STUDIES OF ENTEROVIRUSES AMONG LEBANESE CHILDREN

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HUMAN ENTEROVIRUSES

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ABSTRACT

A total of 222 stool specimens from healthy Lebanese children were tested for the presence of enteroviruses by inoculation of HeLa, primary human amnion, continuous human amnion cell cultures and Swiss albino suckling mice.

Eighty-three virus strains were isolated from 75 positive specimens. Twelve of these isolates were polioviruses (7 type 1, 5 type 3); 17 Coxsackie group B viruses (2 type 1, 15 type 3); and 31 ECHO viruses (19 type 1, 9 type 6, 2 type 12 and 1 type 14). Twenty-three isolates were not identified.

HeLa cell cultures were found to be superior to primary, continuous human amnion cells and suckling mice, for isolation of polio- and Coxsackie group B viruses from stool specimens. The ECHO virus isolates were obtained only by the utilization of primary human amnion cell cultures. Twelve isolates obtained by intracerebral inoculation of suckling mice could not be identified.

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INTRODUCTION

The Picornavirus Group was created in accordance with the decision of the International Subcommittee on Virus Nomenclature (1963)[1] to classify major groups of viruses on the basis of common biochemical and biophysical properties. The following subgroups were recognized:[1]

A. Picornaviruses of human origin
   1. Enteroviruses
      a. Polioviruses
      b. Coxsackie viruses A
      c. Coxsackie viruses B
      d. ECHO viruses
   2. Rhinoviruses
   3. Unclassified

B. Picornaviruses of lower animals

The name enterovirus was first used by the Committee on the Enteroviruses (1957)[2] because of the many similarities in physical properties, disease producing capacities, epidemiologic characteristics and isolation or cultivation methods observed in these viruses.

The enteroviruses are antigenically distinct small

(15-30 mu in diameter) viruses, obtained from stools of children with inapparent, febrile illnesses and/or serious paralytic infections. These viruses are insensitive to ether and desoxycholate salts and contain a ribonucleic acid core. They are relatively stable at 4°C and at room temperature. A recent finding indicates that the presence of magnesium cation seems to enhance their resistance to thermal inactivation.[3,4,5,6,7,8]

The classification of enteroviruses according to the Committee on the Enteroviruses (1957),[2] is presented below. This includes 59 antigenically distinct viruses.

Poliovirus group
  Type 1, 2, 3

Coxsackie virus group
  Group A, types 1-23
  Group B, types 1-6

ECHO virus group
  Types 1-27

More recently, the Committee on Enteroviruses (1962)[9] suggested a new classification (Table I),

indicating that all enteroviruses be placed in a single large group and designated by simple numerical system according to their antigenic types. This classification has not received unanimous recognition at the present time.

The distribution of enteroviruses in the general population of the neighboring Arab and non-Arab countries, including Egypt, Turkey, Israel and others, has been determined by various investigators. Preliminary studies by Garabedian et al. (1964)[10] and Matossian et al. (1964)[11] indicated that polioviruses as well as Coxsackie and ECHO viruses were widely distributed in the population of Lebanon, and especially among children. The purpose of this present investigation is to determine more extensively the distribution of these viruses among the healthy Lebanese children, and to indicate the technics which can be utilized most advantageously in carrying on this work.

TABLE I

Human Enteroviruses: Type, Numbers and Synonyms*

Enterovirus No.	Synonym
1	Poliovirus type 1
2	2
3	3
4	Coxsackie A1
5	A2
6	A3
7	A4
8	A5
9	A6
10	A7
11	A8
12	A9
13	A10
14	A11
15	A12
16	A13
17	A14
18	A15
19	A16
20	A17
21	A18
22	A19
23	A20, 20A, 20B
24	A21 (Coe)
25	A22
26	A24
27	Coxsackie B1
28	B2
29	B3
30	B4
31	B5
32	B6
33	ECHO-1, 8
34	2
35	3
36	4
37	5
38	6, 6', 6"
39	7
40	9 (Coxsackie A23)
41	11
42	12
43	13
44	14
45	15
46	16
47	17
48	18
49	19
50	20
51	21
52	22
53	23
54	24
55	25
56	26
57	27
58	28 (JH-1, 2060)
59	29 (JV 10)
	30 (Bastianni)**
	31 (Caldwell)
	32 (PR 10)

*Cross reactivity has been observed between Coxsackie viruses A3 and 8, A11 and 15, and A13 and 18. Although a separate enterovirus number has been assigned to each, further study of their antigenic relationships may show that each pair should be classified under a single enterovirus number.

**Recently recognized ECHO types (Melnick, J.L. et al. Science 12: 153-4, 1963).

A HISTORICAL REVIEW OF THE TECHNICS USED IN ENTEROVIRUS ISOLATION

The polioviruses are the earliest and most extensively studied group among enteroviruses because of their ability to produce epidemics of paralytic illness and death in humans, specially among children.[3,12] Experimental production of paralytic poliomyelitis in monkeys was first reported by Landsteiner and Popper (1908) by intracerebral (IC) inoculation of pathologic specimens from humans who died of poliomyelitis. Serial monkey to monkey passages of this infectious agent were carried out during the same period by Flexner and Lewis. By 1910, this pathogen was considered to be a filtrable virus. Furthermore, it was demonstrated that the blood of monkeys surviving experimental infection with this virus neutralized its infectivity.[12] The laboratory study of polioviruses was facilitated by Armstrong (1939), who adapted the Lansing strain of poliovirus to laboratory mice. In 1938 Reed and Muench established the methodology of finding the 50% infectivity end-point in biologic assays, thus facilitating quantitative determinations of the infectivity doses of pathogens.[12]

