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Detection of Astroviruses, Enteroviruses, and Adenovirus Types 40 and 41 in Surface Waters Collected and Evaluated by the Information Collection Rule and an Integrated Cell Culture-Nested PCR Procedure

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We evaluated the use of an integrated cell culture-reverse transcription-PCR (ICC-RT-PCR) procedure coupled with nested PCR to detect human astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface water samples that were collected and evaluated by using the Information Collection Rule (ICR) method. The results obtained with the ICC-RT-PCR–nested PCR method were compared to the results obtained with the total culturable virus assay–most-probable-number (TCVA-MPN) method, the method recommended by the U.S. Environmental Protection Agency for monitoring viruses in surface and finished waters. Twenty-nine ICR surface water samples were analyzed. Viruses were concentrated by using filter adsorption-beef extract elution and organic flocculation techniques, and then the preparations were evaluated for viruses by visualizing cytopathic effects in the Buffalo green monkey kidney (BGMK) cell line. In the ICC-RT-PCR–nested PCR technique we used Caco-2 cells to propagate astroviruses and enteroviruses (ICC step), and we used BGMK cells to propagate adenovirus types 40 and 41, as well as enteroviruses. Fifteen of the 29 samples (51.7%) were positive for astrovirus as determined by the ICC-RT-PCR–nested PCR method, and eight of these samples (27.5%) contained infectious astrovirus. Seventeen of the 29 samples (58.6%) were positive for enteroviruses when the BGMK cell line was used, and six (27.6%) of these samples were determined to be infectious. Fourteen of the 29 samples (48.3%) were positive for adenovirus types 40 and 41, and 11 (37.9%) of these samples were determined to be infectious. Twenty-seven of the 29 samples (93.1%) were positive for a virus, and 19 (68.9%) of the samples were positive for an infectious virus. Only 5 of the 29 samples (17.2%) were positive as determined by the TCVA-MPN method. The ICC-RT-PCR–nested PCR method provided increased sensitivity compared to the TCVA-MPN method.

Surface water and groundwater continue to be affected by fecal contamination originating from a variety of human-associated sources in both rural and urban areas (20). Numerous studies have documented the presence of enteric viruses in both raw water and treated drinking water (2, 9, 10). In 1996, the Environmental Protection Agency enacted the Information Collection Rule (ICR). The ICR required that all water utilities that serve more than 100,000 households monitor their source water for viruses every month for 18 months (6). According to the ICR, the total culturable viruses had to be detected and enumerated by the total culturable virus assay–most-probable-number (TCVA-MPN) method (6). This method detects viruses on the basis of expression of viral cytopathic effects (CPE) in Buffalo green monkey kidney (BGMK) cells. However, the total level of viral contamination is greatly underestimated when the BGMK cell line is used alone. Several enteric viruses, including adenovirus types 40 and 41, do not produce CPE during their replication cycles, while other enteric viruses, such as the human astroviruses, cannot replicate in the BGMK cell line.

No single cell culture system can be used for all human enteric viruses. Some human enteric viruses replicate in cell cultures without producing apparent CPE (4, 15), and many of the epidemiologically important enteric viruses cannot be propagated in cell cultures (4, 21). Human astroviruses cannot replicate in the BGMK cell line, and for both astroviruses and adenoviruses proteolytic enzymes must be present for infections to occur in permissive cell lines. Adenovirus infections occur year-round, and there is little or no seasonal variation in shedding (3). Adenoviruses, particularly types 40 and 41, are considered second only to rotaviruses as the primary causes of gastroenteritis in children (3). These epidemiologically important viruses are not detected by the TCVA-MPN method.

Astroviruses are small (diameter, 28 to 30 nm), nonenveloped, positive-sense, single-stranded RNA viruses (13). Astrovirus (HAst) infections occur worldwide, mainly in young children and the elderly. By the age of 10 years, 80% of the population shows evidence of past infection (11). Outbreaks of astrovirus-associated gastroenteritis are being reported with increasing frequency (14). Astroviruses are transmitted via the fecal-oral route, and outbreaks have been associated with the consumption of sewage-polluted shellfish (11), as well as the ingestion of water from streams polluted with feces (5). In a previous study 70% of the environmental samples analyzed from areas in South Africa were positive for human astroviruses (12).

