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Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood

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Abstract

Highly sensitive seminested RT-PCR systems for the specific detection of genotype I and II small round structured viruses (SRSVs) were developed based on the nucleic acid information deposited in the databanks. SRSVs could be detected in 10^7-fold dilutions of three different stool samples. In addition, a rapid and simple purification protocol for enteric viruses from seafood tissues was elaborated using poliovirus (PV) as model. The virus isolation and viral RNA purification include the following steps: elution of the viruses from the seafood tissue with glycine buffer, their concentration by PEG-precipitation, lysis of viral particles with guanidine hydrochloride and viral RNA isolation using a silica based membrane. The detection limit was 3 to 30 $TCID_{50}$ of poliovirus in 1.25 g of seeded seafood tissues without marked food matrix differences, whereas SRSV viruses were 10- and 100-fold better detected in mussels than in shrimps and oysters, respectively. The newly developed purification method, which was shown to remove potential RT-PCR inhibitors present in mussel tissue samples, was applied in a small market survey. 15 mussels, 15 oysters and 12 shrimps were examined for the presence of Hepatitis A virus (HAV), Enterovirus (EV), Rotavirus (RV) and SRSV using specific RT-PCR detection systems. The finding of three oyster samples positive for Rotavirus demonstrated the successful application of our method for the detection of enteric viruses in naturally contaminated seafood samples. The rapid isolation method might be suitable for application in routine testing laboratories and will help to improve public health controls for seafood. © 1997 Elsevier Science B.V.

Keywords: Enteric viruses; Small round structured viruses; Seafood; Shellfish; RT-PCR

1. Introduction

Enteric viruses are important human pathogens, which are able to persist in the environment and include adenoviruses, rotaviruses, astroviruses, caliciviruses, hepeviruses, enteroviruses, and hepatoviruses. Outbreaks of viral diseases have been associated with the consumption of water as well as food including sewage-contaminated shellfish (Richards, 1987). Since viruses cannot be completely eliminated by sewage treatment plants (Block and Schwartzbrod, 1989), they are dispersed into the

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environment and can lead to water-borne outbreaks (Hedberg and Osterholm, 1993). Shellfish such as oysters, cockles and mussels have been implicated unequivocally in food-borne outbreaks of enteric viruses (Dowell et al., 1995; Kohn et al., 1995). Shellfish are filter feeding organisms and can concentrate the low number of virus paricles present in contaminated coastal areas. The depuration process applied to oysters, which is highly effective in removing bacterial pathogens, has little or no effect on enteric viruses (Caul et al., 1993). Food-borne outbreaks can also occur after contamination of food by infected food handlers.

In many outbreaks of viral gastroenteritis the causative agent is a small round structured virus (SRSV), which belongs to the caliciviruses (Caul et al., 1993; Dowell et al., 1995; Kohn et al., 1995; Pontefract et al., 1993). SRSVs, also known as Norwalk or Norwalk-like viruses, are the major worldwide cause of food-borne outbreaks of acute, epidemic nonbacterial gastroenteritis in adults and older children (Hedberg and Osterholm, 1993; Kapikian and Chanock, 1990). The genome characterization of Norwalk and related viruses (Dingle et al., 1995; Jiang et al., 1993; Lambden et al., 1993) enabled their detection in human stools by RT-PCR (Green et al., 1993; Moe et al., 1994). A problem of the nucleic acid based detection of SRSVs is the fact that they form a large group of genetically diverse viruses (Ando et al., 1995). In this study all in the GenEMBL databank available sequence information were used to develop two new seminested RT-PCR systems for the specific and highly sensitive detection of genotype I and II SRSV.

Public health controls are hampered by the absence of rapid and simple detection methods for these viruses present in shellfish and other contaminated food. In addition, nucleic acid based detection of enteric viruses in shellfish has been obstructed by the presence of RT-PCR inhibitors. It could be shown, that these inhibitors can be removed successfully by guanidinium isothiocyanate (GIT) extraction (Chomczynski and Sacchi, 1987; Shieh et al., 1995). This work describes a fast and reliable purification procedure for enteric viruses from seafood, which might be suitable for application in routine testing laboratories.

