J. Hyg., Camb. (1974), **72**, 407
Printed in Great Britain

A study of foot-and-mouth disease virus strains by complement fixation

II. A comparison of tube and microplate tests for the differentiation of strains

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(*Received* 28 *September* 1973)

SUMMARY

Several foot-and-mouth disease virus strains were examined by complement-fixation tests in microplates and in tubes. It was established that the two systems are comparable, although greater reproducibility is obtained with tube tests. While microplate tests are a satisfactory method for the differentiation of strains, tube tests provide a more precise method for the identification of small antigenic differences.

INTRODUCTION

In a previous paper (Forman, 1974) a model was presented for the fixation of complement by foot-and-mouth disease virus (FMDV) antigen/antibody mixtures. On the basis of results obtained from tests in tubes, it was suggested that virus strains could best be differentiated by the comparison of the titres of an antiserum when tested with homologous and heterologous antigens. The serum titre was expressed as the dilution of serum which would fix a defined amount of complement in the presence of an optimum amount of a particular antigen.

The principles demonstrated in that paper were applied to the differentiation of several FMDV strains by complement-fixation tests in both tubes and microplates.

MATERIALS AND METHODS

Reagents

The diluent (veronal-buffered saline), complement and haemolytic system were prepared in the manner previously described (Forman, 1974).

The antigens were the 140S particles of strains of FMDV grown as described in the previous paper (Forman, 1974) and purified by the method described by Brown & Cartwright (1963). The strains used were as follows: (i) two type O strains, O_6 (OVI) and O_1 Lombardy (O_1 Lom), which are classified by the World Reference Laboratory as distinct subtypes; (ii) two type A strains, A6003 and A6900, which had been compared by complement fixation previously by Guerche *et al.* (1972); (iii) three type Asia 1 strains, being a parent strain, Asia 1 C_2 and two derived strains, Asia 1 387 and Asia 1 415 which had been selected under mutagenic conditions for altered electrophoretic mobility. These strains had earlier undergone extensive comparative studies (Priston, 1972).

Antisera to the type O and type A strains were produced in guinea pigs in the manner described by Brooksby (1952), using live guinea-pig adapted virus. For

the Asia 1 strains, antisera were produced by inoculating guinea pigs once only with crude tissue culture harvests and bleeding them 28 days later.

Protocol of the tests

The tube tests were performed as described in the previous paper (Forman, 1974). To compare two strains, each antigen was used in a series of twofold dilutions over a predetermined range which would demonstrate an optimum dilution giving maximal fixation. Each serum was tested at two dilutions against each antigen and the amount of complement fixed with the optimum dilution of antigen at each dilution of serum was calculated. The titre of an antiserum with an antigen was then determined graphically, as previously described (Forman, 1974), as the dilution of serum which would fix 0·5 50 % haemolytic doses of complement (C'H50) with an optimum amount of antigen.

The microplate tests were performed in wells in plastic plates (Linbro–Biocult, Biocult Laboratories Ltd, Glasgow) in a final volume of 125 μl. Reagents were added in the following order and volume: (i) antigen, 25 μl.; (ii) complement, 25 μl.; (iii) antiserum, 25 μl.; (iv) haemolytic system, 50 μl.

The conditions used were designed as far as possible to parallel those used in tube tests. Thus, the reagents were added in the same order, all in 1/20 of the volume used in tubes. All dilutions were made in bottles and the reagents added to the plates with calibrated dropping pipettes. Mixtures were incubated in a hot-air incubator at 37° C. for 60 min. before the addition of sensitized erythrocytes, which was followed by a further incubation at 37° C. for 45 min. and then centrifugation at 600 *g* for 5 min. to sediment unlysed cells. It was established that primary fixation under the conditions used was similar to fixation in tubes after 30 min. in a water bath at 37° C. The tests were read by visual estimation of the sizes of deposited erythrocyte buttons.

The fixed dose of complement used in the microplate tests was 5 C'H50, established by pretitration in plates and equivalent in concentration to 5 C'H50 in the tube test. Control mixtures were included, with complement at 5, 2·5 and 1·25 C'H50 alone and with the antigens and antisera at all the dilutions used in the test.

