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A comparison of some immunological methods for the differentiation of strains of foot-and-mouth disease virus

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SUMMARY

Two FMDV strains which had been previously differentiated by complement-fixation were compared by guinea-pig protection test, kinetic neutralization and micro-neutralization tests. It was found that these tests, which have not been previously applied by the methods described, were all capable of FMDV strain differentiation. Similar differences were found by all methods, which suggests that comparisons made by cross-CF, cross-neutralization or cross-protection involve measurement of the same antigen/antibody interactions.

INTRODUCTION

The detection of antigenic differences between foot-and-mouth disease virus (FMDV) strains within a type has received considerable attention because of its importance in the field, especially in relation to the identification of new strains and the selection of vaccine strains. The final criterion for the differentiation of two strains should be the difference in their performance in cross-protection tests in livestock. However, such tests are too expensive for routine use and other methods have been used. Complement-fixation (CF) and serum neutralization have been the most widely applied techniques, and efforts have been made to correlate the results with cross-protection.

Traub & Möhlmann (1946) showed that immunogenically different strains could also be differentiated by CF. Groups of cattle vaccinated with one strain withstood homologous challenge but were only partially protected against challenge with a heterologous strain. Similar qualitative results have also been obtained by other workers (Henderson, Galloway & Brooksby, 1948; Martin, Davies & Smith, 1962; Hyslop, Davie & Carter, 1963; Hedger & Herniman, 1966). Quantitative cross-protection tests have also been devised, based on the determination of heterologous and homologous 50% protective dose (PD50) values and their use has been described in cattle (Munțiu, Dohotaru, Bercan & Tomescu, 1965) and in guinea-pigs (Fontaine *et al.* 1966; Moosbrugger *et al.* 1967).

In this paper are described comparative tests carried out with two FMDV strains

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which had previously been differentiated by CF, to demonstrate differences by kinetic serum neutralization tests, by micro-neutralization tests and by a guinea-pig cross-protection test.

MATERIALS AND METHODS

Viruses

Two type A FMDV strains A6003 and A6900 were used. They had been compared by Guerche *et al.* (1972) by CF and by protection tests in cattle and had also undergone CF comparisons in this laboratory (Forman, 1974). The viruses were obtained from Dr Prunet as infective cattle tongue epithelium.

Antisera

Antisera to the two strains were prepared by the hyper-immunization of guinea-pigs with live, guinea-pig-adapted virus as described by Brooksby (1952). These antisera were used for all the serological comparisons and had also been used previously for the CF comparisons (Forman, 1974).

The guinea-pig protection test

Viruses were grown on BHK monolayers, inactivated with acetylethyleneimine (0·05 % for 30 hr. at 26 °C.) and concentrated and purified as described by Brown & Cartwright (1963). The quantities of purified virus obtained after sucrose density gradient purification were estimated by optical density (O.D.) measurements at 259 nm. on the basis of the finding of Bachrach, Trautman & Breese (1964) that 1 O.D. unit = 132 μg./ml. of purified virus.

Vaccines were prepared in threefold dilution series by suitably diluting the virus in 0·04 M phosphate buffer at pH 7·6, containing 0·1 % bovine albumin powder (Armour Pharmaceutical Co. Ltd, Eastbourne) and 0·1 % saponin. Guinea-pigs of approximately 600g. body-weight were inoculated subcutaneously with a 1 ml. dose of vaccine. For each dilution of each vaccine, groups of 8 guinea-pigs were inoculated for homologous or heterologous challenge.

At 28 days after vaccination, the guinea-pigs were challenged with homologous or heterologous virus by inoculating them intradermally in one tarsal pad with an estimated $10^{2\cdot0}$ guinea-pig 50 % infective doses (GPID50) and where specific lesions of foot-and-mouth disease were observed at sites other than the inoculation site over the next 7 days, a positive result was recorded.