The development of tissue culture technics and their successful use in isolation and cultivation of viruses helped in the more detailed study of enteroviruses. Enders, Weller

and Robbins (1949, 1950, 1951)[13,14,15] in three consecutive years were the first to demonstrate the successful cultivation of the Lansing strain of polioviruses in human non-neural embryonic tissue cultures (TC) and showed their cytopathic effect (CPE) in cell cultures. They isolated several poliovirus strains from human sources by the employment of cell cultures. Syverton et al. (1951) propagated polioviruses in monkey and human testicular tissues.[12] Likewise, by the use of monkey testicular cell cultures, Ledinko et al. (1952) succeeded in developing a quantitative method for virus neutralization tests.[12] Dulbecco (1952)[16] facilitated the preparation of cell monolayers by the use of trypsin. The successful propagation of the HeLa cell line obtained from a patient, Helen Larson, with carcinoma of the cervix, was carried out by Scherer et al. (1953).[17] This cell line had the advantage of being transferable from one bottle to another giving rise to homogenous cell culture and was also susceptible to cytopathic effect of polioviruses. Thus, it could be used for the isolation, propagation and neutralization tests of polioviruses. During the same period the development of monkey kidney (MK) cell cultures by various investigators, facilitated the isolation of many new enteroviruses which were hitherto unknown.[18,19]

The Coxsackie viruses were accidentally isolated by Dalldorf and Sickles (1948)[20] from children with clini-

cal poliomyelitis in the Coxsackie village in New York. These viruses produced paralysis and death of suckling mice. Similar viruses were isolated within a short time by other investigators. Gilford and Dalldorf (1951),[21] demonstrated that these new isolates produced two types of histopathologic changes in mice. Some viruses, classified as group A, produced paralysis and death together with wide-spread myositis in suckling mice, others, classified as group B, produced paralysis and death due to the necrosis of the intrascapular fat pads and inflammation of the central nervous system. Subsequently, Sickles et al. (1955)[22] demonstrated that Coxsackie viruses could also be cultivated in animal cell cultures producing CPE. Finally, Dalldorf et al. (1959)[23] indicated that certain human diseases were associated with Coxsackie viruses.

By the introduction of MK and primary human amnion cell cultures additional new viruses were isolated along with polioviruses which could not be identified as poliovirus or as Coxsackie virus.[18,24] To classify these new viruses, The National Foundation of Infantile Paralysis, appointed a committee called the Committee on the ECHO viruses (1955).[25] This committee named the new viruses as enteric, cytopathogenic human, orphan viruses (ECHO).

EPIDEMIOLOGY OF ENTEROVIRUS INFECTIONS

The overt manifestations of enterovirus diseases are varied. Similar clinical syndromes may be associated with a number of different enteroviruses. Most human enterovirus infections are characterized by the absence of any signs of clinical illness. Table II represents various clinical syndromes associated with human enteroviruses.[6] A detailed discussion of clinical illnesses associated with enterovirus infections is reported by Ashkenazi and Melnick (1962).[26]

The reports of Melnick (1957);[27] Expert Committee on Poliomyelitis, WHO (1958);[28] Gelfand (1962);[29] Ashkenazi and Melnick (1962);[26] Kalter (1962);[30] Plotkin *et al.* (1962);[7] Plager (1962);[31] Wenner (1962)[5] and others, demonstrate that enterovirus infections are world wide in distribution. Although, all the possible modes of transmission have not yet been definitely demonstrated, yet there is enough epidemiologic evidence to indicate that the portal of entry of enteroviruses is the oropharynx, the intestinal tract is the primary site of multiplication and feces the vehicle of dissemination. Furthermore, there seems to be a close relationship between the incidence of enterovirus infections and the susceptibility of the host. Children up to four years of age are usually the most susceptible group to entero-

TABLE II

Association of Enteroviruses with Human Disease

Enteroviruses	Associated Disease
Polioviruses, Types 1-3	Summer febrile illness Aseptic meningitis Paralysis (flaccid to muscle weakness)
Coxsackie Viruses, Group A, Types 1-19	Summer febrile illness Aseptic meningitis (types A-2, 4, 9) Paralysis (?) (A-7, 9) Herpangina
Coxsackie Viruses, Group B, Types 1-5	Summer febrile illness Aseptic meningitis Paralysis (?) (types B-3, 4, 5) Pleurodynia Myocarditis or encephalomyocarditis in neonatal period and early childhood (types B-2, 3, 4)
ECHO Viruses, Types 1-24	Summer febrile illness Aseptic meningitis (types 2-6, 9, 14, 16) Paralysis (?) (types 2, 4, 6, 16) Summer rash (types 2, 4, 9, 16) Summer diarrhea of infants and children (type 18 and others)

virus infections, and children of lower socioeconomic groups are exposed to enteroviruses earlier than those of higher socio-economic groups. The incidence of enterovirus infections seems to be related to changes in seasonal factors like humidity and temperature. For example, in temperate countries during the warmest months there appears to be a higher incidence of infection, whereas in tropical countries the infection rates are relatively constant throughout the year.

ISOLATION, PROPAGATION, AND IDENTIFICATION OF HUMAN ENTEROVIRUSES

Evidence for the presence of enteroviruses in a community may be obtained by the demonstration of specific antibodies in the population and/or by the isolation of the pathogens from clinical cases of the disease as well as from healthy members of the general population.[28,29]

In general, to demonstrate specific enterovirus antibodies, two methods are used: the complement fixation (CF) and neutralization tests (NT). The CF test is not used as frequently as the neutralization test due to the technical difficulties involved, such as the need for pure and large quantities of active virus as specific antigen. The presence of CF antibodies indicate recent infections because of the tendency of such antibodies to disappear within a period of months; while neutralizing antibodies may persist for several years. However, conclusive evidence for the presence of any enterovirus in a community may be obtained only by the isolation of the specific virus from the human host and its identification in the laboratory.[4,5,28,29,32]

Enteroviruses are most frequently isolated from stools and pharynx of ill as well as healthy individuals for

a period of few weeks following infection; the pharyngeal carrier rates being much shorter. Less frequently, they may be demonstrated from the blood during the viremic stage of infection. On rare occasions, enteroviruses may be isolated from the cerebrospinal fluid, cardiac muscle, and brain tissue.[26,27,28,29,31]

