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DETECTION OF ENTEROVIRUSES AND ADENOVIRUSES IN COASTAL WATERS OF SW GREECE BY NESTED POLYMERASE CHAIN REACTION

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Abstract—One hundred and twenty coastal water samples, from twelve different bathing sites along the Achaia coastline (SW Greece) were analyzed for enteroviruses and adenoviruses using nested polymerase chain reaction, during a five month period (June–October), in 1995 and 1996. When nested PCR was used for detection of enteroviruses, 21 (17.5%) were found positive. On the other hand, using cell culture, only 11 (9.2%) were positive for enteroviruses with a mean number of 2.9 PFU/10 l. In addition, 34 (28.3%) samples were positive by nested PCR detection for adenoviruses. In half of the sites, both enteroviruses and adenoviruses were not detected, while in the remaining half of the sites the above mentioned viruses were periodically detected. Moreover, thirty samples from non-bathing areas were also analyzed as reference samples. The comparison between the presence of faecal bacterial indicators and the presence of enteroviruses and adenoviruses did not show any significant correlation ($P > 0.05$). The PCR methodology was found highly successful in detecting both viruses in coastal waters of our region. The implementation of this molecular technique in laboratories monitoring environmental samples, despite the existence of some difficulties is expected to resolve some problems of detection of pathogen environmental microorganisms. © 1998 Elsevier Science Ltd. All rights reserved

Key words—enteroviruses, adenoviruses, nested PCR, coastal waters, detection

INTRODUCTION

The sanitary quality of sea water along bathing beaches relies on the detection of *Total coliforms* (TC), *Faecal coliforms* (FC) and *Faecal Streptococci* (FS) as indicators of faecal pollution (Anon, 1976). Apart from the three main aforementioned bacterial organisms used to evaluate the possibility of faecal contamination of recreational waters, the E.U. Directive (Anon, 1976) lists a number of microbial standards including the absence of enteroviruses in 10 l water in 95% of the samples examined. The same E.U. Directive (Anon, 1976) specifies that enteroviruses should be monitored when they are suspected to be present. Wyer *et al.* (1995) suggest a poor statistical relationship between bacterial indicators of faecal pollution and enteroviruses in coastal waters resulting in a low predictive capability. The ratio between numbers of pathogens including enteroviruses and numbers of indicator organisms is variable spatially and temporally support Wyer *et al.* (1995).

The enteroviruses (*polioviruses*, *coxsackievirus types A and B*, *echoviruses*) can cause a variety of illnesses ranging from gastroenteritis to myocarditis and aseptic meningitis (Melnick, 1990). Numerous studies have documented the presence of enteroviruses in coastal and recreational waters (Tiemessen *et al.*, 1989; Abbaszadegan *et al.*, 1993; Kopecka *et al.*, 1993; Tsai *et al.*, 1993; Puig *et al.*, 1994). Enteroviruses, in the environment, pose a public health risk because they can be transmitted via the fecal–oral route through contaminated water and low numbers are able to initiate an infection in humans. The U.S. Environmental Protection Agency describes the enteric viruses group itself as the most meaningful, reliable and effective index for environmental monitoring (Karaganis *et al.*, 1983).

Enteroviruses have been recorded in seawater in many parts of the Mediterranean. According to available literature, isolates include all three serotypes of polioviruses, serotypes 1–5 of Coxsackie B virus and serotypes 1, 7 and 30 of Echovirus (Krikelis *et al.*, 1985a,b, 1986), Hepatitis A virus (Papaevangelou, 1992), and a large number of either untyped or unspecified serotypes (Petrilli *et al.*, 1980; Krikelis *et al.*, 1987; Maini *et al.*, 1990). Seven serotypes of adenoviruses (Nos. 1, 2, 3, 4, 5, 7 and 15) have also been isolated (Krikelis *et al.*, 1986) together with a number of unidentified isolates.

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On the other hand, adenoviruses are the only human enteric viruses containing DNA and they are human pathogens. Their presence in coastal waters and their role as originators of gastroenteritis have probably been underestimated (Irving and Smith, 1981; Hurst *et al*., 1988). Numerous studies have also documented the presence of adenoviruses in coastal and recreational waters (Girones *et al*., 1993; Puig *et al*., 1994; Enriquez *et al*., 1995; Enriquez and Gerba, 1995). Serotypes 40 and 41 are called fastidious (Flewett *et al*., 1975) because of the difficulty of their isolation, and both serotypes are etiological agents of infantile gastroenteritis (Uhnoo *et al*., 1984; Tiemessen *et al*., 1989).

