Detection of human enteric viruses in stream water with RT-PCR and cell culture

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ABSTRACT

A multiplex RT-PCR method was used to measure virus occurrence at five stream water sites that span a range of hydroclimatic, water-quality, and land-use characteristics. The performance of the molecular method was evaluated in comparison with traditional cell culture and *Escherichia coli* membrane filtration assays. The study incorporated multiple quality controls and included a control for virus recovery during the sampling procedure as well as controls to detect potentially false-negative and false-positive data. Poliovirus recovery ranged from 16 to 65% and was variable, even in samples collected within the same stream. All five sites were positive for viruses by both molecular and cell culture-based virus assays. Enteroviruses, reoviruses, rotaviruses, and hepatitis A viruses were detected, but the use of the quality controls proved critical for interpretation of the molecular data. All sites showed evidence of faecal contamination, and culturable viruses were detected in four samples that would have met the US Environmental Protection Agency's recommended *E. coli* guideline for safe recreational water.

Key words | enteric virus, quality control, RT-PCR, stream water

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INTRODUCTION

More than 100 types of human pathogenic viruses may be present in faecal-contaminated waters (Havelaar *et al*. 1993). These viruses cause a variety of waterborne diseases, including gastroenteritis, infectious hepatitis, meningitis, respiratory disease, and eye infections (Cukor & Blacklow 1984; Melnick 1984; Lebaron *et al*. 1990). The fact that enteric viruses cause disease in individuals who are exposed to contaminated water has been shown directly through outbreaks associated with recreational use and inadequately treated drinking water (Baron *et al*. 1982; Gray *et al*. 1997; Levy *et al*. 1998; Kukkula *et al*. 1999), and indirectly through studies of swimming-related disease (Cabelli *et al*. 1979, 1982; van Asperen *et al*. 1998). Typically, bacterial indicators of sewage and animal wastes, such as total or faecal coliforms, *Escherichia coli* (*E. coli*), enterococci or bacteriophages, are used to determine the sanitary quality of water and the public-health risk from waterborne disease (Cabelli *et al*. 1982; Havelaar *et al*. 1993). Although use of these faecal indicators has significantly reduced the risk of illness, these surrogates are not always reliable indicators of the presence of human pathogenic viruses (Gerba *et al*. 1979; Rose *et al*. 1987; Griffin *et al*. 1999). Improved and validated methods for direct detection of viruses in water are needed to further protect human health in these situations.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR) methods often detect more viruses in environmental waters than do cell culture methods. In a study of groundwater samples from 448 sites from 35 states, 141 sites (31.5%) tested positive for the presence of enteric viruses by RT-PCR, whereas only 21 sites (4.7%) tested positive for infectious viruses by cell culture (Abbaszadegan *et al*. 1999*a*, 1999*b*). In an RT-PCR analysis of 11 surface water samples from North Carolina, nine were positive for enteric viruses by use of an immunocapture concentration and purification method and four

Table 1 | Description of stream water sampling sites

NAWQA unit	Abbreviation	Location	Land use	Population density (per km^2)	Average annual temperature (°C)
Lake Erie–Lake St Clair Basin	LERI	Cuyahoga River at Cleveland, OH	Urban and agricultural	2,849	8.4
Puget Sound Basin	PUGT	Thornton Creek near Seattle, WA	Urban	15,540	11.2
Eastern Iowa Basin	EIWA	Iowa River near Rowan, IA	Agricultural row crop and confined animal	16	7.8
Santee River and Coastal Drainage	SANT	Cow Castle Creek, SC	Agricultural	544	17.2
Kanawha-New River Basin	KANA	Clear Fork at Whitesville, WV	Mining; rural residential with septic systems	88	9.5

were positive by cell culture infectivity (Schwab *et al.* 1996). Studies of human waste-impacted sites have demonstrated a high rate of human enteric virus detection by RT-PCR. A report on canals and near-coastal waters of the Florida Keys found that 95% of the samples collected contained enteroviruses, hepatitis A virus (HAV), and/or Norwalk-like viruses (Griffin *et al.* 1999). A European study of six river water samples found that all six were positive for enteroviruses, rotaviruses, and Norwalk-like viruses (Gilgen *et al.* 1997).

