ENTEROVIRUSES IN SINGAPORE

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Since 1957, our department has been studying the prevalence and incidence of poliomyelitis in Singapore. In a study of 410 notified cases of paralytic poliomyelitis between 1959-1964, 236 polioviruses were identified from 260 isolates (Lee *et al.*, 1965). Twenty-four (9.3%) other cytopathogenic agents could not be typed by the methods then employed.

A survey in 1960 of normal children between the ages of six months and four years resulted in the isolation of 10 polioviruses, 3 coxsackieviruses and 61 other cytopathogenic agents from 371 children who lived in the city, and 24 polioviruses, 4 coxsackieviruses and 48 other cytopathogenic agents from 269 children who lived in rural areas (Lee *et al.*, 1964). A total of 109 (75%) out of 146 isolates were not identified. A similar survey in 1964 of children between 1 and 6 years old, showed 4 of 60 isolates from 300 children who lived in squatter areas and 10 of 86 isolates from 373 children who lived in flats were polioviruses (Kleevens and Lee, 1966). A total of 132 (90.4%) cytopathogenic agents were not polioviruses.

This paper reports the typing of these unidentified viruses and similar isolates made in subsequent years from cases of poliomyelitis, aseptic meningtis and viral encephalitis.

MATERIALS AND METHODS

Tissue cultures

Primary monkey (MK) tissue cultures in 12 cm. test tubes and 16-oz. sani-glass bottles were prepared from *Cynomolgus macaca irus*. Each tube was seeded with approximately 200,000 cells suspended in 1.0 ml. Hanks's growth medium (Hanks's balanced salt solution with 0.22% sodium bicarbonate, 4% ox serum, 0.5% lactalbumen hydrolysate, 250 unit/ml. of penicillin and 250 µgm/ml. of streptomycin). Each bottle was seeded with 20.0 ml. of similar MK cell-suspension. Cultures were ready for use after incubation at 37°C. for 4-5 days. Before use, the growth medium was replaced with Earle's maintenance medium (EMM). The EMM contained Earle's balanced salt solution with 0.22% sodium bicarbonate, 0.5% lactalbumen hydrolysate and antibiotics as above.

Immune-sera

Type specific antisera against coxsackieviruses and echoviruses were produced in *Cynomolgus macaca irus* monkeys. Each bottle of MK culture was seeded with 0.5 ml. of prototype virus. The tissue culture infectivity dose (TCID) of the prototype viruses ranged from 10^4 to 10^7 per 0.1 ml. The cultures were incubated at 37°C. and examined for cytopathogenic effect (CPE) daily. When CPE was seen throughout the cell sheet, the infected culture fluid was removed and clarified by centrifugation at 3,000 rpm for 30 minutes. The supernatant fluid was mixed with an equal volume of arlacel-bayol adjuvant (1 part arlacel to 9 parts bayol). The mixture was emulsified with an electric stirrer at room temperature. The stability of the emulsion was checked by its retention of droplet-form when dropped into a beaker of water. The emulsion was kept at —20°C. until used.

A 1.0 ml. to 2.0 ml. antigen-adjuvant was injected into the thigh muscle of each monkey on 4 successive occasions at intervals of 7 to 10 days. The neutralizing antibody titre of a blood sample was assayed in MK cultures 7-10 days after the last inoculation. In some instances, booster doses were required to attain a satisfactory antibody titre. The monkeys were then exsanguinated and the serum obtained stored in 5.0 ml. aliquots at —20°C.

Serum-pools

The "Intersecting Serum Scheme" described by Schmidt *et al.*, 1961, was adopted. Twenty-five sera were put into 10 pools according to the scheme shown in Fig. 1.

Virus Isolation

Faecal samples collected in paper cartons or metal containers were kept frozen at —20°C. until passaged.

SERUM-POOLS FOR IDENTIFICATION COXSACKIE AND ECHO VIRUSES

	A	B	C	D	E
F	1	2	4	5	6
G	7	8	9	10	11
H	12	13	14	15	17
I	18	19	22	23	26
K	I	II	III	IV	V

Numerals 1, 2, 4, etc. = Echovirus type 1, 2, 4, etc.
Roman letters I, II, III, etc. = Coxsackie B1, 2, 3, etc.

