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Comparative complement-fixation studies with subtype strains of foot-and-mouth disease virus

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SUMMARY

A comparison was made between a macrotechnique in tubes and a microtechnique in plastic plates for complement-fixation tests, using strains of three subtypes of the Asia 1 type of foot-and-mouth disease (FMD) virus. The results obtained with these techniques were found to be comparable and delineated the antigenic relationships of the three strains employed. The microtechnique was considered to be both economical with reagents and capable of similar accuracy and reproducibility to that of the standard method in tubes. It was concluded that the antigenic analysis of subtype strains of FMD virus can be conveniently carried out by the use of the microtechnique as described.

INTRODUCTION

Complement-fixation reactions using foot-and-mouth disease (FMD) virus systems have been studied extensively (Traub & Möhlmann, 1946; Brooksby, Galloway & Henderson, 1948; Brooksby, 1952; Graves, 1960). Such studies have generally been made by means of macrotechniques which require relatively large volumes of each reagent. An economical use of reagents with certain other virus systems has been achieved when the reagent volumes have been reduced to comparatively small amounts; for example, with influenza (Fulton & Dumbell, 1949; Fulton, 1951), bluetongue (Kipps, 1956) and Teschen viruses (Darbyshire & Dawson, 1963).

The application of similar microtechniques to FMD virus systems has not been recorded previously. The following paper describes the application of a microtechnique of complement fixation to the investigation of antigenic relationships of strains of three subtypes of the Asia 1 type of FMD virus. At the same time, a detailed comparison was made of such a method with an existing technique done in tubes and previously used to identify both type and subtype differences of FMD virus (Brooksby, 1952; Davie, 1964).

MATERIALS AND METHODS

Antigens

Three strains of Asia 1 type FMD virus were investigated. The strains were obtained from the collection of the World Reference Laboratory and were, respectively, Pak 1/54 (a Pakistan strain which was the first recognized Asia 1 type), Isr 3/63 (an Israeli field strain from Yokneam), and Isr 1/57 (the first Asia 1 type recognized in Israel in 1957). The antigens were each produced in one of several different systems as described, namely:

Pig kidney cells. Monolayer cultures of the IB-RS-2 (clone 60) cell line of porcine kidney (de Castro, 1964) were produced in bottles, using a growth medium consisting of Hanks's saline, 3% lactalbumin hydrolysate, antibiotics (neomycin, 65 i.u./ml., mycostatin, 60 i.u./ml. and penicillin, 600 i.u./ml.) and 10% ox serum. The cultures were washed, inoculated with virus and allowed to adsorb for 30 min. at 37° C. They were then overlaid with a maintenance medium consisting of Earle's saline with 5% lactalbumin hydrolysate and antibiotics as above, left for up to 48 hr. and harvested when approximately 90% of the cell sheet showed a cytopathic effect. Cell debris was removed by low-speed centrifugation and the supernatant used as antigen.

Baby hamster kidney cells. Monolayer cultures of baby hamster kidney cells were prepared with the BHK 21 (clone 13) cell line. The cultures were prepared and used as described above.

Guinea-pig materials. Guinea-pigs, weighing 500–700 g., were inoculated intradermally into the metatarsal pads with virus. Vesicular lesions developed within 24 hr. The vesicle epithelium and fluid were subinoculated into further guinea-pigs at the same site and after several such passages the epithelium and vesicle fluids were each subsequently harvested to provide pad epithelium and vesicle fluid antigens, respectively. The pads were ground in 0·04 M phosphate buffer, pH 7·6, to give a 1/10 suspension which was clarified by low-speed centrifugation. The vesicle fluid was also clarified and was suitably diluted for use.

Antisera

The antisera were available from the World Reference Laboratory and had been prepared in guinea-pigs as described previously (Davie, 1964). After approximately 4 weeks, immune guinea-pigs were re-inoculated intradermally with a suspension of fresh infected pads (1/5) or tissue culture virus with saponin as adjuvant. Serum was collected 10 days later, pooled, filtered through a Seitz EK filter and stored at −20° C. until required.