In the present study, a new multiplex RT-PCR method (Fout *et al.* 2003), previously tested on groundwater samples, was evaluated by assessing the presence of enteric viruses in 30 samples collected from five stream water sites. Sites were chosen to represent a variety of water-quality conditions throughout the United States. In addition, a new procedure for measuring matrix effects of water quality on virus recovery was tested, and the importance of quality controls in the interpretation of RT-PCR data is discussed.

METHODS

Sampling locations and site information

Sampling locations were selected from established US Geological Survey (USGS) National Water-Quality Assessment Program (NAWQA) sites located in major hydrologic systems of the United States. To provide wide geographic coverage of the United States and to span a range of hydroclimatic and land-use settings, one site each within the Lake Erie-Lake St Clair Basin (LERI), the Kanawha-New River Basin (KANA), the Santee Basin and Coastal Drainage (SANT), the Puget Sound Basin (PUGT), and the Eastern Iowa Basin (EIWA) were chosen (Table 1). Each site was sampled three times to include a range of streamflows; at least one sample from each site was collected during or after a storm event. Climatic and land-use data were obtained for each site. Human population density data, compiled for 1990, were obtained from the US Bureau of Census (1991); densities were calculated using countywide coverages and applying densities as a percentage of the county in the basin. Average annual temperatures were obtained from the US Department of Commerce (1995).

Sample collection

Three water samples were collected at each site between December 1997 and May 1998. Temperature, pH and turbidity were measured in the stream with a multiparameter water quality meter before sampling for viruses.

Water samples were collected for viruses using a portable, self-contained sampling apparatus with control valves. The sampling apparatus contained a regulator module, a cartridge-housing module with 1MDS cartridge filter (Cuno Inc., Meriden, CT), and a discharge module as specified by Fout *et al*. (1996). A 10-μm polypropylene pre-filter cartridge (Parker Hannifin Corp., Cleveland, OH) was placed in front of the cartridge-housing module if turbidity exceeded 75 nephelometric turbidity units (NTU). A single metering module was used to reduce the pH of waters with values greater than 8.0 though the addition of 0.1 M HCl and to seed samples with poliovirus. A double metering module was used when both pH reduction and virus seeding were required. After each use, the sampling apparatuses were cleaned with dilute Liquinox and disinfected with a 0.1% bleach solution, followed by dechlorination with a sterile 0.005% sodium thiosulphate solution.

Two 100-l stream water samples were collected for each sampling event. Samples were collected from a bridge by pumping water through a length of sterile tubing connected to the intake of the sampling apparatus, using standard sampling guidelines (Fout *et al*. 1996). Water was collected at several locations within a cross-section of the steam to ensure a representative sample. Immediately following the collection of the first sample, a second seeded sample was collected by adding the contents of one vial of Sabin poliovirus vaccine (ORIMMUNE, Lederle Laboratories, Pearl River, NY) diluted in 1 l of water to the sample during filtration using the injector module. The discharge of the seeded sample was treated overnight with 2.25 l of household bleach. The next day residual chlorine levels were checked, and additional bleach was added if the levels were below 5 ppm. Following adequate disinfection, the bleach was neutralized with thiosulphate and the water disposed of via a sanitary sewer. After collection of the sample set, cartridge housings were placed on ice and shipped to the analytical laboratory.