To compare two virus strains in a microplate test, each antigen was used in a series of twofold dilutions, as in the tube tests, and tested against each antiserum in a series of 1·5-fold or twofold dilutions. The serum titre was determined as the dilution of serum which fixed four of the five C'H50 with an optimum amount of antigen, i.e. the highest dilution of antiserum in which 50 % of the sensitized erythrocytes remained unlysed. Where necessary, interpolation between two wells containing more than 50 % and less than 50 % unlysed cells was made by expressing the antiserum titre as the geometric mean of the two dilutions.

Calculation of strain differences

To obtain a quantitative estimate of the relation between two strains, the method used was that described by Ubertini *et al.* (1964) and popularly applied since then to FMDV strain differentiation.

Table 1. *Comparative complement-fixation tests in tubes and in microplates*

(*a*) Microplate tests – the numbers represent a visual score of the percentage of erythrocytes remaining unlysed: 4 = 100 %; 3 = 75 %; 2 = 50 %; 1 = 25 %.

Antigen (reciprocal of dilution)		Antiserum (reciprocal of dilution) A6003 16	32	64	128	256	512	A6900 16	32	64	128	256	512
A6003	32	4	4	4	4	—	—	4	4	4	—	—	—
	64	4	4	4	4	—	—	4	4	4	—	—	—
	128	4	4	4	4	2	—	4	4	4	2	—	—
	256	4	4	4	4	2	—	4	4	4	1	—	—
	512	2	2	2	2	—	—	—	—	—	—	—	—
A6900	32	4	4	4	—	—	—	4	4	4	4	—	—
	64	4	4	4	—	—	—	4	4	4	4	1	—
	128	4	4	4	—	—	—	4	4	4	4	1	—
	256	3	1	1	—	—	—	2	3	3	2	—	—
	512	—	—	—	—	—	—	—	—	—	—	—	—

(*b*) Tube tests – the results are shown as the percentage of lysis in each mixture.

Antigen (reciprocal of dilution)		Antiserum (reciprocal of dilution) A6003 160	200	520	650	A6900 240	300	520	650
A6003	256			21·4	58·8	32·7	58·1		
	512			15·0	46·1	10·9	36·0		
	1024			27·3	54·7	17·7	35·7		
	2048			54·7	67·7	50·0	57·6		
	4096			70·4	76·4	71·0	73·4		
A6900	128	23·7	50·8					42·4	68·7
	256	22·2	47·1					19·9	50·2
	512	32·5	53·7					15·7	40·7
	1024	55·2	66·2					39·1	49·8
	2048	71·0	75·4					64·5	69·2

For two antigens, A and B, and their respective antisera, a and b the following values are determined: r_1 (antiserum a) = Ba/Aa, where Ba represents the reciprocal of the titre of antiserum a with the heterologous antigen and Aa represents the reciprocal of the homologous titre of the same antiserum; similarly, r_2 (antiserum b) = Ab/Bb.

The antigenic relation between the strains (R) is then determined by the formula $R = 100\sqrt{r_1 . r_2}\,\%$.

RESULTS

Comparative fixation patterns in tube and microplate tests

In all the tests carried out, the pattern of fixation in microplates was comparable to that in tubes, as illustrated by Table 1. An optimum dilution of antigen was always demonstrable in tube tests. However, in microplate tests the antigen optimum was often represented by a range of dilutions, probably owing to the semi-quantitative nature of the end-point determinations in microplates.

https://doi.org/10.1017/S0022172400023640 Published online by Cambridge University Press

Table 2. *Serum titres and values for* r *and* R *obtained in microplate tests*

(Parameters are shown as mean values with the range in brackets: (1), OVI *v.* O_1 Lom (three tests, two in duplicate); (2), A6003 *v.* A6900 (two tests, one in duplicate and one in triplicate).)