The limited sensitivity of detection of some enteric viruses by conventional tissue culture methods has prompted the search for new procedures, such as molecular techniques. Amplification of virus sequences in a cell culture followed by detection by reverse transcription-PCR (RT-PCR) both in-

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creases the number of copies of target nucleic acids and includes an infectivity assay (17). In this study we used an integrated cell culture (ICC)-RT-PCR procedure coupled with nested PCR to detect enteroviruses, human astroviruses, and adenovirus types 40 and 41 in 29 surface water samples and optimized this technique.

MATERIALS AND METHODS

Virus types and cell lines. Astrovirus serotype 2 (HAst-2; kindly provided by Dorsey Bass) was propagated in Caco-2 cells as previously described (1). Briefly, the astrovirus HAst inoculum was preactivated with a solution containing 10 μg of trypsin (catalog no. T-0134; Sigma) per ml. Caco-2 cells were washed and infected with the inoculum for 90 min, and 5 ml of serum-free maintenance medium containing 5 μg of trypsin per ml was added. Poliovirus LsC-1 was propagated in BGMK cells grown in Eagle minimal essential medium (MEM) containing L-15, HEPES, and 8% NCS. The infection procedure described by Pinto et al. was used to propagate adenovirus types 40 and 41 in BGMK cells (15, 16); the procedure used was the same procedure that was used for astrovirus HAst.

ICR surface water samples. Twenty-nine surface water samples were filtered and collected by using type 1 MDS Zetapor cartridge filters (Cuno) and the method described in the Environmental Protection Agency (6). Viruses were eluted from the filters with beef extract (BBL Beef extract Powder V) (pH 9.5) and were concentrated by organic flocculation (6). Each sample concentrate was passed through a beef extract-treated 0.22-μm-pore-size syringe filter to remove any microbial contaminants prior to inoculation of a cell culture.

TCVA-MPN method. Twenty-nine samples were evaluated by using BGMK cells between passages 117 and 250 and the ICR (6). A portion of each sample was rapidly thawed. The inoculum volume was determined by dividing the assay sample volume by 20. Ten flasks were then inoculated with an amount of sample equal to the amount of inoculum previously calculated. If there was no evidence of cytotoxicity and if at least three flasks were negative for CPE after 7 days, another aliquot was thawed and inoculated along with the amount described above into 10 new flasks containing confluent monolayers of BGMK cells. The negative controls consisted of 0.15 M sodium phosphate buffer (Na_2HPO_4) at pH 7.0 to 7.5. A positive control consisting of 20 PFU of poliovirus LsC-2 per ml was inoculated into three flasks. Five milliliters of serum-free cell culture maintenance medium was added to each flask after the infection incubation step. Each flask was examined daily for CPE for the first 3 days and then every second day for a total of 14 days.

Flasks were freeze-thawed when more than 75% of the monolayer exhibited CPE. All other flasks were frozen on day 14 even if no CPE were observed, and all flasks were subsequently thawed. Ten percent of each thawed medium was used as the inoculum for a second passage. Flasks in which CPE developed were scored (for both the first passage and the second passage). The contents of second-passage flasks that exhibited CPE were passaged a third time and scored as well. The resulting numbers were then entered into a MPN program to determine the MPN/100 liters.

Surface water sample infection protocol for enteroviruses. Confluent monolayers of BGMK cells in T-25-cm^2 flasks were washed three times with serum-free Eagle MEM. A 500-μl portion of each sample concentrate was inoculated into each flask. The flasks were incubated for 90 min at 37°C and were rocked every 20 min. After incubation, 5 ml of serum-free Eagle MEM was added to each flask, and the cells were to incubated for 5 days at 37°C.

Surface water sample infection protocol for astroviruses. Portions (500 μl) of each sample concentrate were inoculated onto semiconfluent monolayers of Caco-2 cells between passages 90 and 100 in T-25-cm^2 flasks. It has been found previously that Caco-2 cells support growth of many viruses, including enteroviruses, rotaviruses, adenoviruses, and especially astroviruses (17). The infection protocol used was previously described (1). The resulting flasks were also used to detect enteroviruses.