In nature, enteroviruses can also be isolated from house flies, mosquitoes and cockroaches, but these have not been proven to be vectors. Virus isolation from fresh water, sea water, swimming pools, soil, shoe dust, fomites have been reported on few occasions. However, evidence is lacking to incriminate these as possible sources of infection. For studying the presence of enteroviruses in an area samples of stools and sewage are utilized.[7,27,28,29]

Generally, enteroviruses grow in cell cultures prepared from primate tissues. Table III demonstrates the susceptibility of different cell cultures and experimental animals to infections with various enteroviruses.[4]

In practice, MK and HeLa cell cultures are mostly used for poliovirus isolation due to their availability, easy manipulation and greater susceptibility. The monkey is the only susceptible experimental animal to poliovirus infection. However, polioviruses can be adapted to multiply

TABLE III

Enteroviruses: Propagation in Monkey Kidney (MK) and Human Cell Cultures, and Pathogenicity for Animals

	MK	HeLa and other human cells	Pathogenicity	
			Mice	Monkeys
Polio 1-3	+	+	0[a]	+[b]
Coxsackie: A	0[c]	0[d]	+[c]	0[e]
B	+	+	+[c]	0[e]
ECHO	+	0[d]	0[f]	0[e]

[a] Some strains of each type have been adapted to mice.

[b] Attenuated strains used for oral vaccine produce mild localized lesions when inoculated intraspinally and almost no lesions when inoculated intracerebrally.

[c] Coxsackie A9 and B strains grow readily in MK cells; some strains grow poorly in mice and fail to produce disease in term.

[d] Some strains grow preferentially in, or have been adapted to, human cell cultures.

[e] Coxsackie A7 produces a severe polioencephalomyelitis in monkeys; other Coxsackie and ECHO strains produce mild lesions in the central nervous system resembling mild poliomyelitis.

[f] While the prototype and other strains of ECHO-23 are not pathogenic for mice, a number of other strains, especially after passage in monkey kidney cells, produce paralysis in mice (severe Coxsackie-type myositis).

in mice, and in cell cultures of various animal origin.[7,28]

Many Coxsackie viruses can be isolated on cell cultures of primate origin. Whereas all Coxsackie group B viruses grow in MK and HeLa cell cultures, only few Coxsackie group A viruses grow in these or other cell cultures. Group A Coxsackie viruses can best be propagated in Swiss albino suckling mice. Other experimental animals susceptible to Coxsackie viruses are hamsters, ferrets and guinea pigs.

For isolation of ECHO viruses, human primary amnion and MK cells have been found to be the most suitable. No experimental animal has so far been demonstrated to be susceptible to ECHO viruses.[5,32] Kelly et al. (1962)[33] indicated that MK cell cultures were more suitable for isolation of polioviruses and Coxsackie group B viruses from sewage than other cell cultures. However, ECHO viruses grew better in human primary amnion cells.

MATERIALS AND METHODS

1. Stool Suspensions[34,35]

Stool specimens from healthy Lebanese infants and children, were collected from the orphanage of Saint Vincent de Paul and the American University Hospitals in Beirut, during the year of 1962. The specimens were stored at -20°C in paraffin-layered paper boxes. A total of 222 specimens from newborn infants and children up to the age of 14 years were thus collected. Before use, the samples were thawed and 1-2 grams of stools were shaken with glass beads in few ml. of Hanks' balanced salt solution (BSS), pH 7.3. To the homogeneous mixture more BSS was added to make a 10-15% suspension. This was centrifuged for 15 minutes at 2000 r.p.m. and the supernate recentrifuged for 30 minutes at 4000 r.p.m. The supernate obtained after second centrifugation was treated with 500 units of penicillin and 500 ugm of streptomycin per ml., and kept for three hours at room temperature, after which a sterility test was carried out. The non-sterile suspensions were treated a second time with the same amount of antibiotics and tested again for sterility. Sterile stool suspensions were stored in screw capped tubes at -20°C and used for inoculation of cell cultures and suckling mice.

2. Cell Cultures

a. HeLa cell cultures. The HeLa continuous cell line available at the Department of Bacteriology and Virology, A.U.B., was originally obtained from Dr. R.R. Gutekunst, at NAMRU 3 Egypt, in May 1961.

b. Continuous human amnion cells. The continuous human amnion cell line was the U-line, obtained from Laboratorium Voor Microbiologue Der Rijksuniversiteit, Utrecht, Holland, in 1962.

c. Primary human amnion cells. The primary human amnion cell cultures were prepared in this laboratory from healthy placentae obtained from the delivery suite of the American University Hospital. The technic followed was that of Duncan and Bell (1961)[36] with some modification. Each placenta was placed, at the time of delivery, in a large sterile vessel containing 200 ml. of BSS (pH 7.3), and it was used before 3 hours. The placenta was suspended from the cord, the amnion stripped off and washed four times with BSS, to remove the large clots of blood from the membrane. Pieces of membrane were examined microscopically and only those that contained healthy cells were used for cell preparations. The washed amniotic membrane was treated with 200 ml. of 0.25% tripsin (pH 7.8) in GKN solution

for 30 minutes at 37°C water bath. The trypsin-treated membrane was next placed in a large flask containing 200 ml. of fresh trypsin solution, 0.25% at pH 7.8, and agitated with a magnetic stirrer at 35°C. The mixture was examined every 15 minutes until most of the cells were in suspension. Usually 60-90 minutes were sufficient to obtain a good cell suspension. At the end of this period the flask was shaken vigorously for one minute. The suspension was filtered through two layers of gauze and the cells were washed once with BSS. One ml. amounts of cell suspension, containing approximately $1x10^5$ cells in nutrient medium, were distributed into TC tubes and incubated at 37°C in inclined position. Cellular monolayer growth was observed usually within six days.