The comparative survival of viruses and indicator bacteria in the marine environment has shown that bacteria released in the marine environment may be undetectable within a few days while enteric viruses may be present in an infectious state at detectable levels for several months (Wheeler, 1990). This situation leads to the conclusion of inadequacy of current bacterial indicators as a monitoring tool for pollution by viruses.

The detection of viral pathogens by cell culture is very complex and not all groups of viruses can be isolated on regular cell lines. For this reason, PCR was used which provides very sensitive, specific, and rapid detection of viruses in a variety of environmental samples (Jothikumar *et al*., 1992; Abbaszadegan *et al*., 1993; Kopecka *et al*., 1993). Nested PCR amplification was applied to ensure the specificity of detection and to increase the amplification signal, providing the method with very high sensitivity especially for environmental samples which contain low numbers of viruses (Puig *et al*., 1994). As with all PCR determinations, nested PCR for the detection of adenoviruses and enteroviruses should be used by experienced personnel because of the high risk for samples cross contamination associated with this procedure.

The aim of this study was to detect enteroviruses and adenoviruses in coastal waters in our region, a popular tourist destination, with the application of a molecular technique (nested PCR). In addition, we compared the presence of the two viral indicators with the presence of fecal bacterial pollution by testing the three common bacterial indicators (TC, FC, FS) in our geographical area, in order to verify if bacterial indicators could serve as indexes of the virological quality of sea water (Wyer *et al*., 1995).

MATERIALS AND METHODS

Viruses and cells

Adenoviruses were provided by Dr. Annika Allard from the University of Umea, Sweden. Polioviruses type 1, 2, 3 were also provided by Professor Dalaina from the University of Thessaloniki, Greece. The Vero cells used for enteroviruses propagation were provided by Institute Pasteur of Athens, Greece.

Coastal water sample collection

The presence of adenoviruses and enteroviruses in coastal waters was examined for two years, 1995 and 1996, during a five-month period (June–October) each year.

Sea water samples were collected from twelve different sites in South Western Greece, Achaia district. The sites were chosen by the Ministry of Environment and were located all along the Achaia district ("clean sites" where bathing was allowed). The other sampling sites, where bathing was prohibited ("dirty sites"), were chosen by our laboratory, as reference samples. Sampling was made once a month by experienced personnel.

400–1000 l of water were concentrated by filtration through an electropositive MK cartridge filter (AMF CUNO, Meriden, Conn.). Following sampling, the filters were placed in Ziploc plastic bags and shipped on ice to the laboratory for processing. Concurrently, 1 l of the sample was collected for the bacteriological analysis. Separate filters, filter housings, and tubing were used for each site.

Filter elution and concentration

The viruses adsorbed to the filter, were eluted with 0.25 N Glycine buffer, pH 9.5, containing 3% beef extract (BBL, Beckton, Dickinson, U.S.A.). The eluates were adjusted to neutral pH with 1 N HCl.

The 1-l volume of elutes was re-concentrated by organic flocculation (Katzenelson, 1977). The resulting 30 ml volume of viral suspension was then recovered and treated as described by Puig *et al*. (1994).

Decontamination of the samples

Samples were decontaminated by the use of chloroform and then penicillin G sodium, and streptomycin added each at a final concentration of 100 U/ml (Puig *et al*., 1994).

Cell culture technique

Enteroviruses detection by cell culture was carried out by infecting confluent Vero cells in Petri dishes with 1 ml of concentrated, decontaminated sample. The positive samples were then measured using the plaque forming unit test (PFU) (Hurst and Gerba, 1979; Hurst and Goyke, 1983; Dahling and Wright, 1986).

For the detection of enteroviruses, both cell culture and PCR technique were performed while no cell culture technique was performed for adenoviruses detection because of the difficulties of culturing these viruses.