Sample processing

All samples were processed within 72 h of sample collection at the laboratory using the procedure of Dahling (2002). Briefly, viruses were eluted from the 1MDS filter with 1.5% beef extract, pH 9.5 (BD, Franklin Lakes, NJ). Viruses were concentrated from the beef extract eluate by the addition of celite (Ohio Valley Specialty Chemical, Marietta, OH) to 0.1% (w/v), followed by immediate pH adjustment to 4.0 with 1 M HCl to allow virus binding to the celite. After stirring for 10 min, the celite was collected by vacuum filtration onto a pre-filter (7.5 cm diameter; Millipore Corporation, Bedford, MA). Viruses were eluted from the celite with 80 ml of 0.15 M Na_2HPO_4, pH 9.0–9.5 by gravity filtration. The concentrated eluate was neutralized to pH 7.0–7.5, passed through a 0.22-μm sterilizing filter (Gelman Sciences, Ann Arbor, MI), and stored at −70°C for subsequent analysis by RT-PCR or cell culture infectivity assay. A second aliquot of beef extract was added to the cartridge housing and held overnight. The next day, viruses were eluted and concentrated using the same procedure as for the first eluate. The two eluates were combined, and a portion was shipped to US EPA for cell culture assays. The remaining aliquot was treated to remove inhibitors of PCR and then analysed for viruses by multiplex RT-PCR.

Samples were processed for inhibitor removal and analysed by RT-PCR in batches. Each batch consisted of five stream samples and a negative-process control. The negative-process control was prepared by processing sterile water as if it were a field sample. Briefly, viruses present in 32 ml of concentrated eluate were centrifuged through a 30% sucrose cushion at 131,500 × *g* for 4.5 h at 10°C. Pellets were resuspended in 200 μl of phosphate buffered saline (PBS) containing 0.2% bovine serum albumin (BSA; USB, Cleveland, OH) and extracted with an equal volume of a mix containing 0.01% dithiozone (diphenyl thiocarbazone, Fisher Scientific, Pittsburgh, PA), 0.01 M 8-hydroxyquinoline (Fisher Scientific), butanol, methanol, and trichloroethane (0.1/0.9/1/0.25/0.25, v/v). The dithiozone and 8-hydroxyquinoline solutions were prepared in chloroform. The aqueous phase was then concentrated to approximately 40 μL using Microcon-100 filter units (Amicon, Bedford, MA). The concentrated samples were stored at −70°C until further analysis by RT-PCR.

RT-PCR analysis

Each sample was analysed for viruses using two multiplex RT-PCR reactions. Reaction A detected enterovirus,

reovirus and rotavirus, while reaction B detected HAV and Norwalk virus. Assays were developed for enteroviruses, HAV, Norwalk virus, and rotaviruses because members of these groups have caused waterborne disease. Reoviruses were included because they are common in environmental waters and because they cause culture-positive results on BGM cells (Spinner & Di Giovanni 2001; Dahling 2002). The reactions used virus-specific oligonucleotide primers as shown in Table 2. All primers and hybridization probes were synthesized and trityl-specific purified by The Great American Gene Company (Ramona, CA).

A sample volume of 5 μL was added to RT reactions containing 10 mM tris, pH 8.3, 50 mM KCl, 1.5 mM (reaction A) or 1.75 mM (reaction B) $MgCl_2$, 0.67 mM of each deoxyribonucleotide triphosphate (dNTP), and 1.67 μM of the appropriate RT primers (Table 2). Viral RNA, if present, was released from virus particles by heating at 99°C for 5 min, followed by immediate cooling on ice. Following the addition of 1 μL of MuLV reverse transcriptase (PE Applied Biosystems, Foster City, CA) and 0.75 μL of RNasin (Promega, Madison, WI), reverse transcription was performed at 43°C for 60 min. Reverse transcriptase was then inactivated by heating to 95°C for 5 min.

PCR was performed by bringing final conditions to 10 mM tris, pH 8.3, 50 mM KCl, 3.0 mM (reaction A) or 2.75 mM (reaction B) $MgCl_2$, 0.5 μM of the appropriate PCR primers (Table 2), and five units of AmpliTaq Gold polymerase (Applied Biosystems) in a total volume of 100 μL Samples were amplified by 40 cycles of denaturation at 95°C for 60 sec, followed by annealing and amplification at 59°C for 130 sec and then a final 15-min extension at 72°C.

