly vaccinated bi-annually with an inactivated vaccine in Kenya. This vaccination schedule has, since its inception in 1968, resulted in a considerable decrease in both the number of disease outbreaks (Chema, 1975) and of virus carrier animals (Anderson, Doughty & Anderson, 1974*b*) and consequently such outbreaks have only occurred occasionally. However, it has been necessary on two occasions in the last 5 years to incorporate new variants in the vaccine in order to provide satisfactory immunity to the new strain.

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METHODS

Virus strains

For this study variants of the immunological types O, A and SAT 2 were selected. These had been isolated in primary calf thyroid monolayers (Snowdon, 1966) from tongue epithelial samples collected from diseased animals in regularly vaccinated herds.

Strain differentiations

The selected strains were compared by three *in vitro* methods:

(a) *Micro complement-fixation* (Darbyshire, Hedger & Arrowsmith, 1972). Cross fixation ratios (r) were obtained for the reaction of both the field isolate and the vaccine strain with the respective homologous and heterologous antisera and the product of these ratios ($r_1 \times r_2$) calculated as described by Davie (1964). These are expressed as R values, where $R = 100\sqrt{(r_1 r_2)}$ as described by Ubertini *et al.* (1964).

(b) *Neutralization*. Sera from herds with no history of FMD but vaccinated at least four times, were assayed in the micro cell metabolic inhibition test (Anderson *et al.* 1974b) for antibody to both the homologous vaccine strain and the infecting field isolates using increasing test virus inputs of 10, 100 and 1000 TCID₅₀. The antibody concentrations of at least 50 sera were determined and from the resultant regression lines, the geometric mean titre at a virus input of 100 TCID₅₀ obtained for each strain. Antibody titres are expressed as the log₁₀ of the reciprocal serum end-point dilution.

(c) *Radial immunodiffusion* (Mancini, Carbonara & Heremans, 1965). The method used was that described for the foot-and-mouth disease virus system by Cowan & Wagner (1970) and used to distinguish strains of FMDV by Lobo, Cowan, Trautman & Hanson (1974). Antiserum produced against the 146 S particle of each strain was incorporated in equal volumes of buffered agar to give a final twofold range of antiserum dilutions from 1/20 to 1/160. Each volume was poured onto a glass microscope slide and when set, a suitable pattern of wells of internal diameter 3.6 mm was cut. The antigens used were grown in BHK monolayers, concentrated by precipitation with buffered 50% ammonium sulphate and purified in discontinuous caesium chloride gradients. The 146 S particle concentration was determined spectrophotometrically as described by Bachrach, Trautman & Breese (1964). Suspensions of purified antigen of each strain were prepared so that they all contained the same antigen concentration and a standard volume was added to each well. This varied between tests from 10 to 17 μ l per well and the antigen concentrations were of the order of 3.0–8.0 μ g per well. The diameters of the precipitin rings were read after 72 h and plotted against reciprocal serum dilutions on log-log paper according to Cowan & Wagner (1970). The ratio of the slopes for the heterologous reaction to the homologous reaction was calculated for each serum and the cross-ratio products and R values obtained in the same way as for the complement-fixation test.

The antisera used in these tests contained entirely IgG antibody and took an

average of 6 weeks to prepare. This is the limiting factor in the time necessary to compare a troublesome field variant with the vaccine strain. In an attempt to cut down the time required, the tests with the type A strains were repeated using IgM antibody. This was prepared by immunizing 12 guinea-pigs with inactivated 146 S antigen using 0.25 mg saponin per dose as the adjuvant.

At 12 days after vaccination the guinea-pigs were bled out and the globulin fraction precipitated with 50% ammonium sulphate pH 6.5 and resuspended in isotonic saline. After dialysis against 0.1 M Tris-HCl buffer in 0.2 M NaCl pH 8.0 the IgM was separated in a Sephadex G-200 column using the same buffer. The IgM-containing fractions were concentrated by dialysis against 50% polyethylene glycol, assayed spectrophotometrically at 280 nm and found to contain about 2200 µg protein per ml. They were standardized to the same protein content and incorporated in the gel in twofold dilution series over a range of 1000 to 62.5 µg protein/ml.

