DETECTION OF INDIGENOUS ENTERIC VIRUSES IN RAW SEWAGE EFFLUENTS OF THE CITY OF ATHENS, GREECE, DURING A TWO YEAR SURVEY

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ABSTRACT

In a two-year survey of enteric viruses in urban sewage effluents of Athens, Greece, during 1982-1983, indigenous Enteroviruses and Adenoviruses have been detected. Enteroviruses, (Polio, Coxsackie B and Echo) were recovered on Vero or BGM cells by inoculation of samples, after a pre-concentration step utilizing glass powder. Adenoviruses were recovered on Hep2 cells after being precipitated with protamine sulfate. The viral content was in the order of $10^{-10}$ cytopathogenic units (CPU) per litre of sample. Seasonal fluctuations of the various serotypes recovered were observed. The peak of Adenoviruses was found in early spring whereas Enteroviruses peaked in late summer-early fall. The most frequently recovered serotypes within each group were: Polio III (47%), Coxsackie B5 (56%), Echo 7 (61%) and Adeno 7 (43%). Similar isolation frequencies, except for Polio, were found in humans as well. As regards Polioviruses, all strains recovered from sewage were found to be vaccine-related by the intratypic serodifferentiation test.

KEYWORDS
Enteric viruses, Indigenous, Raw sewage effluents, Concentration, Glass powder, Protamine sulfate, Vaccine related Polioviruses.

INTRODUCTION

Human enteric viruses excreted in stools of infected individuals in a community are normally present in domestic sewage. The disposal of sewage in many countries is traditionally practised by discharging effluents directly into the flowing streams, rivers, lakes or to the sea (Berg, 1975; Dor et al., 1976; WHO SCIENTIFIC Group 1979). Increasing demands on available water resources, however, make recycling of domestic wastewater inevitable. Indeed recycling of domestic effluents and sludge from sewage treatment plants, for irrigation and fertilization of crops is practised today in certain countries (Katzenelson et al, 1976; Teitsch et al., 1980, Ward et al., 1982) and viruses have been recovered from such practices.

The presence of enteric viruses in environmental waters and in sewage irrigated crop-land, carries an obvious risk of transmission of viral diseases in humans (Bryan et al., 1974; Melnick et al., 1978; IAWPRC, Study Group on Water Virology, 1983). Therefore, it is important for public health planning that information on the presence of viruses in sewage effluents and environmental waters, be made available. Numerous studies on the subject have been carried out in many
countries (Nestor et al., 1976; Payment et al., 1979; Walter et al., 1982; Fattal et al., 1983), but no previous work of this nature has been done in our country. The present study reports the findings of a two year survey of enteric viruses in raw sewage effluents in the city of Athens, Greece.

MATERIALS AND METHODS

Sewage samples. Grab samples of raw sewage effluents were collected from the central sewer of Athens, at the point of outlet, located at the cape of Keratsini. Sampling was carried out at biweekly intervals, approximately the same time, between 6-7h in the morning. Samples were placed in 10 litre volume sterile containers and transported to the laboratory within an hour after collection for processing.

Sample processing. Enteroviruses were concentrated from raw sewage effluents by an adsorption-elution procedure, as described in detail by Schwartzbrod et al. (1978). Briefly, this method involves the following: a 10 litre volume sample is acidified to pH 3.5 by the dropwise addition of 2N HCl and a solution of 0.5M AlCl3 to obtain a final concentration of 5·10-4 M. The acidified sample is filtered under negative pressure, through a fluidized column of glass powder of particle diameter 100-200 μm (ref. Sovirel 4585-10) at an approximate rate of 500 ml min⁻¹. After filtering all the sample, adsorbed virions on glass particles are eluted with 100 ml of glycine buffer (0.05 M) supplemented with 1.5% beef extract at pH 10.6. Eluates are neutralized immediately after collection to pH 7.2 and decontaminated by filtration through a filter membrane of Ø 0.45μm (Millipore HAWP 025) pretreated with MEM plus 2% PCS to avoid electrostatic adsorption of virions.

Adenoviruses were concentrated by a precipitation procedure using protamine sulfate. The technique initially described by England (1972) and modified by Ridinger et al. (1982), briefly involves the following: to one litre clarified sewage effluent is added egg albumin (Sigma chem. co. A5378), to give a final concentration of 0.15%. The pH is adjusted to 8.3 with 2N NaOH and protamine sulfate (Sigma P4020) is added to a final concentration of 0.01%, followed by incubation at 37°C for 30 min. The precipitate form is collected by centrifugation at 8000 g for 20 min. To elute the viruses the precipitate is first suspended in 3.0 ml of 1M NaCl solution and is further diluted 1:8 for isotonicity. The pH is adjusted to 7.2 and trypsin solution (Difco trypsin 1:250) is added at a final concentration of 100μg/ml, followed by incubation at 37°C for 30 min, in order to digest the complex of virus-protamine. Decontamination is achieved by filtration as described for Enteroviruses.