2. Material and methods

2.1. Viruses

The poliomyelitis vaccine 'Poloral Berna' (Serum Institut, Berne) consists of three poliovirus strains called Sabin I (2 000 000 $TCID_{50}$/ml), II (200 000 $TCID_{50}$/ml), and III (600 000 $TCID_{50}$/ml).

Since SRSVs cannot be cultivated in cell cultures, the only source of SRSVs in high concentration are stool samples of infected patients. Four stool samples (P1-A-11879, P1-B-11860, P2-A-11885 and P2-B-12359) were kindly provided by Dr. T. Ando, CDC, Atlanta. The designations P1-A, P1-B and P2-A, P2-B stand for oligonucleotide probes based on the sequences of genotype I and genotype II SRSV strains, respectively (Ando et al., 1995).

2.2. Food samples

Frozen mussels ('Cozze' from Italy), fresh mussels (*Mytilus edulis*), oysters (Portuguese oyster (*Crassostrea angulata*) and European oyster (*Ostrea edulis*) imported from Brittany (France) and shrimps (from Thailand) were purchased from local food suppliers and from the state control laboratory of the Canton Berne (Switzerland).

2.3. Viral RNA isolation from stool samples

Viral RNA from stool samples was isolated using RNeasy and QIAmp HCV kit (Qiagen, Hilden, Germany). Viral particles from 50 μl stool samples were lysed and RNA was purified on the silica-based membranes according to the manufacturer's protocol. Finally, the RNA was eluted in 50 μl of DEPC treated water.

2.4. Virus purification from seafood

For seeding experiments 1.25 g of homogenized tissue of shrimps (Black Tiger), oysters (Portuguese) and mussels (*Mytilus edulis*) were seeded with 150 μl of tenfold dilutions in water of either poliovirus vaccine or stool sample P2-A-11885 containing SRSV genotype II. After 15 min of incubation an eight-fold volume of 50 mM glycine buffer (pH 9.5) was added and the mixture was shaken at room

temperature for 30 min, followed by 15 min of centrifugation at 13 500 × *g* and 4°C. Polyethylene glycol 6 000 (PEG 6 000 [Fluka, Buchs, Switzerland] as a 50% [wt/vol] solution in phosphate-buffered saline) was added to a final concentration of 8% to the aqueous phase. The mixture was shaken for 1 h at 4°C and centrifuged for 20 min at 11 000 × *g* at 4°C. The PEG-pellet was resuspended in 300 µl of 5 M guanidine hydrochloride (GuHCl). After incubation for 10 min at room temperature and centrifugation at 12 000 × *g* for 10 min, 140 µl of the supernatant was used for RNA isolation with the QIAmp HCV kit as described above.

Market samples (mussels, oysters and shrimps) of 6 to 22 g were shucked and homogenized for 1 min in an eight-fold volume (not exceeding 100 ml) of 50 mM glycine buffer (pH 9.5) in a laboratory blender. After shaking at room temperature for 30 min the homogenate was centrifuged for 2 min at 3 000 × *g*. The supernatant (11 ml) was recentrifuged at 13 500 × *g* and treated with PEG as described above.

2.5. Computer analysis

RNA sequences from the RNA polymerase and capsid region of SRSVs were accessed from the GenEMBL data bank and aligned using the GCG programs PILEUP and PRETTY allowing the identification of conserved regions among SRSVs. RT-PCR-primers were designed within conserved regions by use of the OLIGO 5.0 Primer Analysis Software (NBI, Plymouth, MN 55447-5434). No cross-homologies of potential primers for SRSVs with databank entries were revealed by the use of the GCG FastA program.

2.6. Oligonucleotides

Oligonucleotides used for the detection of the different enteric viruses (Table 1) had been synthesized by either Anawa (Wangen, Switzerland), Schmidheini (Balgach, Switzerland), or MWG-Biotech (Ebersberg, Germany) and stored freeze-dried at −20°C. All PCR primers 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 (SRSV) 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 (Gilgen et al., 1995; Goswami et al., 1993; Hyypiä et al., 1989; Monceyron and Grinde, 1994; Rotbart, 1990; Zoll et al., 1992). The system for group A Rotaviruses is an adaptation of published primer sequences (Gouvea et al., 1990; Le Guyader et al., 1994).