The preparation of viral particles for nucleic acid extraction

The procedure proposed by Puig *et al*. (1994) was followed with some modifications: The viral suspension (28 ml) was centrifuged ($48\,400 \times g$; 225 min; 4°C). Viruses retained in the pellet were eluted by the addition of 5 ml of 0.25 N Glycine Buffer, pH 9.5, on ice for 30 min and then 25 ml of PBS double concentrated was added to the elute. The sample was then centrifuged at $12\,000 \times g$ for 15 min at 4°C. The viral suspensions obtained, were ultracentrifuged at $171\,360 \times g$ for 1 h at 4°C in order to obtain pellets of the viral particles which were resuspended in 0.2 ml of PBS and kept from at −80°C until the nucleic acid extraction.

Nucleic acid extraction

Nucleic acid extraction was performed according to Boom *et al*. (1990) method, with the modifications proposed by Puig *et al*. (1994). The nucleic acids suspension was kept at −20°C until RT-PCR amplification.

cDNA synthesis of enterovirus RNA

The protocol proposed by Puig *et al*., 1994 was followed: The reaction mixture for reverse transcription had a total volume of 10 μl and contained 5 μl of nucleic acids extracted plus 1 × RT buffer (Promega, Madison, U.S.A.) (5× buffer contained 250 mM Tris–HCl, pH 8.3 at 42°C, 250 mM KCl, 50 mM $MgCl_2$, 50 mM DTT, 2.5 mM spermidine), deoxynucleoside triphosphates at 200 μM each, 8 U of AMV RT (Promega, Madison, U.S.A.) and 2.5 μM external primer Ent2 for enterovirus (Puig *et al*., 1994). The reaction mixture was incubated at 95°C for 5 min before the addition of the enzyme and RNAsin (Promega, Madison, U.S.A.). The temperature cycle was set for 30 min at 42°C and then 5 min at 95°C.

Enzymatic amplification of DNA and cDNA

The primers used for the detection of adenoviruses and enteroviruses were the ones proposed by Puig *et al*. (1994). Their specificity was tested and evaluated on several different adenovirus and enterovirus types (Girones *et al*., 1993; Puig *et al*., 1994).

For enzymatic amplification, 10 μl of adenoviruses extracted viral DNA or 10 μl of the enteroviruses cDNA solution was used. Amplification was carried out in a 50 μl reaction mixture containing 50 mM KCl, 10 mM Tris–HCl (pH 9.0 at 25°C), 1.5 mM $MgCl_2$, 0.01% gelatin (w/v), 0.1% Triton X-100, dNTPs at 200 μl each adenovirus primers at 0.08 μM or each enterovirus primer at 0.5 μM and 2 U of Taq DNA polymerase (Promega, Madison, U.S.A.). Thermal cycling of the amplification mixture was performed in a programmable heat block PTC-200 (MJ Research, Watertown, U.S.A.).

In all PCR assays, the first cycle in denaturation was carried out for 2 min at 94°C. The conditions for amplification consisted of denaturation at 92°C for 90 s, annealing at 55°C for 90 s and extension at 72°C for 120 s. Thirty thermic cycles were used followed by a final elongation step at 72°C for 10 min. External primers were used in the first 30 cycles of amplification.

One micro-liter of the PCR product was further added to a new batch of 50 μl of PCR mixture containing each nested primer pair at 0.16 μM for adenovirus detection and 0.20 μM for enterovirus detection in a new 30 cycle amplification (Puig *et al*., 1994). The second PCR reaction (nested PCR) was allowed to proceed for 30 cycles. The primer concentration and sequences where those adapted by Puig *et al*. (1994).

RT and PCR mixtures without DNA were used as negative controls. Twelve micro-liters of the amplified DNA mixture of the first and nested PCR were analyzed by electrophoresis on an ethidium bromide stained 1.5% Seakem Agarose (FMC Bioproducts, Rockland, U.S.A.) and on a 3% Nuisive GTG agarose gel (FMC Bioproducts, Rockland, U.S.A.) respectively, and photographed under UV light. The product of the first PCR had a length of 301 bp, whereas, that from the second nested was 143 bp long.

Bacterial culture

Three bacterial indicators (Total coliforms, Fecal coliforms and Faecal streptococci) according to EU Directive (Anon, 1976) were monitored in the coastal water samples. For each analysis the membrane filtration method was used and 100 ml were assayed. The bacteria were detected by the conventional methods proposed by Standard Methods (APHA, 1992).

