EVALUATION OF VIROLOGICAL QUALITY OF SEWAGE FROM FOUR BIOLOGICAL TREATMENT PLANTS BY A NESTED-PCR TECHNIQUE

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Abstract. In order to determine the virological quality of sewage from four biological treatment plants in Greece (two in the city of Athens and two in the city of Patras), ninety-two raw sewage samples were analyzed for the presence of enteroviruses and adenoviruses, during the period of October 2000 to February 2003. A nested-PCR method was used in order to increase the sensitivity of virus detection. Enteroviruses were detected in 43 samples (47%) and adenoviruses in 75 samples (81.5%) of raw sewage by nested PCR. The more frequent isolation of adenoviruses in raw sewage indicates their stability as virological indicators of the pollution of the environment and their more persistence in sewage.

Keywords: adenoviruses, enteroviruses, sewage, nested-PCR

1. Introduction

Enteric viruses have been associated with outbreaks of waterborne nonbacterial gastroenteritis and are of important concern for public health (1). Significant number of viruses can be isolated from feces and urine of humans as well as from sewage and polluted waters (8, 15).

The presence of enteroviruses in the environment is a public health hazard even when very few viral particles are present (12). Human enteroviruses infect millions of people worldwide each year. In the United States, enteroviruses are responsible for 30,000 to 50,000 meningitis hospitalizations per year as a result of 30 million to 50 million infections (10).

Adenoviruses are also pathogenic to humans and their presence in the environmental samples (polluted waters) may cause infections. Like rotaviruses, adenoviruses are causative agents of gastroenteritis (3, 16). Adenoviruses are the only human enteric viruses to contain DNA. Many adenoviruses serotypes are difficult to culture in regular cell lines. For this reason and because adenoviruses are slow growing, their presence in polluted water and their role as originators of gastroenteritis have probably been underestimated (12).

The development of nucleic acid-based methods has facilitated the detection of viruses that replicate poorly or not at all in cell cultures (11). In the present study, a nested RT-PCR method, developed by Puig et al., (1994), was used with minor modifications (11).

This study was the first research effort in Greece to detect enteroviruses and adenoviruses in raw sewage of four biological treatment plans (two in the city of Athens and two in the city of Patras, Greece) in order to evaluate the virological quality of sewage of these plants (17).

2. Methods

2.1 VIRUSES

Adenoviruses (Ad2 prototype) and Ad40 and Ad41 were provided by Dr. Annika Allard from the University of Umea, Sweden, Polioviruses type 1,2,3 were provided by Prof. Dalaina from the University of Thessaloniki, Greece.

2.2 SAMPLE COLLECTION

The presence of enteroviruses and adenoviruses in raw sewage was examined for three years (2000-2003). Sampling took place once a month by experienced personnel. Samples of 1L of raw sewage were collected and placed in sterile containers. Following sampling, the containers were shipped on ice to the laboratory and stored frozen at –70^{o}C.

2.3 RECOVERY OF VIRAL PARTICLES FOR NUCLEIC ACID EXTRACTION

The methods used for the recovery of viral particles were chosen on the basis of studies (5, 11, 12). Briefly, 40 ml sample was ultracentrifuged (229,600 x g for 1h at 4^{o}C) to pellet all the viral particles together with any suspended material. The sediment was eluted by mixing it with 5ml of 0.25 N glycine buffer (pH 9.5) on ice for 30 min, and the suspended solids were separated by centrifugation (12,000x g for 15min) after the addition of 5ml of 2 X PBS. The viruses were then pelleted by ultracentrifugation (229,600 x g for 1h at 4^{o}C), resuspended in 100μl of 1 X PBS, and stored at –70^{o}C.

2.4 NUCLEIC ACID EXTRACTION

The method proposed by Boom et al., 1990 with the modifications proposed by Puig et al., 1994 was applied. The procedure used 50 μl of viral suspension. The sample was added to a mixture of 40μl of the silica particle suspension and 990μl of lysis buffer (120g of GuSCN in 100ml of 0.1 M Tris-HCl, pH 6.4, with 22ml of 0.2M EDTA adjusted with NaOH to pH 8.0 and 2.6g of Triton X-100 added) left for 10 min at room temperature, and washed twice in 1ml of washing buffer, twice more in ethanol 70% and once in acetone. The pellet obtained after the complete evaporation of acetone was re-suspended in 50 μl of 10mM Tris-HCl, pH 7.6, 0.1 mM EDTA, pH 8.0-1mM DTT with Rnase inhibitor (Promega, Madison, U.S.A) (5U μL^{-1} final concentration) in order to allow nucleic acid elution from silica particles. The resulting supernatant was used in cDNA synthesis for enteroviruses and PCR amplification for adenoviruses.