RESULTS

TYPE A STRAINS

Strains of the A/K₃ subtype group

Field observations

Strains belonging to the A/K₃ subtype group have been responsible for a number of disease outbreaks in herds regularly vaccinated with a strain belonging to the A/K₁ subtype group (Anderson *et al.* 1974a). This vaccine strain was itself isolated from a field outbreak in 1966 and has been used successfully since that time. The A/K₃ strain has been responsible for primary outbreaks in four herds and secondary spread to eight more herds. The immune status of individual animals in a herd varies according to their age, the younger animals having received fewer doses than adults. A survey of the age composition of the herds involved in this study showed that an average of 20% of each herd was less than 1 year old and at best would have been vaccinated twice, while about 45% were 2 years or under and would have had three to four doses. Consequently, the ability of the infecting strain to cause disease in the adults is presumably indicative of a marked antigenic difference between itself and the vaccine strain. Antibody assays of sera collected from uninfected farms that had the same vaccination regimen as the diseased herds indicated that the homologous neutralizing antibody titres of yearlings was 1.5 with 55–60% having titres of 1.35 or greater and therefore considered, from data collected during laboratory challenge tests, to be immune to a normal field challenge. Adult animals had titres of 1.8 to over 2.0 and most could be considered immune to the homologous strain. In all the herds infected the clinical disease was severe, particularly in European breeds and cross-bred Zebus with spread to most animals in the herd. There has also been a recurrence of the disease due to this strain on three of the infected farms.

(i) Zebu animals aged 2–3 years with antibody titres against the vaccine strain of over 2.0 were introduced 9 months after the first outbreak when it was known that 26% of the previously infected cattle were still virus carriers (mean infectivity titre of their oesophageal-pharyngeal samples was less than 10 TCID₅₀ per ml).

Table 1. *Comparative results obtained in the complement-fixation, neutralization and radial immunodiffusion tests for two type A field strains against the A/K₁ vaccine strain*

| Field isolate | Complement-fixation test | | | Geometric mean serum neutralization titre of A/K ₁ vaccinated animals against | | RID R value (%) |
|--------------------------------|--------------------------|------------------|-------------------|--|----------------------------|-----------------|
| | Number of tests | Mean R value (%) | Between-test s.d. | Homologous subtype | Heterologous field isolate | |
| A/K ₃ subtype group | 20 | 16 (4-29) | ± 7.7 | 2.33 | 1.31 | 29 |
| A16/74 | 4 | 49 (44-58) | ± 6.2 | 2.33 | 1.74 | 50 |

Only 6% of this group became infected and the lesions were mild. No further outbreaks have occurred and 2 years later there were no detectable virus carriers.

(ii) Zebu animals 1½ years old and twice vaccinated with A/K₁ strain were introduced to the farm 5 months after the outbreak and mixed with recovered animals. Only 50% could be considered immune to the vaccine strain, the group mean titre being 1.3. They showed mild symptoms of the disease 4 months later and just 2 weeks after receiving another A/K₁ dose. One year later no virus carriers could be found on the farm.

(iii) Ten per cent of a group of 2-year-old Zebu animals with antibody titres to the A/K₁ strain of 1.8 were affected 15 months after the outbreak. The source of the virus could not be determined.

From these field observations it may be concluded that this is a strain which readily infected animals immune to the A/K₁ strain. In addition, the virus carriers resulting from the original outbreak appeared to be able to infect partially immune cattle introduced many months after the outbreak.

Laboratory subtyping

The results of the *in vitro* tests comparing the A/K₃ strains with the A/K₁ vaccine strain are summarized in Table 1. The complement-fixation test gave a mean R value of 16% for 20 separate tests with a between-test standard deviation (s.d.) of ± 7.7%. The mean values for the cross-fixation ratios obtained in two of these tests were: $r(A/K_1) = 0.17 \pm 0.15$, using the same antiserum for each test and $r(A/K_3) = 0.15 \pm 0.05$, using antisera prepared against four different A/K₃ field isolates.