Surface water sample infection protocol for adenovirus types 40 and 41. The infection protocol used for adenovirus types 40 and 41 was similar to the astrovirus infection protocol described above; however, confluent monolayers of BGMK cells were used. The infected flasks were incubated for 5 days at 37°C.

Enterovirus RT-PCR–nested PCR protocol. Enterovirus RNA was detected in infected monolayers of Caco-2 and BGMK cells by the RT-PCR–nested PCR method. The RT primer and PCR primers used have been described by Puig et al. (18). The RT primer sequence was 5′-ATTGTCACCATAAGCAGCCA-3′, and the PCR primer sequence was 5′-CGGTACCTTTGTACGCCTGT-3′. Eleven microliters of an infected cell suspension was heated at 99°C for 8 min to disrupt the virions and then placed on ice. The RT-PCR procedure used was adapted from the procedure described by Grinde et al. (8). An RT reaction mixture was added to 70 μl of a PCR master mixture as described by Grinde et al. (8). The mixture was initially denatured at 95°C for 5 min and then subjected to 35 cycles consisting of 99°C for 1 min, 55°C for 1 min, and 72°C for 1 min. The final extension step consisted of 72°C for 7 min.

The nested PCR was performed immediately after the RT-PCR was performed. One microliter of each RT-PCR mixture was added to a new PCR tube containing 90 μl (final volume) of a solution containing 8 mM $MgCl_2$, 10 μl of 10× buffer (Perkin-Elmer), each deoxynucleoside triphosphate at a concentration of 1 mM, 1 μM primer 5′-TCCGGCCCCTGAATGCGGCTA-3′, and 1 μM primer 5′-GAAACACGGACACCCAAAGTA-3′. The following temperature program was used: 35 cycles consisting of 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Twelve microliters of each sample was placed on a 1.8% agarose gel and visualized by ethidium bromide staining. The RT-PCR primers used yielded 534-bp amplicon. The nested primers yielded a 138-bp amplicon. Molecular weights were determined by comparison with a 1-kb DNA ladder (Life Technologies).

Astrovirus RT-PCR–nested PCR protocol. An astrovirus ICC-RT-PCR–nested PCR procedure was performed by using suspensions of infected Caco-2 cells as described above; the RT primer used was 5′-GTAAGATTCCCAGATTGGT-3′, and the PCR primer used was 5′-CCTGCCCCGAGAACAACCAAG-3′. The RT primer was used in the RT procedure, and both primers were used in the PCR procedure. The procedure used was similar to the enterovirus RT-PCR procedure, except that the following PCR temperature profile was used: a hot start consisting of 95°C for 5 min, followed by 35 cycles consisting of 95°C for 30 s, 56°C for 30 s, and 72°C for 30 s and a final extension step consisting of 72°C for 7 min.

The procedure used was essentially the procedure described above (enterovirus nested PCR protocol); however, the primers utilized were primers A1 (5′-CCTTGCCCCGAGCCAGAA-3′) and A2 (5′-TATTCACAAACTTATGGCAA-3′). Each sample was visualized on a 1.8% agarose gel stained with ethidium bromide. The RT-PCR primers yielded 243- and 193-bp amplicons. These primers amplified a 243-bp region for wild-type human astroviruses and a smaller 193-bp amplicon for astroviruses that had an adaptive deletion. The nested primers yielded a 143-bp amplicon.

Adenovirus RT-PCR–nested PCR protocol. Adenovirus nucleic acid was detected with primers that are specific for adenovirus types 40 and 41. The primers used were the following primers described by Puig et al. (18): hexAA1885 (5′-GCCGCAGTGGTCTTACATGCACATC-3′) and hexAA1913 (5′-CAGCACGCCGCGGATGTCAAAGT-3′). An 11-μl sample from each infected flask was denatured with 0.5 μl of 0.05 M EDTA at 99°C for 8 min. A 90-μl (final volume) mixture containing the following constituents was added to the denatured sample: 7 mM $MgCl_2$, 10 μl of 10× buffer (Perkin-Elmer), each deoxynucleoside triphosphate at a concentration of 1 mM, and each primer at a concentration of 1 μM.