3. White Swiss Mice

White Swiss mice, obtained from United States Naval Research Unit No. 3, Cairo, 1951 were bred in this laboratory.

4. Nutrient Media[34]

a. For HeLa and continuous human amnion cells

Lactalbumin hydrolysate - BSS solution (0.5%)	70 ml.
Yeastolate solution (1%)	10 ml.

Glucose solution (10%)	5 ml.
Rabbit serum	15 ml.
Penicillin	200 units/ml.
Streptomycin	200 ugm/ml.

Sodium bicarbonate solution (2.8%), a sufficient quantity to produce a pH of 7.4.

The medium was filtered through Seitz filter, and incubated for 72 hours at 37°C to test for sterility. Antibiotics were added just before use.

b. The nutrient medium for propagation of primary human amnion cells was essentially the same as that used for HeLa cells with the exception that 20% human serum was used in place of rabbit serum.

5. Maintenance Media

a. Maintenance medium for HeLa and continuous human amnion cells.[34]

BSS	90 ml.
Glucose solution (10%)	5 ml.
Rabbit serum	5 ml.
Penicillin	200 units/ml.
Streptomycin	200 ugm/ml.

Sodium bicarbonate solution (2.8%), a sufficient quantity to produce a pH of 7.4.

b. Maintenance medium for primary human amnion cells.[36]

Lactalbumin hydrolysate - BSS solution (0.5%)	90 ml.
Glucose solution (10%)	5 ml.
Calf serum	5 ml.
Penicillin	200 units/ml.
Streptomycin	200 ugm/ml.

Sodium bicarbonate solution (2.8%) a sufficient quantity to produce a pH of 7.4.

6. Hanks Balanced Salt Solution (BSS)[34]

I. Balanced Stock Salt Solutions

Solution A.-

(1)

Sodium chloride	480.00 gm.
Potassium chloride	24.00 gm.
Magnesium sulfate ($MgSO_4.7H_2O$)	12.00 gm.

Dissolve in one liter of bi-distilled water.

(2)

Calcium chloride ($CaCl_2.2H_2O$)	10.56 gm.

Dissolve in 500 ml. bi-distilled water.

(1) and (2) were mixed and brought up to three liters. Chloroform 5 ml. was added as preservative and stored at $4^{o}C$.

Solution B.-

Potassium phosphate monobasic (KH_2PO_4)	3.60 gm.
Sodium phosphate, dibasic ($Na_2HPO_4.12H_2O$)	9.12 gm.
Dextrose	60.00 gm.
Phenol red (water soluble)	1.20 gm.

Dissolved in one liter of bi-distilled water and brought up to three liters. Chloroform, 5 ml. was added as preservative and stored at 4°C.

II. Working Balanced Salt Solution

Sol. A	30 ml.
Sol. B	30 ml.

Bi-distilled water a sufficient quantity to make 600 ml.

Autoclaved at 15 lb. pressure for 15 minutes and stored at 4°C.

7. Lactalbumin Hydrolysate - Balanced Salt Solution[34]

Stock BSS solution A	30 ml.
Stock BSS solution B	30 ml.
Lactalbumin hydrolysate	3 ml.

Bi-distilled water a sufficient quantity to make 600 ml.

Autoclaved at 15 lb. pressure for 15 minutes and stored at 4°C.

8. GKN Solution[34]

a. GKN Solution. Formula for (10 X)

Glucose ..... 20 gm.
Sodium chloride ..... 160 gm.
Potassium chloride ..... 2000 ml.
40 ml. of 1% phenol red and 6-8 ml. of chloroform were added and stored at 4°C.

b. Working Solution. The stock solution was diluted 10 times with bi-distilled water autoclaved at 15 lb. pressure for 15 minutes and stored at 4°C.

9. Glucose Solution (10%)[34]

Dextrose ..... 10 gm.
Bi-distilled water a sufficient quantity to make ..... 100 ml.
Autoclaved at 15 lb. pressure for 15 minutes and stored at 4°C.

10. Antibiotics[34]

Penicillin, 10,000 units and 10,000 ugms of streptomycin per ml. in sterile bi-distilled water was distributed in 2 ml. amounts in screw-capped tubes and stored at -20°C.

11. Trypsin Solution[34]

Lyophilized Difco - trypsin (1:250) vials were used to prepare the trypsin solution for dispersing HeLa and continuous human amnion cells. A 1% stock solution was prepared in GKN and stored at -20°C. Before use, it was diluted to 0.05% with GKN (pH 7.8). Solutions of 0.25% trypsin in GKN were prepared directly from the lyophilized powder. This was used for dispersing primary human amnion cell cultures. Before use the pH was adjusted at 7.8.

12. Tissue Culture Bottles and Tubes

The TC tubes and bottles used for these determinations were obtained from Kimble Glass Company, U.S.A.

13. Rabbit Serum

Rabbits weighing 4-6 Kgm. were bled to death by direct heart puncture. The blood was kept at room temperature for few hours; the clot was broken and the bottles were kept overnight at 4°C. Next day, the serum was separated from the clot by centrifugation, filtered through Seitz filter, inactivated for 35 minutes at 60°C, and kept at -20°C until use.

14. Human Serum

Human blood was obtained from the Blood Bank at

the American University Hospital. The serum was separated from the clot following the procedure described above.

15. Calf Serum

Calf blood was obtained from the slaughterhouse, Beirut. The serum was separated from the clot following the procedure described above.

16. Antisera

The antisera for poliovirus types 1, 2, 3, Coxsackie virus group B types 1-5 and ECHO virus types 1-3, 5-19 were obtained from Microbiological Associates, U.S.A.

17. Isolation Methods[34,35,37]

After thawing, each stool suspension was centrifuged for 10 minutes at 2000 r.p.m., and 0.2 ml. amounts were inoculated into 3 tubes of monolayer cell cultures. When human primary amnion cells were used for isolation purposes, the monolayer cells were washed three times with BSS (pH 7.3) to remove traces of human serum used in growth medium. The inoculated tubes were kept for 1 hour at 37°C for viral adsorption. Each tube was next examined for any toxic reaction. This was apparent when the cell monolayers showed degenerative changes.

