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Three-step isolation method for sensitive detection of enterovirus, rotavirus, hepatitis A virus, and small round structured viruses in water samples

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Abstract

Control of drinking or bathing water quality in respect to viral contamination remains an unsolved problem. A highly sensitive isolation protocol was developed for concentration and detection of different enteric viruses from water samples. The three-step isolation procedure combines filtration with a positively charged nylon membrane, ultrafiltration and clean-up of the viral RNA with a silica based membrane. Detection of the viral RNA is accomplished by reverse-transcription polymerase chain reaction (RT-PCR). Detection limits were determined to be one 50% tissue culture infective dose ($TCID_{50}$) of seeded coxsackievirus B2 or hepatitis A Virus per litre of tap water by RT-PCR compared to two orders of magnitude lower sensitivity for culture in the case of coxsackievirus B2. The isolation procedure is highly sensitive, easy to perform and allows the detection of different human pathogenic virus groups in one water sample. The application of the isolation procedure to six river water samples and subsequent detection with nested or semi-nested PCR revealed enterovirus in 6/6 (100%), rotavirus in 6/6 (100%), hepatitis A virus in 0/6 (0%), small round structured virus genotype I in 6/6 (100%) and small round structured virus genotype II in 2/6 (33%) of the samples. These findings suggest that first, we have developed a very sensitive detection procedure and second, that river water in Switzerland-where most of the wastewater is handled by sewage treatment plants-shows a high contamination rate with enteric viruses.

Keywords: Enteric viruses; RT-PCR; Detection; Surface water

1. Introduction

Enteric viruses nave been associated with many outbreaks of waterborne nonbacterial gastroenteritis and are a public health concern (Hedberg and Osterholm, 1993). In contrast to the bacterial water quality, viral water quality, concerning either bathing or drinking water, is in Switzerland and most other countries not routinely analysed for the presence of pathogenic enteric viruses. The reason for this mainly results from the lack of useful, easy, fast and

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reliable methods to concentrate and detect these human pathogens. Human enteric viruses are able to persist in the environment due to their extreme resistance to unfavourable conditions (Hedberg and Osterholm, 1993; Moore, 1993). After replicating in the gastrointestinal tract, enteric viruses are excreted into sewage and may be dispersed into the environment since most sewage treatment processes are not able to completely remove them (Block and Schwartzbrod, 1989). Transmission may occur via sewage-polluted water, sewage, well- or groundwater, shellfish and sewage- or sludge-amended soils (Metcalf et al., 1995). The most important waterborne viruses belong to the genus of the enteroviruses (EV), causing a variety of symptoms ranging from benign summer illness to myocarditis and aseptic meningitis (Rotbart, 1995), to hepatitis A virus (HAV), to the group A rotaviruses (RV) and to the only recently recognized small round structured viruses (SRSVs), the latter two being the principal cause of nonbacterial gastroenteris either mainly affecting children (RV) or adults (SRSVs) (Blacklow and Greenberg, 1991). Other enteric viruses being implicated in outbreaks of waterborne disease are hepatitis E virus (HEV) and astrovirus (Metcalf et al., 1995). Since usually only a few viral particles are present in water samples, it is necessary to concentrate the viruses from a large volume of water avoiding the co-isolation of inhibitors. Traditionally, detection of viruses had been done in cell culture, which poses numerous problems: sensitivity is low, the method is tedious, labour- and cost-intensive. Moreover, some enteric viruses, such as HAV or SRSVs, are difficult or impossible to cultivate. Reverse transcription polymerase chain reaction (RT-PCR) is fast and highly sensitive and has been successfully applied to detect enteric viruses in environmental samples (Abbaszadegan et al., 1993; Jothikumar et al., 1993, 1995; Khan et al., 1994; Kopecka et al., 1993; Le Guyader et al., 1994; Ma et al., 1995; Puig et al., 1994; Straub et al., 1994; Tsai et al., 1993). Sophisticated methods such as antigen- or RNA-capture for the isolation of HAV or EV have been developed (Deng et al., 1994; Gajardo et al., 1995; Graff et al., 1993; Monceyron and Grinde, 1994). Such methods often lack the ability to detect different enteric viruses in one water sample. Protocols for the simultaneous isolation and detection with multiplex-PCR have been presented (Tsai et al., 1994). Although such methods are time- and cost-efficient, they often lack high sensitivity. Recently, we developed a magnetic bead-based isolation procedure for the isolation of enteroviral RNA from water samples (Gilgen et al., 1995). In the present study, a simple and sensitive isolation procedure that can be used for the isolation and detection of many water-transmitted enteric virus is described. The three-step concentration procedure was combined with RT-PCR in order to detect different enteric viruses in environmental water samples.