2.5 ENZYMATIC AMPLIFICATION

The reaction mixture for reverse transcription contained 5μl of the nucleic acids extracted plus 1.5mM $MgCl_2$, 1XPCR buffer II (Perkin-Elmer Roche, Inc.) containing 10mM Tris-HCl (pH 8.3 at 25^{o}C), 50mM KCl, 200μM concentrations of each deoxynucleotide triphosphate and the corresponding external primer for enterovirus (0.35μM Ent2) in a total volume of 10μL. The reaction mixture was incubated at 95^{o}C for 5 min before the addition of 50U of Moloney Murine Leukemia Virus reverse transcriptase (Perkin-Elmer Roche, Inc.) and 10U of Rnase inhibitor (Perkin-Elmer Roche, Inc.). Temperature was cycled as follows:30min at 42^{o}C and 5min at 95^{o}C.

For a typical one step reaction, 10μl of external viral DNA was used for adenoviruses and 10μl of the c DNA solution was used for enteroviruses. Amplification was carried out in a 50μl reaction mixture containing 1.5mM $MgCl_2$, 1XPCR buffer II (Perkin-Elmer Roche, Inc.) containing 10mM Tris-HCl (pH 8.3 at 25^{o}C), 50mM KCl, 200μM concentrations of each deoxynucleotide triphosphate, 0.08μM each adenovirus primer or 0.15μM of the enterovirus primer and 2U of Ampli Taq DNA polymerase (Perkin-Elmer Cetus). Thermal cycling of the amplification mixture was performed in a programmable heat block (PTC-200, MJ Research, U.S.A). In all PCR assays, the first cycle of denaturation was carried out for 4min at 94^{o}C.

The conditions for the amplification were denaturing at 92^{o}C for 90s, annealing at 55^{o}C for 90s and extension at 72^{o}C for 120s.

One micro-liter was further add to a new batch of 50μl of PCR mixture containing each nested primer pair at 0.16μM for adenovirus detection and 0.20μM for enterovirus detection in a new 30 cycle amplification (11). The results were analysed by gel electrophoresis on a 2% NuSieve GTG plus 1% Seakem ME agarose gel (FMC Bioproducts, Rockland, Maine) that was stained with ethidium bromide.

3. Results

Table 1 illustrates the results obtained by the analysis of raw sewage samples. Enteroviruses were detected in 43 samples (47%) and adenoviruses in 75 samples (81.5%) of raw sewage (from four biological treatment plants in Greece). Table 2 illustrates the positive samples (%) of the analysis of raw sewage from the four biological treatment plants in Greece (sampling areas 1, 2, 3 and 4). In Figures 1-2 the bands obtained by nested-PCR in positive samples for adenovirus and enterovirus are shown.

4. Conclusions

In this study, 92 raw sewage samples collected monthly in 2000-2003, from four biological treatment plants in Greece were analyzed. The results obtained by nested PCR amplification showed that the adenoviruses were the most often detected human viruses. They were present in 75 (81.5%) of the samples. On the other hand enteroviruses were present in 43(47%) of the samples.

Enteroviruses and adenoviruses were chosen because they have been used as a parameter for evaluating the viral pollution of the environment (13). Adenoviruses are the only human enteric viruses to contain DNA and are also important human pathogens (7). Enteroviruses can be isolated and quantified as PFU in cell culture although many adenoviruses serotypes are difficult to culture in regular cell lines. Our purpose was to perform a complete procedure for virus concentration from raw sewage and the detection of the specific viruses by amplification of DNA and cDNA with the appropriate primers, in order to overcome the problems of the cultivation methods concerning mostly adenoviruses. The results of our study indicate that adenoviruses detection by PCR is a better index for human contamination than detection of the presence of enteroviruses. One of the reasons is the high number of samples that are positive for adenoviruses but negative for enteroviruses. A second reason is that adenoviruses are more stable in various environments (including wastewater) than enteroviruses (4, 6, 9, 14).

The detection of enteroviruses and adenoviruses in raw sewage with this specific, fast and sensitive method is a valuable indicator of quality control of the process made by the biological treatment plant as it can be used for the examination of treated effluents as well as raw sewage samples.

Samples No	Sampling Area	Dates	Enteroviruses Nested PCR	Adenoviruses Nested PCR
1	4	10/2000	+	+
2	3	10/2000	+	+
3	1	10/2000	+	+
4	2	10/2000	+	+
5	1	11/2000	+	+
6	2	11/2000	+	+
7	4	11/2000	-	+
8	3	11/2000	+	+
9	1	12/2000	+	+