Complement

The complement was normal guinea-pig serum. A number of adult guinea-pigs were starved for 24 hr. and the pooled serum collected and treated with Richardson's preservative (Richardson, 1941) before storage at 4° C. in suitable volumes. During all tests the range of dilutions of complement was kept in an ice bath.

Haemolytic indicator system

The indicator system used in the tests performed in plastic plates consisted of a 0·7 % suspension of washed sheep red cells previously sensitized with an equal volume of a 1/800 dilution of haemolytic equine serum (Burroughs Wellcome & Co.). The haemolytic indicator system used in the tests carried in tubes was a 1·5 % sensitized suspension of sheep red cells. In each case the dilution of haemolysin chosen ensured excess and was the equivalent of at least four haemolytic doses.

Complement-fixation tests

Two techniques of performing complement-fixation tests were investigated and these were done simultaneously, using the same reagents for each method. All the antisera were first inactivated by heating at 56° C. for 30 min.

The first technique essentially followed that developed originally by Brooksby (1952) using test tubes. The practical details of the test have been described (Davie, 1964) and were two-dimensional titrations of complement against antisera in the presence of a fixed dilution of antigen. Suitable controls were incorporated in each test and all reagents were maintained at 4° C. when the tests were being set up. Antiserum, complement and antigen mixtures were incubated in a water bath at 37° C. for 30 min. The indicator system was added (1 ml.) to each tube and the tubes re-incubated at 37° C. for a further 30 min. All the tubes were briefly centrifuged and the degree of haemolysis determined as the optical density of the released haemoglobin in a colorimeter.

The second method was performed in disposable plastic micro plates (Cooke Engineering Co., U.S.A.). For the tests in the microplates 5 volumes of 25 μl. each were used. The procedure of the test was essentially that of Fulton & Dumbell (1949) and Fulton (1951). Two-dimensional titrations, varying both complement and antiserum, were done in the presence of a fixed dilution of antigen and full controls were incorporated in each test. The antiserum, complement and antigen mixtures were incubated at 37° C. for 30 min., during which the plates were covered to reduce evaporation. The haemolytic indicator system was then added to each well as a 50 μl. drop and the plates sealed with transparent tape and re-incubated at 37° C. for 30 min.; the plates were gently agitated from time to time to resuspend any unlysed red cells in the mixtures. The plates were then centrifuged at 500 ***g*** for 5 min. to deposit unlysed red cells and examined.

In the microtechnique, the degree of lysis in the indicator system was estimated from the pattern of unlysed cells deposited in each well. The complement dilution interval was 0·18 $\log_{10}$ and the transition of complete lysis to no lysis in any particular row was sufficiently rapid to enable the point of 50 % lysis to be conveniently determined by inspection or, where necessary, by interpolation.

RESULTS

The method of calculating the results obtained with the test in tubes and adopted here has been given in principle by Davie (1964). In the microtechnique the amount of complement fixed by each serum dilution in the presence of antigen