2. Materials and methods

2.1. Virus samples

Poliovirus vaccine strains and a hepatitis A virus vaccine strain used for first experiments were provided by Dr. R. Glück (Seruminstitut, Berne, Switzerland). Hepatitis A virus strain 18F (10^6 $TCID_{50}$ per ml) was a kind gift of Dr. M. Weitz (Institute of Clinical Microbiology and Immunology). Coxsackievirus B2 (10^7 $TCID_{50}$ per ml) were cultured on Vero cells. As positive controls for the RT-PCRs of RV and SRSVs, viruses or RNA isolated from stool samples obtained by courtesy of the institute of medical microbiology of the University of Berne and by Dr. T. Ando (CDC Atlanta, Georgia, USA) were used, respectively.

Sequence data accessed from the EMBL/Genbank have the following accession numbers: X00596 (poliovirus type Sabin 3); M59808 (hepatitis A virus strain 18F); K02033 (human WA rotavirus gene 9); M87661 (norwalk Virus).

2.2. Enterovirus culture

0.15 ml of each specimen were inoculated onto Vero cells and MRC-5 cells (human embryonic lung). Cultures were incubated for ten days at 36°C and examined daily for typical CPE. CPE positive specimens were typed by neutralization using the standard Melnick antiserum pools.

2.3. Concentration procedure

One litre tap water samples were seeded with serial dilutions of CVB2, HAV or a SRSV gtII

containing stool sample. The samples were vigorously shaken and filtered through a positively charged 0.45 μm pore size membrane (Zetapore; AMF-Cuno, Meriden, Connecticut). In addition, a prefilter (Millipore AP 2504700, Bedford, Massachusetts) was used to prevent clogging of the membrane with particles present in the water samples. The prefilter was discarded and the Zetapor membrane transferred to a 50 ml centrifuge tube. Three ml of 50 mM Glycine NaOH, pH 9.5, containing 1% beef extract, were added. After shaking at 500 rpm and room temperature for 20 min in order to elute the viruses, two ml of the virus-containing buffer were adjusted to pH 8 with about 20 μl of 1 M HCl and concentrated to 100 μl by use of a Centricon-100 microconcentrator (Amicon, Inc., Beverly, Mass.) at 1000 × g according to Tsai (Tsai et al., 1993). The retentate was adjusted to 140 μl with PBS, the viruses were lysed and the RNA isolated using the QIAamp HCV kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. The eluted RNA was dried on a speed-vac, redissolved in 10 μl of diethyl-pyrocarbonate (DEPC) treated water per projected RT reaction and subjected to reverse transcription.

2.4. River water samples

One litre water samples were collected on October 12 and 23, 1995, from different sites of the river Aare, Berne. They were processed according to the protocol described above. The RNA was isolated, dried on a speed-vac and dissolved in 50 μl of DEPC-treated water. For every virus group to be detected, 10 μl of RNA were subjected to RT. We used tap water from our laboratory and an autoclaved water sample from one sample site as negative controls.

2.5. Oligonucleotides

Oligonucleotides used for the detection of the different enteric viruses are described in Table 1. Oligonucleotides had been synthesized by either Anawa (Wangen, Switzerland), Schmidheini (Balgach, Switzerland), or MWG-Biotech (Ebersberg, Germany) and stored freeze-dried at − 20°C. All the PCR systems are located in highly conserved regions of the viral genomes, allowing the simultaneous detection of either different serotypes (EV, RV), different members of a genetic group (SRSVs) or different strains of one virus species (HAV).