Statistical analysis

The statistical analysis was performed using the student's *t*-test and comparing the presence of viral indicators and the existence or absence of faecal pollution according to EU guidelines among the samples. Any sample of which its bacterial pollution exceeded the EU guidelines was considered a faecally polluted sample.

RESULTS

Virus contamination

In our study, 120 water samples from bathing sites (''Clean'') and 30 water samples from non-bathing sites (''Dirty''), were analyzed for the presence of enteroviruses and adenoviruses.

''Clean'' areas. Twenty one (17.5%) out of 120 water samples were enterovirus positive by nested PCR while the corresponding number for adenoviruses was 34 (28.3%). Only 11 samples (9.2%) were positive for enteroviruses by cell culture technique with a mean concentration of 2.9 PFU/10 l. In two samples, enteroviruses were detected after the first amplification step while the rest were detected only when nested PCR was performed. The number of adenoviruses positive samples after the first amplification step was higher (5.8%) (Table 1).

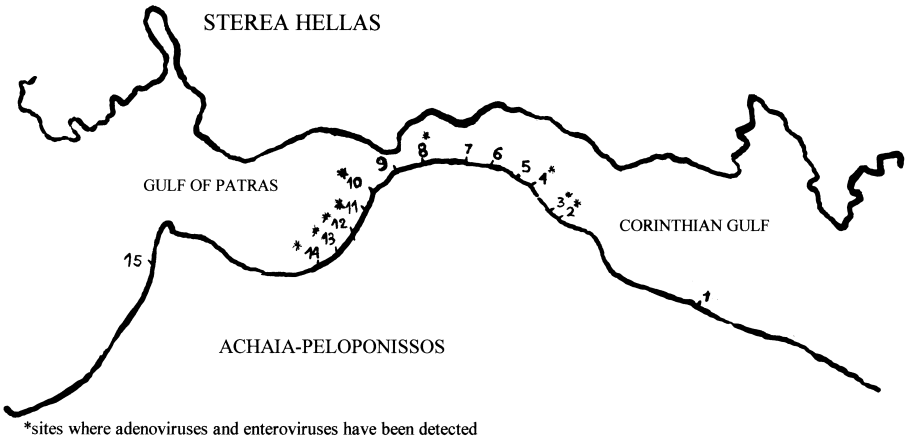
The Plate 1 depicts the sites where samples were taken. The monthly distribution of enteroviruses and adenoviruses in 1995 and 1996 in coastal waters along the Achaia district is shown in Table 2.

In site 2, enteroviruses were present in all but October 1995 and September 1996 samples while adenoviruses were present in all samples during the above mentioned period. In samples of the sites No. 3, 4, 8, 10, 11, enteroviruses and adenoviruses were found periodically. Enteroviruses were mostly present in coastal water samples collected in June during both years whereas adenoviruses isolation was at random preference (Table 2).

Table 1. Positive samples for enteroviruses, adenoviruses and faecal bacteria indicators of coastal water samples in Achaia district during a five month period (June–October) in 1995 and 1996

	Enteroviruses			Adenoviruses		F. pollution[d]
Sites	1st PCR	nPCR[a]	PFU/EV[b]	1st PCR	nPCR[a]	(CFU[c]/100 ml)
''CLEAN''	2 (1.7%)	21 (17.5%)	11 (9.2%)	7 (5.8%)	34 (28.3%)	14 (11.7%)
''DIRTY''	6 (20%)	25 (83.4%)	12 (80%)[e]	11 (36.7%)	27 (90%)	16 (53.3%)

[a]nPCR = nested Polymerase Chain Reaction.
[b]PFU/EV = Plaque forming Units formed by enteroviruses/10 l sample.
[c]CFU/100 ml = Colony Forming Units/100 ml sample.
[d]F. Pollution = Faecal Pollution according to EU regulations ($TC > 10^4$ CFU/100 ml, $FC > 2 \times 10^3$ CFU/100 ml, and $FS > 10^2$ CFU/100 ml).
[e]Only 15 out of 30 samples were checked because of the toxicity of the rest of the samples.