The nested procedure used was essentially the procedure described above; however, the primers utilized were primers hexAA1893 (5′-GCCACCGAGACGTACTTCAGCCTG-3′) and hexAA1905 (5′-TTGTACGAGTACGCGGTATCCTCGCGGTC-3′). Each sample was visualized on a 1.8% agarose gel stained with ethidium bromide. The PCR primers yielded a 301-bp amplicon. The nested primers yielded a 143-bp amplicon.

RT-PCR–nested PCR analysis of sample concentrates. Two 10-μl aliquots were obtained from each sample concentrate, and two direct RT-PCR–nested PCR were performed with each sample concentrate. In one reaction 10^2 RT-PCR units of virus was seeded into the concentrate as a quality control measure in order to evaluate inhibition of the reaction by agents present in samples. The other reaction was a negative control reaction performed to demonstrate that viral detection was not possible without amplification in cell culture.

For the samples that were positive for virus as determined with sample concentrates and cell culture supernatants, 1:5 dilutions of each sample concentrate and cell culture supernatant were prepared and assayed for virus. The dilutions were then compared. If the sample concentrate was negative after dilution but the cell culture supernatant remained positive, the sample contained infectious virus. If both the sample concentrate and the cell culture supernatant were positive after dilution, it was not possible to determine if the sample contained infectious virus. Positive PCR results were determined by visualization of amplicons on 1.8% agarose gels stained with ethidium bromide (10 μg/ml).

Quality control of the amplification methods. To reduce the chance of sample contamination by extraneous amplified DNA molecules, we used certain precautions, such as using aerosol-resistant tips, treating pipettors with ethanol between sample collection procedures, and decontaminating instruments between reaction sets. Additionally, negative controls consisting of mock-infected cell culture supernatants and sample water were included in each reaction set (a negative control was defined as an amplification reaction mixture that had the same components as a test sample tubes but contained no virus). Two positive controls containing viral samples and a cell culture-positive control were included in each reaction set.

RESULTS

TCVA-MPN analysis. A total of 29 water samples were analyzed for enteroviruses by the TCVA-MPN procedure performed with BGMK cells, as recommended by the ICR. Five of the 29 samples (17.2%) exhibited CPE.

Detection capabilities of the ICC-RT-PCR–nested PCR procedure with ICR samples. A total of 29 water samples were analyzed by the ICC-RT-PCR–nested PCR procedure for enteroviruses, human astroviruses, and adenovirus types 40 and

TABLE 1. Surface water results for detection of human astroviruses

Site	Concentrate			ICC with Caco-2 cells			ICR TCVA-MPN/100 liters[d]
	RT-PCR[a]	Nested PCR		RT-PCR[d]	Nested PCR		
		10^{1}[b]	1:5[c]		10^{1}[e]	1:5[f]	
1	−[g]	−	NA	−	−	NA	
2	−	−	NA	−	+	NA	
3	+	+	+	+	+	+	2.11 (0.08–9.41)[h]
4	−	−	NA	−	−	NA	
5	−	+	+	−	+	+	
6	−	−	NA	−	+	NA	
7	−	−	NA	−	+	NA	
8	−	−	NA	−	−	NA	1.03 (0.18–3.48)
9	−	−	NA	−	−	NA	2.35 (0.8–7.43)
10	−	−	NA	−	+	NA	
11	−	−	NA	−	−	NA	
12	I	I	I	−	+	NA	
13	−	−	NA	+	+	NA	
14	−	−	NA	−	+	NA	
15	I	I	I	+	+	NA	
16	I	I	I	+	+	NA	
17	−	+	−	−	+	+	
18	−	−	NA	−	−	NA	
19	−	−	NA	−	−	NA	
20	−	−	NA	−	−	NA	1.03 (0.19–3.47)
21	−	−	NA	−	−	NA	
22	−	+	−	+	+	+	
23	−	−	NA	−	−	NA	3.23 (0.18–7.39)
24	I	I	I	−	+	NA	
25	I	I	I	−	+	NA	
26	−	−	NA	−	−	NA	
27	−	−	NA	−	−	NA	
28	−	−	NA	−	−	NA	
29	−	−	NA	−	−	NA	

[a] One of 29 samples (3.4%) was positive.
[b] Four of 29 samples (13.7%) were positive.
[c] Two samples were positive.
[d] Five of 29 samples (17.2%) were positive.
[e] Fifteen of 29 samples (51.7%) were positive.
[f] Four samples were positive.
[g] −, negative; +, positive; NA, not applicable; I, inhibitory for the RT-PCR–nested PCR as determined by seeded experiments.
[h] The values in parentheses are confidence intervals.

