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Microneutralization tests for serological typing and subtyping of foot-and-mouth disease virus strains

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SUMMARY

A microneutralization test for serotyping of FMD viruses is described. It is based on earlier observations by Booth, Rweyemamu & Pay (1978) that dose–response relationships in quantal microneutralizations often deviated from linearity. The typing test described therefore utilizes undiluted virus preparations. In about 90% of samples a positive typing was obtained in contrast with about 50% for the complement fixation test. The test was also found to be susceptible to minimal quantities of heterotypic viral contamination.

For strain differentiation the microneutralization test was carried out as a checkerboard test. When compared with the complement fixation test it was found to be more specific. The necessity to utilize virus-neutralization test systems for comparing FMD virus strains particularly for the purpose of vaccine selection is emphasized. The two dimensional microneutralization test has been applied to a study of comparing FMDV vaccine strains for Europe, South America, the Middle East and East Africa.

INTRODUCTION

The diagnosis and control of foot-and-mouth disease by vaccination demands the recognition of the existence of seven distinct sero-types and a plurality of subtypes of the virus (Brooksby, 1968). Complement fixation tests have been the most widely employed in the serological typing and subtyping of foot-and-mouth disease virus (Brooksby, 1952; Davie, 1964; Graves, 1960*a*; Forman, 1974). Other methods less widely used include immunodiffusion (Graves, 1960*b*; Brown & Crick, 1958; Cowan & Wagner, 1970; Lobo *et al.* 1974), kinetics of neutralization (Forman, 1975; Rweyemamu, Booth & Pay, 1977*a*; Rweyemamu *et al.* 1977*b*; Martinsen, 1971), plaque reduction (Capstick, Sellers & Stewart, 1959; McVicar & Sutmoller, 1974), and fluorescent antibody tests (Sugimura & Eissner, 1976).

In this communication we describe the application of microneutralization tests to routine typing and subtyping of foot-and-mouth disease virus. We present evidence that the technique is more sensitive than the complement fixation test. Factors that affect the dose–response relationship in the microneutralization test have been described previously (Booth *et al.* 1977) as have criteria for recognizing statistically significant differences between strains (Rweyemamu, Pay & Parker, 1977*c*).

MATERIALS AND METHODS

Virus strains and antisera

The methods for preparation of virus antigens and antisera have been described previously (Booth *et al.* 1978; Rweyemamu *et al.* 1977*a*).

Microneutralization typing test

Doubling dilutions from 1/4 to 1/512 of the seven type specific antisera in Eagle's cell growth medium were made in tissue culture grade microtitre plates, using 0·05 ml diluting loops and to each of these was added 0·05 ml undiluted test virus sample. The serum–virus mixtures were left at room temperature for 1 h. Then 0·05 ml of $1{\cdot}5 \times 10^6$ BHK monolayer cells per ml was added to each well. Suitable controls were set up for cell growth, virus cytopathogenicity and possible serum cytotoxicity.

The plates were incubated at 37 °C and examined under the microscope for cytopathic effect (CPE) at 24 h after inoculation. If CPE was extensive in the virus controls the plates were stained; otherwise the test was read at 48 h after inoculation. The stain consisted of 0·1 % crystal violet in 10 % formalin.

A positive typing was indicated by the presence of a row of intact, stained cell monolayers corresponding to the serial dilutions of only one of the seven type specific antisera.

Microneutralization subtyping test

The test was carried out as a two dimensional checkerboard titration in microtitre plates as described previously (Booth *et al.* 1978). Serial twofold dilutions of antisera were mixed with a range of 0·5 log-step dilutions of the test virus in equal volumes of serum and virus (0·05 ml each) and incubated for 1 h at room temperature. BHK 21 monolayer cells were added in 0·05 ml volumes at a seeding rate of $7{\cdot}5 \times 10^4$ cells per cup. The plates were incubated at 37 °C for 2 days; then the medium was tipped off into a bowl of 2 % citric acid and the monolayers were stained with 0·1 % crystal violet in 10 % formol-saline.

CPE was indicated by cell lysis and, therefore, only uninfected monolayers were fixed and took up stain. Virus neutralization was marked by the presence of residual monolayers. At each virus dose tested the corresponding level of neutralization was determined by the method of Karber (1931). Neutralization titres corresponding to virus doses of less than 0·5 $\log_{10}$ TCID 50 were not included in determining the final antibody titre. A regression curve was calculated for serum titre against virus dose using the Wang 2200 desk top computer and the neutralizing antibody titre was determined as the final dilution of antiserum which neutralized exactly 100 TCID 50 of the virus. As previously described (Booth *et al.* 1978) the plot of log serum titre against virus dose was sometimes curvilinear. Such curves were often marked by lack of progressive decrease in neutralizing titre with increasing virus dosage at high virus inputs. Such values were therefore excluded from the regression curve calculation and the regression line was only fitted to the middle portion of the curve.