The RT-PCR systems for the detection of enteroviruses and hepatitis A virus are based on primers described previously (Goswami et al., 1993; Hyypiä et al., 1989; Monceyron and Grinde, 1994; Rotbart, 1990; Zoll et al., 1992), the system allowing the detection of group A rotaviruses is an adaptation of published sequences (Gouvea et al., 1990; Le Guyader et al., 1994). All the primers have been checked for specificity with the GCG software. Two seminested PCR systems for the detection of the two SRSV genotypes (Lew et al., 1994; Wang et al., 1994) have been developed in our laboratory (Häfliger, 1995) by alignments of all the sequences of SRSVs available on the EMBL/Genbank.

2.6. Reverse transcription

Ten μl of RNA were reverse transcribed by incubation for 60 min at 41°C followed by five min at 95°C. In a final volume of 20 μl, reaction conditions were 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM each dNTP, 1.25 μM antisense primer described in Table 1 (EV03, RV1, HAV4, SRI-1 or SRII-1, respectively), 10 U RNasin (Promega, Madison, WI) and 100 U M-MLV reverse transcriptase (Promega). RNA used for rotavirus RT was denatured for four min at 94°C in the presence of 5% DMSO and chilled on ice for ten min.

2.7. PCR

Oligonucleotides and conditions for the different PCR systems are described in Table 1 and Table 2. In order to improve sensitivity and specificity, either seminested or nested PCR systems were applied, resulting in amplicon lengths listed in Table 2. The completed RT reaction (20 μl) was mixed with 80 μl of PCR mixture (final concentration: 10 mM Tris-HCl (pH 8.8), 50 mM KCl, 0.1% Triton-X-100, 2 μg ml^{-1} BSA, 200 μM each dNTP, 2 U of *Taq* Polymerase (Promega), 0.25 μM of the first primer pair and optimal $MgCl_2$ concentration as indicated in Table 2). Two μl of the first reaction were used as a template for the second round of PCR, using conditions and primer pairs (each primer at concen-

Table 1
Primer oligonucleotides used throughout this study

Virus and oligonucleotide	Region	Sequence 5′ → 3′[a]	Polarity[b]	Localization[c]
Enterovirus	5′ UTR			
EV03		ATT GTC ACC ATA AGC AGC CA	−	601–582
EV05		CAC GGA CAC CCA AAG TA	−	563–547
EV06		CAA GCA CTT CTG TTT CCC CGG	+	164–184
EV09d		dig-TCC TCC GGC CCC TGA ATG CG	+	448–467
Rotavirus group A	VP7 gene			
RV1		GTC ACA TCA TAC AAT TCT AAT CTA AG	−	1061–1036
RV2		CTT TAA AAG AGA GAA TTT CCG TCT G	+	3–27
RV3		TGT ATG GTA TTG AAT ATA CCA C	+	50–71
RV4		ACT GAT CCT GTT GGC CAW CC	−	395–376
Hepatitis A virus	3D			
HAV1		TTT GGT TGG ATG AAA ATG GTT	+	6305–6325
RAV2		CAA CCT GTC CAA AAG ATG AAT	+	6416–6436
HAV3		ACC AAC ATC TCC GAA TCT TA	−	6648–6629
HAV4		ATT CTA CCT GCT TCT CTA ATC	−	6716–6696
SRSV genotype I	Capsid			
SRI-1		CCA ACC CAR CCA TTR TAC AT	−	5659–5640
SRI-2		AAA TGA TGA TGG CGT CTA	+	5344–5361
SRI-3		AAA AYR TCA CCG GGK GTA T	−	5584–5566
SRSV genotype II	RNA pol			
SRII-1		CGC CAT CTT CAT TCA CAA A	−	5357–5339
SRII-2		TWC TCY TTY TAT GGT GAT GAT GA	+	4844–4866
SRII-3		TTW CCA AAC CAA CCW GCT G	−	5046–5028

[a] Mixed bases in degenerated primers: W = A/T, Y = C/T, K = G/T, R = A/G, dig: digoxigenine.
[b] Polarity, −: negative, +: positive.
[c] Nucleotide positions for EV, rotavirus, HAV and both genotypes of SRSVs are in reference to the genomes of poliovirus type Sabin 3 (X00596), human WA (serotype 1) rotavirus (K02033), HAV strain 18F (M59808), and norwalk virus (M87661), respectively.