41. Fifteen of the 29 samples (51.7%) were positive for astroviruses when the Caco-2 cell line was used (Table 1). However, if an ICC step was not used, only 13.7% of the sample concentrates were positive for astroviruses. If a nested PCR was not performed, only 17.2% of the cell culture supernatants and only 3.4% of the sample concentrates were positive. A nested PCR was required to increase the assay sensitivity and to increase the specificity.

Five of the 29 samples (17.2%) were positive for enteroviruses as determined by the ICC-RT-PCR–nested PCR method when Caco-2 cells were used (Table 2). However, when BGMK cells were used instead of Caco-2 cells, 17 of the 29 samples (58.6%) were positive for enteroviruses (Table 3). Only 44.8% of the sample concentrates were positive for enteroviruses when no cell culture step was used. When no nested PCR was used, no sample concentrates were positive, and 6 of the 29 samples (20.6%) (BGMK cells) or 2 of the 29 samples (6.8%) (Caco-2 cells) of cell culture supernatants were positive as determined by RT-PCR alone.

Fourteen of the 29 samples (48.3%) were positive for adenovirus types 40 and 41 when ICC-RT-PCR–nested PCR was used with the BGMK cell line for viral amplification (Table 4). Three of the sample concentrates (10.3%) and four of the cell culture supernatants (13.7%) were positive for adenoviruses as determined by RT-PCR–nested PCR performed with the sample concentrates. No sample concentrate was positive as determined by RT-PCR alone, and only 13.7% of the cell culture supernatants were positive as determined by RT-PCR alone.

Dilution of samples for astrovirus analysis. All samples that were positive as determined by the ICC method, including sample concentrates, were serially diluted (1:5) to determine if viral replication was taking place. We found that 8 of the 29 samples were positive for infectious astroviruses. Five sample concentrates inhibited the RT-PCR, and two samples were positive after dilution of both the sample concentrate and the cell culture supernatant; therefore, the infectious nature of the astroviruses present could not be determined, as shown in Table 5.

Dilution of samples for Enterovirus analysis. We found that 6 of the 29 samples contained infectious enteroviruses (the data include both the BGMK data and the Caco-2 data). Another 13 samples (both sample concentrates and cell culture supernatants) were positive after two serial dilutions. Only one sample concentrate inhibited the RT-PCR–nested PCR per-

TABLE 2. Surface water results for detection of enteroviruses with the Caco-2 cell line

Site	Concentrate			ICC with Caco-2 cells			ICR TCVA-MPN/100 liters[d]
	RT-PCR[a]	Nested PCR		RT-PCR[b]	Nested PCR		
		10^{1}[b]	1:5[c]		10^{1}[d]	1:5[c]	
1	−[e]	−	NA	−	+	NA	
2	−	−	NA	−	−	NA	
3	−	−	NA	−	−	NA	2.11 (0.08–9.41)[f]
4	−	−	NA	−	−	NA	
5	−	−	NA	−	−	NA	
6	−	−	NA	−	−	NA	
7	−	−	NA	−	−	NA	
8	−	−	NA	−	−	NA	1.03 (0.18–3.48)
9	−	−	NA	−	−	NA	2.35 (0.18–7.43)
10	−	−	NA	−	−	NA	
11	−	−	NA	−	−	NA	
12	I	I	I	−	−	NA	
13	−	−	NA	−	−	NA	
14	−	−	NA	−	−	NA	
15	I	I	I	−	−	NA	
16	I	I	I	−	−	NA	
17	−	−	NA	−	−	NA	
18	−	−	NA	−	−	NA	
19	−	−	NA	−	−	NA	
20	−	+	+	−	+	+	1.03 (0.19–3.47)
21	−	−	NA	−	−	NA	
22	−	−	NA	−	−	NA	
23	−	+	+	−	+	+	3.23 (0.18–7.39)
24	I	I	I	+	+	NA	
25	I	I	I	+	+	NA	
26	−	−	NA	−	−	NA	
27	−	−	NA	−	−	NA	
28	−	−	NA	−	−	NA	
29	−	−	NA	−	−	NA	

[a] None of the 29 samples tested was positive.
[b] Two of 29 samples (6.8%) were positive.
[c] Two samples were positive.
[d] Five of 29 samples (17.2%) were positive.
[e] −, negative; +, positive; NA, not applicable; I, inhibitory for the RT-PCR–nested PCR as determined by seeded experiments.
[f] The values in parentheses are confidence intervals.