In comparing pairs of virus strains using antiserum to one of them the degree of their serological relatedness was expressed as the ratio:

$$r = \frac{\text{heterologous neutralization titre}}{\text{homologous neutralization titre}}.$$

The two-way-cross relationship between pairs of strains was denoted by the value:

$$R = 100\sqrt{(r_1 \times r_2)}.$$

The complement fixation test (CFT)

The 'chess-board' type test was carried out in microtitre plates as described previously for virus subtyping (Forman, 1974; Rweyemamu *et al.* 1977*b*). Fixation was for 1 h at 37 °C using three haemolytic doses of complement. Guinea pig antisera raised to purified, 146S antigens were reacted with pelleted antigens for the subtyping test. For virus typing, antigens were tested as unconcentrated tissue culture virus harvests.

RESULTS

Foot-and-mouth disease virus typing

For the typing method virus was tested, usually undiluted, at titres as high as 6·5–8·0 log p.f.u./ml. The incentive for examining the use of undiluted virus stocks in serotyping of FMD virus by the neutralization reaction stemmed from our earlier observation that the dose–response relationship in microneutralization tests often deviated from linearity at high virus inputs (Booth *et al.* 1978). By testing undiluted stock virus against homotypic and heterotypic antisera it was possible regularly to demonstrate clear specific neutralization only with homotypic antiserum.

It was observed, however, that sometimes homotypic antisera failed to type viruses of widely different subtype. In such cases typing was achieved by the use of either homotypic antiserum from a more closely related subtype or homologous antiserum. This suggested that even at high virus doses, as employed in the typing test, the microneutralization test was still largely strain specific. This was tested by carrying out ten blocks of homologous and heterologous neutralizations using undiluted virus stocks as in the typing test. From the resultant homologous and heterologous serum titres r values were calculated. The same strains were also tested in the complement fixation subtyping test. The subtype relationships obtained in the two tests are compared in Table 1. Of the 39 pairs of r values obtained using either the subtyping CFT or the high virus–dose microneutralization typing test, 90% were within 4-fold range of each other. It was surprising that the CFT subtyping test was apparently no more specific than the microneutralization typing test.

Table 1. *A comparison of the strain specificity of the microneutralization typing test using undiluted virus stocks with the complement fixation subtyping test*

(*a*) *r* values

Serum	Virus	... A_5 Westwald	A_5 France 1/68 (Allier)	A Pando (1970)	A_{30} Uruguay	A_{24} Cruzeiro	A_{25} Argentina	A_{26} Argentina	A_{27} Col 1/67	A_{22} Iraq 24/64	A_{22} Mahmatli
A_5 Westwald	MN	1·00	0·44	0·09	0·13	0·20	0·20	0·20	0·20	0·13	0·13
	CF		0·99	0·18	0·27	0·58	0·58	0·28	0·89	0·25	—
A_5 France 1/68 (Allier)	MN	1·50	1·00	0·30	0·13	0·30	0·44	0·67	0·44	0·30	0·30
	CF	0·97		0·12	0·40	—	—	—	0·79	—	—
A Pando (1970)	MN	1·50	1·00	1·00	1·00	0·45	0·45	0·45	0·45	< 0·30	< 0·30
	CF	0·20	0·26		0·35	0·17	< 0·15	0·24	< 0·26	—	—
A_{30} Uruguay	MN	1·48	1·00	1·00	1·00	0·44	0·67	0·67	0·67	< 0·44	< 0·44
	CF	0·37	0·38	0·39		—	0·15	0·21	0·40	—	—
A_{24} Cruzerio	MN	0·55	0·36	0·11	0·25	1·00	0·82	0·55	0·36	0·36	0·36
	CF	0·31	—	0·07	—		0·35	0·63	—	—	—
A_{25} Argentina	MN	0·44	< 0·44	< 0·44	0·44	0·67	1·00	0·44	< 0·44	< 0·44	< 0·44
	CF	0·38	—	0·14	0·30	0·36		—	—	—	—
A_{26} Argentina	MN	0·67	< 0·67	< 0·67	< 0·67	0·67	< 0·67	1·00	< 0·67	< 0·67	< 0·67
	CF	0·40	—	0·10	0·20	0·55	—		0·35	—	—
A_{27} Col 1/67	MN	2·22	1·00	0·44	0·44	0·67	0·67	0·67	1·00	< 0·44	< 0·44
	CF	0·78	0·49	0·31	0·32	—	—	0·13		—	—
A_{22} Iraq 24/64	MN	0·45	< 0·20	< 0·20	0·20	0·30	0·30	0·30	0·20	1·00	1·00
	CF	0·06	—	—	—	—	—	—	—		—
A_{22} Mahmatli	MN	1·00	1·00	0·45	0·68	0·68	1·00	0·68	0·45	1·00	1·00
	CF	—	—	—	—	—	—	—	—		

MN, Microneutralization of high virus dose by serial dilutions of serum.
CF, Chessboard complement fixation test using 3 MHD50 of complement.