Table 2
Primer pairs, annealing-temperatures, $MgCl_2$ concentrations, and length of amplicons for all the PCR-systems used in this study

Virus	Primer-pair	Annealing temperature	$MgCl_2$ concentration[a]	Length of amplicons[b]
Enterovirus	EV03/EV06	55°	3.5 mM	438 bp
	EV05/EV06	55°	3.0 mM	400 bp
Rotavirus	RV1/RV2	55°	1.5 mM	1059 bp
	RV4/RV3	55°	3.5 mM	346 bp
Hepatitis A Virus	HAV4/HAV1	55°	3.0 mM	412 bp
	HAV3/HAV2	55°	2.5 mM	233 bp
SRSV gtI	SRI-1/SRI-2	50°	3.5 mM	316 bp
	SRI-3/SRI-2	50°	2.0 mM	241 bp
SRSV gtII	SRII-1/SRII-2	55°	3.5 mM	514 bp
	SRII-3/SRII-2	50°	1.5 mM	203 bp

[a] The optimal $MgCl_2$ concentration was determined experimentally (data not shown).
[b] The lengths of the amplicons are in reference to the genomes of Poliovirus type Sabin 3 (X00596), human WA (serotype 1) rotavirus (K02033), HAV strain 18F (M59808), and norwalk virus (M87661), respectively.

tration of 0.5 μM) as described in Table 2. The reaction mixes were overlaid with 80 μl of mineral oil (Sigma). Cycling was done on a PHC-1 cycler (Techne, Princeton, NJ) with one min at 94°C, followed by 25 cycles at 94°C for 30 sec, annealing for 30 sec according to Table 2, 72°C for 60 sec for the first round and 40 cycles with the same cycling profile for the second, seminested or nested PCR. In addition, an endextension step was performed at 72°C for three min after the first and the second PCR.

2.8. Analysis of PCR products

Aliquots of the second reaction (10 μl) were analysed on 1.5% agarose gels. The products were visualized by ethidium bromide staining and UV transillumination. To unequivocally identify the EV amplicons to be of enteroviral origin, Southern blot hybridization was performed using the 5′-digoxigenin labelled oligonucleotide EV09d, homologous to a highly conserved region of the amplicons. PCR products, separated by agarose gel electrophoresis, were transferred to positively charged nylon membranes (Boehringer, Mannheim, Germany) as described by the manufacturer. The membranes were incubated in a prehybridization solution containing 5X SSC, 1% blocking reagent (Boehringer), 0.1% N-lauroylsarcosin sodium salt (Sigma) and 0.02% SDS at 45°C for two h. Hybridization was performed overnight in prehybridization solution with added digoxigenin-labeled oligonucleotide at a final concentration of 125 pmol ml^{-1} at 45°C. Membranes were washed twice with 2X SSC containing 0.1% SDS at room temperature for 5 min and two times with 5X SSC, 0.1% SDS at 47°C for 10 min. Detection of hybridizing bands was done with anti-digoxigenin-AP conjugate, Fab fragments (Boehringer), using a colour reaction according to the manufacturer's protocol.

2.9. RFLP-typing of the amplicons

Further typing of rotavirus isolates was achieved by digestion with restriction analysis using either *Alu*I, *Cfo*I, or *Hae*III. Ten to 30 μl of amplicons were directly incubated with 1 U μl^{-1} enzyme and analysed on 4% agarose gels. The restriction fragment pattern was compared with sequence data for different serotypes and strains accessible on the EMBL/Genbank.

3. Results

3.1. Isolation procedure

Our three-step isolation procedure includes two subsequent concentrations followed by virus lysis and RNA-isolation. The first concentration step is based on adsorption of viral particles to a positively charged nylon membrane. The subsequent elution step with a high pH buffer containing beef extract was empirically optimized. Initial experiments had shown that addition of beef-extract improved the recovery rate at least ten times and did not have any inhibitory effects on RT-PCR. Beef extract, often used to elute viruses but suspected to show inhibitory effects on PCR (Schwab et al., 1995), seems to be removed either partly at the ultrafiltration or following denaturation with the guanidine thiocyanate (GuSCN) containing virus lysis and RNA isolation buffer included in the QIAamp HCV kit.