2.7. Reverse transcription

Ten µl of isolated RNA were reverse transcribed using Moloney murine leukemia virus (M-MLV) reverse transcriptase by incubation for 60 min at 41°C (EV, RV, HAV) or 45°C (SRSV), followed by 5 min at 95°C. In a final volume of 20 µl the reaction conditions were 50 mM Tris–HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, 10 mM DTT, 0.5 mM dNTPs, 1.25 mM 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 4 min at 94°C in the presence of 5% DMSO and chilled on ice for 10 min.

2.8. PCR

In order to improve sensitivity and specificity, either seminested or nested PCR systems were applied, resulting in amplicon lengths listed in Table 2. Ten µl of the completed RT reaction was mixed with 40 µl PCR mixture (final concentration: 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.1% Triton-X-100, 2 mg/ml BSA, 200 mM dNTPs, 2 U of Taq Polymerase (Promega), 0.25 mM of the first PCR-primer pairs 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 concentration of 0.5 mM) as described in Table 2 in a final volume of 100 µl. The reaction mixes were overlaid with 80 µl of mineral oil (Sigma, St. Louis, MO). Cycling was done on a PHC-1 thermal block (Techne, Princeton, N.J.) with the following temperature program: denaturation at 94°C for 1 min; 25

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	*VP7gene*			
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
HAV2		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
Parameters of PCR systems for the detection of enteric viruses

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-4	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.

cycles at 94°C for 30 s, annealing according to Table 2 for 60 s, 72°C for 60 s; end-extension at 72°C for 3 min; 40 cycles with the same cycling profile for the second round of PCR.

2.9. Analysis of PCR products

Ten μl aliquots of the second PCR reaction were analysed on 1.5% agarose gels. The products were visualized by ethidium bromide staining and UV transillumination. To identify unequivocally the EV amplicons as being of enteroviral origin, Southern blot hybridization was performed using the 5′-digoxigenin labelled oligonucleotide EV09d, homologous to a highly conserved internal region of the EV-amplicons. PCR products, separated on a 1.5% agarose gel, were transferred (2–10 h) to positively charged nylon membranes (Boehringer 1209 299, Mannheim, Germany) with 0.4 M NaOH using the capillary transfer method recommended by the manufacturer. The membranes were incubated in a prehybridization solution containing 5 × SSC, 1% blocking reagent (Boehringer), 0.1% *N*-lauroylsarcosin sodium salt (Sigma) and 0.02% SDS at 45°C for 2–10 h. Hybridization was performed for 12–16 h in prehybridization solution with added digoxigenin-labeled oligonucleotide at a final concentration of 125 pmol/ml at 45°C. Membranes were washed twice with 2 x SSC containing 0.1% SDS at room temperature for 5 min and two times with 5 x SSC, 0.1% SDS at 47°C for 10 min. Detection of hybridizing probes was carried out with anti-digoxigenin alkaline phosphatase conjugate Fab fragments (Boehringer) according to the manufacturer's protocol.

3. Results

3.1. RT-PCR systems for the detection of SRSVS

The 3′-part of the RNA polymerase region and the capsid region of SRSV genomes were chosen for the computer aided selection of RT-PCR primers. The computer alignments confirmed that SRSVs fall into two genotypes (Ando et al., 1994; Green et al., 1994; Lew et al., 1994; Wang et al., 1994). Since the nucleotide identity between genotype I and II is at best 65%, two different RT-PCR systems had to be designed for the detection of the known human pathogenic SRSVs. The RT-PCR primers for the detection of genotype I and II SRSVs were selected within the 5′-part of the capsid and the 3′-part of the RNA polymerase region, respectively (Table 1).

In order to achieve a high sensitivity of the PCR based detection system, the newly developed RT-PCR systems were optimized with respect to annealing temperatures and Mg^{2+} concentrations (Table 2) by using viral RNA extracted from stool samples as templates (see Section 2).

The sensitivity of the two optimized RT-PCR systems was determined by using 10-fold serial dilutions of stool samples in water. Viral RNA of samples containing genotype I and II SRSV (P1-A, P2-A and P2-B) were detectable up to a dilution of 10^{-7} (Fig. 1). Since RT was performed with 10 μl, we assume that the number of viral genomes in 1 g stool sample must have been higher than 10^9. In addition the genotype specifity of the RT-PCR systems was tested. The genotype I-specific system detected the genotype I viruses of sample P1-A, whereas the viruses of sample P2-A, P2-B (genotype II) were detected only by the genotype II specific primer set. Viral RNA of sample P1-B could be amplified only with the genotype II-specific system after reverse transcription at 41°C (data not shown).