Tubes showing any cellular degeneration were discarded. To the remaining tubes, 0.8 ml. of maintenance medium was added. The inoculated tubes were observed daily for apparent CPE and pH changes for a period of thirteen days. Cell cultures showing CPE within 13 days, were stored at -60°C. They were next thawed, centrifuged and the supernate was re-inoculated into fresh cell monolayers. The cells were observed for a period of 10 days. Tubes showing CPE were stored at -60°C. The supernate from these was used for identification of virus.

For isolation of Coxsackie and other enteroviruses 24-hour old mice were inoculated using one litter of mice for each stool suspension. The mice were inoculated intracerebrally and subcutaneously at the intrascapuler region, using 0.03 ml. amounts for each site. Animals dying within 24 hours were discarded. Those showing paralysis and/or death within 15 days were stored at -60°C. For use, the brain and muscle tissues from the legs and thoracic region were removed and a 10-15% suspension prepared in BSS (pH 7.2). A second passage was carried out using a new litter of mice. Mice showing paralytis and/or death in the second passage were collected and processed as in the first passage. The viral suspension from the second passage was stored at -60°C and used for identification of virus.

18. The Neutralization Tests[34,35,38]

The methodology of neutralization tests used in these determinations was essentially that of Godtfredsen, and Von Magnus (1959)[35] with some modification.

Viruses isolated from stools on HeLa and continuous human amnion cells were first tested with poliovirus hyperimmune sera, types 1, 2 and 3. If no neutralization occurred, they were tested with Coxsackie virus group B antisera, types 1-5.

Isolates from stools on human primary amnion cells were first tested with ECHO virus hyperimmune sera, types 1-3 and 5-19. If no neutralization occurred they were tested with poliovirus types 1, 2, 3 and Coxsackie virus group B, types 1-5 antisera.

The isolates from stools in suckling mice were tested with Coxsackie virus group B types 1-5, and with Coxsackie group A, type 9 antisera.

The technics followed in poliovirus and Coxsackie virus neutralization tests were identical. Equal amounts, 0.3 ml., of antiserum and the isolate were mixed, kept for one hour at room temperature, and the mixture inoculated each into 2 cell culture tubes in 0.2 ml. amounts.

Tubes were incubated for 30 minutes at 37°C, after which 0.8 ml. maintenance medium was added. They were then re-incubated in inclined position. Uninoculated cell cultures, virus inoculated cell cultures and cell cultures containing antiserum alone, were kept as controls. Neutralization tests with ECHO viruses were carried out using the same technic, except that the virus-antiserum admixtures were kept for 1 hour at 4°C.

Two technics were followed for identifying the isolates from primary human amnion cells. The first was the technic used by Schmidt *et al.*[38] where pools of antisera were prepared, each two pools containing a common antiserum in both vertical and horizontal positions. Thus, two pools showing neutralization of the isolate would indicate the presence of the virus specific to the common antiserum in both pools. However, the fact that many of the isolates were neutralized in the presence of more than two pools of antisera, necessitated testing each isolate with individual type of antiserum. The second technic used was to prepare several pools, each containing three types of antisera. The pool showing neutralization was next tested separately with individual types constituting the pool.

The neutralization tests in mice were carried

out in the following manner. Equal volumes of undiluted antiserum and isolate were mixed in a test tube. The admixture was left for one hour at room temperature and inoculated in 0.03 ml. amounts intracerebrally into suckling mice. Mice receiving the same quantity of isolate intracerebrally, were kept as controls.

RESULTS

Enterovirus isolation studies were carried out from 222 human stool samples using 3 different cell lines and suckling mice. Seventy-five positive stool samples yielded 99 virus strains (Table IV).

A comparison of the sensivity of four technics utilized for isolation of enteroviruses is presented in Table V. These results indicate that of 219 stool specimens inoculated into tubes of HeLa cells, 30 showed CPE, 133 showed no change and 56 showed toxic degeneration of cells. Furthermore, of 30 viruses isolated, 10 were polioviruses, 17 were Coxsackie group B viruses and 3 could not be identified. Of the 10 polioviruses isolated, 5 were type 1, and 5 type 3. Of the 17 Coxsackie viruses isolated, 2 were group B, type 1, and 15 group B, type 3.

Of 212 stool samples inoculated into tubes of continuous human amnion cells, 14 showed CPE, 114 showed no change and 84 showed toxic degeneration of cells. Of 14 viruses isolated, 5 were polioviruses, 5 were Coxsackie viruses and 4 could not be identified. Of 5 polioviruses isolated, 3 were type 1, and 2 type 3. Of the 5 Coxsackie virus isolates, 1 was group B, type 1 and 4 group B, type 3.

Of 222 stool samples inoculated into primary human amnion cells, 43 showed CPE, 145 showed no change and 34 showed toxic degeneration of cells. Of 43 viruses isolated, 3 were polioviruses, 3 Coxsackie viruses, 31 ECHO viruses, and 6 could not be identified. All 3 poliovirus isolates were type 3. Of 3 Coxsackie isolates, 2 were group B type 1, and 1 was group B type 3. Of 31 ECHO virus isolates, 19 were type 1, 9 type 6, 2 type 12 and 1 was type 14.

Of 121 stool specimens inoculated into suckling mice, 12 caused paralysis and/or death. These viruses could not be identified.

The total number and types of enterovirus isolates from 222 stool samples by utilization of 3 different cell lines and suckling mice, is presented in Table VI. Of 83 isolates 12 were polioviruses, 7 type 1, and 5 type 3; 17 Coxsackie viruses, 2 group B type 1, and 15 group B type 3; and 31 ECHO viruses, 19 type 1, 9 type 6, 2 type 12, and 1 type 14. A total of 23 viruses were not identified with the available antisera.