	Antigen	Antiserum	Serum titre	*r*	*R* (%)
(1)	OVI	OVI	1072 (994–1220)	0·54 (0·44–0·66)	40 (35–47)
	O_1 Lom	OVI	575 (441–661)		
	O_1 Lom	O_1 Lom	562 (397–661)	0·30 (0·30)	
	OVI	O_1 Lom	166 (118–196)		
(2)	A6003	A6003	272 (256–372)	0·36 (0·25–0·50)	42 (30–60)
	A6900	A6003	97 (91–102)		
	A6900	A6900	288 (256–324)	0·50 (0·35–0·71)	
	A6003	A6900	145 (128–162)		

Table 3. *Serum titres and values for* r *and* R *obtained in tube tests*

Parameters are shown as mean values with the range in brackets: (1), OVI *v.* O_1 Lom (4 tests); (2), A6003 *v.* A6900 (3 tests); (3), Asia 1 C_2 *v.* Asia 1 415 (3 tests); (4), Asia 1 C_2 *v.* Asia 1 387 (2 tests); (5), Asia 1 415 *v.* Asia 1 387 (2 tests).

	Antigen	Antiserum	Serum titre	*r*	*R* (%)
(1)	OVI	OVI	3548 (2564–3846)	0·37 (0·34–0·42)	30 (28–32)
	O_1 Lom	OVI	1318 (1097–1429)		
	O_1 Lom	O_1 Lom	1862 (1724–1887)	0·26 (0·23–0·26)	
	OVI	O_1 Lom	447 (427–485)		
(2)	A6003	A6003	589 (552–602)	0·28 (0·28–0·30)	37 (36–38)
	A6900	A6003	166 (153–178)		
	A6900	A6900	562 (538–658)	0·49 (0·47–0·52)	
	A6003	A6900	295 (281–311)		
(3)	Asia 1 C_2	Asia 1 C_2	195 (180–213)	1·00 (0·97–1·04)	83 (82–84)
	Asia 1 415	Asia 1 C_2	195 (174–221)		
	Asia 1 415	Asia 1 415	145 (127–152)	0·69 (0·67–0·71)	
	Asia 1 C_2	Asia 1 415	105 (94–122)		
(4)	Asia 1 C_2	Asia 1 C_2	195 (180–213)	0·95 (0·95)	101 (100–101)
	Asia 1 387	Asia 1 C_2	191 (177–202)		
	Asia 1 387	Asia 1 387	85 (79–100)	1·07 (1·06–1·08)	
	Asia 1 C_2	Asia 1 387	95 (86–108)		
(5)	Asia 1 415	Asia 1 415	145 (127–152)	0·64 (0·62–0·65)	80 (78–83)
	Asia 1 387	Asia 1 415	87 (81–94)		
	Asia 1 387	Asia 1 387	85 (79–100)	1·00 (0·99–1·00)	
	Asia 1 415	Asia 1 387	89 (78–99)		

Where inhibition of fixation in antigen excess was not observed in microplate tests, the demonstration of an optimum amount of antigen was accepted on the basis of at least two antigen dilutions indicating the same maximum antiserum end-point.

The results shown in Table 1 also indicate that in both systems the optimum dilution of an antigen was generally very similar for two different antisera, always being within 1 twofold interval for both. This was a consistent finding in all tube and microplate tests.

Reproducibility of results

Tables 2, 3 summarize the results of microplate and tube tests respectively. The ranges and mean values of the serum titres and of r and R were obtained from the values for individual tests and, where microplate tests were performed with replicates, the values for r and R were derived from all combinations of the appropriate replicate titres within each test.

The range of variation in the results was lower in tube tests than those in microplates. The maximum variation from a mean value for R, as a percentage of the mean, was 7 % in tube tests and 43 % in microplate tests. The values for R in comparative tests were slightly greater, but not significantly so, in microplate tests than in tube tests.

It is apparent that the range of variation in a serum titre in tube tests was greater than the variation in a value for R. Apart from the OVI antiserum of which a number of different pools were used, the greatest variation in serum titres in the tube tests was observed with the Asia 1 antisera. These exhibited large and variable pro-complementary effects and compensation for this by assuming that the activity was additive to that of complement was apparently inadequate. The values of r and R were affected much less by these errors, since they are proportional values.