TABLE 3. Surface water results for detection of enteroviruses with the BGMK cell line

Site	Concentrate			ICC with BGMK cells			ICR TCVA-MPN/ 100 liters[g]
	RT-PCR[a]	Nested PCR		RT-PCR[d]	Nested PCR		
		10^{1}[b]	1:5[c]		10^{1}[e]	1:5[f]	
1	−[h]	−	NA	+	+	NA	
2	−	+	+	−	+	+	
3	−	+	+	−	+	+	2.11 (0.08–9.41)[i]
4	−	−	NA	−	−	NA	
5	−	+	+	−	+	+	
6	−	−	NA	−	−	NA	
7	−	−	NA	−	−	NA	
8	−	+	+	+	+	+	1.03 (0.18–3.48)
9	−	−	NA	−	−	NA	2.35 (0.18–7.43)
10	−	+	−	−	+	+	
11	−	+	−	−	+	+	
12	I	I	I	−	+	NA	
13	−	+	+	−	+	+	
14	−	+	−	+	+	+	
15	I	I	I	−	−	NA	
16	I	I	I	−	−	NA	
17	−	−	NA	−	−	NA	
18	−	+	+	−	+	+	
19	−	+	+	−	+	+	
20	−	+	+	−	+	+	1.03 (0.19–3.47)
21	−	−	NA	−	−	NA	
22	−	−	NA	+	+	NA	
23	−	+	+	+	+	+	3.23 (0.18–7.39)
24	I	I	I	−	−	NA	
25	I	I	I	−	−	NA	
26	−	−	NA	−	−	NA	
27	−	−	NA	−	−	NA	
28	−	+	+	+	+	+	
29	−	−	NA	−	+	NA	

[a] None of the 29 samples tested was positive.
[b] Thirteen of 29 samples (44.8%) were positive.
[c] Ten samples were positive.
[d] Six of 29 samples (20.6%) were positive.
[e] Seventeen of 29 samples (58.6%) were positive.
[f] Fourteen samples were positive.
[g] Five of 29 samples were positive.
[h] −, negative; +, positive; NA, not applicable; I, inhibitory for the RT-PCR–nested PCR as determined by seeded experiments.
[i] The values in parentheses are confidence intervals.

formed with the sample concentrate, even after dilution (Table 5).

Dilution of samples for adenovirus analysis. We found that two adenovirus samples inhibited PCR–nested PCR. For one sample both the sample concentrate and the cell culture supernatant were positive after serial dilution; therefore, we could not determine if viral replication took place. However, 11 of the original 14 positive samples were positive for infectious virus.

Cumulative results. The 29 samples which we used were randomly selected from 1,100 archived ICR samples. Twenty-seven of the 29 samples contained at least one of the viruses revealed by the method used. Hence, 93.1% of the samples were positive for virus (infectious and/or noninfectious virus) as determined by the ICC-RT-PCR–nested PCR method. One sample was positive as determined by the TCVA-MPN method but negative as determined by the molecular approach. Only one sample was not positive for any virus as determined by the ICC-RT-PCR–nested PCR and TCVA-MPN methods.

Nineteen of the original 29 samples (65.5%) contained infectious virus, compared to five samples (17.2%) that were positive as determined by the TCVA-MPN method. Some samples were positive for members of more than one infectious virus group. As shown in Table 5, 26 infectious virus-positive tests were obtained for the 19 positive samples. The sample from site 22 was positive for infectious enteroviruses, astroviruses, and adenovirus types 40 and 41 but negative as determined by the TCVA-MPN method.

DISCUSSION

The ICC-RT-PCR–nested PCR method described here included an infectivity assay, a sensitive RT-PCR, and a nested PCR in order to eliminate false-positive results, to increase amplification signals, and to provide a method with high levels of sensitivity and specificity. This method allowed us to detect small numbers of viral contaminants in ICR surface water sample concentrates (18, 19).

