A MICRO-CULTURE METHOD FOR THE TYPING OF ENTEROVIRUSES

S.K. LAM

Department of Medical Microbiology, University of Malaya, Kuala Lumpur, Malaysia.

INTRODUCTION

Lim and Benyesh-Melnick (1960) described a neutralization method for the typing of coxsackie and echoviruses in primary monkey kidney tube cultures using immune serum pools. The following year Schmidt *et al.*, (1961) devised the intersecting serum scheme for the identification of echoviruses by neutralization in primary monkey kidney tube cultures. In this scheme, each type-specific immune serum was incorporated into two different pools, and identification was made by demonstrating neutralization of the virus by the two pools sharing a common type-specific immune serum.

This paper reports the use of a micro-culture method for the typing of enteroviruses by the standard WHO Intersecting Serum Pools following the successful application of the micro-culture method for the cultivation of enteroviruses (Lam, 1972).

MATERIALS AND METHODS

Viruses: Stock viruses used are shown in Table 1. They were passaged once in primary *Cynomolgous* monkey kidney cells and harvested when maximum cytopathic effects were observed. Enterovirus isolates were obtained from stool specimens of normal individuals who had no history of polio vaccination. Isolation was performed in tube cultures of primary monkey kidney cells or LLC-MK_2 cells, a stable rhesus kidney cell line. After the second passage, the isolates were diluted tenfold and typed by the micro-culture method using the WHO enterovirus intersecting serum pools.

Table 1

Stock Viruses and their neutralization patterns.

Stock Virus	Neutralization Pattern
Polio 1	Pools 1 and 7
Polio 3	1 and 9
Cox. A7	1 and 10
Cox. A9	1 and 11
Cox. B1	1 and 13
Cox. B3	2 and 8
Cox. B5	2 and 10
Cox. B6	2 and 11
ECHO 3	3 and 7
4	3 and 8
5	3 and 9
6	3 and 10

Cells: The details for the method of preparing the cells in micro-culture are given elsewhere (Lam, 1972).

Briefly, LLC-MK_2 cells were grown in 4 oz. screw-capped bottles in Leibovitz L-15 medium enriched with 3% foetal calf serum and 10% tryptose-phosphate broth. A cell concentration of 3×10^5 cells per ml was prepared and used in micro-culture on standard Perspex WHO pattern haemagglutinating trays. The overlay medium consisted of 1.5% carboxymethyl-cellulose in double-strength growth medium. After incubation for the appropriate period, the infected cultures were stained with naphthalene-black.

Neutralization tests: The dose of stock virus used in the neutralization test was determined by preliminary titration as the lowest dilution to give confluent plaques.

0.4 ml of this virus dose was mixed with an equal volume of each of the thirteen immune serum pools. The mixtures were incubated at 37°C for 1 hour with occasional shaking. A virus control (VC) was prepared using 0.4 ml of growth medium instead of serum pool. 0.2 ml of each virus-serum mixture was then dispensed in triplicates into a WHO haemagglutinating tray followed by 0.2 ml of cell-suspension. Cell controls (CC) were set up at both ends of the tray. After the cells had been allowed to settle for 4 hours at 37°C, 0.4 ml of overlay medium was added to each well and the tray re-incubated.

WHO Enterovirus Intersecting Serum Pools: The composition of the serum pools is shown in Table 2. Each pool was made up with 6 or 7 immune sera and each serum was diluted in the pool to contain 50 antibody units per 0.1 ml. Just prior to testing, an appropriate volume of each of the serum pools was inactivated at 56°C for 30 minutes.

Table 2

Composition of WHO enterovirus intersecting serum pools.

Pool	7	8	9	10	11	12	13
1	P1	P2	P3	A7	A9	A16	B1
2	B2	B3	B4	B5	B6	E1	E2
3	E3	E4	E5	E6	E7	E9	E11
4	E12	E13	E14	E15	E16	E17	E18
5	E19	E20	E21	E22	E23	E24	E25
6	E26	E27	E29	E30	E31	E32	E33

RESULTS

Stock viruses were coded prior to typing. The neutralization patterns bore out the identity of the coded viruses as shown in Table 1.

Table 3

Identification of enterovirus isolates.