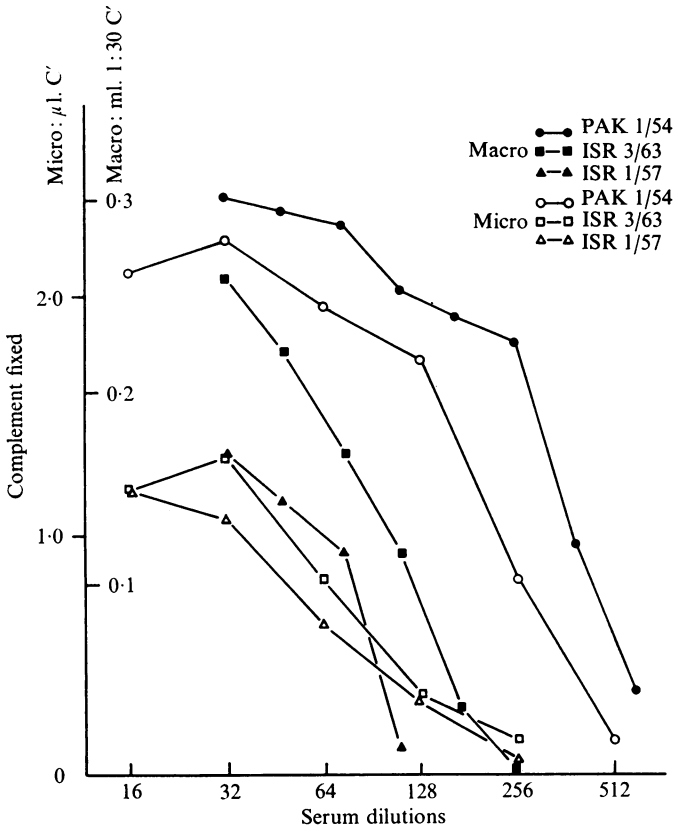

Fig. 1. Complement-fixing activity of three strain-specific antisera with a guinea-pig vesicular fluid antigen (Pak 1/54) using two methods.

was calculated after Fulton (1951). For each method, the amounts of complement fixed by antigen and a particular antiserum were drawn graphically; an example is given in Fig. 1.

It will be seen that the general shapes of the curves for both methods are comparable. In those parts of the curves which approached linearity, especially in the range of the higher serum dilutions, the amounts of complement fixed were used to calculate a mean value of the reaction slope. For this, the method proposed by Bradish, Brooksby & Tsubahara (1960) was adopted, using the formula

$$B = \frac{V_c - V_0}{v_i},$$

where V_c is the total volume of complement required for 50 % lysis, V_0 is the volume of complement required by the anticomplementary reagents, and v_i is the volume of serum used in the test over the dilution series employed. In this manner, the value of the amount of complement which could be fixed by each antigen with undiluted antiserum was calculated.

The values obtained when each antigen was tested against either the homologous or the heterologous antisera in turn are illustrated in Fig. 2, and represent the

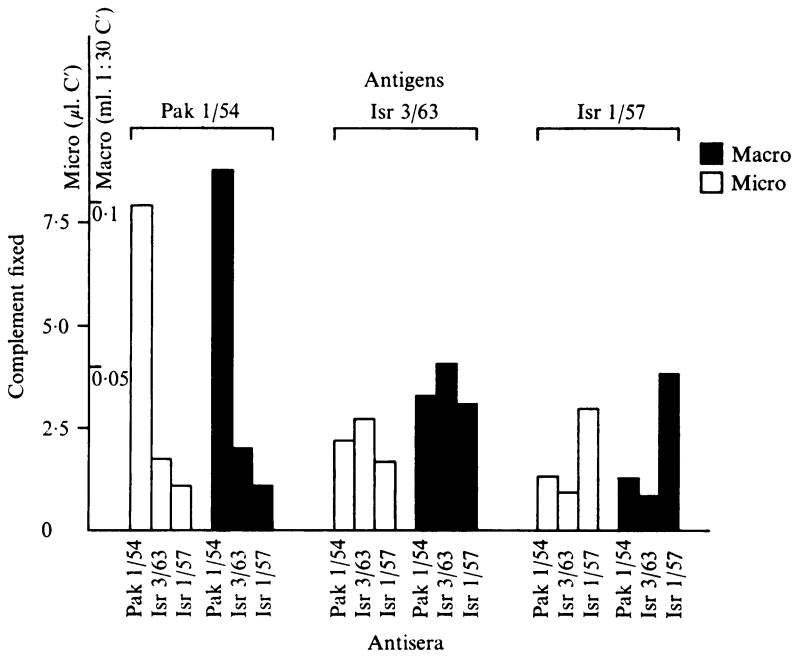

Fig. 2. Complement-fixing activity of three guinea-pig vesicular fluid antigens with homologous and heterologous antisera using two methods.

