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# The typing of enteroviruses in tissue culture by neutralization with composite antiserum pools

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Because of the many known types of enterovirus in existence (Report, 1962) identification of newly isolated strains by specific neutralization is becoming increasingly difficult. Neutralization tests with individual type specific antisera are so uneconomical in tissue culture and time that the use of pooled antisera initially is advantageous.

The preparation of antiserum pools, in which a given antiserum is included in more than one pool, so that strains can be identified from the pattern of positive results obtained in a single set of neutralization tests, has already been reported (Lim & Benyesh-Melnick, 1960; Schmidt, Guenther & Lennette, 1961). Although results are obtainable fairly quickly such methods are expensive in their use of antisera.

In the scheme which is now described virus identification is done in two stages. The identity of a virus is established less rapidly than in the other methods referred to, but the scheme has the advantages of economy in tissue culture and sera, of easy extension to cover more viruses and of allowing each virus to be neutralized by a specific antiserum on two separate occasions. It appears to work satisfactorily on most occasions but any uncertain results necessitate repetition and, if necessary, full neutralization tests with individual antisera. The methods of preparation of the composite antiserum pools and the procedures for virus typing are first described. They are followed by some results of virus identification by this typing scheme.

## PREPARATION OF THE ANTISERUM POOLS

The scheme described provides for the identification of twenty-seven viruses: polioviruses 1–3; Coxsackie viruses A 9 and B 1–6; ECHO viruses 1–3, 5–7, 9, 11–16, 19, 22–24. The antisera were prepared in rhesus monkeys or rabbits by immunization with prototype viruses. The titre of an antiserum, based on a volume of 0·1 ml., was the highest dilution which would neutralize about 100 tissue-culture doses (TCD 50) of the homologous prototype virus, when this was suspended in 0·1 ml. of maintenance medium. The amount of serum in such a dilution then constituted one unit of antibody. In the titrations 0·3 ml. of serial fourfold dilutions of serum were mixed with 0·3 ml. of virus suspension containing about 300 TCD 50. After remaining 1 hr. at room temperature 0·2 ml. volumes of the mixtures were inoculated into duplicate tubes of rhesus monkey kidney cultures.

Control titrations to check the correctness of the virus dose were made at the same time. Cultures were incubated at 36–37° C. and examined microscopically at intervals. Titres, based on the neutralization of the cytopathic effect (C.P.E.), were calculated from the readings on the sixth day as 50 % end-points by the method of Kärber (1931). All titres quoted refer to initial serum dilutions before the addition of virus. All the sera were tested for neutralizing antibody to the other viruses in their group. Only those with little or no heterologous antibody were used.

Table 1. *Enterovirus pool* 1

| Antiserum | Serum titre | Dilution containing about 50 units/0·1 ml. | Dilution for use in the pool |
|---|---|---|---|
| Poliovirus 1 | 1,280 | 1/25 | 1/8 |
| Poliovirus 2 | 10,000 | 1/200 | 1/67 |
| Poliovirus 3 | 2,560 | 1/50 | 1/17 |

Table 2. *The four major composite antiserum pools*

| Enterovirus pool (EP) number | Antisera contained in the pools |
|---|---|
| EP 1 | Polioviruses 1–3 |
| EP 2 | Coxsackie B 1–6 |
| EP 3 | ECHO 1–3, 5–7, 9, 11 and Coxsackie A9 |
| EP 4 | ECHO 12–16, 19, 22–24 |

Table 3. *Association of* EP 3 *with pools* A–F

| Pool identity | Antisera in pool |
|---|---|
| A | ECHO 1, 5, 9 |
| B | ECHO 2, 6, 11 |
| C | ECHO 3, 7, Cox A9 |
| D | ECHO 1, 2, 3 |
| E | ECHO 5, 6, 7 |
| F | ECHO 9, 11, Cox A9 |

Table 4. *Association of* EP 4 *with pools* G–M

| Pool identity | Antisera in pool |
|---|---|
| G | ECHO 12, 15, 22 |
| H | ECHO 13, 16, 23 |
| J | ECHO 14, 19, 24 |
| K | ECHO 12, 13, 14 |
| L | ECHO 15, 16, 19 |
| M | ECHO 22, 23, 24 |

The composition of the pools was designed so that about 50 units of each antiserum were present in 0·1 ml. of a pool. In making the pools equal volumes of all the sera were mixed, the requisite initial serum dilutions being obtained by multiplying that dilution which contains 50 units by the number of sera in the pool. As an example Table 1 shows how enterovirus pool 1 was prepared.