Virus Isolates	Neutralization patterns	Identity
EVS 780	Pools 1 and 7	Polio 1
786	Pools 1 and 7	Polio 1
834	Pools 1 and 7	Polio 1
841	Pools 1 and 7	Polio 1
EVS 840	Pools 1 and 8	Polio 2
1131/2	Pools 1 and 8	Polio 2
1133/2	Pools 1 and 8	Polio 2
136	Pools 2 and 9	Cox. B4
EVS 760	Pools 2 and 9	Cox. B4
767	Pools 2 and 9	Cox. B4
EVS 790	Pools 2 and 11	Cox. B6
792	Pools 2 and 11	Cox. B6

Enterovirus isolates from normal individuals were also identified by this method and the results are shown in Table 3. Of the 12 isolates tested, 4 were identified as polio 1 virus, 3 were coxsackie (Cox.) B4 and 2 were coxsackie B6. The results of the identifications by the serum pools were confirmed by neutralization with the corresponding homologous sera alone by the microculture method.

Fig. 1 shows a typical plate infected with serial tenfold dilution of an enterovirus isolate (EVS 790). At 10^{-1} and 10^{-2} dilutions

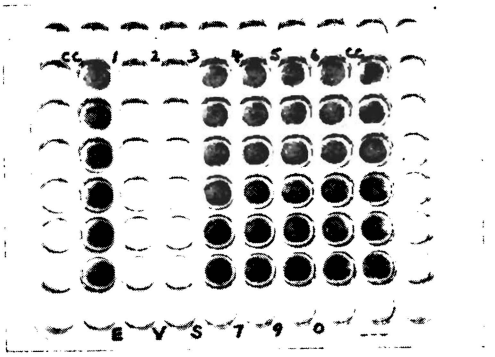

Fig. 1—Plate infected with serial dilutions of enterovirus isolate EVS 790.

of virus, there were confluent plaques (100% cytopathic effect). At 10^{-3} dilution of virus, there was little evidence of cell destruction. 10^{-2} dilution of this virus was then used for the neutralization test.

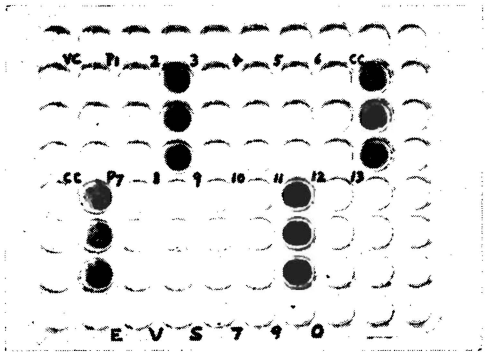

Fig. 2—Neutralization pattern with enterovirus isolate EVS 790 against the WHO serum pools.

Fig. 2 shows the neutralization pattern of EVS 790 using the 13 serum pools. The virus control (VC) shows 100% cell destruction as evidenced by non-staining by naphthalene black while the cell controls (CC) at both ends of the tray are stained. The virus is shown to be neutralized by Pool 2 and Pool 11, giving it the identity of coxsackie B6. This was confirmed by neutralization of the isolate by coxsackie B6 antiserum alone.

DISCUSSION

The method described above has been shown to give consistent results not only for stock viruses but also for enterovirus isolates. It is economical on time and materials and the simplicity of the test lends itself to the large scale typing of enteroviruses in epidemiological survey. It has been found possible to type up to ten virus isolates against the 13 serum pools in a single day.

The use of the micro-culture method for the primary isolation of enteroviruses from stool specimens has yielded only limited success owing to non-specific cytopathic effect caused by the inoculum on LLC-MK_2 cells. Other cell lines may prove more fruitful. However, once the virus has been isolated in tube cultures, there is little problem in typing the isolate by this simple and efficient method.

SUMMARY

The use of the intersecting serum pools for the typing of enterovirus has been in practice in many laboratories. This paper reports how such pools could be used in a simple microculture method, thus saving on time and materials which are important factors in epidemiological survey in underdeveloped and developing countries. Using this method, it has been possible to type up to ten virus isolates against 13 serum pools in any single day.

ACKNOWLEDGEMENTS

The author wishes to thank Dr. J.L. Melnick, Baylor University College of Medicine, Houston, Texas for providing the stock viruses and Dr. Lee Liang Hin, Department of Bacteriology, University of Singapore, for the serum pools. Thanks are also due to Mrs. Lingga Teo for her excellent technical assistance and to Dr. Dora Tan of the Institute for Medical Research, Kuala Lumpur for supplying the enterovirus isolates.

REFERENCES

LAM, S.K., (1972). A micro-culture method for the study of enteroviruses. *Asian J. Med.*, *8* : 247.

LIM, K.A. and BENYESH-MELNICK, M., (1960). Typing of viruses by combinations of antiserum pools. Application to typing of enteroviruses (Coxsackie and Echo). *J. Immun.*, *84* : 309.

SCHMIDT, N.J., GUENTHER, R.W. and LENNETTE, E.H., (1961). Typing of ECHO virus isolates by immune serum pools: The "Intersecting Serum Scheme". *J. Immun.*, *87* : 623.