J. Hyg., Camb. (1967), **65**, 67
Printed in Great Britain

Genetic marker studies of poliovirus

I. Natural variation

BY YVONNE E. COSSART
Virus Reference Laboratory, Central Public Health Laboratory, Colindale Avenue, London, N.W. 9

(*Received* 12 *October* 1966)

Intratypic variation of poliovirus was first recognized by Ramos-Alvarez & Sabin (1954) when they isolated from the faeces of healthy children naturally attenuated strains of poliomyelitis types 2 and 3 which were avirulent for monkeys by intracerebral inoculation. Antigenic variation, extensively studied by Wenner, Archetti & Dubes (1959) occurs in each serotype, but all strains of a single type share major antigens to such a large extent that special methods such as the kinetic neutralization test (McBride, 1959) or plaque inhibition (Wecker, 1960) are required to demonstrate the differences.

Dubes & Chapin (1956) were able to select variants of type 1 which replicated almost as well at 30° as at 36° C. and suggested that this alteration was accompanied by attenuation of the strains. This was applied (Sabin, Hennessen & Winsser, 1954) in the artificial production of attenuated strains for use in poliovirus vaccines. Lwoff (1962) developed the 'reproductive capacity temperature' (R.C.T.) marker test and pointed out that the naturally virulent prototype strains were able to grow as well at the 'fever temperature' of 40° as at 37° C., but were almost completely inhibited at 30° C. The attenuated vaccine strains behaved in converse fashion. He suggested that this ability to grow at 'fever' temperature is causally related to virulence.

Many other genetic marker tests have since been described. Some depend on the tissue culture host range (Kanda & Melnick, 1959; Kelly, 1962); some on resistance to physical agents such as heat (Stanley, Dorman, Ponsford & Larkin, 1956); others on growth requirements, e.g. for cystine (Dubes & Chapin, 1958); while an interesting group (Bengtssen, Philipson, Persson & Laurent, 1964) measures adsorption of the infective particles to charged molecules such as aluminium salts (Wallis, Melnick, Terry & Wimberley, 1962) or dextran sulphate (Takemoto & Kirschstein, 1964).

These tests have mostly been applied to prototype strains, including both naturally derived and vaccine strains, and relate especially to type 1. Their immediate practical value is to act as *in vitro* indicators of the relative virulence of different strains and to detect the possible derivation of strains from live attenuated vaccine.

Tests designed to measure virulence are usually compared with the monkey neurovirulence test as standard (Sabin & Lwoff, 1959). It has been shown (Benyish-Melnick & Melnick, 1959) that there is good correlation between the R.C.T. test

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and monkey neurovirulence for prototype strains, but vaccine trials (Koprowski *et al.* 1960; Magrath, Boulgar & Hartley, 1964) have revealed a tendency for this laboratory character of the attenuated strains to revert towards that of the wild strains after quite short periods of intestinal passage in man. This finding has given impetus to the study of properties which do not vary directly with virulence, and so may be more useful as genetic markers in epidemiological studies.

Although there has been much work on vaccine progeny, little information is available about the natural variation of polioviruses with respect to these 'marker' properties. It was therefore decided to examine strains collected in this laboratory before the introduction of vaccination by three tests: (*a*) the R.C.T._{40} test (Lwoff, 1962), (*b*) serodifferentiation by a modification of the Wecker technique (Nakano & Gelfand, 1962), and (*c*) (for type 1 strains only) inhibition of growth by dextran sulphate (Cossart, 1966). The R.C.T._{40} and serological tests were chosen because they have been applied extensively in other laboratories, while the dextran sulphate inhibition was included as an economical method of measuring an adsorption property.

MATERIALS AND METHODS

Strains

The viruses included in the study may be divided into the following groups:

Type 1. (*a*) 1953: obtained from clinical cases of poliomyelitis which occurred in different areas of the United Kingdom (Goffe, 1955)—21 strains. (*b*) 1957: isolated from clinical cases of poliomyelitis in that year—34 strains.

Type 2. 1953: obtained from cases of poliomyelitis in the epidemic of that year—18 strains.

Type 3. 1953–54. Only a few strains were available, reflecting the sporadic occurrence of type 3—5 strains.

The isolations had been carried out in primary monkey kidney cell culture and strains were then stored at -30° C. The earliest pass available was used provided its titre was $10^{5\cdot5}$ TCD 50/0·1 ml. or more. The passage numbers and clinical diagnosis are listed in the tables.