From the results, the PD50 value was calculated for each vaccine for homologous and heterologous challenge by the method of Reed & Muench (1938). A value for r was determined for each vaccine, being the proportion PD50 (homologous)/PD50 (heterologous). From the values of r_1 and r_2 for the two vaccines, the cross-protection relationship, R(CP), was determined for the two viruses using the formula of Ubertini *et al.* (1964) in a manner analogous to that described for the CF test (Forman, 1974), i.e. $R(\mathrm{CP}) = 100\sqrt{(r_1.r_2)}$.

Sera obtained from the guinea-pigs 21 days after vaccination were assayed for serum neutralizing antibody activity in microplates (Linbro/Biocult, Biocult

Laboratories, Glasgow). The sera were diluted in the plates from a starting dilution of 1/4 and tested with a dose of $10^{1.5}$ TCID50 of homologous virus per well. Other conditions were the same as described for the conventional cross-neutralization test (see below).

The kinetic neutralization test

Kinetic neutralization tests were performed by mixing virus and antiserum and taking samples, at fixed time intervals, which were immediately diluted and subsequently titrated for plaque production in tissue culture.

The diluent was phosphate buffered saline, pH 7·4, with 0·1 % bovine albumin powder (PBSA). Viruses were diluted to an initial infectivity of between 3×10^5 and 8×10^5 p.f.u./ml. Antisera were inactivated and diluted to a pre-determined concentration which would give a suitable rate of neutralization.

Neutralization was carried out at room temperature. Two viruses were tested against one antiserum in the same test. One ml. of virus was added to 1 ml. of PBSA (to determine the initial infectivity) or to 1 ml. of antiserum dilution. Samples of 0·25 ml. were taken from the virus/antiserum mixtures at fixed intervals and mixed with 24 ml. of diluent at 0° C. Threefold dilutions were then made from these and each dilution used to inoculate 3 IB-RS-2 monolayers in 6 cm. Petri dishes. After allowing 30 min. at 37° C. for absorption of the virus, the monolayers were overlaid with maintenance medium containing 0·9 % agar and incubated for 30 hr. in a 5 % CO_2 atmosphere. The plates were then fixed and stained with a solution of 4 % formaldehyde and 10 % saturated methylene blue in tap water, the overlay removed and plaques counted. The mean infectivity titre for each sample was determined from a weighted mean of dilutions producing between 10 and 100 plaques on each plate.

For each virus-antiserum mixture, the rate of neutralization was determined by the method of least squares, from the infectivity titres of individual samples. A neutralization constant (K) was then determined using the equation:

$$K = D/t \times 2{\cdot}3 \log_{10} V_0/V_t,$$

where D = reciprocal of the antiserum dilution, t = time in minutes and V_0 and V_t represent the infectivities of the mixtures at times 0 and t respectively. Using the values of K for homologous and heterologous reactions obtained in the same test, values for r were determined for each antiserum, being the proportion K (heterologous)/K (homologous). With values for r_1 and r_2 for the two antisera, the kinetic neutralization relationship, $R(KN)$, was determined for the two viruses as described above for $R(CP)$.

The micro-neutralization test

Cross-serum neutralization tests were carried out in microplates using IB-RS-2 cells (de Castro, 1964). The medium used for the dilution of all reagents was 85 % Eagle's, 10 % tryptose phosphate broth, 4 % normal bovine serum and 1 %, w/v, of D-glucose. Sera were inactivated, then diluted in twofold steps in bottles and added to the wells in 50 μl. volumes with dropping pipettes. A 50 μl. drop of virus

was added to each well and the plates were incubated for 60 minutes at 37° C. Viruses were titrated in the same test. After incubation, cells were added in a 50 μl. drop at a concentration of $1{\cdot}5 \times 10^6$/ml. The plates were then sealed with adhesive tape and incubated at 37° C. for 48 hr.

Tests were read by examining the monolayers microscopically or after fixing with formalin and staining with methylene blue. The virus was considered neutralized if more than half of the monolayer in a well was intact. Antibody titres were determined by the method of Kärber (1931) and expressed as the reciprocal of the highest dilution of serum which resulted in neutralization of the virus.