Cultural assay for virus detection. Suspensions of the cell lines Hep2, Vero or BGM were made in growth medium (MEM with 5% FCS plus 100 U Pen. + 100 μg strep/ml) containing approximately 10⁵ cells /ml, and plated into 96 flat bottom wells, tissue culture plates (Falcon 3040). The plates were incubated at 37°C for 24-28h in order to form confluent monolayers. Before inoculating the cell monolayers, the growth medium was discarded and replaced with the virus concentrates (inocula) which were made as follows: Ten ml fractions of each sample concentrate were diluted up to 100 ml with maintenance medium (MEM with 1% FCS plus antibiotics) and were dispensed on to the preformed cell monolayers in the TC plates. To each well was delivered exactly 0.25 ml of diluted inoculum with a semi-automatic Cornwell syringe. The surface of the plates was blotted dry, covered with autoadhesive transparent tape (Falcon 3044 F) and incubated at 37°C. The plates were examined microscopically for evidence of CPE every second or third day. Viral growth was confirmed by at least one subculture of the suspected wells. The whole contents of wells presenting advanced CPE (> 75% of monolayer) were subcultured into the corresponding cells (Hep2, Vero or BGM) grown in test tubes and incubated at 37°C in rolling drums. When complete CPE (4+) developed in the tubes they were frozen until identification of the isolates. Preliminary
grouping of the isolates into Enteroviruses or Adenoviruses was based on the characteristic CPE produced. Final identification of the isolates was performed by the micro-neutralization test in microtitre plates (Melnick et al, 1979), using the Lim Benysh-Melnick pools of antisera for typing Enteroviruses (kindly supplied by the Statens Seruminstitut Copenhagen) while Adenoviruses were typed with monospecific antisera obtained from Microbiological Associates.

In order to estimate the viral content of the samples the total number of positive wells counted in four TC plates was divided by the volume of the inoculum plated and multiplied by the total volume of virus concentrate obtained initially.

RESULTS AND DISCUSSION

A total of 48 samples of raw sewage effluents were examined for detection of viruses, at biweekly intervals, over the two-year survey period under question. Practically every sample tested was found to be positive for virus isolation. The Enteroviruses were detected relatively early during the incubation period, producing the characteristic CPE in the cell monolayers, between the 3rd and 9th day after inoculation. Adenoviruses, on the other hand, were not detected before the 10th day after inoculation. As a result, observations of inoculated HeP2 TC plates for the detection of adenoviruses were extended up to the 25th day after inoculation. Virus concentration levels for each group were calculated separately. The mean concentration values for Enteroviruses (Table 1) were 150 and 200 CPU per litre of sewage sample for 1982 and 1983 respectively, with the corresponding range for each year of 30-900 and 10-750 CPU per litre. Only three samples failed to yield Enteroviruses but they were positive for Adenoviruses.

<table>
<thead>
<tr>
<th>Year</th>
<th>Virus</th>
<th>Concentration (CPU L⁻¹)</th>
<th>Positive samples (n=24)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Mean</td>
<td>Range</td>
</tr>
<tr>
<td>1982</td>
<td>Enterovirus</td>
<td>150</td>
<td>30-900</td>
</tr>
<tr>
<td></td>
<td>Adenovirus</td>
<td>280</td>
<td>150-1200</td>
</tr>
<tr>
<td>1983</td>
<td>Enterovirus</td>
<td>200</td>
<td>10-750</td>
</tr>
<tr>
<td></td>
<td>Adenovirus</td>
<td>500</td>
<td>70-3200</td>
</tr>
</tbody>
</table>

* CPU L⁻¹ = Cytopathogenic Units /litre

Mean concentration values for Adenoviruses were 280 and 500 CPU per litre of sewage sample, respectively for 1982 and 1983 with range values of 150-1200 and 70-3200 CPU per litre. From the above results it is evident that Adenovirus concentration levels in raw sewage effluents of Athens are higher than those recorded for Enteroviruses. Indeed, Adenoviruses constitute the most frequently isolated virus group from the population of Athens, as recorded by the diagnostic Virology Laboratory of our Institute (Kouloumbis et al, 1981). Higher isolation rates for Adenoviruses versus the Enteroviruses have been reported by Irving et al, (1981), during a one-year survey of sewage in the city of Melbourne. Other investigators, however, reported only occasional detection of Adenoviruses (Sellwood et al, 1981, Payment et al, 1983), but this finding may be related first to the concentration method used and second to whether these viruses are endemic in the region or not.