Amplicons of SRSVs can further be characterized by digestion with different endonucleases. The amplicon of P1-A was digested with *Ban*I and a RFLP similar to Norwalk and Southampton virus was obtained. Amplicons of P2-A were digested with *Sau*96 I and the obtained RFLP was similar to Toronto virus (data not shown).

3.2. Purification of enteric viruses from seafood

The main aim of this work was the development of a purification method for enteric viruses from seafood. For the evaluation and improvement of the different purification steps we used mussel and oyster tissues seeded with titrated poliovirus vaccine (PV). The first steps of the purification procedure include the elution of intact virions from the food matrix followed by their concentration. After elution with glycine buffer (Croci et al., 1993), two different virus concentration procedures were tested. PEG precipitation was preferred to the adsorption of viruses to positively charged nylon filters because it

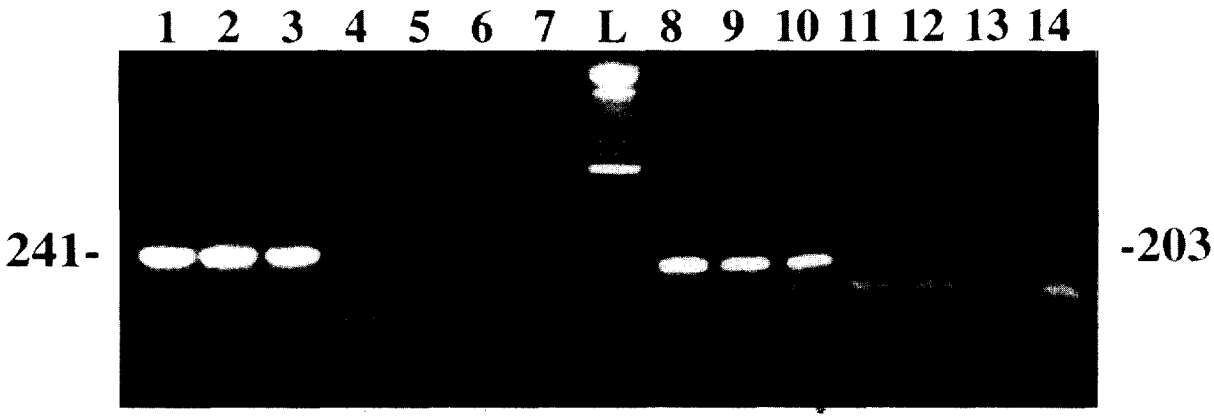

Fig. 1. Sensitivity of the SRSV-specific RT-PCR systems. Detection of SRSV amplicons on an ethidium bromide-stained agarose gel. Lanes: L, 100 bp DNA ladder; 1 to 5 and 8 to 12; 10^5, 10^6, 10^7, 10^8, 10^9 fold dilutions of samples P1-A and P2-B, respectively; 6 and 13, negative control of RT; 7 and 14, negative control of PCR.

was more rapid and the handling was simpler whereas similar recoveries were obtained (data not shown). Initial centrifugation of shellfish homogenates at $3\,000 \times g$ for 15 min previous to PEG precipitation led to dirty PEG pellets, which were difficult to resuspend for RNA extraction. In order to get cleaner supernatants with less insoluble shellfish components prior to PEG precipitation, the centrifugation of shellfish homogenate in elution buffer was finally performed at $13\,500 \times g$. PEG pellets of mussel samples were colorless whereas the pellets of oyster samples were grey, pointing to matrix differences. In addition, the resuspension of PEG pellets was generally easier for mussel than for oyster samples.

The elimination of RT-PCR inhibitors originating from the food matrix by guanidinium extraction was verified by adding treated mussel extract to purified viral RNA in the reverse transcription reaction. No inhibition was observed after addition of processed mussel tissue extracts, whereas RT-PCR was inhibited by untreated mussel extract (data not shown).