Table 1 (*cont.*)

(*b*) *R* values (%)

Serum	Virus	... A_5 Westwald	A_5 France 1/68 (Allier)	A Pando (1970)	A_{30} Uruguay	A_{24} Cruzeiro	A_{25} Argentina	A_{26} Argentina	A_{27} Col 1/67	A_{22} Iraq 24/64	A_{22} Mahmatli
A_5 Westwald	MN	} 100	{ —	—	—	—	—	—	—	—	—
	CF		{ —	—	—	—	—	—	—	—	—
A_5 France 1/68 (Allier)	MN	81	} 100	{ —	—	—	—	—	—	—	—
	CF	98		{ —	—	—	—	—	—	—	—
A Pando (1970)	MN	37	55	} 100	{ —	—	—	—	—	—	—
	CF	19	18		{ —	—	—	—	—	—	—
A_{30} Uruguay	MN	44	36	100	} 100	{ —	—	—	—	—	—
	CF	32	39	37		{ —	—	—	—	—	—
A_{24} Cruzeiro	MN	33	33	22	33	} 100	{ —	—	—	—	—
	CF	42	—	11	—		{ —	—	—	—	—
A_{25} Argentina	MN	30	< 44	< 44	54	74	} 100	{ —	—	—	—
	CF	47	—	< 14	21	35		{ —	—	—	—
A_{26} Argentina	MN	37	< 67	< 55	< 67	61	< 54	} 100	{ —	—	—
	CF	33	—	15	20	59	—		{ —	—	—
A_{27} Col 1/67	MN	67	66	44	54	49	< 54	< 67	} 100	{ —	—
	CF	83	62	< 28	36	—	—	21		{ —	—
A_{22} Iraq 24/64	MN	24	< 24	< 24	< 30	33	< 36	< 44	< 30	} 100	{ —
	CF	12	—	—	—	—	—	—	—		{ —
A_{22} Mahmatli	MN	36	55	< 37	< 55	49	< 66	< 67	< 44	100	} — 100
	CF	—	—	—	—	—	—	—	—	—	

MN, Microneutralization of high virus dose by serial dilutions of serum.
CF, Chessboard complement fixation test using 3 MHD50 of complement.

Table 2. *The effect of heterotypic viral contamination on the efficiency of typing FMD virus by the microneutralization test*

Stock virus dose per cup	Contaminating virus dose per cup	SN_{50} titre A Pando (1970)	SN_{50} titre O_1 BFS 1860
	Expt. I		
A Pando 6·3 log p.f.u.	Diluent	1/64	< 1/4
O_1 BFS 1860 6·2 log p.f.u.	Diluent	< 1/4	1/256
A Pando 6·3 log p.f.u.	O_1 BFS 1860 5·2 log p.f.u.	< 1/4	< 1/4
A Pando 6·3 log p.f.u.	O_1 BFS 1860 4·2 log p.f.u.	< 1/4	< 1/4
A Pando 6·3 log p.f.u.	O_1 BFS 1860 3·2 log p.f.u.	< 1/4	<1/4
A Pando 6·3 log p.f.u.	O_1 BFS 1860 2·2 log p.f.u.	1/16	< 1/4
A Pando 6·3 log p.f.u.	O_1 BFS 1860 1·2 log p.f.u.	1/128	< 1/4
O_1 BFS 1860 6·2 log p.f.u.	A Pando 5·3 log p.f.u.	< 1/4	< 1/4
O_1 BFS 1860 6·2 log p.f.u.	A Pando 4·3 log p.f.u.	< 1/4	< 1/4
O_1 BFS 1860 6·2 log p.f.u.	A Pando 3·3 log p.f.u.	< 1/4	< 1/4
O_1 BFS 1860 6·2 log p.f.u.	A Pando 2·3 log p.f.u.	< 1/4	1/8
O_1 BFS 1860 6·2 log p.f.u.	A Pando 1·3 log p.f.u.	< 1/4	1/128
	Expt. II		
O_1 BFS 1860 5·0 log TCID 50	Diluent	< 1/4	1/16
A Pando 5·3 log TCID 50	Diluent	1/64	< 1/4
A Pando 5·3 log TCID 50	O_1 BFS 1860 5·0 log TCID 50	< 1/4	< 1/4
A Pando 5·3 log TCID 50	O_1 BFS 1860 4·0 log TCID 50	< 1/4	< 1/4
A Pando 5·3 log TCID 50	O_1 BFS 1860 3·0 log TCID 50	< 1/4	< 1/4
A Pando 5·3 log TCID 50	O_1 BFS 1860 2·0 log TCID 50	< 1/4	< 1/4
A Pando 5·3 log TCID 50	O_1 BFS 1860 1·0 log TCID 50	< 1/4	< 1/4
A Pando 5·3 log TCID 50	O_1 BFS 1860 0·0 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 5.3 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 4·3 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 3·3 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 2·3 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 1·3 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 0·3 log TCID 50	< 1/4	1/8
	Expt. III		
O_1 BFS 1860 5·0 log TCID 50	Diluent	< 1/4	1/32
A Pando 5·1 log TCID 50	Diluent	1/32	< 1/4
A Pando 5·1 log TCID 50	O_1 BFS 1860 5·0 log TCID 50	< 1/4	< 1/4
A Pando 5·1 log TCID 50	O_1 BFS 1860 4·0 log TCID 50	< 1/4	< 1/4
A Pando 5·1 log TCID 50	O_1 BFS 1860 3·0 log TCID 50	< 1/4	< 1/4
A Pando 5·1 log TCID 50	O_1 BFS 1860 2·0 log TCID 50	< 1/4	< 1/4
A Pando 5·1 log TCID 50	O_1 BFS 1860 1·0 log TCID 50	< 1/4	<·1/4
A Pando 5·1 log TCID 50	O_1 BFS 1860 0·0 log TCID 50	< 1/4	< 1/4
A Pando 5·1 log TCID 50	O_1 BFS 1860 $\bar{1}$·0 log TCID 50	1/64	< 1/4
A Pando 5·1 log TCID 50	O_1 BFS 1860 $\bar{2}$·0 log TCID 50	1/64	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 5·1 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 4·1 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 3·1 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 2·1 log TCID 50	< 1/4	< 1/4
O_1 BFS 1760 5·0 log TCID 50	A Pando 1·1 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 0·1 log TCID 50	< 1/4	< 1/4
O_1 BFS 1860 5·0 log TCID 50	A Pando 1·1 log TCID 50	< 1/4	1/32
O_1 BFS 1860 5·0 log TCID 50	A Pando 2·1 log TCID 50	< 1/4	1/32