The prototype strains are listed below:

	Naturally occurring	Vaccine
Type 1	Mahoney	Sabin 1
Type 2	YSK	Sabin 2
Type 3	Saukett	Sabin 3

The naturally occurring strains have been passaged in monkey kidney cells in this laboratory since they were obtained as monkey testicular passes from Dr J. E. Salk.

The vaccine strains were obtained from Dr A. B. Sabin and used as the fourth monkey kidney passage in this laboratory.

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Tissue culture

Primary rhesus monkey kidney cell cultures were used for tube titration. Primary or secondary rhesus monkey kidney or primary patas monkey kidney cells were used as monolayers in Petri dishes for plaque assays.

The R.C.T.$_{40}$ *marker test*

This was performed by titrating each strain in parallel at 36° C. and at a higher temperature which differed according to the serotype, 39·8 ± 0·2° C. for types 1 and 2 and 40·3 ± 0·2° C. for type 3. This upper temperature of 39·8° C. was chosen as a compromise between the classical 40° and 39·5° which is recommended by the Biological Standards Control Division, Medical Research Council, who also choose 40·3° C. for type 3 (D. I. Magrath, personal communication). These temperatures are the critical ones at which the Sabin strains are only just completely suppressed while at the same time the wild prototype strains show very slight reduction in titre. The titrations at the higher temperatures were incubated in a well-insulated incubator with a fan to maintain air circulation. Tests with a thermocouple and recording device showed the temperature was maintained within the stated range of ± 0·2° C.

The virus suspensions were titrated in $\log_{10}$ steps and four monkey kidney culture tubes were inoculated with 0·1 ml. of each dilution. Synthetic medium 199 was used for maintenance. Two tubes per dilution were incubated at 36° C. and two at the higher temperature. Cytopathic effects were observed on the 3rd and 6th day and the end-point calculated from the 6th-day reading. Naturally derived and vaccine prototype strains of the appropriate serotype were included in each test. A satisfactory test showed less than 2 $\log_{10}$ TCD50 reduction in titre of the wild strain while the titre of the vaccine strain was reduced by 5 $\log_{10}$ TCD50 or more at the higher temperature (Sabin, 1961).

The serological marker test

This was performed by a modification of the Wecker technique (Nakano & Gelfand, 1962).

Preparation of sera. At weekly intervals rabbits were given intravenous injections of monkey kidney tissue culture fluid containing approximately 10^8 TCD50 of prototype virus in 5 ml. They were bled from the ear 10 days after the last injection. A number of antisera were made against each prototype vaccine and wild strain and those which showed a fourfold or greater difference in titre in standard tube neutralization tests between the vaccine and wild strains of the same serotype were selected for use. These sera were then titrated by the Wecker method and the concentration of serum which was just sufficient to give 95 % plaque reduction of the homologous strain was used in subsequent tests.

Method of the test. Monolayers of monkey kidney cells were grown in 60 mm. plastic Petri dishes (Sterilin). For each strain the medium was poured from six dishes and 0·2 ml. of the virus suspension containing about 30 TCD50 was inoculated into the centre of each dish. Virus was allowed to adsorb during 90 min.

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at 37° C. in the CO_2 incubator and dishes were then overlaid as follows: Two dishes with 'plain' agar (1·5% Bacto Difco agar in Medium 199), two dishes with agar containing the appropriate dilution of antiserum to the wild prototype and two dishes with agar containing antiserum to the vaccine prototype strain of the same serotype. When the agar had set the dishes were inverted and returned to the CO_2 incubator for 2 days. They were then overlaid a second time with agar containing 1/10,000 neutral red (Vital fluorochrome, Gurr). After overnight incubation plaques were counted and the percentage reduction under each serum was calculated.

So that strains could be compared, an index was derived from these values, thus:

$$\text{serological index} = \frac{\%\ \text{plaque reduction under anti-Sabin-strain serum}}{\%\ \text{plaque reduction under anti-wild-strain serum}}.$$

For type 3, however, a strain-specific anti-Saukett serum was not obtained despite repeated attempts, and the results for type 3 strains are given as percentage plaque reduction under anti-Sabin 3 serum. With each test wild and vaccine prototype strains of the same serotype were included, but no correction has been made for variation in the serological index of the control strains.