3.2. Detection limits

In order to specifically detect different enteric viruses, nested or seminested RT-PCR systems were developed. Primers were chosen to hybridize to highly conserved regions of the viral genomes, allowing the simultaneous detection of either different serotypes, different members of one genotype or different strains of one virus species. Detection limits of the RT-PCR systems were determined for the EV and HAV RT-PCR systems by serial ten-fold dilutions of viral RNA. Our nested or seminested systems are capable to detect about 0.01 $TCID_{50}$, for either CVB2 or HAV strain 18F (Table 3). The specificity of the PCR-systems for group A RV and SRSVs were established by use of RNA isolated from different stool samples and proved to be specific and highly sensitive (data not shown). In addition no cross-reactivity between the different examined RT-PCR systems was observed.

The detection limits of our isolation procedure was experimentally determined by seeding one litre tap water samples with serial dilutions of either CVB2, HAV or SRSV gtII. As little as 1 $TCID_{50}$ for CVB2

Table 3
Detection limits of HAV, CVB2 and SRSV gtII RT-PCR system: RT-PCR alone and in combination with isolation from one-litre seeded tap water samples

Virus	RT-PCR Detection limit[a]	Detection limit in one litre water	Efficiency
CVB2	0.01[b]	1.0[b]	10^{-2}
HAV	0.01[b]	1.0[b]	10^{-2}
SRSV gtII[d]	10^{7} [c]	10^{5} [c]	10^{-2}

[a] Detection limits were performed on serial dilutions of purified viral RNA.
[b] $TCID_{50}$.
[c] Reciprocal of last dilution by which virus was still detectable.
[d] Tap water samples were seeded with serial dilutions of SRSV gtII stool samples.

and HAV and a 10^5 dilution of the SRSV gt II stool sample per litre of water could be detected by subsequent RT-PCR and analysis of the amplicons on agarose gels (Table 3).

Finally, the recovery of free RNA from seeded tap water samples using our isolation procedure was tested. One litre tap water samples were mixed with RNA equivalent to 40 or 400 $TCID_{50}$ of poliovirus and immediately subjected to our isolation and detection procedure. 400 $TCID_{50}$ were detectable by RT-PCR followed by Southern hybridization whereas RNA from 40 $TCID_{50}$ tested negative (result not shown). Thus, as expected, free RNA is detectable only at high concentrations.

3.3. Comparison of detection by RT-PCR and by cell culture

The detection of CVB2 were compared between RT-PCR and cell culture following isolation from one litre water samples. One litre tap water samples were seeded with serial dilutions of CVB2 (20 000, 2 000, 200, 20, 2, 0.2, 0.02, 0 $TCID_{50}$) and subjected to our isolation procedure (see Section 2). Previous to the final step of the procedure, i.e. virus-lysis and RNA clean-up, the isolate was adjusted to 300 μl with PBS, 150 μl were used for detection on cell culture and 150 μl were subjected to our RNA clean-up procedure and to RT-PCR with the EV PCR system, respectively. Detection by RT-PCR was by two orders of magnitude more sensitive than cell culture where CPE were observed down to 100 $TCID_{50}$ only (Table 4).

Table 4
Comparison of CVB2 detection in one liter water by RT-PCR and by cell culture

Amount of seeded CVB2 [$TCID_{50}$]	Detection procedure	
	RT-PCR	Cell culture
10^4	+	+
10^3	+	+
10^2	+	+
10^1	+	−
10^0	+	−
10^{-1}	−	−
10^{-2}	−	−
0	−	−

3.4. Isolation of enteric viruses from river water samples

Having achieved a satisfactory detection limit with artificially seeded tap water samples, our isolation and detection system was applied to river water samples collected from the river Aare, Berne. Ten μl aliquots of RNA (i.e. 20% of the total isolated RNA), corresponding to the viral load of about 130 ml from one site, were subjected to RT for each specific virus system. All six sites were positive for EV, RV, SRSV gtI, and two sites were additionally positive for SRSV gtII, but HAV was not detected at any sample site (Fig. 1, Table 5). Southern blot hybridization with the oligonucleotide EV09d which hybridizes to a highly conserved region of the EV genome, confirmed that the EV-amplicons were of enteroviral origin (Fig. 1). The seminested enterovirus PCR system for EV sometimes yields fragments of lengths around 400 bp even from