Serum dilutions were made in Earle's balanced salt solution containing sodium bicarbonate 0·18 %, penicillin 100 units/ml. and streptomycin 100 $\mu$g./ml. The serum pools, distributed in small amounts, were stored at $-30°$ C. until required. For use one container was thawed and kept at 4° C. until finished. No deterioration in titres was observed over periods of 3 months.

For the scheme four major pools of antiserum, labelled enterovirus pool (EP) 1–4 and twelve secondary or intersecting pools in alphabetical order A–M (excluding I)

were prepared. The composition of the major pools is shown in Table 2, pool 1 being concerned with the polioviruses, pool 2 with the Coxsackie B viruses, and pools 3 and 4 with the ECHO and Coxsackie A 9 viruses. The largest number of distinct serotypes occurs among the ECHO viruses, therefore the secondary pools—six linked with EP 3 and six with EP 4—are used in the identification of these agents. This association is shown in Tables 3 and 4.

## VIRUS IDENTIFICATION

Stationary culture tubes ($4 \times \frac{1}{2}$ in.) of primary rhesus monkey kidney, primary human amnion and HeLa cells incubated at 36–37° C. have been used for virus identification. Other types of cell and sizes of tubes may be found just as satisfactory. The susceptibility of the different cells may be a guide to the identity of an unknown virus, which should be typed in the culture system found to be most susceptible. The virus suspension for use in the neutralization tests is prepared by the inoculation of cultures of the specified cells. After inoculation the cultures are examined daily for cytopathic changes. Complete destruction of the cell sheet within 3 days is preferable, and if this is not obtained initially a further passage should be made. When destruction is complete, the cultures are frozen at −30° C., rapidly thawed at 37° C., and harvested. This harvest forms the stock virus suspension for all the neutralization tests and is stored at −30° C. until required. Under these circumstances the approximate titres of the stock suspensions may be estimated as depicted in Table 5. Experience has shown that, within limits, the dilutions suggested are usable without the need for detailed titrations.

Table 5. *Estimation of virus titres*

| Time for complete CPE to occur | Approximate infective virus TCD 50/0·1 ml. | Dilution of stock virus suspension for tests |
|---|---|---|
| 1 day | $10^6$ | $10^{-4}$ |
| 2 days | $10^5$ | $10^{-3}$ |
| 3 days | $10^4$ | $10^{-2}$ |

When a suitable suspension of the unidentified virus is available and a decision is made on the cell cultures to be used, the virus is tested against the major pools of antiserum (EP 1–4). Inhibition of the cytopathic agent by a pool places the unknown virus in one of the four groups. In the tests 0·1 ml. of virus suspension, diluted to contain an estimated 100 TCD 50 of virus (Table 5), is mixed with 0·1 ml. of each of the antiserum pools. After 1 hr. at room temperature, 0·1 ml. of each serum-virus mixture is inoculated into one tissue-culture tube containing 1 ml. of maintenance medium. As neutralization of virus growth is expected in only one culture the other three act as virus controls. The final readings are made on the sixth day.

Once allocated to a group a virus may be identified by the same technique of neutralization with the appropriate antisera in the following manner. For the first group the individual polioviruses 1–3 antisera may be used and for the second

group the individual Coxsackie B viruses 1–6 antisera. For the third group the intersecting ECHO and Coxsackie A 9 antiserum pools A–F, and for the fourth group pools G–M are used. Examples of how the system of neutralization works with pools A–M are shown in Fig. 1 (*a*) and (*b*).