TABLE IV

Enterovirus Isolations from Infant and Children Stool Samples in Lebanon by Utilization of Four Technics

Age group	Laboratory number of stool samples	HeLa TC	Primary human amnion TC	Continuous human amnion TC	Suckling mice
0-11 months	St-20	-	T	P_1	-
	St-49	T	E_1	T	-
	St-139	B_3	T	T	-
	St-175	T	E_1	T	-
	St-190	B_3	B_3	B_3	-
	St-218	-	CPE[c]	ND	-
	St-50	-	E_1	T	-
	St-53	P_3	-	-	-
	St-56	-	E_1	T	ND
	St-58	-	-	T	+
	St-63	T	-	T	+
	St-69	T	E_1	T	-
	St-75	T	E_6	T	-
	St-77[d]	P_3	P'_3	Contam.	+[e]
	St-94[d]	B_3	E_6	B_3	-
	St-163	-	E_1	-	ND
	St-204	-	CPE[c]	T	ND
	St-206	-	E_1	T	ND
	St-207	-	CPE[c]	T	ND

Age group	Laboratory number of stool samples	HeLa TC	Primary human amnion TC	Continuous human amnion TC	Suckling mice
12-23 months	St-26	T	E_1	-	-
	St-38	-	-	-	+[e]
	St-99	-	-	-	+[f]
	St-108	-	-	-	+
	St-174	T	E_1	T	-
	St-181	T	E_1	T	-
	St-202	-	E_1	T	-
	St-54	T	-	-	+
	St-60	T	E_1	T	-
	St-86	-	E_1	-	-
	St-97	B_3	-	-	-
	St-121	-	CPE[c]	CPE[a]	-
	St-125	-	-	CPE[a]	-
	St-134	-	E_{12}	-	ND
	St-200	CPE[a]	E_6	-	ND
2-4 years	St-21	T	T	P_1	-
	St-165	-	E_{14}	-	-
	St-215	P_1	Cont'am.	ND	-
	St-219[d]	P_1	E_6	ND	-
	St-95	B_3	-	-	-
	St-102	B_3	-	B_3	ND
	St-104	B_1	B_1	B_1	ND
	St-106	ND	-	-	+

Age group	Laboratory number of stool samples	HeLa TC	Primary human amnion TC	Continuous human amnion TC	Suckling mice
2-4 years (cont'd)	St-106	ND	-	-	+
	St-114	-	E_6	-	-
	St-115	B_3	-	-	-
	St-117	-	-	-	+
	St-120	-	-	-	+
	St-136	-	E_1	CPE[b]	ND
	St-145	B_3	-	-	ND
	St-147	B_3	-	-	ND
	St-172	-	E_{12}	-	ND
	St-195	P_1	T	T	ND
	St-196	P_3	P_3	P_3	ND
	St-198	-	E_1	-	ND
	St-103	B_3	-	-	-
5-14 years	St-44[d]	B_3	-	-	+[f]
	St-45	B_3	-	-	-
	St-162	CPE[a]	-	CPE[b]	-
	St-100	P_1	-	-	-
	St-130[d]	B_3	E_6	B_3	-
	St-132	B_3	-	-	-
Ages not known (all children)	St-30	T	T	T	+
	St-39	-	E_6	-	-
	St-141	-	E_1	ND	-

Age group	Laboratory number of stool samples	HeLa TC	Primary human amnion TC	Continuous human amnion TC	Suckling mice
Ages not known (all children) (cont'd)	St-142	B_3	-	T	-
	St-143	B_1	B_1	T	-
	St-149	P_3	P_3	P_3	-
	St-159	T	E_1	T	-
	St-177	T	E_1	-	-
	St-191	T	CPE[c]	-	-
	St-210	P_3	-	T	-
	St-211	CPE[a]	-	ND	-
	St-212	T	E_1	T	-
	St-221	-	E_b	T	-
	St-223	T	CPE[c]	T	-
	St-197[d]	P_1	E_6	P_1	ND

P_1 = Poliovirus type 1
B_1 = Coxsackie virus group B type 1
E_1 = ECHO virus type 1
ND = Not done
T = Toxic
CPE= Cytopathic effect positive
- = Cytopathic effect negative
+ = Death or paralysis in mice

[a]Could not be neutralized with poliovirus types 1-3 and Coxsackie virus group B, types 1-5 antisera.

[b]Could not be neutralized with poliovirus types 1-3 antisera.

[c]Could not be neutralized with ECHO virus antisera types 1-3 and 5-19.

[d]Multiple isolations.

[e]Could not be neutralized with Coxsackie virus group B, types 1-5 antisera.

[f]Could not be neutralized with Coxsackie virus group B types 1 and 3 antisera.

TABLE V

Comparison of Four Technics Utilized for Isolation of Enteroviruses

Technic Used	Total Number of Stool Samples				Identification of isolated viruses								
					Poliovirus Types		Coxsackie B virus* Types		ECHO virus Types				Not identified
	Tested	Positive	Negative	Toxic	1	3	1	3	1	6	12	14	
HeLa TC	219	30	133	56	5	5	2	15	ND	ND	ND	ND	3
Human Primary Amnion TC	222	43	145	34	0	3	2	1	19	9	2	1	6
Human Continuous Amnion TC	212	14	114	84	3	2	1	4	ND	ND	ND	ND	4
Suckling mice	121	12	109	0	ND	ND	0	0	ND	ND	ND	ND	12

ND = Not done

*All Coxsackie group B isolates from cell cultures caused death in mice upon IC inoculation.