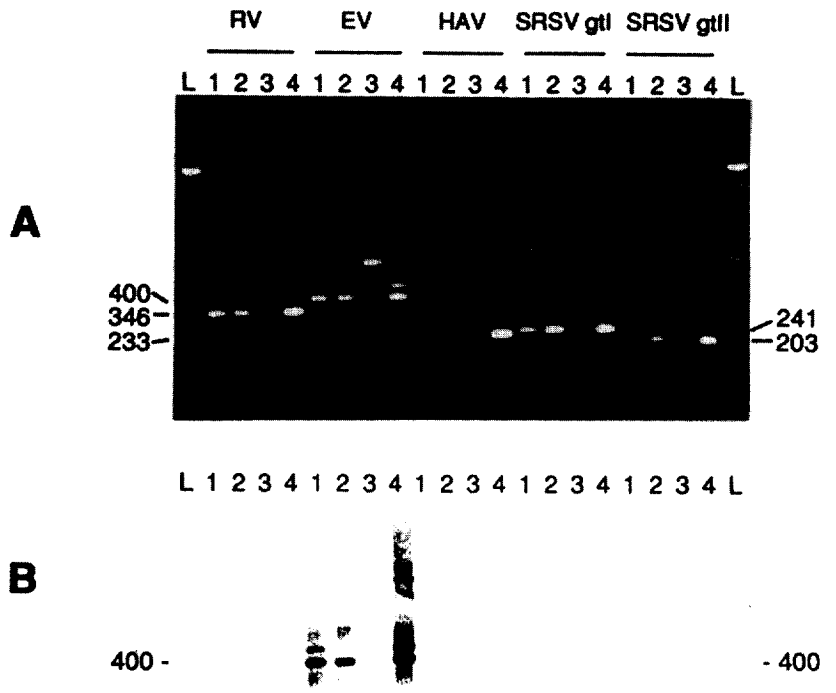

Fig. 1. Detection of enteric viruses in river water. One litre water samples were collected from the river Aare, Berne, Switzerland and the viruses concentrated and detected by RT-PCR as described in Section 2. A: Ethidium-bromide stained agarose gels with amplicons (10μl) specific for rotavirus (RV, 346 bp), enterovirus (EV, 400 bp), hepatitis A virus (HAV, 233 bp), small round structured virus genotype I (SRSV gtI, 241 bp), and small round structured virus genotype II (SRSV gtII, 203 bp). B: Southern blot hybridization with enterovirus specific oligonucleotide EV09d. Lanes: 1, water sampled at site #B; 2, water sampled at site #F; 3, tap water negative control; 4, positive controls of RT-PCR; L, 100 bp DNA standard (Gibco BRL).

Table 5
RT-PCR results of river water analysis

Sampling site	Enterovirus	Rotavirus	Hepatitis A virus	SRSV gtI	SRSV gtII
#A	+	+	–	+	–
#B	+	+	–	+	–
#C	+	+	–	+	–
#D	+	+	–	+	–
#E	+	+	–	+	+
#F	+	+	–	+	+
% positive	100%	100%	0%	100%	33%

Two negative controls for the isolation and detection procedure were used: the first was a sample from site #F, autoclaved for 20 min at 121°C, the second was a tap water sample from our laboratory. Both samples yielded negative results.

samples not containing any viruses. Clearly, Southern blot analysis confirms that the amplicons from the river and positive control samples are of enteroviral origin, whereas the additional bands visible in the negative control and the amplicons from all other examined enteric viruses did not hybridize (Fig. 1).

3.5. Typing of the rotavirus isolates by restriction enzyme digestion

Restriction fragment length polymorphism (RFLP) typing was performed with the amplicons derived from RV PCR in order to further type and distinguish the detected RV. The amplicons were incubated with either *Alu*I, *Hae*III or *Cfo*I and the pattern of the fragments were compared with data accessible on the EMBL/Genbank. Analysis on agarose gels of the amplicons incubated with *Alu*I revealed a pattern dominated by fragments of 199 and 147 bp length (Fig. 2), corresponding to the typical fragments of RV serotype 1. Digested amplicons from two sites yielded additional fragments belonging to the serotypes 3, 4 or 10. All amplicons did not contain any internal restriction sites for *Hae*III or *Cfo*I (results not shown). SRSV yielded restriction fragments that unequivocally determined the amplicons to be of SRSV amplicons origin (results not shown).