SITES WHERE SWIMMING WAS PROHIBITED	SITES WHERE SWIMMING WAS ALLOWED	
12. PATRA (MARINA)	1.EGIRA	7.RODINI
13. PARALIA	2.EGIO (1)	8.ARAHOVITIKA
14. MONODENDRI	3.EGIO(2)	9.RIO
	4.SELIANITIKA	10.AGYIA (1)
	5.LOGGOS	11.AGYIA (2)
	6.LAMBIRI	15.KALOGRIA

Plate 1. Sites along Achaia district where sampling was made during a five month period (June–October) in 1995 and 1996.

In coastal water samples of No. 1, 5, 6, 7, 9 and 15 sites, enteroviruses and adenoviruses were not detected.

Figures 1 and 2 demonstrate representative positive samples for enteroviruses and adenoviruses visualized after agarose gel electrophoresis.

"Dirty" areas. Twenty five (83.4%) out of 30 samples were found positive for enteroviruses by nested PCR whereas 27 (90%) were adenovirus positive. After the first amplification step, six samples (20%) were found positive for enteroviruses and 11 (36.7%) for adenoviruses (Table 1). Using cell culture, 12 out of 15 analyzed (80%) samples were positive for enteroviruses by the PFU technique (mean concentration 3.9 PFU/10 l). Fifteen samples (50%) were very toxic for the Vero cells and thus results could not be obtained.

Bacteriological results

"Clean" areas. Among the water samples analyzed, derived from "clean" sites, 14 (11.7%) out of 120 were positive for faecal contamination according to EU regulations ($TC > 10^4$ CFU/100 ml, $FC > 2 \times 10^3$ CFU/100 ml, and $FS > 100$ CFU/100 ml) (Table 3).

The comparison between the presence of fecal bacterial pollution (according to EU regulations: $TC > 10^4$ CFU/100 ml, $FC > 2 \times 10^3$ CFU/100 ml, and $FS > 100$ CFU/100 ml) and the presence of enteroviruses and adenoviruses did not show any significant correlation ($P > 0.05$).

"Dirty" areas. Sixteen samples out of 30 (53.3%) were faecally contaminated according to E.U. guidelines.

Table 2. Results from the virological analysis of water samples from sites where swimming was allowed, showing a monthly distribution of the detection of enteroviruses and adenoviruses using nested PCR

	6/95		7/95		8/95		9/95		10/95		6/96		7/96		8/96		9/96		10/96	
Site	EV	AD	EV	AD	EV	AD	EV	AD	EV	AD	EV	AD	EV	AD	EV	AD	EV	AD	EV	AD
No. 1 EGIRA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 2 EGIO(1)	+	+	+	+	+	+	+	+	−	+	+	+	+	+	+	+	−	+	+	+
No. 3 EGIO(2)	+	+	−	−	−	+	−	−	−	−	+	+	−	−	−	+	−	+	−	−
No. 4 SELIANITIKA	+	+	+	+	+	+	−	+	−	−	−	+	+	+	−	−	−	−	−	−
No. 5 LOGGOS	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 6 LAMBIRI	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 7 RODINI	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 8 ARAHOVITIKA	+	+	−	+	−	−	+	+	−	−	+	+	−	−	−	−	−	−	−	+
No. 9 RIO	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
No. 10 AGYIA(1)	+	+	−	−	−	−	−	+	−	−	+	+	−	+	+	+	−	+	−	+
No. 11 AGYIA(2)	−	−	−	−	−	−	−	−	−	−	+	−	−	−	−	+	−	−	−	−
No. 15 KALOGRIA	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−

+: Positive sample; −: Negative sample; EV: Enteroviruses; AD: Adenoviruses.

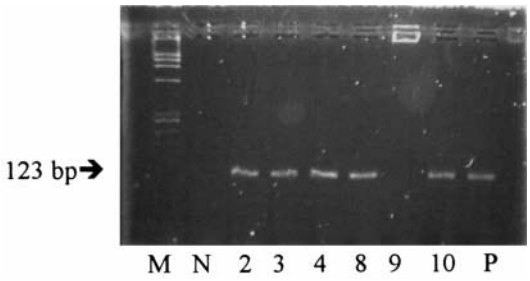


Fig. 1. Agarose gel electrophoresis of a nested PCR product of enterovirus amplification (123 bp) from coastal samples. M: marker φX174 × *Hae*III, P: positive sample with poliovirus 1, N: negative, 2, 3, 4, 8, 9, 10: Coastal water samples.