RT-PCR is a powerful technique for detecting the nucleic acid sequence of an organism and for differentiating between types of enteric viruses, such as enteroviruses, astroviruses,

TABLE 4. Surface water results for detection adenovirus types 40 and 41

Site	Concentrate			ICC with BGMK cells			ICR TCVA-MPN/ 100 liters[g]
	PCR[d]	Nested PCR		PCR[d]	Nested PCR		
		10^{1}[b]	1:5[c]		10^{1}[e]	1:5[f]	
1	−[h]	−	NA	+	+	NA	
2	−	−	NA	−	−	NA	
3	−	−	NA	−	+	NA	2.11 (0.08–9.41)[i]
4	−	−	NA	−	+	NA	
5	−	−	NA	−	+	NA	
6	−	−	NA	−	−	NA	
7	−	−	NA	−	−	NA	
8	−	+	−	+	+	+	1.03 (0.18–3.48)
9	−	−	NA	−	−	NA	2.35 (0.18–7.43)
10	−	−	NA	−	−	NA	
11	−	−	NA	−	−	NA	
12	I	I	I	−	−	NA	
13	−	−	NA	−	−	NA	
14	−	−	NA	−	−	NA	
15	I	I	I	−	+	NA	
16	−	−	NA	−	−	NA	
17	−	−	NA	−	−	NA	
18	−	−	NA	−	−	NA	
19	−	−	NA	−	+	NA	
20	−	−	NA	−	+	NA	1.03 (0.19–3.47)
21	−	+	−	−	+	+	
22	−	−	NA	−	+	NA	
23	−	−	NA	+	+	NA	3.23 (0.18–7.39)
24	I	I	I	−	−	NA	
25	I	I	I	−	−	NA	
26	−	−	NA	−	−	NA	
27	I	I	I	−	+	NA	
28	−	−	NA	−	+	NA	
29	−	+	+	+	+	+	

[a] None of the 29 samples tested was positive.
[b] Three of 29 samples (10.3%) were positive.
[c] One sample was positive.
[d] Four of 29 samples (13.7%) were positive.
[e] Fourteen of 29 samples (48.3%) were positive.
[f] Three samples were positive.
[g] Five of 29 samples (17.2%) were positive.
[h] −, negative; +, positive; NA, not applicable; I, inhibitory for the RT-PCR–nested PCR as determined by seeded experiments.
[i] The values in parentheses are confidence intervals.

TABLE 5. Cumulative results for all viruses and the 29 surface water samples[a]

Virus	No. of positive samples	No. of samples positive for infectious virus	% of samples positive for infectious virus
Astrovirus	15	8	27.5
Enterovirus (BGMK cells)	17	6	20.6
Enteroviruses (Caco-2 cells)	5	1	3.4
Adenovirus types 40 and 41	14	11	37.9
Total[b]	20	26	68.9

[a] When the ICR TCVA-MPN method was used, 5 of the 29 samples were positive, and 5 of the 29 samples (17.2%) were positive for infectious virus.
[b] A total of 29 samples were examined. Samples that were positive for more than one infectious virus were counted once.

rotaviruses, and adenoviruses (2). Important control measures were included to ensure that our data were valid. For each sample that was positive as determined by the ICC-RT-PCR–nested PCR technique, a sample concentrate was seeded with each virus to determine if there were inhibitory substances present. The spiked samples each contained 10 to 100 RT-PCR units of virus. A positive result indicated that no sample inhibition occurred. In addition, the sample concentrate was also evaluated by the RT-PCR–nested PCR procedure to demonstrate that viral detection was not possible without cell culture amplification. For each reaction batch, two negative controls and two positive controls were included. A cell culture negative control (mock-infected cell culture supernatant) and an RT-PCR negative control (UV-treated water) were used to ensure that no sample contamination had occurred. A cell culture positive control and an RT-PCR positive control for each virus were included with every batch. The controls were subjected to the same treatment as the sample concentrates and cell culture lysates.