To compare two strains, each antiserum was diluted in twofold intervals and titrated in eight replicates against two concentrations each of homologous and heterologous viruses, estimated to be $10^{1{\cdot}5}$ and $10^{2{\cdot}5}$ TCID50 per well. Antiserum titres for each concentration of each challenge virus were determined and the titre of each antiserum against $10^{2{\cdot}0}$ TCID50 of each virus was determined by graphical interpolation between the titres for the two challenge doses. Values of r for each antiserum were determined as the proportion of:

$$\frac{\text{titre with heterologous virus}}{\text{titre with homologous virus}}.$$

From values for r_1 and r_2 a value for the cross-neutralization relationship, $R(MN)$, was determined as described above.

RESULTS

The two strains, A6003 and A6900, were compared in a guinea-pig cross-protection test. Purified, inactivated 140S antigen of the two strains contained 300 μg./ml. (strain A6003) and 250 μg./ml. (strain A6900) of virus in the peak fractions of the preparative sucrose density gradients.

Guinea-pigs were challenged 28 days after vaccination with an estimated $10^{2{\cdot}0}$ GPID50 of either the homologous or the heterologous virus. The actual infectivity titres of the two challenge viruses from titrations at the time of challenge were $10^{1{\cdot}9}$ (A6003) and $10^{2{\cdot}1}$ (A6900) GPID50. The results are shown in Table 1.

From the PD50 values, the values for r and R (CP) were calculated and are as follows:

$$r_1 \text{ (vaccine A6003)} = 0{\cdot}33,$$
$$r_2 \text{ (vaccine A6900)} = 0{\cdot}36,$$
$$R\text{(CP)} = 34\,\%.$$

The results of titrations for neutralizing antibody in sera obtained 21 days after vaccination are shown in Table 2. It is evident that the antibody titres were very low and, in fact, around the level of 1PD50 (homologous) for each vaccine, antibody was generally not detectable. However, the method of antibody titration is considered to be relatively sensitive. For comparison, reference antisera were titrated in the same tests, being those used for the serological comparisons. Their antibody titres were 1/4096 (A6003) and 1/3388 (A6900).

Kinetic neutralization tests were carried out with the same two strains. Preliminary experiments were carried out to verify that the rate of neutralization in

Table 1. *Results of homologous and heterologous challenge of guinea-pigs vaccinated with purified 140S antigen of FMDV strains A6003 and A6900*

Challenge virus strain	Vaccine virus strain	Antigen dose (ng)	Proportion of positive reactions	Value of PD 50 (ng)
A6003	A6003	1,111	0/8	33
		370	0/8	
		123	0/8	
		41	3/8	
		14	7/7	
A6900	A6003	10,000	0/8	99
		3,333	0/8	
		1,111	0/8	
		370	0/8	
		123	3/8	
		41	8/8	
		14	7/8	
A6900	A6900	1,111	0/8	90
		370	0/8	
		123	4/7	
		41	5/8	
		14	8/8	
A6003	A6900	10,000	0/7	258
		3,333	0/8	
		1,111	0/8	
		370	2/8	
		123	7/7	
		41	7/8	
		14	8/8	

Table 2. *Neutralization antibody titres of sera from guinea-pigs 21 days after vaccination with purified virus vaccines of the strains A6003 and A6900*

Vaccine virus strains	Antigen dose (ng)	Neutralizing antibody titres		Proportion with positive titres
		Range	Mean	
A6003	10,000	32–128	69	8/8
	3,333	16–45	30	8/8
	1,111	11–45	25	16/16
	370	4–45	11	16/16
	123	< 4–16	—	7/15
	41	< 4–8	—	2/16
	14	< 4–4	—	1/15
A6900	10,000	32–128	59	8/8
	3,333	45–128	76	8/8
	1,111	11–181	36	16/16
	370	8–64	18	15/15
	123	< 4–45	—	8/15
	41	< 4–8	—	2/15
	14	< 4–4	—	1/15