differences observed in reactivity between pairs of antigens and antisera when using either technique. The amounts of complement fixed by such pairs of reagents were used to assess the antigenic relationships between the virus strains by expressing each homologous and heterologous reaction as a ratio. Thus, for example, using antigens A and B with homologous antisera a and b, a ratio, r_A for antigen A would be represented by

$$r_A = \frac{Ab}{Aa},$$

where Ab represents the complement-fixing activity of the heterologous reaction and Aa the complement-fixing activity of the homologous reaction. Similarly, a ratio for antigen B would be derived from

$$r_B = \frac{Ba}{Bb}.$$

The antigenic relationship (R) of the two antigens may be expressed from the product of such ratios as a percentage, by use of the following formula (Archetti & Horsfall, 1950; Chu, Andrewes & Gledhill, 1950; Jordan & Gaylin, 1953; Wenner, Kamitsuka & Lenahan, 1956; Ubertini *et al.* 1964)

$$R = 100\sqrt{(r_A r_B)}.$$

Although, in the present series of experiments, the ratios were calculated as described above, namely from tests between an antigen and a number of antisera in turn, it should, nevertheless, be noted that antigenic comparisons may also be

based on ratios derived from tests of one antiserum against a number of antigens in turn. In the latter instance, the ratio r_a for antiserum a against antigens A and B would then be represented

$$r_a = \frac{Ba}{Aa}.$$

The calculated r values obtained from the various tests with each of the different antigens available in the present studies are presented in Tables 1, 2 and 3, respectively. The R values calculated from these have been given in Tables 4, 5 and 6, and indicate the results obtained with antigens derived from three sources, viz. pig kidney and BHK cells or guinea-pig materials.

Table 1. *Calculated r values using microplate and tube techniques with antigens produced in IB-RS-2 cells*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
			Experiment 1			
Pak 1/54	1·00	1·00	0·84	0·93	0·30	0·28
Isr 3/63	0·23	0·22	1·00	1·00	0·28	0·27
Isr 1/57	0·16	0·16	0·67	0·81	1·00	1·00
			Experiment 2			
Pak 1/54	1·00	1·00	0·72	0·42	0·36	0·36
Isr 3/63	0·25	0·34	1·00	1·00	0·30	0·40
Isr 1/57	0·19	0·24	0·71	0·79	1·00	1·00

Table 2. *Calculated r values using microplate and tube techniques with antigens produced in BHK 21 cells*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
			Experiment 1			
Pak 1/54	1·00	1·00	0·80	0·83	0·30	0·24
Isr 3/63	0·23	0·28	1·00	1·00	0·29	0·21
Isr 1/57	0·20	0·13	0·70	0·74	1·00	1·00
			Experiment 2			
Pak 1/54	1·00	1·00	0·88	0·83	0·29	0·21
Isr 3/63	0·22	0·28	1·00	1·00	0·25	0·22
Isr 1/57	0·17	0·14	0·88	0·80	1·00	1·00

DISCUSSION

A method of comparing minor antigenic differences between strains of FMD virus of the same immunological type was developed by Bradish, Brooksby & Tsubahara (1960) and Bradish & Brooksby (1960). In that method, the product of the cross-fixation ratios of any two antigens was taken to be indicative of the degree of antigenic relationship between strains. A scheme for the taxonomic classification of FMD virus strains into subtypes was thereby shown by Davie (1964) to be applicable. When the value of the product of any two strains was $\geqslant 0{\cdot}5$ it was considered that such strains should be classified within the same subtype group and that those with values of $< 0{\cdot}5$ were different.

At the present time, a similar classification method which is feasible and currently employed is one based on the value of R, derived from the formula given. If R is $\geqslant 70\%$, then the strains under investigation are considered to fall within the same subtype group; if R is $< 70\%$, they are regarded as being of different subtypes.

Table 3. *Calculated r values using microplate and tube techniques with antigens produced in guinea-pigs*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
	Experiment 1*					
Pak 1/54	1·00	1·00	0·91	0·78	0·44	0·28
Isr 3/63	0·22	0·22	1·00	1·00	0·31	0·22
Isr 1/57	0·14	0·14	0·63	0·74	1·00	1·00
	Experiment 2*					
Pak 1/54	1·00	1·00	0·89	0·99	0·36	0·27
Isr 3/63	0·23	0·21	1·00	1·00	0·22	0·26
Isr 1/57	0·21	0·14	0·90	0·71	1·00	1·00

* Experiment 1: vesicular fluid antigen. Experiment 2: pad epithelium.