Initial seeding experiments were performed with 1.25 g mussel homogenate (flesh and fluid) and 150 μl of virus suspensions containing 0.03 to 30 000 $TCID_{50}$ of poliovirus. After isolation of viral RNA and detection by RT-PCR 0.3 $TCID_{50}$ of PV were still detectable (data not shown). Since most virus particles probably do not adsorb to shellfish tissue after seeding, but stay dissolved in the fluid, no elution has to occur to allow further detection. Thus, these initial seeding experiments are likely to have overestimated the recovery of our isolation procedure. Therefore the intervalve water of shellfish was separated before seeding, to get flesh samples, from which the virus had to be eluted by the glycine buffer. With such samples a detection limit of 3 to 30 $TCID_{50}$ per 1.25 g of mussel tissue could be reproduced. The recovery rates of our purification protocol from oysters and from shrimps were determined to be about 30 $TCID_{50}$ of PV (Fig. 2). In contrast, seeding experiments performed with SRSVs using stool sample P2-A yielded different recoveries from the examined food matrices. The best recovery was obtained with mussels (3×10^5-fold dilution), whereas with shrimps and oysters the recoveries were reduced 10- and 100-fold, respectively (Fig. 3). Detection limits of our purification protocol following seeding of seafood tissue with SRSVs are compared in Table 3.

3.3. Application of the detection method in a small market survey

Finally, the newly developed detection procedure was tested with a few seafood samples (3 mussels, 3 oysters, and 4 shrimps) in collaboration with the state control laboratory of the Canton Berne (Switzerland). The samples were analysed for the presence of four different viruses (HAV, EV, RV and SRSV) in our laboratory, whereas the same samples were examined for bacterial contamination by the state

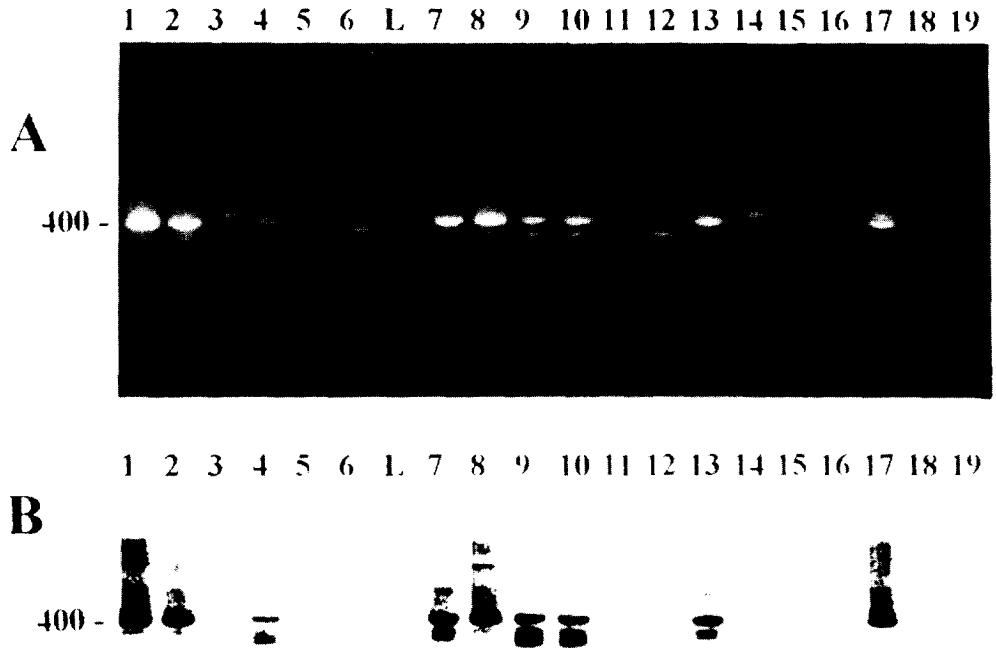

Fig. 2. Recoveries of Poliovirus from artificially contaminated shrimps, oysters and mussels. (A) Detection of PV amplicons on an ethidium bromide-stained agarose gel. Lanes 1 to 6, 1.25 g of homogenized tissue of shrimps seeded with 30 000, 3000, 300, 30, 3 and 0 $TCID_{50}$; lanes 7 to 12, 1.25 g of homogenized tissue of oysters seeded with 30 000, 3000, 300, 30, 3 and 0 $TCID_{50}$; lanes 13 to 16, 1.25 g of homogenized tissue of mussels seeded with 30, 3, 0.3 and 0 $TCID_{50}$; lane 17, RT-PCR positive control; lanes 18 and 19, negative controls of RT and PCR, respectively. (B) Southern blot of gel from panel A, using a PV-specific probe. Lanes are the same as in (A).