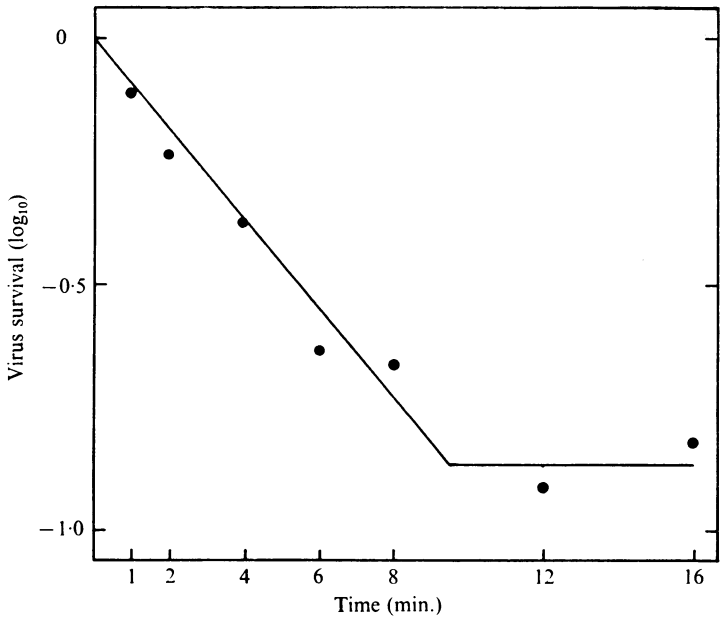

Fig. 1. Kinetic curve of neutralization of A6003 virus by homologous antiserum.

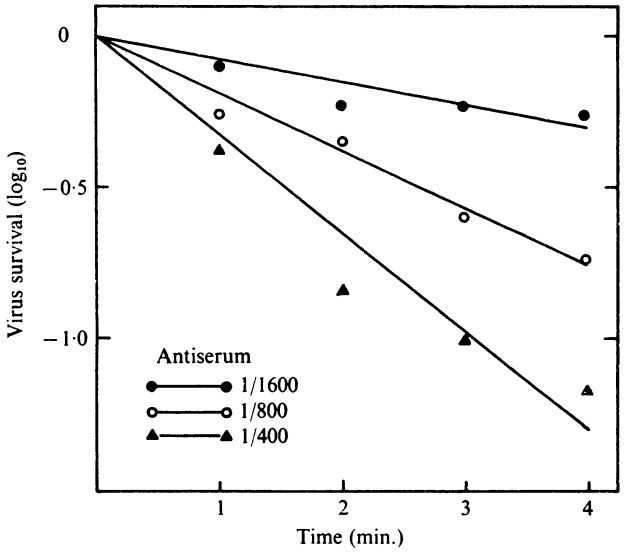

Fig. 2. Kinetic curves of neutralization of A6003 virus by varying dilutions of homologous antiserum.

this system is determined by the principles elucidated by Dulbecco, Vogt & Strickland (1956), on which the validity of the formula used for the calculation of K-values is dependent.

An experiment carried out with A6900 virus and antiserum showed that with a virus concentration of approximately 3×10^5 p.f.u./ml. and antiserum diluted