Table 4. *Calculated R values from tests using antigens produced in IB-RS-2 cells*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
	Experiment 1					
Pak 1/54	100	100	44	44	22	21
Isr 3/63	—	—	100	100	44	42
Isr 1/57	—	—	—	—	100	100
	Experiment 2					
Pak 1/54	100	100	44	43	26	29
Isr 3/63	—	—	100	100	47	38
Isr 1/57	—	—	—	—	100	100

On such criteria, the overall results obtained in the present studies with either complement-fixation method indicate that the strains Pak 1/54, Isr 3/63 and Isr 1/57 of the Asia 1 type of FMD virus are representative of different subtype groups, since the relevant R values are < 70. The reactions of the antigens in all tests were greater with the homologous antisera than with either of the respective heterologous antisera (see Figs. 1 and 2) and it was also demonstrated that each antigen was present in excess when tested with the higher dilutions of any serum.

The profiles of the histograms (see Fig. 2) in all the tests were comparable between the two techniques, which suggests that newly isolated virus strains of unknown antigenic constitution may be compared with existing subtypes using subtype-specific sera by either technique of complement fixation with equal confidence. In the absence of homologous antiserum to a newly isolated FMD virus strain, only a uni-directional test is possible. Nevertheless, a comparison of the histograms obtained with the new virus strain with those constructed from

Table 5. *Calculated R values from tests using antigens produced in BHK 21 cells*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
			Experiment 1			
Pak 1/54	100	100	43	48	24	18
Isr 3/63	—	—	100	100	45	40
Isr 1/57	—	—	—	—	100	100
			Experiment 2			
Pak 1/54	100	100	44	48	25	19
Isr 3/63	—	—	100	100	47	42
Isr 1/57	—	—	—	—	100	100

Table 6. *Calculated R values from tests using antigens produced in guinea-pig tissues*

	Virus					
	Pak 1/54		Isr 3/63		Isr 1/57	
Serum	Micro	Tube	Micro	Tube	Micro	Tube
			Experiment 1*			
Pak 1/54	100	100	45	42	24	19
Isr 3/63	—	—	100	100	44	41
Isr 1/57	—	—	—	—	100	100
			Experiment 2*			
Pak 1/54	100	100	45	46	27	19
Isr 3/63	—	—	100	100	45	43
Isr 1/57	—	—	—	—	100	100

* Experiment 1: vesicular fluid antigen. Experiment 2: pad epithelium.

uni-directional tests using established subtype strains of virus against an identical range of subtype-specific antisera may provide some indication of the antigenic constitution of such an unknown strain, although, in the absence of reciprocal tests, the information thus made available is limited and must be interpreted with caution.

The complement-fixing activity of the same antisera used here in either technique varied between tests, not only when the antigens were produced in the same system and at different times but also with the antigens derived from the different sources. Such variability in antigen potency did not affect the general profiles of the histograms or, to some extent, the r values derived therefrom. The calculated values for R between strains, nevertheless, remained approximately constant between tests, both within a technique and in comparison between techniques (see Tables 4, 5 and 6).

In the microtechnique, the values for R (%) between strains varied by up to 4%; this was in one instance where the Pak 1/54 and Isr 1/57 antigens were derived from IB-RS-2 cells. In the tube test there was little variation except in one case (8%), again with Pak 1/54 and Isr 1/57 antigens from IB-RS-2 cells. There was a close similarity overall between the results for the two techniques; in a total of 18 values calculated for R, there was less than 5% difference in all except four instances, namely, two of 6% and one of 8% and one of 9%.

The present experiments indicate that the results obtained with the microplate method are analogous to those of the technique in tubes. The advantages of the microplate technique include the reduction in the volume of reagents required and the increased technical facility. In an analysis of subtype strains of FMD virus, the production of homologous antiserum may require 6–8 weeks and, consequently, any method which conserves such a reagent would be especially desirable. The use of the microplate method for general subtype analysis of FMD virus strains is accordingly now a regular technique in the World Reference Laboratory.

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