Table 1. *Results of marker tests on prototype strains given as the average of five determinations with the range of values shown in brackets*

Strain	R.C.T.$_{40}$ ($\log_{10}$ difference)*	Serological index†	Dextran marker ($\log_{10}$ difference)
Sabin 1	−6·0 (6·5–5·5)	1·9 (1·0–∝)‡	−2·5 (1·5–3·0)
Mahoney	−0·5 (1·0–+0·5)	0·13 (0–0·7)	−0·3 (+0·5–0·5)
Sabin 2	−6·5 (6·5–6·5)	1·6 (1·31–1·8)	—
YSK	−1·0 (1·5–0)	0·20 (0·12–0·5)	—
Sabin 3	−5·5 (6·0–5·0)	75 (100–65)	—
Saukett	−0·5 (1·5–0)	20 (11–25)	—

* $\log_{10}$ difference at 39·8° C. (types 1 and 2) or 40·3° C. (type 3).
† See method for derivation of the values.
‡ ∝ omitted from calculation of average.

The dextran sulphate inhibition test

This was performed by parallel tube titration of each strain in synthetic medium 199 and in this medium incorporating 0·05% dextran sulphate 2000 (M.W. 2×10^6, Pharmacia). The type 1 virus suspensions were titrated at $\log_{10}$ intervals and two tubes maintained on Medium 199 and two tubes maintained on the dextran medium were inoculated with 0·1 ml. of each dilution. The tubes were incubated at 36° C. and cytopathic effects observed on the 3rd and 6th days. The reduction in titre was calculated from the 6-day end-points. Control titrations of Mahoney and Sabin type 1 virus strains were performed with each batch; less than $10^{0.5}$ reduction in titre of Mahoney virus and more than $10^{5.5}$ reduction in titre of Sabin type 1 virus being accepted.

The range of values obtained with the prototype strains is shown in Table 1.

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RESULTS

Considerable variation has been obtained with naturally occurring strains (Tables 2–5). While the values are mainly comparable with those obtained for the prototype wild strains of the same serotype, the results for some strains in each serotype by each test approach those of the vaccine strains.

Table 2. *Type* 1 *strains isolated during* 1953 *from cases of paralytic poliomyelitis*

Passage no.	Laboratory no.	Titre at 37° C. ($\log_{10}$ TCD50/0·1 ml.)	Log_{10} difference at 39·8° C.	Serological index	Log_{10} difference in 0·05 % dextran
MK 2	4659	6·5	−3·0	0·8	+0·5
	4862	6·0	−4·5	0·5	+0·5
	4863	5·5	−4·0	0·7	+0·5
	5037	6·0	−2·5	0·6	0
	5038	6·0	−2·5	0·1	+1·0
	5045	5·5	−2·0	0·3	0
	5076	6·0	−4·5	0·6	−0·5
	5117	5·5	0	0	+0·5
	5191	6·5	−1·0	0·6	+0·5
	5314	5·5	−4·0	0·6	+1·0
	5382	5·5	−5·5	0	−0·5
	5527	6·5	−5·0	0·6	+1·0
	5747	5·5	−3·0	0·5	0
	6186	6·0	−3·5	0·5	0
	4656	6·5	−2·5	0·5	+0·5
MK 3	5343	5·0	−3·5	—	−1·0
MK 2	6183	6·5	−1·0	1·0	0
	6808	6·0	−2·5	0·6	0
	166	6·0	−0·5	0·5	+0·5
	182	6·0	−1·0	0·3	0
	274	6·0	−2·0	0·2	+1·5

No single strain, however, shows values for all the marker tests of the vaccine type. Indeed a striking feature is the lack of correlation between the tests. The results of the R.C.T.$_{40}$ test are plotted against the serological indices of the type 1 strains (Fig. 1). The random pattern of the points is striking as is their fairly even distribution over the entire field.

Most of the 1957 type 1 strains were isolated from patients with paralytic poliomyelitis, but they also included a number from cases of aseptic meningitis and non-paralytic poliomyelitis as well as from healthy contacts of cases (Table 3). No characteristic pattern of results was shown by any one of these clinical groups.

The R.C.T. test on type 1 strains shows the most striking degree of variation. If the tests are performed at 39·3° C., however, only a few strains continue to show significant reduction (Table 6).