In their recent paper on the same subject, Rweyemamu, Pay & Parker (1976) give an s.d. for r values in the complement-fixation test of 0.21. In our tests with pairs of viruses of types O, A and SAT 2, the overall s.d. for values of r was 0.16. Using the criteria suggested by Pereira (1976) that a new subtype must have values of r of 0.25 or less in both directions, it may be concluded that the A/K₃ strain constitutes a different subtype from the vaccine strain on the basis of the complement-fixation test results and this correlates with the field data.

The r value of 29 obtained in the radial immunodiffusion (RID) test (Table 1)

Table 2. Comparative precipitin ring diameters obtained in the single radial immunodiffusion test with type A strains using IgG antibody

| Antiserum ... | A/K ₁ | | | A/K ₃ | | | A K16/74 | | |
|----------------|------------------|------------------|-------|------------------|------------------|-------|------------------|------------------|-------|
| Antigen ... | A/K ₁ | A/K ₃ | 16/74 | A/K ₁ | A/K ₃ | 16/74 | A/K ₁ | A/K ₃ | 16/74 |
| Serum dilution | | | | | | | | | |
| 1/10 | 6.05 | 7.65 | 7.1 | 6.3 | 5.15 | 5.3 | 6.6 | 7.05 | 6.05 |
| 1/20 | 7.15 | 9.55 | 8.95 | 7.5 | 5.5 | 5.65 | 7.65 | 8.6 | 7.1 |
| 1/40 | 8.75 | 12.6 | 11.1 | 8.95 | 6.05 | 6.4 | 8.9 | 9.4 | 7.7 |
| 1/80 | 10.45 | — | — | 9.95 | 7.1 | 7.9 | — | — | — |
| 1/160 | 11.7 | — | — | — | 8.55 | — | — | — | — |

Table 3. Comparative precipitin ring diameters obtained in the single radial immunodiffusion test with type A strains using IgM antibody

| Antiserum ... | A/K ₁ | | | A/K ₃ | | | A K16/74 | | |
|---------------|------------------|------------------|-------|------------------|------------------|-------|------------------|------------------|-------|
| Antigen ... | A/K ₁ | A/K ₃ | 16/74 | A/K ₁ | A/K ₃ | 16/74 | A/K ₁ | A/K ₃ | 16/74 |
| 1000 | 6.65 | — | 8.55 | 7.55 | 5.7 | 8.55 | 6.0 | 6.0 | 5.9 |
| 500 | 7.65 | — | 10.25 | 9.05 | 6.5 | 9.45 | 7.0 | 7.3 | 6.85 |
| 250 | 9.25 | — | — | — | 7.75 | — | 8.1 | 8.35 | 8.15 |
| 125 | 10.35 | — | — | — | — | — | 10.2 | — | 9.65 |

is similar to that obtained in the complement-fixation test. Details of the diameters of the precipitin rings obtained with the type A strains are given in Table 2 for IgG antibody and Table 3 for IgM antibody. The use of IgM antibody did not allow calculation of an *R* value for the reaction between the A/K₁ and A/K₃ strains because no precipitin ring developed for the A/K₃ antigen against even the highest concentration of A/K₁ IgM antibody. The test did however indicate that there was a marked difference between the two strains.

The neutralization test results (Table 1) on 50 sera from A/K₁ vaccinated animals showed a 10-fold difference in antibody titre to the two strains. The test has a between-test s.d. of 0.3. The regression lines obtained are shown in Fig. 1.

Strain A 16/74

Field observations

This strain caused mild disease in vaccinated animals of less than 2 years of age on three farms while, on a fourth, it affected about 50% of animals of all ages. This last farm had been vaccinated three times with the A/K₁ strain and the homologous antibody titre of 2- to 3-year-old animals was 1.9 with about 80% being considered immune. There was no spread from any of these primary outbreaks to other farms.