TABLE VI

Total Enterovirus Isolates by Utilization of Four Technics

Technics used	Poliovirus Types		Coxsackie B virus Types		ECHO virus Types				Not identified
	1	3	1	3	1	6	12	14	
HeLa	4	2	0	11	0	0	0	0	2[a]
Continuous human amnion	2	0	0	0	0	0	0	0	1[a]+1[b]
Primary human amnion	0	0	0	0	19	9	2	1	5[c]
HeLa and continuous human amnion	1	0	0	3	0	0	0	0	1[a]
HeLa and primary human amnion	0	1	1	0	0	0	0	0	0
HeLa, continuous and primary human amnion	0	2	1	1	0	0	0	0	0
Continuous and primary human amnion	0	0	0	0	0	0	0	0	1[d]
Mice	0	0	0	0	0	0	0	0	12[e]
Total enteroviruses isolated	7	5	2	15	19	9	2	1	23

[a]Not neutralized with poliovirus types 1-3 and Coxsackie virus group B, types 1-5 antisera.

[b]Not neutralized with poliovirus types 1-3 antisera.

[c]Not neutralized with ECHO virus types 1-3 and 5-19 antisera.

[d]Not neutralized with poliovirus types 1-3, Coxsackie virus group B, types 1-5 and ECHO virus types 1-3 and 5-19 antisera.

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[e]Two isolates were not neutralized by Coxsackie virus group B types 1-5 antisera and Coxsackie virus group A type 9 antisera, and two other isolates were not neutralized by Coxsackie virus group B types 1 and 3 antisera.

DISCUSSION

During the last ten years the incidence of paralytic poliomyelitis has been decidedly increased among children of several Middle Eastern countries.[39,40] Interest in this infection has led to the laboratory studies of polioviruses in Egypt, Israel and Lebanon. It is evident from these studies on patients and healthy individuals that all three types of polioviruses circulate freely in the Middle East.[39,40] The infection occurs in infants during the early months of life and maximum incidence of paralytic poliomyelitis is observed between the ages of 6 and 24 months.[39,40] A high percentage of children over the age of five years may be considered immune. In Egypt,[39,40] investigations reveal that during 1955 poliovirus type 2 was the predominant type causing paralytic illness among children. Later, in 1957-1958, type 1 became the predominant type. Israel,[39] in 1950, experienced an epidemic of poliomyelitis, poliovirus type 1 being the predominant strain. The same type was responsible for paralytic poliomyelitis cases until 1956. In 1957, most of the cases were due to types 2 and 3; in 1958, 93% was due to type 1; in 1959, 80% was due to type 3; in 1960, 50% was due to type 1 and finally in 1961, 98% was due to type 1. Likewise, in Cyprus,[39] during an epidemic of poliomyelitis in 1958, poliovirus type 1 was found to be the causative agent.

The reports of Garabedian et al. (1963)[10] and Matossian et al. (1964)[11] indicated that there was an increase in the incidence of paralytic poliomyelitis in Lebanon, reaching epidemic proportions during recent years. Thus serologic studies of 400 infants and adults showed that most of the susceptibles, as determined by the absence of neutralizing antibodies, fall within the age-group of 0-4 years. These findings are in agreement with the high incidence of paralytic poliomyelitis occuring in this age-group.[10,11]

Further serologic and virologic studies on 93 patients with paralytic poliomyelitis showed that all 3 poliovirus types exist in Lebanon and any one of these types may be responsible for the causation of paralytic poliomyelitis. There was, however, a preponderence of poliovirus type 1.[10,11]

Coxsackie and ECHO viruses are also present in the Middle East. Gelfand, (1961)[29] in a report points out the presence of Coxsackie group A, types 1-4, 6, 7, 9, 10; Coxsackie virus group B, types 1-5, and ECHO virus, types 1-9, 11-15, 17-19, in Egypt; and Coxsackie virus group A, types 1-4, 6-9, Coxsackie virus group B, types 1, 3, 4, 5 and ECHO virus, types 2, 9, 14, in Israel. Our studies reveal that Coxsackie and ECHO viruses are likewise present

in Lebanon. The strains isolated in this laboratory from stools belong to Coxsackie virus group B, types 1 and 3, and to ECHO virus types 1, 6, 12 and 14.

A total of 23 viruses isolated from stool samples could not be identified by the use of the available enteroviral antisera (Table VI). Eleven of these viruses were isolated in cell cultures. These isolates may have been enteroviruses or viruses belonging to other groups such as the adenoviruses, herpes simplex and the respiratory viruses that may occasionally be present in human stools.[41] A total of 12 viruses obtained by inoculation of stool samples into suckling mice were not neutralized by Coxsackie virus group B, types 1-5 antisera. These probably belong to group A, Coxsackie viruses or to group B, type 6, against which no antisera were available.

HeLa cell cultures seemed to be superior in yielding poliovirus and Coxsackie group B virus isolates from fecal samples, than continuous human amnion and primary human amnion cell cultures. Likewise, primary human amnion cells were the only susceptible cells that yielded ECHO virus isolates from fecal samples (Table V). Furthermore, it is interesting to note that, although Coxsackie group B viruses were initially not isolated by inoculation of fecal samples into mice, yet these viruses once isolated in cell cultures

caused death in suckling mice upon IC inoculation (Table V). This discrepancy may have been due to the presence in the fecal suspension used for inoculation of an inadequate number of viral particles which *per se* were unable to cause fatal infection in mice. For poliovirus and Coxsackie group B virus isolations the use of HeLa cell cultures seems to be more advantageous than primary or continuous amnion cell lines.

It should be pointed that these investigations do not reflect the seasonal, socio-economic, and other variations effecting the incidence of enterovirus infections in the country. Furthermore, because only a limited number of stool samples were studied, the results reported herein should not be interpreted as representing the entire picture of the whole territory of Lebanon.