Hybridization

RT-PCR results were always confirmed by dot-blot hybridization. Reaction products were bound to Magnagraph nylon membranes. They then were hybridized overnight at 51°C to each of five digoxigenin-labelled (DIG; Roche Applied Science, Indianapolis, IN) probe sets specific for the five virus groups (Table 2). Hybridization conditions were 5 × sodium chloride-sodium citrate buffer (SSC), 1% blocking reagent (Roche Applied Science), 0.1% sarkosyl, 0.05% sodium dodecyl sulphate (SDS) as described by Fout *et al.* (2003). Membranes were washed with optimal concentrations of 0.19 to 0.43 × SSC at 51°C to remove non-specifically bound hybridization probes. Specifically bound probes were detected following incubation with anti-DIG-alkaline phosphatase antibody (Roche Applied Science) and then CDP-Star chemiluminescent substrate (Promega, Madison, WI), as specified by the manufacturers. Hybridization results were recorded on X-ray film.

Quality controls

Two types of negative controls were analysed with each sample batch to detect false-positive results. A RT-PCR negative control was included to determine whether RT-PCR reagents were free from contamination. This control was prepared by adding 5 μL of sterile water to the RT-PCR assay in place of sample eluate. A negative-process control, prepared as described above, was included with each batch to detect cross-contamination occurring during sample processing and analysis.

Two types of positive control were also analysed with each sample batch to determine the degree to which potentially false-negative results occurred. The first type of positive control was prepared by seeding the negative-process control with virus. This control verified that the RT-PCR assay was performing properly. Matrix spike controls, the second type of positive control, were prepared by seeding each stream sample with virus just prior to RT-PCR. These controls were run to demonstrate that inhibitors of the enzymatic amplification had been adequately removed from the environmental samples. Positive controls were seeded with 50 RT-PCR units of poliovirus (Chat strain; 50 RT-PCR units equals 50 times the lowest concentration of virus that can be detected by RT-PCR) and 100 RT-PCR units of reovirus (serotype 3) and rotavirus (Wa strain) for reaction A assays, or 100 RT-PCR units each of HAV (HM-175 strain) and Norwalk virus for reaction B assays, as described in Fout *et al.* (2003).

Hybridization negative and positive controls were run with each hybridization probe used. Negative controls, in which sterile water was substituted for RT-PCR products,

Table 2 | Oligonucleotide RT-PCR primers and hybridization probes

Virus	Name	Sequence (5′→3′)	Reaction[1]
Primers			
Enterovirus	MRD 13	ACC GGA TGG CCA ATC CAA	RT
	MRD 14	CCT CCG GCC CCT GAA TG	PCR
Reovirus	MRD 188	ACG TTG TCG CAA TGG AGG TGT	PCR
	MRD 189	GTG CTG AGA TTG TTT TGT CCC AT	RT
Rotavirus	MRD 154	GCT GGC GTG TCT ATG GAT TCA	PCR
	MRD 155	CAA AAC GGG AGT GGG GAG C	RT
Hepatitis A	MRD 185	CTT CTA ACG TTG CTT CCC ATG TCA G	PCR
	MRD 186	CCA TTT TCC CTC TGT TAG CTT TTC C	RT
	MRD 193	AAT GCC TTC TGG GTC TCC TTG C	PCR
	MRD 194	TCA AAC TCA GCG TTA CTT CTC TGC C	RT
Norwalk	MRD 211	CAA GCC CCC CAA GGT GAA T	PCR
	MRD 212	GGC GCA TGG TTT GTT GAT TTC	RT
Probes			
Enterovirus	MRD 32	ACT ACT TTG GGT GTC CGT GTT TC	
Reovirus	MRD 190	GAC ACT CGT CCT TCA AAT GCG TTA	
	MRD 191	GCG TTG TTA ATC AAG TCC ACG ACC T	
	MRD 192	GCG TTG TTA ATC AAG TCC ACG ATC T	
Rotavirus	MRD 156	GTA ATC ATC GGA ATC AGA CTC TG	
	MRD 157	GTA ATC TTC ATA GTC AGA ATC TGC TT	
	MRD 158	CAT TTT CTG TTC TTA GTT TCA TGT TT	
Hepatitis A	MRD 187	CAT CCA TAG CAT GAT AAA GAG GAG C	
	MRD 195	GAA AAG TCA ATT CTG AAA CTG GCT TC	
Norwalk	MRD 214	CCA GGG GGT ATG CAG GAA AC	