Table 3. *The effect of heterotypic virus mixing on the efficiency of FMD virus typing by the complement fixation test*

Virus sample	CF antibody titre O_1 BFS 1860	CF antibody titre A Pando
O_1 BFS 1860 10^0 + A Pando 10^0	458	458
O_1 BFS 1860 10^0 + A Pando 10^{-1}	458	60
O_1 BFS 1860 10^0 + A Pando 10^{-2}	458	12
O_1 BFS 1860 10^0 + A Pando 10^{-3}	458	12
O_1 BFS 1860 10^0 + A Pando 10^{-4}	458	12
O_1 BFS 1860 10^0 + A Pando 10^{-5}	458	12
A Pando 10^0 + O_1 BFS 1860 10^0	458	458
A Pando 10^0 + O_1 BFS 1860 10^{-1}	27	458
A Pando 10^0 + O_1 BFS 1860 10^{-2}	18	458
A Pando 10^0 + O_1 BFS 1860 10^{-3}	18	458
A Pando 10^0 + O_1 BFS 1860 10^{-4}	18	458
A Pando 10^0 + O_1 BFS 1860 10^{-5}	18	458

Table 4. *Analysis of complement fixation typing results*

FMD virus type	No. of tests	No. positive typing	No. untyped: Anti-complementary	No. untyped: Negative typing	No. untyped: Total	No. heterotypic reaction ⩾50 %
O	89	57	20	10	30	2
(%)	(100)	(64)	(22)	(11)	(34)	(2)
A	164	98	23	29	52	14
(%)	(100)	(60)	(14)	(18)	(32)	(9)
C	42	22	6	12	18	2
(%)	(100)	(52)	(14)	(29)	(43)	(5)
SAT 1	101	48	19	32	51	2
(%)	(100)	(48)	(19)	(32)	(50)	(2)
SAT 2	155	77	33	37	70	8
(%)	(100)	(50)	(21)	(24)	(45)	(5)
SAT 3	71	34	12	19	31	6
(%)	(100)	(48)	(17)	(27)	(44)	(8)
ASIA 1	41	22	3	7	10	8
(%)	(100)	(54)	(7)	(17)	(24)	(22)
All types	663	358	116	146	262	43
(%)	(100)	(54)	(17)	(22)	(40)	(6)

Effect of heterotypic virus contamination on the efficiency of FMD virus typing by the microneutralization test

Three experiments were carried out to assess the value of the microneutralization typing test for determining the purity of virus preparations.

In the first experiment a type O_1 strain BFS 1860 virus at a titre of 6·2 $\log_{10}$ p.f.u./0·025 ml was mixed with a type A strain Pando (1970) at a titre of 6·3 $\log_{10}$ p.f.u./0·025 ml in the following way: neat virus of one strain was mixed in equal volume with serial tenfold dilutions of the other virus. The mixed samples were typed both in the complement fixation test and in the microneutralization.