We demonstrated the effectiveness of the RT-PCR–nested PCR technique compared to the TCVA-MPN method for viral detection. The results of our study clearly show the limited scope of the data gathered by the ICR. We found that the BGMK cell line is an effective cell line for propagating enteroviruses. This was shown by the increase compared with the five positive samples obtained with Caco-2 cells (58.2% compared to 17.2%). Two samples were positive for enteroviruses when Caco-2 cells were used but negative when BGMK cells were used. This can be explained by the fact that not all viruses can infect and replicate in the BGMK cell line. This supports the concept that no cell line is effective for all enteric viruses.

Of the 29 samples tested, 19 (65.5%) contained infectious viruses; 27.5% of the samples contained infectious astroviruses, 20.6% contained infectious enteroviruses, and 37.9% were positive for infectious adenovirus types 40 and 41 (Table 5). Several samples contained one or more infectious viruses. When our method and the two cell lines were used, 27 samples were positive for viruses, compared to the five samples that were positive as determined by the ICR method.

Including the nested PCR technique in the analysis was necessary so that the analysis would be sensitive enough to detect very low numbers of viruses. In a previous study, Green et al. (7) found that it was difficult to detect small round structured viruses by RT-PCR in environmental samples. Frequently, RT-PCR amplicons were visible only because of the sensitivity of the nested RT-PCR technique (7). If the nested PCR procedure had not been included in our assay protocol, there would have been many false-negative results. For example, only one sample concentrate and five cell culture supernatants were positive for astroviruses as determined by RT-PCR alone. These numbers are similar to those obtained for the enteroviruses and adenoviruses. Nested PCR provided the sensitivity necessary to detect very few viral particles in both the cell culture supernatants and the sample concentrates.

An additional advantage of the nested PCR is its increased specificity. In other studies workers have used labeled oligonucleotide probes and hybridization to confirm the identities of RT-PCR products. In these analyses only a single oligonucleotide probe was used. In the nested PCR protocol two specific primers are used, which increases the specificity of the reaction compared with the specificity of a single-probe assay.

The ICR provided data on the concentrations of specific microbes, such as the total culturable viruses. The objective of this study was to develop and determine the effectiveness of an ICC-RT-PCR–nested PCR technique for analyzing surface water samples and to compare the results with the results obtained with the ICR TCVA-MPN technique. Only 5 samples were positive as determined by the TCVA-MPN method, while 19 samples were positive for infectious viruses when the molecular method was used. It is important to note that if the sample concentrates and cell culture supernatants had been diluted further, it may have been possible to show that more samples contained infectious viruses. Additionally, in the ICR method between 10 and 20 ml of each sample concentrate was evaluated by using 20 25-cm^2 flasks. In the ICC-RT-PCR–nested PCR protocol 500 μl of each sample concentrate and only one 25-cm^2 flask were used. If we had used 10 to 20 ml of sample and 20 flasks for the nested PCR, it is likely that many more samples would have been positive for one or more viruses.

It is important to note that one sample was positive as determined by the ICR method but negative for all three viruses as determined by ICC-RT-PCR–nested PCR method. This could be explained by the presence of a reovirus, a virus capable of growing on BGMK cells; such viruses are not detected by the enterovirus primers used in the RT-PCR–nested PCR protocol. Reoviruses are sometimes present in environmental samples in greater numbers than enteroviruses (18).

Our data suggest that analysis of enteroviruses by the TCVA-MPN method greatly underestimates the concentrations of viruses in surface water samples. Viruses such as adenoviruses and astroviruses may not be detected if other monitoring methods are not used for samples. Even when a cell culture lysate is passaged in a second set of cells, as in the ICR TCVA-MPN method, many viruses still do not produce CPE. To elucidate the extent of viral contamination in source water samples, the TCVA-MPN method must be replaced with newer techniques which have the required sensitivity and specificity. It is important to note that with few exceptions, the normal water laboratory of a water utility cannot perform the tests described here. However, water laboratories at academic institutions and private-sector water laboratories that are actively participating in research should have no problem emulating the procedures described here.

In this study we demonstrated the power of the ICC-RT-PCR–nested PCR technique. In future studies researchers must include other epidemiologically important viruses, such as rotaviruses, Norwalk viruses, hepatitis A viruses, and the small round structured viruses, all of which could be examined to obtain an even more detailed picture of viral contamination in surface waters.

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