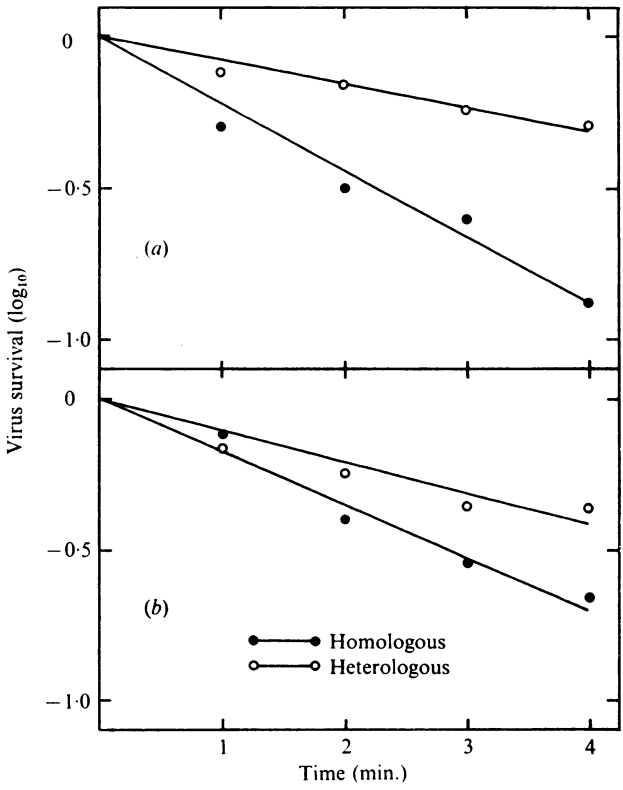

Fig. 3. Kinetic curves of neutralization of (*a*) A6003 virus and (*b*), A6900 virus by homologous and heterologous antisera.

1/1000, the reaction appeared to be of the first order for a period of about 10 min. (Fig. 1). To ensure that neutralization was being measured during the period of first order reaction, subsequent experiments were carried out over 4 min., taking samples at 1 min. intervals.

A further experiment with A6900 virus and antiserum (Fig. 2) demonstrated that the first-order character of the reaction was independent of antiserum concentration. The values of K for the three antiserum dilutions were:

antiserum 1/1600, $K = 276$;
antiserum 1/800, $K = 350$;
antiserum 1/400, $K = 299$.

These values determined for K can be considered the same (within experimental error) and this indicates that the value of K is independent of the antiserum concentration. Consequently, the rate of neutralization was proportional to the concentration of antiserum.

Three experiments were then performed with both of the antisera, A6003 and A6900, to measure the values of K for homologous and heterologous reactions and from these to determine values for r and $R(KN)$. Fig. 3 shows the results of typical

Table 3. *Values for K and r, determined by kinetic neutralization tests with the strains A6003 and A6900*

Antiserum	Expt no.	*K* (homologous)	*K* (heterologous)	*r*
A6003	1	171	49·6	0·29
	2	154	70·7	0·46
	3	152	53·5	0·35
	4	124	50·0	0·40
A6900	1	161	94·3	0·59
	2	271	161·0	0·59
	3	219	117·0	0·53

Table 4. *Micro-neutralization tests with strains A6003 and A6900*

		Antiserum titre			
Expt. no	Antiserum	Homologous	Heterologous	*r*	*R*(*MN*)
1	A6003	1202	513	0·43	33 %
	A6900	3236	851	0·26	
2	A6003	1479	457	0·31	30 %
	A6900	2427	692	0·29	

experiments with each antiserum. The values of K and r, determined from the experiments, are presented in Table 3.

It is apparent from Table 3 that, although individual K values were highly variable, the values of r were rather less so (the maximum range of variation was $\pm 25\%$ of the mean value). From the values for r, mean values for r_1, r_2 and $R(KN)$ were obtained as follows:

$$r_1 \text{ (antiserum A6003)} = 0{\cdot}37,$$
$$r_2 \text{ (antiserum A6900)} = 0{\cdot}56,$$
$$R(KN) = 46\%.$$

Two comparisons were made between the two strains by micro-neutralization tests. The results are shown in Table 4.

The mean values obtained from the tests were:

$$r_1 \text{ (antiserum A6003)} = 0{\cdot}36,$$
$$r_2 \text{ (antiserum A6900)} = 0{\cdot}27,$$
$$R(MN) = 32\%.$$

DISCUSSION

In this paper an attempt has been made to provide some evidence that the differences between strains as detected by CF can be correlated with those differences which are detected on the basis of cross-neutralization or cross-protection. It would appear reasonable to assume that if the differences determined by CF, by neutralization *in vitro* and by protection *in vivo* are of a similar magnitude then in each system the same combinations of antigen and antibody are being detected.