Table 5. *Analysis of microneutralization typing results*

FMD virus type	No. of tests	No. typed: Positive typing	No. typed: 100% CPE initially	No. typed: Total	No. untyped (no CPE)	No. heterotypic reaction ≥50 %
O	217	173	23	196	19	2
(%)	(100)	(80)	(11)	(*90*)	(9)	(1)
A	247	200	30	230	16	1
(%)	(100)	(81)	(12)	(*93*)	(6)	(0·4)
C	85	63	16	79	6	0
(%)	(100)	(74)	(19)	(*93*)	(7)	(0)
SAT 1	91	73	5	78	11	2
(%)	(100)	(80)	(5)	(*86*)	(12)	(2)
SAT 2	96	66	13	79	17	0
(%)	(100)	(69)	(14)	(*82*)	(18)	(0)
SAT 3	123	105	8	113	4	6
(%)	(100)	(85)	(7)	(*92*)	(3)	(5)
ASIA 1	41	34	2	36	3	2
(%)	(100)	(83)	(5)	(*88*)	(7)	(5)
All types	900	714	97	811	76	13
(%)	(100)	(79)	(11)	(*90*)	(8)	(1)

In the second and third experiments the two virus strains were mixed as described for the first experiment, but in addition each virus was titrated in the microculture system in order to determine the virus dose per cup.

The results are summarized in Tables 2 and 3. It is evident particularly from results of the second and third experiments that even minimal levels of heterotypic viral contamination of a test virus renders impossible the serological typing of the virus by the microneutralization test. It was concluded that provided sufficiently high titred sera were used for microneutralization FMD typing, failure to type neat virus would be probably indicative of heterotypic viral contamination of the sample. In contrast only undiluted contaminating virus interfered significantly with the CFT typing (Table 3). This is consistent with the general experience that virus suspensions produced in tissue culture systems cannot be diluted very much before failing to fix complement and as a result the CF test is only likely to detect gross levels of heterotypic virus contamination.

Analysis of FMDV typing results in the complement fixation test

Results of typing in the complement fixation test for 663 tissue culture FMD virus samples are summarized in Table 4. In all, 358 (54 %) tests resulted in positive typing; in 116 cases the samples were anti-complementary and in 146 no complement fixing activity was detectable giving a total of 262 (40 %) in which there was no positive typing result. Significant heterotypic reactions were observed in 43 (6 %) cases. There were no significant variations from type to type of FMD virus.

Table 6. *Comparison of microneutralization and complement fixation FMD typing in parallel tests*

Virus type	No. tested	No. typed in micro-neutralization test	No. typed in complement fixation test
O	27	23	15
(%)	(100)	(85)	(56)
A	23	21	15
(%)	(100)	(91)	(65)
C	11	11	3
(%)	(100)	(100)	(27)
SAT 1	11	10	3
(%)	(100)	(91)	(27)
SAT 2	18	16	3
(%)	(100)	(89)	(17)
SAT 3	10	8	5
(%)	(100)	(80)	(50)
ASIA I	4	4	1
(%)	(100)	(100)	(25)
All types	104	93	45
(%)	(100)	(89)	(43)

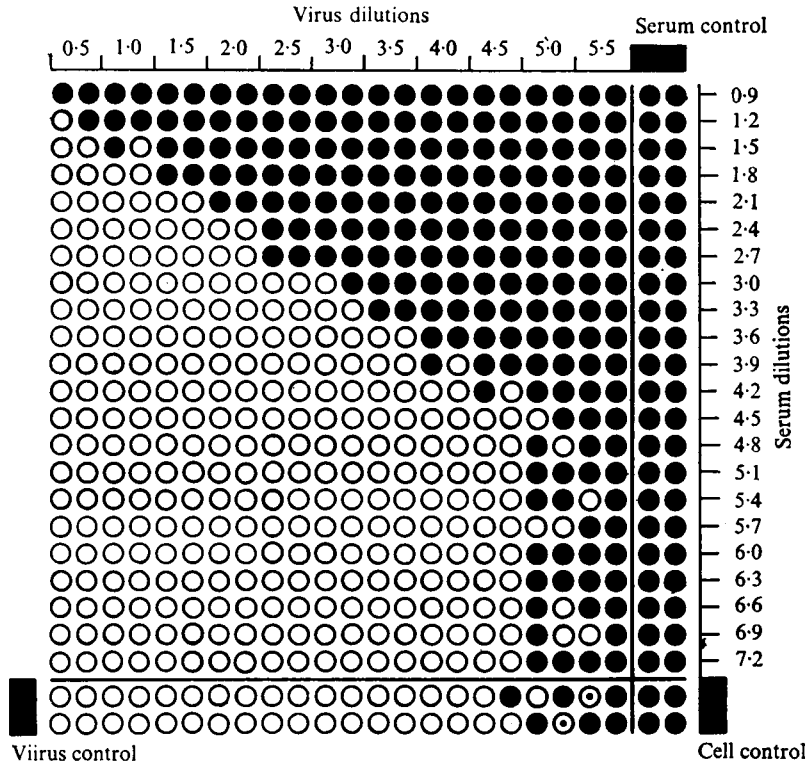

FIG. 1. Two-dimensional microneutralization test used in FMDV subtyping. Serum, C Noville; test virus, C K267/67.