[1]This column indicates whether primers were used at the reverse transcription or PCR steps.

were analysed to determine whether cross-contamination occurred during hybridization. RT-PCR products from each control virus were also used with each probe set. This generated one positive control for each set to verify successful completion of the hybridization reaction and four additional negative controls.

An equipment blank control was performed by pumping 20 l of sterile water though a sampling apparatus 10 days after it had been used for the first LERI sample. The sample was then processed in the same manner as field samples.

Cell culture infectivity assays

Following the completion of the molecular assays, the concentration of poliovirus in seeded samples was measured with a plaque assay technique using Buffalo Green Monkey (BGM) kidney cells as described in Chapter 10 of Berg *et al*. (1984). Unseeded samples and the equipment blank were measured for culturable virus (e.g. many enteroviruses and reoviruses) using BGM cells as described by Dahling (2002) and typed as described in Chapter 12 of Berg *et al*. (1984). Briefly, each sample was inoculated into a single roller bottle. The presence of virus in samples in all bottles that were positive for cytopathic effects was confirmed by re-passage in tubes (Dahling 2002) and by typing.

Determination of *E. coli* concentrations

Water from several locations in a stream cross-section was combined and analysed in the field vehicle for *E. coli* using the mTEC agar membrane-filtration method (US EPA 1986). Samples were analysed in 30-, 10-, 3-, and 1-ml volumes in order to obtain ideal plate counts of 20–80 colonies per plate. If bacteria concentrations were expected to be elevated, a 1/100 dilution of the sample in sterile saline buffer was used for the analysis.

RESULTS AND DISCUSSION

Sample site characteristics

The NAWQA sites chosen to evaluate the utility of a new multiplex RT-PCR method (Fout *et al*. 2003) covered agricultural to urban areas with population densities that ranged from 16 to 15,540 individuals per km^2 (Table 1). Water quality parameters from the sites are shown in Table 3. Water temperatures reflected the winter and early spring sampling conditions, ranging from 0 to 12.6°C. Water pH values ranged from 5.4 to 8.1 and turbidity from 0.7 to 344 NTU. *Escherichia coli* concentrations ranged from 59 to 23,000 colony-forming units per 100 ml.

Quality controls

This study used a virus elution and re-concentration procedure that was shown to recover greater than 90% of poliovirus from groundwater (Dahling 2002). To determine the effects of stream water quality on virus recovery, a new procedure for seeding samples safely in the field was developed. Table 3 shows that recovery ranged from a low of 16% at the LERI site to 65% at the KANA site. Large recovery differences also were observed between individual samples at two of the sites. At the LERI site, the best recovery occurred at the highest turbidity reading. One of the lowest recoveries (18%) was observed in one sample from the EIWA site. The pH of this sample, which was not adjusted during sampling due to sampling problems, was 8.1. Values above 8.0 are known to affect recovery on 1MDS cartridge filters (Sobsey & Jones 1979). Unfortunately, with the limited number of samples in this study it was not possible to determine specific correlations between water quality and virus recovery. However, the importance of virus recovery controls was suggested by the detection of a greater number of virus types in all samples with higher virus recoveries, with the possible exception of samples from the KANA site (Table 3). The variable results obtained with this control suggest a clear need for its use in studies involving quantitative determinations of virus levels in environmental waters.