BIBLIOGRAPHY

1. International Enterovirus Study Group, 1963. Picornavirus group. Virology 19: 114.
2. Committee on the Enteroviruses, 1957. The enteroviruses. Am. J. Publ. Hlth. 47: 1556.
3. Rivers, T.M. and Horsfall, F.L., ed. Viral and Rickettsial Infections of Man. 3rd ed. Philadelphia, J.B. Lippincott Company, 1959, p. 433.
4. Melnick, J.L., 1962. Enteroviruses. Ann. N.Y. Acad. Sci. 101: 331.
5. Wenner, H.A., 1962. The ECHO viruses. Ann. N.Y. Acad. Sci. 101: 398.
6. Syverton, J.T., 1959. An appraisal of the enterovirus problem. Amer. J. Trop. Med. Hyg. 8: 101.
7. Plotkin, S.A., Corp, R.I., and Graham, A.F., 1962. The polioviruses of man. Ann. N.Y. Acad. Sci. 101: 357.
8. Schwerdt, C.E., 1957. Physical and chemical characteristics of purified poliomyelitis virus. Special publications of the New York Academy of Sciences, Vol. V, 157.
9. Committee on Enteroviruses, 1962. Classification of human enteroviruses. Virology 16: 501.
10. Garabedian, G.A., Matossian, R., and Hatem, J., 1963. Poliomyelitis in Lebanon. Leb. Med. J. 16: 216.

11. Matossian, R.M., Garabedian, G.A., Balassanian, N., et Hatem, J., 1964. La poliomyélite paralytique au Liban en 1962. Rév. Méd. Moy. Orient. No. 1, p. 25.

12. Boyd, T.E., 1957. A biography of poliomyelitis. N.Y. Acad. Sci. 5: 39.

13. Enders, J.F., Weller, T.H., and Robbins, F.C., 1949. Cultivation of the Lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science 109: 85.

14. Robbins, F.C., Enders, J.F., and Weller, T.H., 1950. Cytopathogenic effect of poliomyelitis virus in vitro on human embryonic tissues. Proc. Soc. Exptl. Biol. Med. 75: 370.

15. Robbins, F.C., Enders, J.F., Weller, T.H., and Florentino, G.L., 1951. Studies on the cultivation of poliomyelitis virus in tissue culture. V. The directed isolation and serologic identification of virus strains in tissue culture from patients with nonparalytic and paralytic poliomyelitis. Am. J. Hyg. 54: 286.

16. Dulbecco, R., 1952. Tissue culture monolayer. Proc. Nat. Acad. Sci. 38: 747.

17. Scherer, W.F., Syverton, J.T., and Gey, G.D., 1953. Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain

HeLa) derived from an epidermoid carcinoma of the cervix. J. Exptl. Med. 97: 695.

18. Younger, J.S., 1954. Monolayer tissue cultures. F. Preparation and standardization of suspensions of trypsin dispersed monkey kidney cells. Proc. Soc. Exptl. Biol. Med. 85: 202.

19. Dulbecco, R., and Vogt, M., 1954. Plaque formation and isolation of pure lines with poliomyelitis virus. J. Exptl. Med. 99: 167.

20. Dalldorf. G., and Sickles, G.M., 1948. An unidentified filtrable agent isolated from the feces of children with paralysis. Science 108: 61.

21. Gilford, R., and Dalldorf, G., 1951. The morbid anatomy of experimental Coxsackie virus infection. Amer. J. Path. 27: 1047.

22. Sickles, G.M., Mutterer, M., Feorino, P., and Plager, H., 1955. Recently classified types of Coxsackie virus. Proc. Soc. Exptl. Biol. Med. 90: 529.

23. Rivers, T.M., and Horsfall, F.L., ed. Viral and Rickettsial Infections of Man. 3rd ed. Philadelphia, J.B. Lippincott Company, 1959, p. 519.

24. Zitcer, E.M., Fogh, J., and Dunnebacke, T.H., 1955. Human amnion cells for large scale production of poliovirus. Science, 122: 30.

25. Committee on the ECHO Viruses, 1955. Enteric cytopathogenic human orphan (ECHO) viruses. Science, 122: 1187.

26. Ashkenazi, A., and Melnick, J.L., 1962. Enteroviruses - A review of their properties and associated diseases. Amer. J. Clin. Path. 38(3): 209.

27. Melnick, J.L., 1957. ECHO viruses. Special publications of the New York Academy of Sciences. Vol. V, 365.

28. Expert Committee on Poliomyelitis, Second Report, 1958. Wld. Hlth. Org. Techn. Rep. Ser. 145.

29. Gelfand, H.M., 1961. The occurrence in nature of the Coxsackie and ECHO viruses. Progress Med. Virol. 3: 193.

30. Kalter, S.S., 1962. A serologic survey of antibodies to selected enteroviruses. Bull. Wld. Hlth. Org. 26: 759.

31. Plager, H., 1962. The Coxsackie viruses. Ann. N.Y. Acad. Sci. 101: 390.

32. Wenner, H.A., 1962. Problems in working with enteroviruses. Ann. N.Y. Acad. Sci. 101: 343.

33. Kelly, S., Sanderson, W., 1962. Comparison of various tissue cultures for the isolation of enteroviruses. Am. J. Pub. Hlth. 52(3): 1455.

34. Merchant, D.J., Kahn, R.H., and Murphy, W.H. Handbook of Cell and Organ Culture. Minneapolis, Burgess Publishing Company. 1960.

35. Godtfredsen, A., and Von Magnus, H., 1959. Routine diagnosis of enteroviruses using tissue culture techniques. Danish Med. Bull. 6(8):264.

36. Duncan, I.B.R., and Bell, E.J., 1961. Human-amnion-tissue

37. Gamble, D.R., 1962. Isolation of Coxsackie viruses from normal children aged 0-5 years. Brit. Med. J. 1: 16.

38. Schmidt, J.N., Guenther, W.R., Edwin, H.L., 1961. Typing of ECHO virus isolates by immune serum pools. J. Immunol. 87: 623.

39. Regional Committee for the Eastern Mediterranean, WHO, Eleventh Session, Agenda item 14, July 1961. Poliomyelitis.

40. Seal, J.R., 1961. The epidemiological background for use of poliovaccines in the Middle East. J. Egypt. Med. Assoc. 44(4): 275.

41. Timbury, C., Morag, 1962. Virus infections of the intestinal tract. The Practitioner (Symposium on Diseases of the intestinal tract) 189: 317.