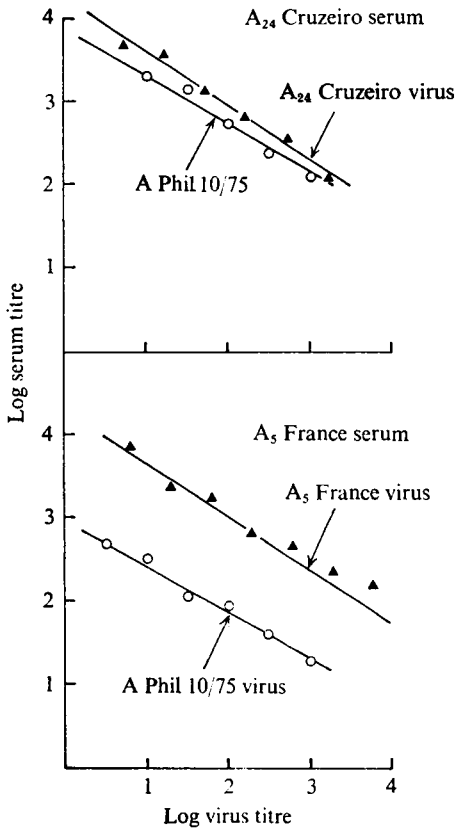

Fig. 2. Strain comparisons by two-dimensional microneutralization.

Analysis of FMDV typing results in the microneutralization test

This analysis is summarized in Table 5. A total of 900 tests have been analysed. Of these 714 (79 %) gave clear positive typing. In 97 (11 %) cases the virus demonstrated apparent non-neutralization which was attributable to the use of very high titre virus against low titre antisera. Such difficulties were easily overcome by repeat tests with either stock virus diluted 1/10 or using stronger homotypic antiserum. In a few cases, however, the apparent non-neutralization could be attributed to contamination of the test virus with heterotypic virus. Four such virus preparations were detected during the course of routine purity check of virus harvests before their release for use in vaccine manufacture or research. Therefore in the microneutralization test a total of 803 out of 900 tests (90 %) resulted in positive typing in contrast with only 54 % in the complement fixation test.

Failure to type on account of poor or no CPE because of lack of infectivity accounted for 8 % and heterotypic reactions were observed in 1 % of the tests.

Comparison of the CFT and microneutralization test for FMD typing on the same samples in parallel tests

A total of 104 samples were typed in parallel in the microneutralization test and in the complement fixation test. As demonstrated in Table 6, in 93 cases (89 %) the

Table 7. *Parameters of the two-dimensional microneutralization test regression line*

	Virus type					
	A	O	C	SAT 1	SAT 2	SAT 3
No. of tests analysed	458	134	37	40	51	12
Coefficient of correlation (with 95% confidence limits)	0·981 (0·885, 0·997)	0·977 (0·865, 0·996)	0·978 (0·893, 0·996)	0·983 (0·887, 0·998)	0·983 (0·918, 0·996)	0·980 (0·922, 0·995)
Pooled S.E. of estimate	0·218	0·192	0·253	0·231	0·211	0·132

microneutralization test resulted in positive typing. In contrast, positive typing in the complement fixation test was obtained only in 45 cases (43%). It is also evident that in the case of types C, SAT 1, SAT 2 and Asia 1 viruses the incidence of positive typing in the complement fixation test was only 17–27% compared with 89–100% in the microneutralization test.

The microneutralization subtyping test

The demonstration of significant serological differences between FMD virus strains requires an accurate serological technique. For this reason we have developed a two-dimensional microneutralization test. Fig. 1 summarizes the layout of the test and the pattern of neutralization observed at each virus dose. Fig. 2 demonstrates the dose–response relationships for homologous and heterologous reactions. As described previously the dose–response curve was not always rectilinear (Booth *et al.* 1978). It was further noted that the shape of the slope was independent of homologous or heterologous reactions but that the level of neutralization corresponding to 100 TCID 50 was always on the linear part of the slope. Since at this level antibody titre was dependent on infective virus dose it was considered imperative to ensure that the neutralizing antibody titre was always assayed against 100 TCID 50 exactly and by employing the full two-dimensional test it was always possible to interpolate the slope at this value.