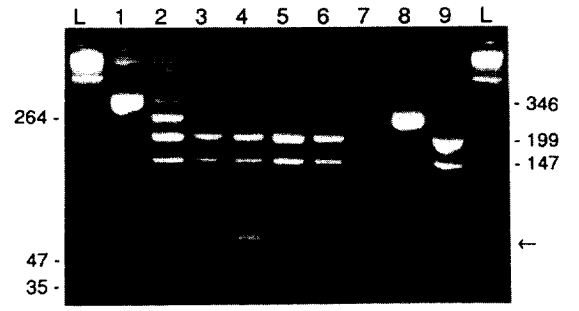

Fig. 2. Typing of the RV amplicons derived from river water samples by restriction enzyme digestion. Agarose gel showing amplicons digested with *Alu*I. Lanes: 1, undigested product; 2, sample site #A; 3, sample site #B; 4, sample site #C; 5, sample site #D; 6, sample site #E; 7, sample site #F; 8, 9, digested amplicons derived from stool samples, suspected to be either serotype 3, 4 or 10 (lane 8) or 1 (lane 9). *L* = 100 bp DNA standard (Gibco BRL). The arrow represents the length corresponding to the primer artefact.

4. Discussion

In many outbreaks of gastroenteritis, the causative agent cannot be determined. Frequently, the reason of the outbreak is suspected to be of viral origin, but due to the lack of sensitive and reliable methods for the detection of viruses in food or water, the assumption can only rarely be confirmed by experimental results. We aimed to develop a sensitive method for the isolation and detection of enteric viruses from small volume water samples. Besides shellfish and person-to-person transmission, waterborne transmission is one of the most prominent transmission routes of these human pathogens (Hedberg and Osterholm, 1993). Performing seeding experiments with different viruses, we determined the detection limit to be 1 $TCID_{50}$ per litre of water. These detection limits are two orders of magnitude less sensitive than the experimentally determined RT-PCR detection limits of 0.01 $TCID_{50}$ using the same viral suspensions. Similar results were obtained for SRSV gtII, where a 10^7 dilution of the stool sample could be detected in direct RT-PCR and a 10^5 dilution by seeding one litre water samples, respectively. Comparison of detection by cell culture and by RT-PCR revealed that the detection limit for cell culture was by 2 magnitudes less sensitive than PCR. This is in agreement with the differences in detection limits on viral suspensions which are by a factor of 100 lower for cell culture compared to RT-PCR. Although free enteroviral RNA was detectable using our isolation procedure, the sensitivity of detection is about three orders of magnitude reduced compared to viral particles. Thus, our isolation protocol will mainly allow the isolation of viruses and not of free viral RNA. In addition, free RNA is likely to be immediately degraded in the environment (Tsai et al., 1995). Since besides infectious, also defective particles are detected, no statement about the infectivity of the detected viruses can be made.

The limiting step in our three-step isolation procedure is considered to be the first filtration with the 0.45 μm membrane. Since the isolation of the

viruses depends on their adsorption to the membrane, it may be difficult to isolate all the present viruses. By use of filter cartridges, this step could further be improved, also allowing the filtration of larger volumes which might be necessary for the control of drinking water. The loss of virus or viral RNA during the second and third steps is expected to be small, since the virus particles are much larger than the used ultrafiltration cut-off and in addition, the 'spin-around' technique for the collection of the retentate makes it well suitable and easy to handle. The use of silica-based membranes for the isolation of viral RNA allows viral lysis with GuSCN, thus preventing degradation of RNA by RNases and allowing RNA isolation and removal of inhibitors without the use of labour-intensive phenol and chloroform extractions or precipitation steps.

The sensitivity of our isolation and detection protocol encouraged its application to environmental samples. By testing six one-litre river water samples for the presence of EV, RV, HAV, and both SRSV genotypes, we were able to detect EV, RV and SRSV gt I in all six samples, and SRSV gt II in two samples. However, no HAV was detectable in any of the samples. The high rate of positive samples is further astonishing since only about 130 ml of water were actually tested for the presence of one virus type (1/3 of the sample is lost following the elution from the membrane filter). In Switzerland, most sewage is treated by wastewater plants, leading to the expectation of a low overall viral load in rivers and lakes. In agreement with our results, the high prevalence of EV and RV in surface water of this country has recently been documented by another group, detecting EV in seven of 18 and RV in nine of 18 one litre surface water samples (Metzler et al., 1996). In 1993, using a less sensitive detection method, we had detected EV in seven of 40 natural bathing and recreational waters (Gilgen et al., 1995).