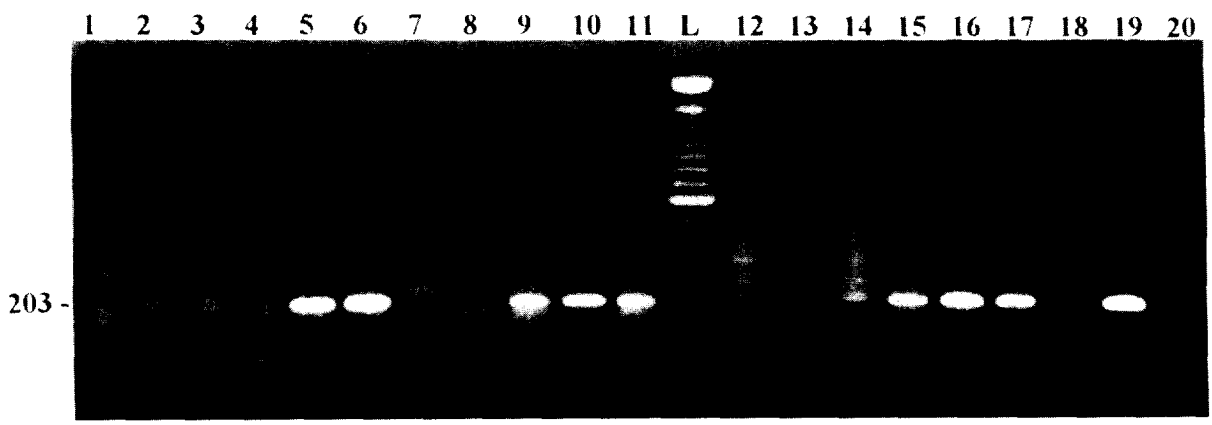

Fig. 3. Recoveries of SRSV genotype II from artificially contaminated shrimps, oysters and mussels with sample P2-A. Lanes 2 to 6, 1.25 g of homogenized tissue of oysters seeded with 3×10^6-, 3×10^5-, 3×10^4-, 3×10^3- and 3×10^2-fold dilutions of stool sample; lanes 8 to 11, 1.25 g of homogenized tissue of mussels seeded with 3×10^6-, 3×10^5-, 3×10^4- and 3×10^3-fold dilutions of stool sample; lanes 13 to 16, 1.25 g of homogenized tissue of shrimps seeded with 3×10^5-, 3×10^4-, 3×10^3- and 3×10^2-fold dilutions of stool sample; lanes 1, 7 and 12; negative controls of oysters, mussels and shrimps, respectively; lanes 17 and 18, positive and negative controls of RT; lanes 19 and 20, positive and negative controls of PCR; L: 100 bp ladder.

Table 3
Comparison of detection limits for Polioviruses and SRSVs in different matrices

Matrix[a]	SRSV[b]	Relative efficiency	PV $TCID_{50}$	Relative efficiency
Stool / virus stock	10^7	1	0.01	1
Mussels	3×10^5	3×10^{-2}	3–30	3×10^{-4}
Oysters	3×10^3	3×10^{-4}	30	3×10^{-4}
Shrimps	3×10^4	3×10^{-3}	30	3×10^{-4}

[a] Viral RNA extraction is described in Section 2.
[b] Reciprocal of last dilution at which virus was detected.

control laboratory according to the Swiss Food Manual (Anonymous, 1988). All samples tested negative for HAV, EV, and SRSVs, but Rotaviruses were detected in all three oyster samples (Fig. 4). In addition all the samples tested negative for *Staphylococcus aureus*, *Escherichia coli* and *Listeria monocytogenes*, confirming that bacterial contamination is not an indicator for viral contamination (Block and Schwartzbrod, 1989; Centers for Disease Control, 1991; Guyer, 1989; Larkin and Hunt, 1982).

The RVs detected in three oyster samples were typed by digestion of the amplicons with *Alu*I, *Cfo*I and *Hae*III. The obtained RFLP pattern was compared with RV sequences from the databank GenEMBL and similarity with serotype 6 bovine rotavirus ROBVP7G was found. Clearly, the used PCR positive control revealed a completely different pattern of restriction fragments excluding cross-contamination of the oyster samples (data not shown). In an additional survey with 8 shrimps, 12 oysters and 12 mussels purchased from local food suppliers no enteric viruses (EV, HAV, RV or SRSV) were detectable.