As expected at terminal virus inputs the estimation of the corresponding level of neutralization was variable and grossly inaccurate. It was, therefore, decided to exclude readings corresponding to virus doses of 0·5 log TCID 50. As previously described at high virus inputs, usually in excess of 3·5 $\log_{10}$ TCID 50, the dose–response curve sometimes became flat (Booth *et al.* 1978). Consequently, neutralization titres corresponding to these higher virus doses were not included in the calculation of the final result if such values did not demonstrate a progressive increment in neutralizing titre with a decrease in virus dose. In such cases the regression line was fitted only to the middle portion of the curve.

Table 7 summarizes the parameters of the two-dimensional microneutralization regression line for 732 tests. It is evident that the application of a regression curve calculation to determine log SN 50 as described is valid. For the six FMD virus types tested the overall coefficient of correlation ranged from 0·977 to 0·983 with a pooled standard error of estimate of 0·132–0·253.

Table 8. *Comparison of type A FMD virus vaccine strains in the two-dimensional microneutralization test*

r values

Virus ... / Serum	A_5 Wester-wald	A_5 France 1/68 (Allier)	A Pando (1970)	A_{30} Uruguay	A_{24} Cruzeiro	A_{25} Argentina	A_{26} Arg. 66	A_{27} Colombia 1/67	A_{22} Iraq 24/64	A_{22} Mah	AK 18/66
A_5 Westerwald	1·00	0·49	0·09	0·16	0·58	0·08	0·35	0·22	0·01	0·02	N.D.
A_5 France 1/68 (Allier)	0·83	1·00	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	—	0.004
A Pando (1970)	0·02	0·02	1·00	0·79	0·03	0·02	0·003	0·01	0·001	0·04	0·001
A_{30} Uruguay	0·03	0·01	0·89	1·00	0·05	0·02	0·02	0·02	0·002	0·04	N.D.
A_{24} Cruzeiro	N.D.	0·12	0·01	N.D.	1·00	0·776	0·251	0·339	0·041	N.D.	0·045
A_{25} Argentina	0·09	0·21	0·04	0·30	0·56	1·00	0·59	0·17	0·02	0·12	N.D.
A_{26} Argentina/66	0·20	0·25	0·14	0·35	1·26	0·38	1·00	0·15	0·07	0·16	N.D.
A_{27} Colombia 1/67	0·41	0·69	0·54	0·47	0·43	0·52	0·31	1·00	0·01	0·02	N.D.
A_{22} Iraq 24/64	0·01	0·001	0·01	0·004	0·14	0·28	0·02	0·01	1·00	1·00	0·030
AK 18/66	N.D.	0·005	0·006	N.D.	0·023	N.D.	N.D.	0·012	0·009	N.D.	1·00

Table 9. *Comparison of type A FMD virus vaccine strains in the two-dimensional microneutralization test*

	R values								
A_5 Westerwald	100								
A_5 France	64								
A Pando	4	100							
A_{30} Uruguay	7	84	100						
A_{24} Cruzeiro	26	2	N.D.	100					
A_{25} Argentina	8	3	11	66	100				
A_{26} Argentina/66	26	2	16	56	47	100			
A_{27} Colombia 1/67	30	7	9	38	30	22	100		
A_{22} Iraq 24/64	1	0·3	0·3	5	7	4	1	100	
AK 18/66	0·4	0·2	N.D.	3	N.D.	N.D.	N.D.	2	100
	A_5 Westerwald	A Pando	A_{30} Uruguay	A_{24} Cruzeiro	A_{25} Argentina	A_{26} Argentina/66	A_{27} Colombia 1/67	A_{22} Iraq 24/64	AK 18/66

An application of the two-dimensional microneutralization test to an evaluation of relationships among some FMDV type A strains

The microneutralization test described above was used in the study of serological relationships among some type A FMD vaccine viruses derived from Europe, South America, the Middle East and East Africa. The r values obtained are summarized in Table 8 and the two-way cross-relationships denoted by R values are given in Table 9. It is interesting to compare the r values in Table 8 with those given earlier in Table 1 for the complement fixation test. Of the 41 pairs of r value obtained in the two-dimensional microneutralization test and the complement fixation test for the same strains 36 % (15 values) demonstrated r values in the CFT which were more than 4-fold greater than those obtained in the microneutralization test. This is not suprising since only the strain specific antigenic determinants located on the trypsin sensitive polypeptide of the intact capsid react in the neutralization test. In contrast the complement fixation test, in addition, reacts with the less specific trypsin resistant antigenic determinants on the capsid as well as the group antigenic determinants on the protein subunits (Wild, Burroughs & Brown, 1969; Cowan, 1973).