For the two strains, A6003 and A6900, the relationships determined by CF (Forman, 1974), by micro-neutralization, by kinetic neutralization and by animal cross-protection were expressed as values for R, $R(MN)$, $R(KN)$ and R(CP) respectively. These relationships are comparable in being derived in the same manner from values for r, which in each case were determined as the proportion of heterologous reaction/homologous reaction, for two antigens reacting with one antiserum (or, in the case of cross-protection, two viruses challenging guinea-pigs inoculated with one vaccine). The values for R, $R(MN)$, $R(KN)$ and R(CP) were respectively 37, 32, 46 and 35 %. Since they are all of a similar order, this provides some support for the validity of using CF to determine the relationships between strains. It is recognized that further comparisons of a similar nature would be desirable but these must inevitably be limited by the high cost of large-scale animal experiments.

It is evident that the guinea-pig protection test described in this paper is an effective method for the detection of strain differences. Animal protection tests suffer from the disadvantages of being costly and time-consuming and of being subject to considerable variation due to differences in individual animal susceptibility. Their greatest advantage is that differences determined by animal protection are possibly more analogous to the situation for which the information is required; viz., the susceptibility of vaccinated animals to challenge in the field. The results obtained with the test suggest that variation in individual animal susceptibility was not an important source of error, since all end-points were clearly defined over a range of only one or two vaccine dilutions.

The use of vaccines prepared from purified 140S antigens for cross-protection tests has not been previously described. Nor has the response of guinea-pigs to challenge after vaccination with such preparations. Mowat (1972) determined the PD50 values for pigs and cattle of purified 140S antigen vaccines prepared from strains of types A, SAT 2 and Asia 1. For pigs, the PD50 values ranged from 5·8 ng. to 14·1 ng., depending on the strain, and for cattle, from 32 ng. to 255 ng. The present results suggest that guinea-pigs require similar doses of 140S antigen to cattle for protection against homologous challenge.

Morgan, McKercher & Bachrach (1970) vaccinated guinea-pigs with purified 140S antigen and measured the development of neutralizing antibody in their sera. They found that only guinea-pigs inoculated with more than 160 ng. of antigen had detectable neutralizing antibody 28 days after vaccination. The present results confirm these findings.

Neutralizing kinetics have been used for strain differentiation within many virus groups; for example, picornaviruses (McBride, 1959; Richter, Macpherson, Campbell & Làbzoffsky, 1972), influenza viruses (Pereira & Tumova, 1967), reoviruses (Munro & Wooley, 1973) and poxviruses (Dunlap & Barker, 1973). Capstick, Sellers & Stewart (1959) studied neutralization kinetics with FMDV but the method has not previously been applied to FMDV strain differentiation. The method was shown to be reasonably reproducible and to demonstrate differences between two strains to a similar extent to CF. From the results of the present work, it would appear that the kinetic neutralization test has no advantage over the

CF test for sensitivity or reproducibility. However, it could prove to be a useful method for the detection of strain differences where antisera suitable for CF are not available.

The micro serum-neutralization test performed in microplates also appeared to provide a satisfactory differentiation between the two strains. It is a simple and rapid technique and for this reason could have a greater application than the kinetics test as described in this paper. For both of these neutralization tests, a more extensive evaluation is required before their reproducibility and their usefulness for FMDV strain differentiation can be fully assessed.

The reasonably close agreement between the results of the cross-protection test, the CF test and the micro-neutralization tests support the conclusions of the other workers referred to earlier. However, much of the earlier work was not carried out on a quantitative basis. Guerche *et al.* (1972) suggested that strains which were distinguishable in cross-protection tests could not be differentiated by CF. However, since their two strains, A6003 and A6900, derived from their cattle experiment were used for the present work in which they were clearly differentiated by both methods, their conclusion is refuted.

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