10	2	12/2000	+	+
11	4	12/2000	+	+
12	3	12/2000	+	-
13	1	1/2001	+	+
14	2	1/2001	-	+
15	4	1/2001	-	+
16	3	1/2001	+	+
17	1	2/2001	-	+
18	2	2/2001	+	-
19	4	2/2001	+	+
20	3	2/2001	-	+
21	1	3/2001	+	-
22	2	3/2001	-	+
23	4	3/2001	+	+
24	3	3/2001	+	+
25	1	4/2001	-	+
26	2	4/2001	+	-
27	4	4/2001	+	+
28	3	4/2001	+	-
29	3	5/2001	-	-
30	4	5/2001	+	+
31	1	5/2001	+	+
32	2	5/2001	+	+
33	1	6/2001	-	-
34	2	6/2001	+	+
35	3	6/2001	-	+
36	4	6/2001	-	+
37	3	7/2001	-	+
38	4	7/2001	-	+
39	1	8/2001	+	+
40	2	8/2001	-	+
41	3	8/2001	+	+
42	4	8/2001	+	+
43	4	9/2001	+	+
44	3	9/2001	+	+
45	1	9/2001	+	+
46	2	9/2001	-	+
47	3	11/2001	-	+
48	4	11/2001	-	+
49	1	12/2001	-	+
50	2	12/2001	-	+
51	3	12/2001	-	+
52	4	12/2001	-	+
53	4	1/2002	-	+
54	3	1/2002	-	+
55	1	1/2002	+	+
56	2	1/2002	+	+
57	3	2/2002	+	+
58	4	2/2002	+	+
59	1	2/2002	+	-
60	2	2/2002	+	+
61	3	3/2002	+	+
62	4	3/2002	+	+
63	1	3/2002	-	+
64	2	3/2002	+	+
65	3	4/2002	-	+
66	4	4/2002	+	+
67	1	4/2002	-	+
68	2	4/2002	-	+
69	3	5/2002	-	+
70	4	5/2002	-	+
71	1	5/2002	+	+
72	2	5/2002	-	+
73	2	6/2002	-	+
74	1	6/2002	-	+
75	2	7/2002	-	+
76	4	7/2002	-	+
77	3	7/2002	-	+
78	3	8/2002	+	+
79	4	8/2002	+	+
80	1	8/2002	-	-
81	3	9/2002	-	+
82	2	9/2002	-	+
83	1	9/2002	-	-
84	4	10/2002	-	+
85	2	10/2002	-	-

86	1	10/2002	-	-
87	1	12/2002	-	-
88	2	12/2002	-	-
89	1	1/2003	-	+
90	2	1/2003	-	-
91	2	2/2003	-	-
92	1	2/2003	-	-

Table 1. Results from the analysis of sewage samples during 2000-2003
+: Positive samples; -: Negative samples; 1: Rio, Patras; 2: City of Patras; 3: City of Athens; 4: Metamorfosi, Athens

Sampling Area	Enteroviruses Positive Samples %	Adenoviruses Positive Samples %
1	45.83	66.67
2	41.67	75.00
3	50.00	86.36
4	54.54	100.00

Table 2. Positive samples % from the four biological treatment plants in Greece.1: Rio, Patras; 2: City of Patras; 3: City of Athens; 4: Metamorfosi, Athens

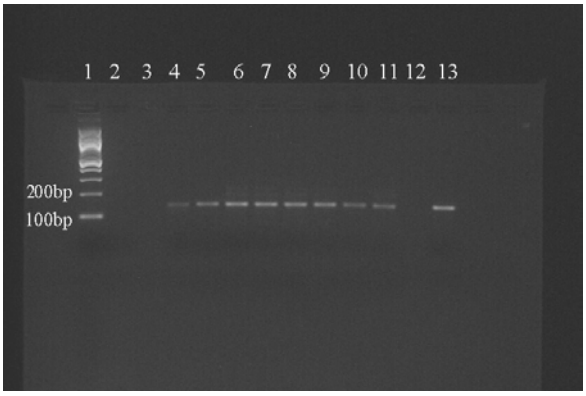

Figure 1. Electrophoresis in agarose gel showing the amplified PCR-product 143bp of nested-PCR of adenoviruses. Lane 1: Marker 100bp DNA Ladder, Lane 2: Negative control, Lane 4-11: Positive sewage samples for adenovirus, Lane 13: Positive control.

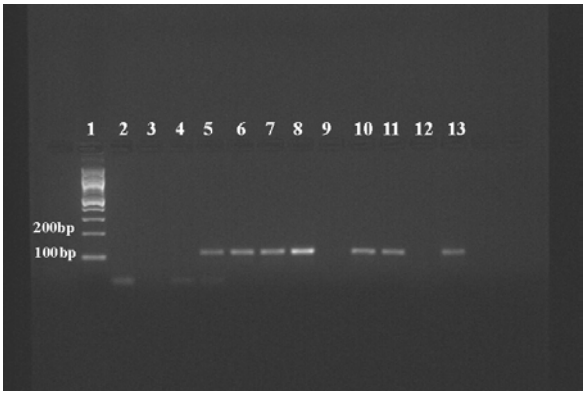

Figure 2. Electrophoresis in agarose gel showing the amplified PCR-product 123bp of nested-PCR of enteroviruses. Lane 1: Marker 100bp DNA Ladder, Lane 2: Negative control, Lane 4-11: Positive and Negative sewage samples for enterovirus, Lane 13: Positive control.

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