Fig. 1.

A 10% faecal suspension was prepared in sterile distilled water containing 2,000 units penicillin and 2,000 µgm streptomycin per ml. The particulate matter in the faecal suspension was allowed to settle out in a 4°-8°C. refrigerator. The supernatant fluid from each specimen was clarified by centrifuging twice at 4°C. at 3,500 rpm for 30 minutes. A 0.1 ml. volume of the supernate was inoculated into each of 3 tubes of MK culture containing 1.0 ml. of EMM. Specimens not immediately passaged were ampouled and stored frozen at —20°C. The inoculated tubes were incubated at 37°C. in roller drums and were examined daily for CPE. Tissue culture fluids from tubes showing CPE were ampouled in 1.0 ml. volume and stored at —20°C. prior to identification.

Tubes showing no CPE after 14 days of incubation were regarded as virus negative.

Identification of Viruses

The isolates were tested for viability by passages in MK cultures. Virus suspensions with low infectivity titres were given serial tissue culture passages.

A 0.3 ml. virus suspension (100-1000 TCID per 0.1 ml.) was added to 0.3 ml. of pooled antiserum and 0.3 ml. EMM. The virus-serum mixture was agitated and put into a 37°C. water-bath for 1 hour. A 0.2 ml. volume of each mixture was inoculated into each of 2 tubes of MK cultures. A few uninoculated tubes provided the normal cell control. The cultures, placed in a roller drum incubated at 37°C., were examined for CPE daily.

The final reading was taken 2 days after the virus control showed CPE. Where a partial neutralisation was encountered, the virus was retested at a lower TCID. In some instances, the virus was retested against each type belonging to the pool which gave partial neutralisation.

Serology

Sera obtained from patients in the acute phase of illness and during convalescence were tested against prototype viruses homotypic to the isolates from these patients. The sera were tested at five-fold dilutions ranging from 1 in 2 to 1 in 1250. The neutralisation end-point was recorded 2 days after the virus control showed CPE. The results are shown in Table IV.

Serological tests were not undertaken in the survey of normal children.

RESULTS

Some isolates from the earlier years were nonviable when tested in MK cultures. Of the 75 cytopathogenic agents from clinical cases, 4 were coxsackieviruses, 62 echoviruses and 9 remained unidentified (Table I). Four coxsackieviruses, 100 echoviruses and 25 unidentified viruses were found in 129 isolates from normal children (Table II). The prevalence of each type of enteroviruses in both groups studied is shown in Table III.

The acute and convalescent sera from 4 patients showed neutralising antibody titres which suggest a recent echovirus infection. In 2 instances, the convalescent antibody titres were more than 1 in 1250 (Table IV).

DISCUSSION

Echoviruses 1(8), 2, 4-9, 11(19), 14, 15, 17 18 and coxsackievirus A9 were isolated from cases provisionally diagnosed as poliomyelitis, aseptic meningitis and viral encephalitis. The commonest of the echoviruses was echovirus 9 (29%). Nine of 75 isolates from these cases were not identified by our serum-pools. Echoviruses 1(8), 2, 4, 6-9, 11(19), 12-15, 18, 26 and coxsackieviruses B2 and B5 were found in normal children. The commonest echoviruses in these children were echovirus 6 (15%), echovirus 7 (28%) and echovirus 13 (19%). Twenty-five of 129 isolates remained unidentified.

Echovirus 9 which represented 29% of 62 echoviruses isolated from clinical cases was found only in 4% of 100 echoviruses in normal children. Echoviruses 5 and 17 present in 4.8% and 3.2% respectively of echoviruses isolated from cases of clinical disease were not isolated from normal children. On the other hand, echoviruses 12, 13 and 26 which represented

TABLE I

COXSACKIE AND ECHO VIRUSES ISOLATED FROM CLINICAL CASES

Virus Isolates	Number of Isolates in Year										
	1957	1958	1959	1960	1961	1962	1963	1964	1965	1966	Total
ECHO											
1(8)	2	1		1	2			3	1		9
2						1	1				3
4	1						1				2
5		1					3				3
6		1		2				1			4
7							2		2		5
9		1	1	1		9	3		3	1	18
11(19)	2				2		1	1			7
14				1					1		2
15							2				2
17				2							2
18				2	1				1	1	5
COXSACKIE A9	1		2	1							4
UNKNOWN	1	1	1	1			1	2	2		9
TOTAL	7	5	4	11	5	10	14	7	10	2	75