Concerning the identification of Enteroviruses it was possible to type 253 field-isolates which were distributed into three groups as follows: 88 strains (35%)

TABLE 1. Virus Concentrations and Positive samples (%)
were Polioviruses, 101 strains (40%) were Coxsackie B viruses and the other 64 strains (25%) were Echo viruses. Ten different serotypes of these viruses were found (table 2), among which are included all three Polio serotypes, five Coxsackie B serotypes and only three Echo serotypes, namely E1, E7, and E30. Although a changing pattern of serotype prevalence is seen with the Coxsackie B viruses and Echo viruses from 1982-1983, this is not so for Polioviruses, which remain almost unchanged for the two years. Greece has utilized the Sabin vaccine (O.P.V., trivalent) to immunize all preschool age children for nearly 20 years now. Thus all three Polio serotypes should normally be present in domestic sewage. Indeed, all three Polio serotypes have been found, but Polio III is the most prevalent (47%).

TABLE 2. Identification of Enterovirus Field Isolates

<table>
<thead>
<tr>
<th>Year</th>
<th>Polioviruses</th>
<th>Coxsackie B viruses</th>
<th>Echo viruses</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>I II III</td>
<td>1 2 3 4 5 6</td>
<td>7 30</td>
</tr>
<tr>
<td>1982</td>
<td>13 13 21</td>
<td>2 10 5 10 45 -</td>
<td>- 8 1</td>
</tr>
<tr>
<td>1983</td>
<td>12 9 20</td>
<td>8 5 1 7 8 -</td>
<td>2 51 2</td>
</tr>
<tr>
<td>Total</td>
<td>25 22 41</td>
<td>10 15 6 17 53 -</td>
<td>2 59 3</td>
</tr>
</tbody>
</table>

However, in a four-year study of Enteroviruses, 1979-1982, in the population of Athens, (Krikelis 1983, Doctoral Thesis), it was found that among the Polioviruses type II was the most frequently isolated serotype (60%). It cannot be said with certainty which parameters contribute to this discrepancy, but it could possibly be due to greater resistance of Polio III in the environment. All Polioviruses isolated were tested by the intratypic serodifferentiation method of Van Wezel et al. (1979) in order to characterize their origin. Indeed all strains, except two, (data not shown) were found to be vaccine related or Sabin-like. The two strains, one type I and one type II, were intermediate between the prototype wild strains of Mahoney and Mef-1 and the corresponding attenuated Sabin strains.

Among the Coxsackie B viruses we were able to detect the first five serotypes (Coxsackie B6 has never been isolated either from humans or from sewage in our laboratory) and serological surveys showed that only 3% of sera tested were positive (Krikelis et al., 1981). Serotype Coxsackie B5 was the most prevalent (56%) in 1982 among the Echo and the Coxsackie viruses isolated. As regards the Echo viruses only three serotypes have been detected in sewage. Serotype Echo 7 was very frequent (61%) during 1983. Interestingly enough the Coxsackie B and Echo viruses found in sewage were isolated from humans during the same study period as well.

Concerning the Adenoviruses 118 field isolates have been identified (table 3) which were distributed into seven different serotypes. The most prevalent serotypes were Adeno 7 (43%) and Adeno 2 (31%). We failed to detect Adeno 6 either from sewage or from humans. Adeno 4 and Adeno 15 were the least frequently isolated serotypes. It is worth mentioning that chronologically the Adenoviruses prevail in the community of Athens between the months of January to May practically every year. The highest concentration levels of these viruses in the sewage effluents were found in the months of March and April. The Enteroviruses on the other hand prevail during the summer-autumn months in the population and peak values in the sewage effluents were found in the months of August and September.

In conclusion, from the results obtained over the two-year survey period
of cultivatable Enteric viruses in raw sewage effluents, 18 different serotypes have been detected by the concentration methods used, which to a greater or lesser extent reflect those viruses circulating in the population of Athens.

**TABLE 3. Identification of Adenovirus Field Isolates**

<table>
<thead>
<tr>
<th>Year</th>
<th>Adeno serotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 2 3 4 5 6 7 15</td>
</tr>
<tr>
<td>1982</td>
<td>3 16 5 - 4 - 17 2</td>
</tr>
<tr>
<td>1983</td>
<td>5 20 4 3 4 - 34 1</td>
</tr>
<tr>
<td>Total</td>
<td>8 36 9 3 8 - 51 3</td>
</tr>
</tbody>
</table>

Mean concentration levels for both groups do not exceed 1000 viral cytopathogenic units per litre of sample and a range of values from as low as 80 to as high as 3950 CPU per litre have been recorded. These results can be very valuable in viral epidemiological studies for our country and can also be used when developing strategies for the management of domestic effluents as well as recreational and other environmental waters.

**ACKNOWLEDGEMENTS**

The authors wish to acknowledge the assistance of Dr. Ir. A. L. van Wezel and Mr. A. G. Hazendonk, Rijksinstituut voor de Volksgezondheid, Bilthoven, in performing the intratypic serodifferentiation of the Poliovirus strains and for providing our laboratory with the strain specific antisera. To Dr. C. H. Mordhost, Statens Serum Institut, Copenhagen for kindly providing the enterovirus pools of antisera we extend our thanks. Thanks are also due to Mrs. H. Afendaki for her excellent technical assistance.

This work was partly supported by the Ministry of Research and Technology Project no. 81082.

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