Subtyping

The results of the *in vitro* tests are given in Table 1. The *R* value found in four replicate complement-fixation tests was 49 with an s.d. of ± 6.6. In the RID tests, values of 50–57 were obtained with IgG and IgM antibody respectively. Details of the precipitin ring diameters are given in Tables 2 and 3. The neutralization tests

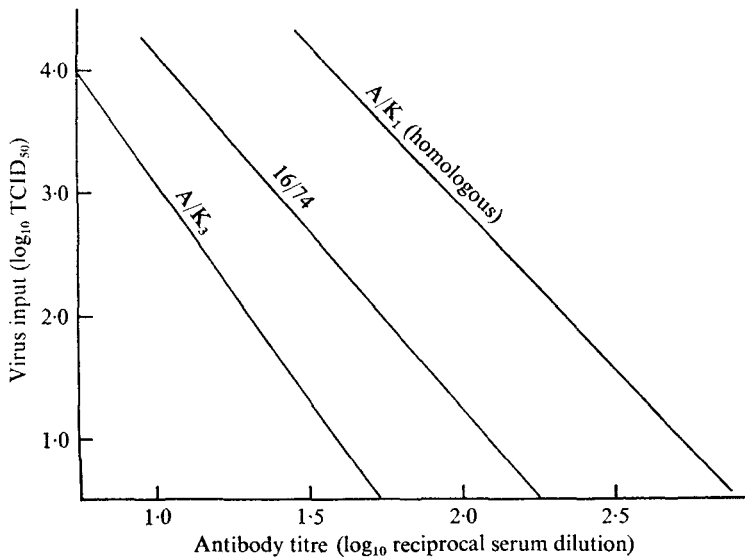


Fig. 1. Regression analysis of mean serum neutralizing antibody titres of A/K₁ vaccinated animals *v.* two outbreak strains.

Table 4. Comparative results obtained in the complement-fixation, neutralization and radial immunodiffusion tests for two type O field isolates against the O/K₁ vaccine strain

| Field isolate | Complement-fixation test | | | Geometric mean serum neutralization titre of O/K ₁ vaccinated animals against | | RID R value (%) |
|---------------|--------------------------|------------------|-------------------|--|----------------------------|-----------------|
| | Number of tests | Mean R value (%) | Between-test s.d. | Homologous subtype | Heterologous field isolate | |
| 204/73 | 8 | 39 (28-54) | ± 7.8 | 1.95 | 1.40 | 36 |
| 124/74 | 4 | 42.5 (35-51) | ± 6.6 | 1.90 | 1.65 | 33 |

gave a 2.5-fold difference in the mean titres against the homologous vaccine strain and the infecting strain. These differences correlate with a strain which caused a limited number of outbreaks with some spread within the herd particularly in the younger animals, but with little spread to other herds.

TYPE O STRAINS

Field observations

The two field strains examined, O 204/73 and O 124/74, are examples of O strains, all showing similarity to each other in *in vitro* tests, which have caused primary outbreaks on nine farms in the past 3 years. In all cases the disease has been confined to animals of under 2 years of age with humoral antibody titres of 1.4 with only about half of this age group considered to be immune. The lesions were frequently mild and there was generally little spread to other herds.

Table 5. Comparative results obtained in the complement-fixation, neutralization and radial immunodiffusion tests for SAT 2 field isolates against the SAT 2/K₂ vaccine strain

| Number of tests | Complement-fixation test | | Geometric mean serum neutralization titre of SAT 2/K ₂ vaccinated animals against | | RID R value (%) |
|-----------------|--------------------------|-------------------|--|----------------------------|-----------------|
| | Mean R value (%) | Between-test s.d. | Homologous subtype | Heterologous field isolate | |
| 12 | 33 (14-53) | ± 13 | 1.6 | 1.1 | 39 |

Subtyping

The comparative results obtained in the three tests are given in Table 4. The two strains included in this study are antigenically similar having mean *R* values of 61 and 70 in the complement-fixation and RID tests respectively. In comparison with the vaccine strain, O/K₁, the complement-fixation test gave a mean *R* value over 12 tests of 41 with an s.d. of ± 7.2. The RID test gave a value of 35. The mean neutralization titres of sera from 50 O/K₁ vaccinated cattle showed a 3.5- and 2.2-fold difference respectively against the two field strains. These O strains have resulted in rather more primary outbreaks than the A 16/74 strain described above; there was some spread within the infected herds and an occasional secondary outbreak.