TABLE II

COXSACKIE & ECHO VIRUSES ISOLATED FROM SURVEY OF NORMAL CHILDREN

Virus Isolates	Number of Isolates from place & Year of Study				
	Paediatrics 1959	Odin Square 1960	Ulu Bedok & Bedok 1960	Bukit Ho Swee 1964	Total
ECHO					
1(8)	4	1	2	2	6
2	1	1		2	6
4		1			1
6			2	14	15
7				26	28
9	3			1	4
11(19)	1	2		1	4
12		1			1
13		2		17	19
14		1		6	7
15		1	1	1	3
18			1	1	2
26				4	4
COXSACKIE B2		1	2		3
B5	1				1
UNKNOWN	1	7	3	14	25
TOTAL	11	18	11	89	129

TABLE III

COXSACKIE & ECHO VIRUSES FROM CLINICAL CASES AND NORMAL CHILDREN

Virus Isolates	Number of Isolates from	
	Clinical Cases 1957 to 1966	Normal Children 1959, 1960 and 1964
ECHO		
1(8)	9	6
2	3	6
4	2	1
5	3	0
6	4	15
7	5	28
9	18	4
11(19)	7	4
12	0	1
13	0	19
14	2	7
15	2	3
17	2	0
18	5	2
26	0	4
COXSACKIE		
A9	4	0
B2	0	3
B5	0	1

TABLE IV

NEUTRALISING ANTIBODY IN PATIENTS' SERA

Patient's No.	Serum/ Virus	Neutralising Antibody Titres 1/2	1/10	1/50	1/250	1/1250	Serum control	Virus control
17 (1963)	AS/E9	—	+	+	+	+	—	+
	CS/E9	—	—	—	—	+	—	
123 (1963)	CS/E7	—	—	—	+	+	—	+
230 (1963)	AS/E5	+	+	+	+	+	—	+
	CS/E5	—	—	—	+	+	—	
310 (1965)	AS/E8	—	—	+	+	+	—	+
	CS/E8	—	—	—	—	+	—	
402 (1965)	CS/E9	—	—	—	—	—	—	+
404 (1965)	AS/E18	—	—	—	—	—	—	+
	CS/E18	—	—	—	—	—	—	

Serum/Virus = Serum tested against virus named.
AS = Acute Serum
CS = Convalescent Serum
— = no CPE (Neutralised)
\+ = CPE

1%, 19% and 4% of echoviruses in normal children were not associated with cases of clinical disease. Coxsackievirus A9 was isolated from clinical cases while coxsackievirus B2 and B5 from normal children only.

Sera from patients showed neutralising antibody titres suggestive of recent infection by the echovirus types used in the tests.

The use of a single cell line (MK tissue cultures) for virus isolation could have limited the range of enteroviruses found in the surveys. The isolation rate might have been greater had cultures showing negative CPE been given further tissue culture passages. However, with procedures presently adopted, the data show a difference in the prevalence and types of enteroviruses in these 2 groups of subjects. Further tests such as virus isolation from cerebral spinal fluid and post mortem materials from clinical cases and serological survey of both groups of people would have been helpful in establishing the role of these viruses in the population.

SUMMARY

A report of some coxsackieviruses and echoviruses isolated in Singapore is given. Further investigations are required to establish the significance of these enteroviruses in viral encephalitis, aseptic meningitis and other central nervous system involvements in the island.

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REFERENCES

1. Kleevens, J.W.L., & Lee, L.H. (1966): "The Occurrence of Enteroviruses in Children from Modern Flats and Squatter-Dwellings in Singapore," Singapore Med. J. 1, 7.
2. Lee, L.H., Lim, K.A., & Tye, C.Y. (1964): "Prevention of Poliomyelitis in Singapore by Live Vaccine," Brit. Med. J. 1, 1077.
3. Lee, L.H., Lim, K.A., & Kanagaratnam, K. (1965): "The Poliomyelitis Immunization Programme in Singapore," Singapore Med. J. 6, 2, 89.
4. 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.