DISCUSSION

It is useful to estimate the occurrence of enteroviruses and adenoviruses in seawater, in order to assess the public health risk associated with exposure to these pathogens. Enteroviruses are important etiological agents of viral gastroenteritis. The enteric adenoviruses Ad40, Ad41, are of particular importance since they constitute a major cause of infantile gastroenteritis, second in importance after rotaviruses (Rousell *et al*., 1993) and are the only human enteric DNA viruses.

In Greece, during the bathing season, a large amount of gastroenteritis has been documented. Although there is no epidemiological evidence, the possibility exists that viruses are the etiological agents responsible for this gastroenteritis.

As even a very low number of virus particles may lead to infection when swallowed (in our cases the mean number was low 2.9 PFU/l), the danger of infection as a result of bathing in virally polluted waters is therefore not negligible (Katzenelson, 1977). Previous studies by Krikelis *et al*. (1985a, 1987) reported a total virus range in coastal waters of 5 to 145 CPU/l.

The pollution caused by enteroviruses in various environmental waters is well documented (Melnick, 1990; Muschillo *et al*., 1994). According to our results, in 17.5% of the samples from bathing areas in our region enteroviruses were found with nested PCR while only half of the samples (9.2%) were found positive for enteroviruses with cell culture techniques.

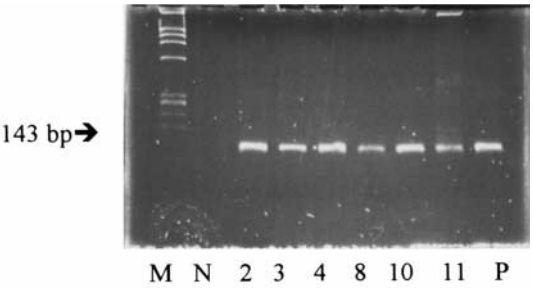


Fig. 2. Agarose gel electrophoresis of a nested PCR product of adenovirus amplification (143 bp) from coastal samples. M: marker φX174 × *Hae*III, P: positive sample with adenovirus 41, N: negative, 2, 3, 4, 8, 10, 11: Coastal water samples.

There are numerous problems associated with traditional cell culture. The sensitivity of detection by cell culture is low and the method is labor intensive, tedious and expensive (Morris and Waite, 1980; De Leon and Sobsey, 1991). Moreover, some enteric viruses, such as hepatitis A virus (HAV), rotaviruses and Norwalk virus, are difficult or impossible to cultivate. Molecular techniques can circumvent the disadvantages of the traditional cell culture method. Nested PCR has been successfully used to detect enteroviruses, polioviruses, rotaviruses, Norwalk virus, adenoviruses and HAV from stool, oysters and environmental samples (Rotbart, 1990; De Leon *et al*., 1992; Jiang *et al*., 1992; Kopecka *et al*., 1993).

The three-step concentration method described, used by us, was successfully performed after removing or inactivating the inhibitors for the PCR and RT-PCR as Puig *et al*. (1994) suggested. The nested PCR protocol that was followed, significantly increased the sensitivity of the method. It should also be pointed out that this method can be completed in 48 h, so it can serve as a powerful tool in well-organized public health laboratories.

Our study demonstrated that adenoviruses are more often detected than enteroviruses in coastal waters. Apart from their better stability and survival than that of enteroviruses in sea water (Enriquez *et al*., 1995), adenoviruses constitute the most frequently isolated virus group from the population of Greece as recorded by the Diagnostic Virology Laboratory of the Institute Pasteur in Greece (Kouloumbis *et al*., 1981). Krikelis *et al*. (1987) also reported higher amounts of adenoviruses than enteroviruses in domestic sewage in Athens. Our results are also in agreement with those of Puig *et al*. (1994) who reported a greater presence of adenoviruses in waters than enteroviruses.

The comparison between the bacterial faecal pollution (exceeding EU guidelines) and the presence of viral indicators gave no statistically significant correlation ($P > 0.05$). It seems that in our geographical region, the presence of enteric viruses is not linked to the presence of *Escherichia coli* or other microorganisms used as indicators in Public Health. Our results are, also, in agreement with those of Wyer *et al*. (1995) who reported poor statistical relationships between concentrations of bacterial indicators of sewage pollution and enterovirus in marine waters. On the contrary, our results are in conflict with those of Merrett-Jones *et al*. (1991) who suggested a highly significant correlation between enterovirus and indicator bacteria concentration and with Gilgen *et al*. (1995) whose findings confirmed that virus contamination was found only in samples that were positive for *E. coli*.