DISCUSSION

A serological test for routine foot-and mouth disease virus typing needs to be relatively simple and reliable. The complement fixation test offers the advantages of simplicity, versatility, rapidity of results and can be used to type inactivated as well as infectious virus preparations. However, we have found the test to be unreliable for quality control on type specificity of tissue culture virus stocks. For this purpose the serological test (*a*) requires to offer definitive results in the majority of cases on the original test sample without further passage of the virus; (*b*) should be sensitive to small quantities of specific antigen; and (*c*) should be affected by a very low grade of heterotypic virus contamination. As is evident from Table 4, in 40 % of 663 samples it was not possible to get a definitive typing in the complement fixation test on account of antigen preparation being either anti-complementary or not containing sufficient quantities of antigen. In contrast a positive typing could be obtained for 90 % of the samples with the microneutralization test. When the same virus samples were tested in the two systems in parallel a positive typing was obtained for 89 % of the samples in the microneutralization test whilst in the complement fixation test only 45 % of the same samples gave definite typing. The microneutralization test, was therefore, clearly more sensitive than the CFT.

Another disadvantage of the CFT for FMD virus typing of tissue culture virus harvests is that these preparations contain at least two antigens, the 12S subunits and the virus infection-associated antigen, both of which have strong heterotypic reactions (Rowlands, Cartwright & Brown, 1969; Cowan & Graves, 1966; Cowan, 1973). Such reactions accounted for 6 % in our series. Although heterotypic reactions have been observed in neutralization tests (Cottral, 1972) these only account for a small proportion. In our case they accounted for only 1 % of the

tests. It was further demonstrated in this study that minimal heterotypic virus contamination interfered with specific typing in the microneutralization test. This finding, coupled with the fact that the microneutralization test is being carried out using undiluted stock virus and is very sensitive for specific antigen, renders the test suitable for both routine diagnosis and for quality control on the type specificity and purity of virus stocks. The test is relatively simple to carry out and to interpret; it is economical in materials and does not require accurately standardized reagents as is needed for the complement fixation test.

The suitability of the two-dimensional microneutralization test for foot-and-mouth disease virus strain differentiation has been demonstrated in the results described in this and previous communications (Booth *et al.* 1978; Rweyemamu *et al.* 1977*b*, *c*). In order to estimate antibody titre reasonably accurately, as is demanded for intratypic comparisons of FMD virus strains, it was found necessary to carry out the test as a checkerboard since at the conventional 100 TCID 50 virus input, neutralization titre was directly proportional to infective virus dose. We have found simpler antibody assay systems that employ a single dilution of virus containing approximately 100 TCID 50 to be inadequate. In practice, the virus dose employed in the test in such cases could range between 30 and 300 TCID 50 and at such limits the corresponding neutralizing titre may vary by as much as $\pm$ 0·44 log SN50 (unpublished data). In order to minimize between-test variations homologous and heterologous determinations were carried out as matched pair tests.

The microneutralization test described, in common with other neutralization test systems, has been shown to be more strain specific than the complement fixation test (Rweyemamu, Booth & Pay, 1975; Rweyemamu *et al.* 1977*b*, *c*). For this reason, the fact that the reaction involves exclusively the antigenic determinant on the virus capsid which is responsible for the D-antigenicity of FMD virus (Brown & Smale, 1970) and that neutralizing antibody is related to immunity (Martin & Chapman, 1961; Lucam, Fedida & Dannacher, 1964; Stellman *et al.* 1968; Pay, 1972; Pay & Parker, 1977), we have preferred the use of neutralization test systems to complement fixation tests for comparison of FMD virus strains especially for the purpose of selecting suitable vaccine viruses. The two-dimensional microneutralization test described readily renders itself for routine application. It should be noted, however, that criteria for declaring pairs of strains different as measured in the neutralization tests are different from those laid down for the complement fixation test (Pereira, 1977; Rweyemamu *et al.* 1977*c*). Similarly the respective intrinsic errors are different. In our hands, for homologous and heterologous comparisons the microneutralization test was found to have an overall standard deviation per pair difference in titre of 0·32 but for the complement fixation test it was 0·21 (Rweyemamu *et al.* 1977*c*).

As an example of the application of the two-dimensional microneutralization test in strain differentiation we have employed it in a study of the serological relationships among type A FMD vaccine viruses (Tables 8 and 9). It is evident that the two strains belonging to the A_{22} subtype were quite distinct from any other type A strain as was the East African strain AK 18/66. Between the other

viruses there were varying degrees of cross-reactions. The data in Tables 8 and 9 indicate that the vaccine strains probably fall into four distinct groupings when taking into account criteria proposed by Pereira (1977) and the intrinsic error of the test system (Rweyemamu *et al.* 1977*c*).

Group I. Comprising, on one hand, the A_5 virus strains and, on the other, four of the South American subtypes A_{24}, A_{25}, A_{26}, A_{27}.

Group II. Consisting of the two strains previously isolated from Uruguay, South America, A Pando, A_{30}.

Group III. The A_{22} viruses.

Group IV. Represented by the East African strain AK 18/66.

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