4. Discussion

Molecular methods to detect enteric viruses in seafood and water will help to elucidate the potential hazard of these agents for public human health. Nested or seminested RT-PCR systems are among the most sensitive detection methods available today with the theoretical potential to detect as few as one copy of a viral genome. In addition, such systems are highly specific. Since the human pathogenic SRSVs can be grouped into two distinct genotypes, we aimed to develop two different RT-PCR detection systems in order to be able to detect most human

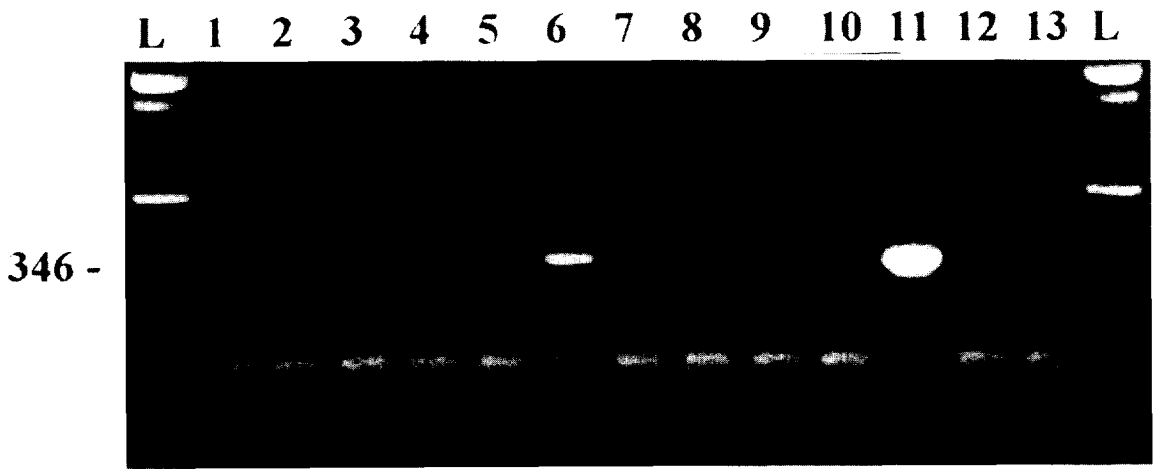

Fig. 4. Detection of group A Rotavirus in seafood samples from market survey. Seafood samples (6–22 g) were analyzed for the presence of HAV, EV, SRSV and RV. Detection of RV RT-PCR products on an ethidium bromide stained agarose gel. Lanes 1 to 3, mussels; lanes 4 to 6, oysters; lanes 7 to 10, shrimps; lane 11, positive control (RNA isolated from stool sample); lanes 12 and 13, negative control of RT and PCR, respectively.

pathogenic SRSVs. Our systems proved to be highly sensitive and extrapolation to the amount of viral nucleic acid present in examined stool samples pointed to $\geq 10^9$ copies of SRSV genomes present in 1 g stool. This number is considerably higher than previous estimates based on electron microscopy (10^5 to 10^6/g (Kapikian and Chanock, 1990)) or RT-PCR ($\geq 10^7$/g (Atmar et al., 1995)). Although the reason for this discrepancy is not known, possible explanations are the presence of amplifiable free viral RNA in stool or in infected cells shed to the stool or more efficient removal of potential RT-PCR inhibitors. Interestingly, the genotype I-specific RT-PCR system failed to detect a stool sample previously identified as genotype 1 SRSV-positive (P1-B), which could be detected by the genotype II-specific system, albeit with reduced sensitivity. Similar results were obtained in another laboratory (J. Noel, CDC Atlanta, personal communication). Sample P1-B contains SRSVs which are homologous to the strains uk1-6 and uk1-7 and therefore, are likely to be genetically distinct from other genotype I strains (Ando et al., 1995). Clearly, further DNA sequence analysis will help to elucidate the molecular basis for this discrepancy.