(*a*) Pools A–F

| | A | B | C |
|---|---|---|---|
| D | Echo 1 | Echo 2 | Echo 3 |
| E | Echo 5 | Echo 6 | Echo 7 |
| F | Echo 9 | Echo 11 | Cox. A9 |

(*b*) Pools G–M

| | G | H | J |
|---|---|---|---|
| K | Echo 12 | Echo 13 | Echo 14 |
| L | Echo 15 | Echo 16 | Echo 19 |
| M | Echo 22 | Echo 23 | Echo 24 |

Fig. 1. Neutralization patterns obtained with intersecting antiserum pools. ▥, Neutralization by pools B and E indicates that the agent is an ECHO 6 virus. ▤, Neutralization by pools J and K indicates that the agent is an ECHO 14 virus.

## RESULTS

To assess the value of the scheme a series of 115 viruses was tested. All the reported experiments were done in stationary cultures of primary rhesus monkey kidney cells maintained with medium 199 containing 0·22 % sodium bicarbonate, penicillin and streptomycin. The technique of neutralization has been described. The series consisted of ninety-six recently isolated viruses previously identified by standard methods, seventeen prototype strains and two unidentified strains as shown in Table 6A and B. Of ninety-seven viruses (Table 6A) which could have been identified eighty-seven (90 %) were successfully typed at the first attempt. Later, in repeat tests, some of the remaining viruses (Table 6A) were successfully identified by the use of a smaller dose of virus. Of the eighteen viruses (Table 6B) which did not have their corresponding antisera in the pools none were neutralized. Thus no false positive identification results were obtained.

## DISCUSSION

The use of composite pools of antiserum and only a single tissue-culture tube for each serum-virus mixture is essentially a minimal screening method in the attempted identification of a large proportion of newly isolated enteroviruses. This scheme differs from the methods already reported. In particular complete virus identification requires two stages instead of one. Each stage consists of a set of neutralization tests; the first places the virus in one of the four main groups (EP 1, 2, 3 or 4) and the second establishes the precise type. Although a two-stage method has the disadvantage that it takes up to 12 days to identify a virus, it is favoured by an economy in the use of tissue cultures and by its minimizing the wastage of antiserum. It also includes the important safeguard of allowing each virus to be neutralized by a specific antiserum on two separate occasions before final identification.

Table 6. *Results of neutralization tests with composite antiserum pools*

| | Virus* | No. tested | No. identified | No. not identified |
|---|---|---|---|---|
| A. Viruses typable by the composite antiserum pools | Poliovirus type 1 | 3† | 2 | 1 |
| | Poliovirus type 2 | 1† | 1 | — |
| | Poliovirus type 3 | 3† | 2 | 1 |
| | Coxsackie B1 | 1† | 1 | — |
| | Coxsackie B2 | 1 | 1 | — |
| | Coxsackie B3 | 1† | 1 | — |
| | Coxsackie B4 | 6 | 5 | 1 |
| | Coxsackie B5 | 2 | 2 | — |
| | Coxsackie B6 | 1† | 1 | — |
| | Coxsackie A9 | 8 | 8 | — |
| | ECHO 1 | 11 | 11 | — |
| | ECHO 2 | 3 | 3 | — |
| | ECHO 3 | 1† | 1 | — |
| | ECHO 5 | 2 | 2 | — |
| | ECHO 6 | 13 | 11 | 2 |
| | ECHO 7 | 2 | 2 | — |
| | ECHO 9 | 2 | 2 | — |
| | ECHO 11 | 5 | 4 | 1 |
| | ECHO 12 | 3 | 3 | — |
| | ECHO 13 | 3† | 3 | — |
| | ECHO 14 | 10 | 10 | — |
| | ECHO 15 | 2† | 1 | 1 |
| | ECHO 16 | 2† | 1 | 1 |
| | ECHO 19 | 8 | 6 | 2 |
| | ECHO 22 | 1 | 1 | — |
| | ECHO 23 | 1† | 1 | — |
| | ECHO 24 | 1† | 1 | — |
| | Totals | 97 | 87 | 10 |
| B. Viruses not typable by the composite antiserum pools | ECHO 4 | 2 | — | 2 |
| | ECHO 17 | 2† | — | 2 |
| | ECHO 18 | 1† | — | 1 |
| | ECHO 20 | 2† | — | 2 |
| | ECHO 21 | 2 | — | 2 |
| | ECHO 25 | 3 | — | 3 |
| | ECHO 26 | 1† | — | 1 |
| | ECHO 27 | 1† | — | 1 |
| | Adenovirus type 1 | 2 | — | 2 |
| | Unidentified‡ | 2 | — | 2 |
| | Totals | 18 | — | 18 |