Other quality controls also proved important. An equipment blank was run during the study to ensure that the apparatus washing and disinfection procedure was adequate. Unexpectedly, this quality control was positive for enterovirus by RT-PCR and for poliovirus by cell-culture analysis (Table 4). The sampling apparatus used in the blank had been used for a field sample 10 days

Table 3 | Water-quality and virus recovery characteristics

NAWQA unit	Sample date	Temp. (°C)	Water pH	Turbidity (NTU)	Streamflow	*E. coli*[1]	Poliovirus recovery[2]	Virus detected[3]
LERI	12/19/97	4.8	7.5	< 70	Low	1,800	16	E, R
	2/18/98	6.0	7.6	282[4]	High	23,000	58	E, R, Ro,[5] H[5]
	3/4/98	5.8	7.3	16	Medium	ND[6]	ND	E
PUGT	1/12/98	4.5	8.1 (7.4)[7]	7	Medium	98	52	E
	2/9/98	8.9	7.9	8	Medium	240	59	E, R, Ro
	3/9/98	8.7	7.9	32	High	520	ND	E
EIWA	1/29/98	0	7.7	6	Low	90	59	E, R[5]
	3/6/98	5.6	8.1	6	Low	59	18	E
	5/1/98	12.3	7.8	ND	Medium	73	ND	E
SANT	12/16/97	8.9	5.8	23	Medium	210	35	E, R,[5] Ro
	1/14/98	12.5	6.2	< 25	Medium	150	42	E, R, Ro,[5] H
	2/4/98	11	5.4	ND	High	2,800	ND	E
KANA	12/10/97	5.6	7.9	0.7	Low	620	65	R
	3/18/98	7.0	7.7	5.9	Medium	173	ND	H
	4/17/98	12.6	7.5	344	High	1,500	ND	E, H

[1]Colony-forming units per 100 ml.
[2]Percent virus recovery is based on total PFU in seeded samples divided by 2.78×10^6, the number of PFU in a Sabin vaccine vial.
[3]Viruses detected: culture (unseeded sample only)—E, enterovirus; RT-PCR—R, reovirus, Ro, rotavirus, H, hepatitis A.
[4]A pre-filter was added prior to the 1MDS filter for samples with turbidities greater than 70 NTU.
[5]Virus was detected in both samples of a sample set.
[6]ND, not determined.
[7]Water pH values above 8.0 were adjusted with 0.1 N HCl to the value in parentheses.

earlier, but it had been thoroughly washed with detergent and disinfected using a standard recommended procedure (Fout *et al*. 1996). As a result, the molecular data obtained for enteroviruses were called into question and were not reported here. This positive equipment blank control suggests that mere implementation of accepted disinfection practices may not be adequate to assure that complete viral disinfection has occurred. In retrospect, the configuration of the sampling apparatus may have contributed to the positive results. The components of the apparatus (including tubing) were fixed in place for convenience of carrying, perhaps making it difficult for the bleach to reach all surfaces. However, no other viruses were detected in the equipment blank by RT-PCR, and infectious poliovirus was also not identified in any unseeded sample, indicating that carryover of infectious virus is rare.

Seeded negative-process controls, hybridization positive controls, and matrix spikes were quality-control samples included in this study to assess the degree to

Table 4 | Virus analyses of stream water samples

	Number of positive samples[1]				
Site	**Culturable enteroviruses**	**Reoviruses**	**Rotaviruses**	**HAV**	**Norwalk**
LERI	3 (CB1, NT)[2]	2	2	2	0
PUGT	3 (CB1, NT)	1	1	0	0
EIWA	3 (CB1, NT)	2	0	0	0
SANT	3 (CB1, NT)	3	3	1	0
KANA	1 (NT)	1	0	2	0
Equipment blank	1 (Poliovirus)	0	0	0	0
Total[3]	13 (87)	9 (30)	6 (20)	5 (17)	0

[1]A sample was considered positive for enteroviruses if an isolate from the unseeded sample did not type as a poliovirus and for other viruses if samples were positive by RT-PCR. Potentially false-positive samples were excluded from the count.
[2]CB1=coxsackievirus B1; NT=not typable; RT-PCR results for enteroviruses were excluded because of the positive equipment blank.
[3]The total number of stream water samples that were positive. The percent of these samples that are positive are given in parentheses. The percent is based upon 15 unseeded samples for enteroviruses and 30 seeded and unseeded samples for other viruses.

which potentially false-negative results occur. These controls showed that 23% of the stream samples analysed gave potentially false-negative (and thus unacceptable) results (data not shown). On a site basis, the amount of false-negative results ranged from 7% at the SANT site to 40% at the KANA site. Hybridization positive controls were always positive and thus acceptable for all six analytical groups, indicating the proper completion of the hybridization step every time.