In the microplate tests of OVI *v.* O_1 Lom, 1·5-fold serum dilutions were used. Since the range of variation of serum titres within tests was similar to those for tests of A6003 *v.* A6900, in which twofold serum dilutions were used, it would appear that there is no advantage in using the closer dilution interval.

Antigenic relations of the strains

From tests in tubes, the mean value for R between OVI and O_1 Lom was 30 % and between A6003 and A6900 was 37 %. In both these comparisons, antigenic differences were detectable with either serum but were quantitatively different, i.e. the values of r_1 and r_2 were unequal.

Two of the Asia 1 strains appeared to be identical (Asia 1 C_2 versus Asia 1 387, R = 101 %) and the third strain, Asia 1 415, was different but held a similar relation to the other two (Asia 1 C_2 *v.* Asia 1 415, R = 83 %, Asia 1 387 *v.* Asia 1 415, R = 80 %).

There was very little difference in the titres of either the Asia 1 C_2 or the Asia 1 387 antisera when tested against any of the three strains, so that antigenic differences between the strains was detectable only with the Asia 1 415 antiserum. The two values for r obtained with this antiserum were of the same order (Asia 1 415 *v.* Asia 1 C_2, r_1 = 0·69; Asia 1 415 *v.* Asia 1 387, r_1 = 0·64), which confirms the virtual identity of the Asia 1 C_2 and Asia 1 387 strains.

As the antisera to the Asia 1 strains were prepared using crude tissue culture antigens, it was possible that antibody to BHK cellular material could react with contaminating cellular debris. However, the method of antigen purification used should eliminate host material and there was no fixation of complement by any of the three antisera, tested at the lowest dilutions used, against a similarly purified heterologous (type O) antigen.

DISCUSSION

The comparative results of tube and microplate tests illustrate the essential difference between the two methods. Microplate tests, while simple and rapid to perform and to replicate, are considerably less reproducible than tube tests which, however, require considerable care in the preparation and pretitration of reagents. It would appear that the error in microplate tests would be quite acceptable for the routine differentiation of field strains of FMDV if the tests were sufficiently replicated. However, the tube test provides a sensitive and accurate method for the detection of small antigenic differences.

Guerche *et al.* (1972) found that the two type A strains, A6003 and A6900, could not be differentiated by complement fixation. Since they found that the two strains were immunologically distinct, they concluded that complement fixation was an unsatisfactory method for detecting immunological variants. These authors used a complement-fixation test in tubes in which the concentrations of antigen, antiserum and complement were all varied. Their results were not entirely consistent with the model of fixation described previously (Forman, 1974) but this could be explained by their use of crude tissue culture harvests as antigens, since these will contain at least three complement-fixing antigens, probably at different concentrations and with different strain specificities (unpublished data). The value for R determined in this study ($R = 37\%$) was of a similar order to that of the two type O strains which are accepted as different sub-types (OVI *v.* O_1 Lom, $R = 30\%$). It can only be concluded from this that the two strains, A6003 and A6900, are serologically distinct and this is consistent with the finding of Guerche *et al.* (1972) that the two strains are immunologically different.

The differences between the type Asia 1 strain 415 and the two other strains, C_2 and 387, were small but reproducible and in the light of the interrelations between the three strains would appear to be significant. It was demonstrated that the close similarities were not the result of fixation of complement by contaminating non-viral antigens. Nor is it likely that viral antigens other than 140S were reacting, since other unpublished work suggests that such antigens – in particular, 12S sub-units resulting from degradation of the virion – are present in such preparations in only very small amounts. These differences could, however, not be correlated with any of the other criteria by which the strains have been compared (R. Priston, 1972; work to be published).

The author thanks Dr R. Priston for providing the Asia 1 antigens and antisera and Mr W. Carpenter for valuable technical assistance. The helpful advice of Dr J. B. Brooksby and Dr H. G. Pereira is gratefully acknowledged.

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