DISCUSSION

This study was undertaken to obtain a base-line for interpreting the results of genetic marker tests on newly isolated strains of poliovirus and especially for differentiating wild strains from vaccine strains and their progeny. The substantial

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degree of variation which has been found with strains from pre-vaccine years suggests that caution is needed in drawing conclusions from the results of these tests. Any one test is insufficient to classify a strain, but with only one or two additional tests a reasonably reliable estimate can be made.

Table 3. *Type* 1 *strains isolated during* 1957

(First passage in monkey kidney tissue culture in all cases.)

Laboratory no.	Clinical illness	Titre at 37° C. ($\log_{10}$ TCD 50/0·1 ml.)	$\log_{10}$ difference at 39·5° C.	Serological index	$\log_{10}$ difference in 0·05 % dextran sulphate
15	P.P.	7·0	−7·0	0·1	−1·0
17	P.P.	7·0	−5·5	0·1	0
20	P.P.	6·0	−0·5	0·2	+0·5
55	P.P.	7·0	−5·0	0·4	+1·0
73	Enc.	6·5	−0·5	0·1	+1·0
77	P.P.	7·5	−4·5	0	0
80	P.P.	6·0	−6·0	0·3	0
88	P.P.	6·5	−4·5	0·2	0
91	P.P.	6·5	−4·0	0	0
123	P.P.	7·5	−4·0	0·4	0
124	A.M.	7·5	−2·0	0·9	+0·5
126	P.P.	7·0	−1·5	0	+0·5
137	P.P.	7·5	−3·0	0·1	+0·5
140	A.M.	6·5	−5·5	0·1	0
142	P.P.	6·0	−4·0	0·2	−1·0
145	P.P.	6·5	−3·0	0·4	0
166	Contact	6·5	−1·0	0·3	0
169	P.P.	6·5	−4·0	0·8	0
170	P.P.	6·5	−4·0	0·3	+0·5
174	P.P.	7·0	−2·0	0·3	0
175	P.P.	6·0	−4·5	0·5	+0·5
198	P.P.	7·0	−5·5	0·6	+0·5
206	A.M. Contact	6·5	−2·5	0	0
209	P.P.	7·0	−5·5	0·5	+1·0
210	P.P.	7·5	−2·5	0·1	−1·0
211	Enc.	7·5	−2·5	0·5	+0·5
212	P.P.	6·5	−1·5	0·2	0
220	P.P.	6·5	−1·0	0·2	−0·5
222	P.P.	6·0	−3·5	0·5	−0·5
245	P.P.	6·5	−1·0	0·6	−0·5
369	N.P.P.	7·5	−2·0	0·3	+0·5
403	P.P.	7·5	−2·0	0·4	0
422	P.P.	7·0	−1·5	0·3	0
425	P.P.	7·5	−2·0	0·4	+0·5

Enc. = encephalitis, N.P.P. = non-paralytic poliomyelitis, P.P. = paralytic poliomyelitis A.M. = aseptic meningitis.

The striking alteration in the behaviour of type 1 strains when the upper temperature in the reproductive capacity temperature test is reduced by only 0·5° C. emphasizes the arbitrary nature of these tests. The original derivation of Sabin's vaccine strain LSc 2ab from Mahoney by successive selection of clones able to grow at suboptimal temperatures (Sabin *et al.* 1954) probably accounts

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Table 4. *Type 2 strains isolated in 1953 from cases of paralytic poliomyelitis*

(Second passage in monkey kidney tissue culture in all cases.)

Laboratory no.	Titre at 37° C. ($\log_{10}$ TCD 50/0·1 ml.)	Log_{10} difference at 39·8° C.	Serological index
4659	6·0	−3·0	0
4702	6·5	−6·5	1·6
4895	5·5	−1·0	2·0
4956	5·5	−1·5	0
4957	6·0	−5·0	2·1
5003	6·0	−4·0	5·4
5148	5·5	0	7·0
5196	6·5	−1·0	0·8
5346	5·5	−5·5	0·5
5526	6·0	−6·0	2·5
5599	6·0	−2·0	0·7
5616	6·5	−3·0	0·7
5796	6·5	−2·5	1·5
5993	5·5	0	1·8
4669	6·5	−1·5	2·8
5883	6·0	−1·0	0
6224	6·0	−0·5	0·6
6958	7·0	−2·0	1·2