The typing of detected viruses will help to elucidate infectious routes in epidemiological outbreaks. Typing may be performed by DNA-sequencing, digestion with restriction enzymes or hybridization with serotype-specific probes. DNA sequence analysis may be hampered by the fact that the amplicons will often represent several different virus serotypes thus leading to ambiguous DNA sequence information. A different way to distinguish different isolates is RFLP typing, leading to a specific fragment pattern. This pattern may also be influenced by virus mixtures, but may easier be interpreted than a sequencing reaction of non-homogenous amplicons. Computer aided DNA sequence analysis or RFLP data comparison of isolates with data banks will finally lead to the classification and identification of the detected viruses from water samples. Recently, genotyping of group A rotaviruses from sewage had been done with serotype-specific primers and enabled the detection and identification of serotypes 1, 2 and 3 (Gajardo et al., 1995).

Using RFLP, the isolated RV serotypes were typed. Clearly, the bands of 147 and 199 bp (*Alu*I digestion) indicate the presence of serotype 1 in all six samples. The presence of serotype 1 is not further astonishing, since this is the most prevalent serotype isolated from children as well as from environmental samples (Gajardo et al., 1995; Kapikian and Chanock, 1985; Ushijima et al., 1994). In samples from sites #A and #F, additional bands of 264, 47 and 35 bp reveal the presence of either serotype 3, 4 or 10. In all but one of the samples a band with the length of the uncut product of 346 bp is visible. This may either be an additional, undigested serotype or the band is due to incompletely digested product. Thus, RFLP typing might in some cases be of limited value since serotype differences can not always be resolved by this technique.

River water free of any viral contaminations can not be the ultimate aim, nor is it possible to strive for it. However, the introduction of different quality standards as used in bacterial water control (Swiss Federal Office of Public Health, 1991) seems necessary. Such a standard will be difficult to define, since already only a few viral particles are able to provoke an infection. In order to have the analytical tools for the control of these standards, it will be necessary to introduce quantitative methods, either based on cell-culture or RT-PCR. It will also be indispensable to get more information about the health risk posed by these viruses present in river water. Hence the comparison of RT-PCR with cell culture for viruses amenable to culture (EV, RV, HAV) in connection with epidemiological studies will yield useful data.

A rough quantification of the viral load is possible by serial dilution of the isolated RNA. Using this strategy, a 1:20 dilution of the isolated EV RNA

from one sample site was found to still yield a positive result in RT-PCR, implying that the viral load at this site was at least by a factor of 20 higher than our experimentally determined detection limit of 1 $TCID_{50}$ per litre of water (result not shown). Although such a dilution method gives reliable results, it is cost- and labour-intensive.

Techniques based on molecular biology are particularly promising procedures for rapid monitoring viral contamination in order to determine the origin of waterborne viral outbreaks and epidemiological association with disease. Although PCR has the two main disadvantages that quantitation is difficult and that no statement about the infectivity of the detected viruses can be made, this powerful tool will be very important in term of surveillance of water quality. Concerning drinking water quality, it is obvious that no enteric virus should be detectable in one-litre water samples. So far, water samples from our laboratory always tested negative.

This study presents a rapid and efficient PCR-based method to simultaneously detect four important enteric viruses commonly transmitted via water and food (Hedberg and Osterholm, 1993; Metcalf et al., 1995). This method represents a practical approach for the identification of viruses in water samples, allowing the concentration of viral RNA to the microlitre range. The presented protocol is fast, easy and highly sensitive. Whereas other protocols often use dilution steps that do decrease sensitivity (Abbaszadegan et al., 1993; Jothikumar et al., 1993; Tsai et al., 1993), our protocol uses 2/3 of the viral load of one litre to be detected in RT-PCR. Such a procedure has a great potential in quality control of either surface or drinking water, allowing the subsequent detection of different virus species in one water sample after analysing the isolated RNA by different specific RT-PCR systems. Clearly, the detection of enteric viruses in the environment or in drinking water will help to prevent viral gastroenteritis outbreaks.

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