TYPE SAT 2 STRAINS

Field observations

Immunization against the SAT types is often problematical because of the low protective power of the vaccines (Pay & Schermbrucker, 1974; Mowat, Prince, Owen & Taylor, 1975). With this reservation in mind we have compared two identical field strains SAT 2 162/72 and 176/74 with the two vaccine strains in use, namely SAT 2/K₁ and SAT 2/K₂. These field strains have caused severe outbreaks in herds vaccinated particularly with the SAT 2/K₂ strain.

Subtyping

The results obtained with these strains against the SAT 2/K₂ vaccine strain are given in Table 5. The mean *R* value of 12 tests, nine with 162/72 and three with 176/74, was 33 with an s.d. of ± 13. The cross-fixation ratios for the tests using 162/72 only showed a smaller variation and were: $r(\text{SAT } 2/K_2) = 0.28 \pm 0.22$, and $r(162/72) = 0.30 \pm 0.22$.

The *R* value obtained in the RID test was 39. That the two field isolates were identical was shown by *R* values of 61 and 77 obtained in the two tests.

The difference in neutralizing antibody titres in sera from adults given SAT 2/K₂ vaccine against two field strains, 162/72 and 28/73, and the other vaccine strain, are shown in Fig. 2. There was a threefold difference in titre between the field strains and the homologous vaccine strain.

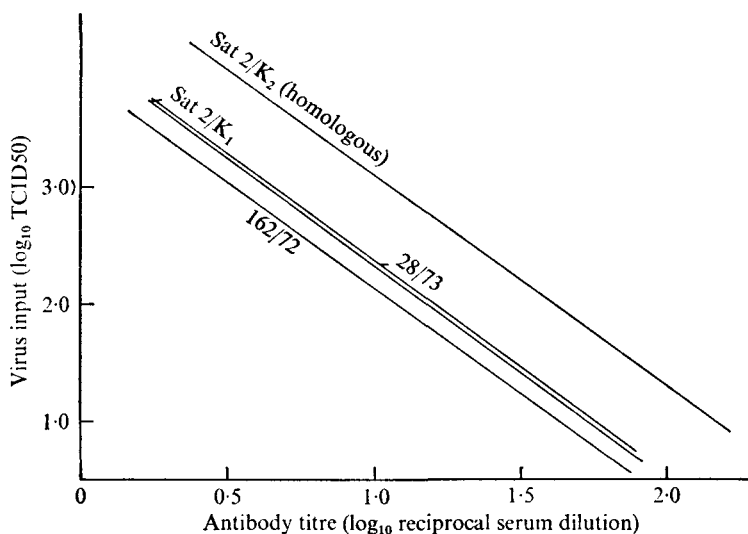


Fig. 2. Regression analysis of mean serum neutralizing antibody titres of SAT 2 K₂ vaccinated animals *v.* SAT 2 K₁ vaccine strain and two field strains.

DISCUSSION

The object of this study was to try to relate the infectivity of field strains of FMD virus for vaccinated herds with antigenic differences found in *in vitro* tests, and to assess the significance of these differences for control of the disease by vaccination. The results reported here show that the field strain that was highly infective for vaccinated cattle had an *R* value in the complement-fixation test of less than 30, with values of *r* of less than 0.25 in both directions. Strains which were less infective but which nevertheless caused primary outbreaks had *R* values of between 30 and 42.

Neutralization tests on sera from vaccinated cattle indicated that 3-fold or greater differences in antibody titre to the homologous vaccine strain and the infecting field strain could not be ignored. Using the data published by Rweyemamu *et al.* (1976) this correlates with a value for *r* of 0.3 in their cross-neutralization tests.

From the practical control point of view it has been found necessary to include two of the strains involved in this study in the vaccine used routinely in Kenya, namely the A/K₃ group and a member of the SAT 2 K176/74 group. The results serve to emphasize again the need for the continuous survey of subtypes present in the field so that vaccine strains used keep pace with the, albeit slow, antigenic drift in field strains.

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