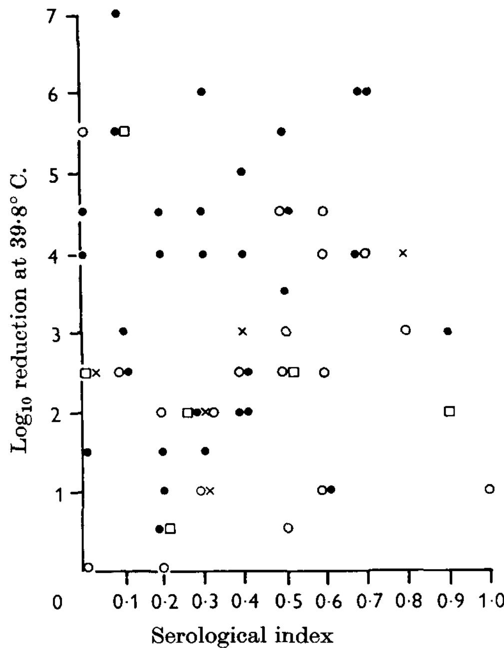

Fig. 1. Correlation of marker tests, type 1. □, 1957 aseptic meningitis; ●, 1957 paralytic polio; ×, 1957 contacts; ○, 1953 paralytic polio.

for the characteristic behaviour of the vaccine strain. There is no evidence in this series that there is any correlation between clinical virulence and the R.C.T.$_{40}$ test for naturally occurring strains.

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Table 5. *Type 3 strains isolated from cases of paralytic poliomyelitis in 1953–55*

(Second passage in monkey kidney tissue culture in all cases.)

Laboratory no.	Titre at 37° C. (log_{10} TCD50/0·1 ml.)	Log_{10} difference at 40·3° C.	Serological marker (% difference)
4748	5·5	−5·5	0
4814	7·0	−3·5	−17
5192	5·0	−2·5	+10
43	7·0	−5·5	−33
666	5·5	−2·5	−25

Table 6. *Comparison of the reproductive capacity of type 1 strains at 39·8° and 39·3° C.*

Strain no.	Titre at 36° (log_{10} TCD50/0·1 ml.)	Log_{10} reduction at 39·8°	Log_{10} reduction at 39·3°
4659	6·0	3·0	0
4863	5·5	4·0	2·0
5076	6·0	4·5	2·5
5314	5·5	4·0	1·0
5382	5·5	5·5	3·0
5527	6·5	5·0	2·0
5745	5·5	3·0	0
6186	6·0	3·5	1·5
5343	5·0	3·5	0·5
15	7·0	7·0	3·0
17	7·0	5·5	1·0
55	7·0	5·0	0
77	7·5	4·5	0·5
80	6·0	6·0	5·5
88	6·5	4·5	4·5
91	6·5	4·0	1·0
123	7·5	4·0	1·0
137	7·5	3·0	4·0
140	6·5	5·5	3·0
142	6·0	4·0	3·0
145	6·5	3·0	2·0
168	6·5	4·0	0
170	6·5	4·0	1·5
174	7·0	7·0	1·0
175	6·0	4·5	4·0
209	7·0	5·5	6·0
222	6·0	3·5	3·5
Mahoney	6·5	0·5	0
Sabin 1	6·0	6·0	4·5

Two points of practical importance emerge. First, the R.C.T.$_{40}$ test may be useful as a marker test in epidemiological studies, when subgrouping of naturally occurring strains may be attempted by varying the temperature over the range 39–40° C. Secondly, very strict temperature control is necessary to obtain reproducible results which can be compared with those in other laboratories.

The variation found with the serological and dextran markers is similar to that described (Karzon, Pollock & Barron, 1959) for other enteroviruses.

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SUMMARY

The properties of naturally occurring poliovirus strains of each serotype have been studied using three marker tests: the R.C.T.$_{40}$ test, intratypic serodifferentiation and, for type 1 strains only, inhibition by dextran sulphate.

The results show that while most strains resemble the prototype strain of the same serotype, considerable natural variation exists, especially in the ability to grow at temperatures between 39° and 40° C.

No correlation has been found between the results of the tests on individual strains and it is concluded that no single test is sufficient to characterize a strain.

I wish to acknowledge the helpful advice of Dr A. D. Macrae, Virus Reference Laboratory, and also to thank Miss Marjorie Bennett for her technical assistance.

The strains used in this study were isolated in the Virus Reference Laboratory by the late Dr A. P. Goffe, Dr A. D. Macrae and Miss J. Stokes.

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