From each batch, a negative-process control (processed through inhibitor removal) and a RT-PCR negative control were assayed to determine the degree to which false-positive PCR reactions occurred. Presumptively false-positive enterovirus results were obtained with 7% of the unseeded samples. However, due to the positive equipment blank, the molecular data for enteroviruses were not reported in Table 4. In the first three of the six RT-PCR assays performed in the study, there were no false-positive results with the other virus groups. In the last three assays all reovirus samples showed presumptively false-positive results. The only other false-positive result was from a HAV reaction.

The rapidity with which potentially false-positive reactions developed for the reovirus primer set was surprising. In spite of keeping post- and pre-PCR assays separate, all samples processed after the date of the second round of PCR were affected. The cause of the cross-contamination could not be determined, but likely relates to the robustness of the reovirus primer set. This set, which generates the smallest PCR fragment size of 126 base pairs, appears to be very insensitive to environmental inhibitors in that potentially false-negative results are usually not observed (data not shown).

Culturable virus assay

The unseeded samples from each of the five stream water sites were analysed using a cultural assay. Culturable viruses were detected in 87% of the unseeded samples

(Table 4). Coxsackievirus B1 was identified in 53% of the positive samples and was a commonly circulating enterovirus at the time of this study (CDC 2000). Isolates from the remaining positive samples could not be typed, indicating that they were likely either a mixture of human enteroviruses or reoviruses.

RT-PCR assay

RT-PCR has distinct advantages and disadvantages when compared with cultural methods. The major advantage is that many of the viral pathogens that cannot be cultured or those that are difficult to culture can be detected with RT-PCR. In addition, results can be obtained in a much shorter time frame than results from cultural procedures. The major disadvantage is that RT-PCR does not distinguish between infectious and non-infectious virus particles. In this study, water samples from five NAQWA sites were analysed for enteroviruses, HAV, Norwalk virus, reoviruses and rotaviruses. After exclusion of potentially false-positive results based upon quality controls, reoviruses were detected in 30% of all samples, rotaviruses in 20% and HAV in 17% (Table 4). Overall, a total of 13 (43%) of the samples and 100% of the sites were found to be positive by RT-PCR for one or more of these viruses.

The numbers of virus-positive samples and the types of viruses detected varied among the sites sampled (Tables 3 and 4). Infectious enteroviruses were found at all sites, with the KANA site having the lowest incidence. The KANA site is a mining site located about 3 km upstream from a town's drinking water intake. Wastewater treatment plants from housing developments and straight pipes from septic tanks are sources of potential stream contamination. Reoviruses were also detected at this site, and it was one of two sites with detections of HAV in two samples.

The EIWA site is an agricultural site with a basin-wide proliferation of large-scale hog confinement facilities. The site has a low population density and therefore a low potential for human enteric virus contamination. The low potential is supported by the result that only reoviruses were found at this site by RT-PCR assay.

The SANT site is another agricultural site with some cattle, pig, and chicken facilities within the basin. In this study, samples from this site had the most virus detections. Fifty percent of the samples were positive for reoviruses and rotaviruses and one (17%) was positive for HAV.

Six detections of virus were found by the RT-PCR assay in samples from the LERI site. This site receives waste from both urban (domestic and industrial) and agricultural land use. Faecal indicator concentrations are often elevated during periods of high flow as a result of wastewater effluents and combined sewer overflows. Samples were collected in December through March when wastewater effluents were not being disinfected (disinfection of wastewaters entering the site is required from May 1 to Oct. 15).