Table 3. Results from the virological and bacteriological analysis of water samples from bathing sites, showing a monthly distribution of the detection of enteroviruses using cell culture techniques and of bacterial indicators using traditional culture methods

Site	6/95				7/95				8/95				9/95				10/95			
	TC	FC	FS	EV	TC	FC	FS	EV	TC	FC	FS	EV	TC	FC	FS	EV	TC	FC	FS	EV
1	0	0	30	0	6	3	3	0	0	0	0	0	0	0	1	0	1	0	0	0
2	87	16	30	3	10^6	10^6	98	6	8	0	10	1.2	10	11	60	0	25	5	10	0
3	10	4	6	0	7	3	95	0	0	0	13	0	20	12	15	0	20	10	12	0
4	50	40	50	3.6	22	4	1	0	12	3	3	0	25	5	10	0	30	2	10	0
5	20	8	11	0	0	0	0	0	13	0	1	0	12	5	8	0	5	0	3	0
6	84	27	100	0	1	0	0	0	10	1	3	0	5	1	0	0	1	1	0	0
7	27	0	1	0	4	0	2	0	18	2	0	0	15	3	5	0	12	0	0	0
8	56	18	120	0	45	36	56	0	10^5	10^5	10^3	0	10^5	10^5	10^5	3	120	40	50	0
9	6	5	1	0	4	4	48	0	6	1	0	0	8	2	0	0	2	1	0	0
10	26	16	50	0	54	32	68	0	10^5	10^5	30	0	250	160	40	0	200	150	30	0
11	40	30	20	0	45	14	46	0	10^5	10^5	40	0	50	40	20	0	42	35	20	0
15	3	0	2	0	30	0	0	0	4	4	0	0	4	0	0	0	2	0	0	0
Site	6/96				7/96				8/96				9/96				10/96			
	TC	FC	FS	EV	TC	FC	FS	EV	TC	FC	FS	EV	TC	FC	FS	EV	TC	FC	FS	EV
1	30	0	5	0	1	1	0	0	25	0	12	0	30	5	0	0	0	0	0	0
2	60	25	45	3	30	28	40	0	42	20	25	3	100	25	80	0	0	0	6	0
3	10^6	10^6	120	8	12	5	21	0	30	8	40	0	10^6	0	10^6	0	0	0	13	0
4	14	0	5	0	0	0	38	1.8	30	3	20	0	3	1	1	0	32	0	13	0
5	8	5	1	0	0	0	0	0	0	0	1	0	3	0	0	0	13	0	1	0
6	18	2	3	0	22	11	33	0	5	0	4	0	4	0	1	0	10	1	3	0
7	8	0	13	0	3	0	10	0	15	2	24	0	2	0	4	0	18	2	0	0
8	200	80	112	4.8	11	5	8	0	18	3	7	0	18	0	7	0	10^3	10^3	100	0
9	0	0	3	0	0	0	0	0	0	0	35	0	0	0	4	0	6	1	0	0
10	26	16	50	0	10	8	25	0	10^5	10^5	40	2	26	16	50	0	10^5	10^5	40	0
11	180	60	20	0	20	15	28	0	26	14	25	0	22	10	90	0	26	14	120	0
15	4	0	5	0	0	0	1	0	4	4	0	0	0	0	15	0	4	3	0	0

TC (Total coliforms cfu/100 ml), FC (Faecal coliforms cfu/100 ml), FS (Faecal streptococci cfu/100 ml), EV (enteroviruses PFU/10 L). Sites are the same as shown in Table 2.

Since faecal indicators for monitoring the health hazards in recreational waters, in our region, have low predictive capability for the presence of viruses and since viruses are pathogenic microorganisms with public health concerns, the available methodology for the detection and enumeration of these pathogen microorganisms should be implemented in laboratories dealing with marine pollution on a routine basis. This implementation faces a number of problems (very small size of the viruses, difficult concentration techniques, uncultured viruses). A probable solution to these problems is associated with the use of molecular techniques, which have so far yielded satisfactory results in the detection of pathogen environmental microorganisms.

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