* Previously identified by neutralization with individual antisera.
† Including one prototype strain.
‡ Not neutralized by antisera to ECHO 1–28, Coxsackie A7, A9, B1–6, polioviruses 1–3.

In its present form the scheme can be used for the identification of twenty-seven enteroviruses, but it can be extended to cover more viruses with only a very small increase in the tissue-culture requirement. For example, the preparation of a fifth major pool and its corresponding intersecting pools could provide coverage for a further nine enteroviruses and yet only increase the tissue culture demand by a single tube.



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The scheme is flexible in that, if the identity of the virus is suspected from its growth characteristics in different cell cultures, some or all of the major pools may be omitted and the virus may be typed with the intersecting pools or the individual antisera. An important requirement is the use of high titre antisera in the pools. This is to ensure that the necessary initial concentration of a serum which is used to provide 50 units of antibody in the final mixture does not contain any heterologous antibody. Nor should it be likely to inhibit viruses non-specifically thereby giving false positive results. In the results reported these requirements have been met.

In the tests, although an estimated 100 TCD 50 of virus in 0·1 ml. is mixed with an equal volume of serum only half of this serum–virus mixture is transferred to the culture tube. Thus if no neutralization occurs only about 50 TCD 50 of virus is introduced into the culture; however, this should be sufficient to demonstrate cytopathic change. Although theoretically the whole 0·2 ml. of mixture should be inoculated into the culture a measured 0·1 ml. amount gives satisfactory results. The amount of virus suggested for use in virus–serum mixtures is an estimate, which may well err on occasions. Failure of specific inhibition of a virus may be due to too great a virus concentration or to a mixture of viruses—a problem this scheme does not solve. It may also be because the unidentified viruses are prime strains (Melnick, 1958) which have a broader antigenic structure and are therefore not neutralized to the same extent by antiserum to the prototype viruses.

As this method is essentially a screening procedure to identify many viruses and to allow those not readily recognized to be examined more thoroughly, it is important that no false positive results should be obtained. The results show that during the testing of 115 viruses no agent was wrongly identified.

## SUMMARY

A scheme is described for the identification of enteroviruses in tissue culture by neutralization with composite antiserum pools. The method for making these pools is given. Antisera to twenty-seven enteroviruses were included in the pools which were used to examine 115 viruses consisting of ninety-eight recently isolated viruses and seventeen prototype strains. The results indicate that this scheme provides a useful screening method for identifying enteroviruses. It has proved to be practicable, time saving and very economical in tissue culture.

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## REFERENCES

Kärber, G. (1931). Beitrag zur kollektiven Bhandlung pharmakologischer Reihenversuche. *Arch. exp. Path. Pharmak.* **162**, 480.

Lim, K. A. & Benyesh-Melnick, M. (1960). Typing of viruses by combination of antiserum pools. Application to typing of enteroviruses (Coxsackie and ECHO). *J. Immunol.* **84**, 309.

Melnick, J. L. (1958). Advances in the study of enteroviruses. *Progr. med. Virol.* **1**, 59.

Report (1962). Classification of human enteroviruses. *Virology*, **16**, 501.

Schmidt, N. J., Guenther, R. W. & Lennette, E. H. (1961). Typing of ECHO virus isolates by immune serum pools. The 'intersecting serum scheme'. *J. Immunol.* **87**, 623.