The PUGT site is an urban land-use site located in a densely populated area served by city sewer lines. Urban storm water runoff significantly affects water quality in the stream, but this basin is not affected by combined sewer overflows or point sources of wastewater effluent. Although this site has the highest human population density among the sites sampled, reoviruses and rotaviruses were detected in the RT-PCR assay in only one sample.

All the streams showed a considerable level of faecal pollution, except those from EIWA, where all the samples were below 100 colony-forming units/100 ml of *E. coli* (Table 3). *Escherichia coli* concentrations generally increased with increasing streamflow, turbidity and temperature. Water pH did not affect concentrations of *E. coli*. The number of samples collected was not sufficient to correlate virus findings with water quality factors.

The viruses that were detected in this study included enteroviruses, HAV and rotaviruses. While the positive findings of the RT-PCR assay do not prove the presence of infectious viruses, the fact that infectious enteroviruses were found via cell culture suggests the possibility that infectious HAV and rotaviruses may have been present. Although health effects were not evaluated in this study, the presence of infectious enteric viruses in such waters has the potential for causing illness in recreational users and in those who use the water for irrigation. Based upon studies of health effects among swimmers in recreational areas (Cabelli *et al*. 1982), the US EPA has recommended that fresh waters are safe for recreational use when *E. coli*

levels are below 126 colony-forming units per 100 ml (Federal Register 1986). In this study, infectious enteroviruses were found in all four unseeded samples collected from sites where *E. coli* levels were below this recommended level (Tables 3 and 4). In addition, reovirus was detected in both samples collected from the first sampling event at the EIWA site. This suggests that indicator bacteria alone may not always adequately reflect the viral quality of waters. However, widespread application of virus monitoring procedures is not currently practical and would likely have to await a reduction in the total cost of virus assays.

CONCLUSIONS

Molecular and cell culture methods were used in this study to assess virus occurrence in five NAWQA stream water sites. Poliovirus recovery, which averaged 45% (Table 3), was measured by collecting a poliovirus-seeded sample with each sample set. Samples were processed and analysed for the presence of enteroviruses, reovirus, rotavirus, HAV and Norwalk virus. All sites were positive for the presence of *E. coli*, an indicator of faecal contamination, and for viruses by both cultural and molecular methods. Overall, 87% of unseeded samples contained culturable viruses. Quality control data were critical in the interpretation of molecular results, in that potentially false-positive and false-negative results, and a positive equipment blank, were detected during the study. After excluding potentially false-positive results, RT-PCR assays identified reovirus in 30% of the samples, rotavirus in 20%, and HAV in 17%. Based upon study results, the following quality controls are suggested for virus occurrence studies in environmental waters:

- equipment blanks be included as a regular quality control in environmental assays for enteric viruses;
- the level of HOCl used for disinfection of sampling apparatuses be increased from 0.1% to 0.525% (i.e. a 1:10 dilution of household bleach);
- seeded recovery controls be run using diluted Sabin poliovirus and sampling apparatuses that are dedicated to this use;
- standard positive and negative controls, negative-process controls and matrix spike controls be included with all RT-PCR assays.

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ABBREVIATIONS AND NOTATION

BGM	Buffalo Green Monkey kidney cells
BSA	bovine serum albumin
DIG	digoxigenin
dNTP	deoxyribonucleotide triphosphate
EIWA	Eastern Iowa Basin
HAV	hepatitis A virus
KANA	Kanawha-New River Basin
LERI	Lake Erie-Lake St Clair Basin
NAWQA	National Water Quality Assessment Program
NTU	nephelometric turbidity units
PBS	phosphate-buffered saline
PUGT	Puget Sound Basin
RT-PCR	reverse transcription–polymerase chain reaction
SANT	Santee Basin and Coastal Drainage
SDS	sodium dodecyl sulphate
SSC	sodium chloride/sodium citrate buffer ($20\times = 3$ M and 0.3 M, respectively)
US EPA	United States Environmental Protection Agency
USGS	United States Geological Survey
v/v	volume/volume
w/v	weight/volume

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