For the survey of seafood, a simple and efficient purification protocol for enteric viruses was devised. Our recoveries of seeded PV and SRSV are within the range reported by others (Atmar et al., 1993; Jaykus et al., 1993). Interestingly, a remarkable variability in recovery rates was observed with oysters, which gave less good results than mussels and shrimps. The reason for this matrix differences is not known, but was already reported by others (Bouchriti et al., 1994; Lees et al., 1995).

We applied our purification method in a small market survey and three oyster samples were found to be positive for rotaviruses demonstrating, that our developed protocol is suitable for the detection of enteric viruses in naturally contaminated shellfish. In contrast to SRSVs, which have often been implicated in shellfish associated outbreaks of gastroenteric diseases, relatively little is known about RV transmission by shellfish (Ansari et al., 1991; Blacklow and Greenberg, 1991).

This study describes a highly sensitive and specific RT-PCR based system for the detection of SRSVs. This simple and efficient purification protocol for enteric viruses from seafood followed by specific RT-PCR detection will enable to detect enteric viruses in food samples in order to investigate foodborne outbreaks of viral diseases.

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References

Ando, T., Mulders, M.N., Lewis, D.C., Estes, M.K., Monroe, S.S., Glass, R.I., 1994. Comparison of the polymerase region of small round structured virus strains previously classified in three antigenic types by solid-phase immune electron microscopy. Arch. Virol. 135,(1–2) 217–226.

Ando, T.S., Monroe, S.S., Gentsch, J.R., Jin, Q., Lewis, D.C., Glass, R.I., 1995. Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and Southern hybridization. J. Clin. Microbiol. 33,(1) 64–71.

Anonymous (1988) Swiss Food Manual (Schweizerisches Lebensmittelbuch). Mikrobiologie, 5th ed., vol. 2, chapter 56. Eidgenössische Drucksachen- und Materialzentrale, Berne, Switzerland.

Ansari, S.A., Springthorpe, V.S., Sattar, S.A., 1991. Survival and vehicular spread of human rotaviruses: possible relation to seasonality of outbreaks. Rev. Infect. Dis. 13, 448–461.

Atmar, R.L., Metcalf, T.G., Neill, F.H., Estes, M.K., 1993. Detection of enteric viruses in oysters by using the polymerase chain reaction. Appl. Environ. Microbiol. 59, 631–635.

Atmar, R.L., Neill, F.H., Romalde, J.L., Le Guyader, F., Woodley, C.M., Metcalf, T.G., Estes, M.K., 1995. Detection of Norwalk virus and Hepatitis A virus in shellfish tissues with the PCR. Appl. Environ. Microbiol. 61, 3014–3018.

Blacklow, N.R., Greenberg, H.B., 1991. Viral gastroenteritis. New Engl. J. Med. 325,(4) 252–264.

Block, J.C. and Schwartzbrod, L., 1989. Viruses in water systems—Detection and Identification. VCH Publishers, Inc., New York.

Bouchriti, N., Goyal, S.M., Elmarrakchi, A., Jellal, M., 1994. Comparison of three methods for the concentration of poliovirus from Moroccan shellfish. J. Food Prot. 57,(11) 996–1000.

Caul, E.O., Sellwood, N.J., Brown, D.W., Curry, A., Humphrey, T.J., Hutchinson, D.N., Kurtz, J.B., Palmer, S.R., Riordan, T., Sharp, I.R., 1993. Outbreaks of gastroenteritis associated with SRSVs. PHLS Microbiol. Dig. 10,(1) 2–8.

Centers for Disease Control, 1991. Gastroenteritis associated with consumption of raw shellfish—Hawaii, 1991. Morbid. Mortal. Weekly Rep. 40, 303–305.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156–159.

Croci, L., De Medici, D., Divizia, M., Gabrieli, R., Toti, L., Pana, A., 1993. Recovery of poliovirus type 1 from experimentally contaminated shellfish: Evaluation of different methods. Wat. Sci. Technol. 27, 45–48.

Dingle, K.E., Lambden, P.R., Caul, E.O., Clarke, I.N., 1995. Human enteric Caliciviridae: the complete genome sequence and expression of virus-like particles from a genetic group II small round structured virus. J. Gen. Virol. 76, 2349–2355.

Dowell, S.F., Groves, C., Kirkland, K.B., Cicirello, H.G., Ando, T., Jin, Q., Gentsch, J.R., Monroe, S.S., Humphrey, C.D., Slemp, C., Dwyer, D.M., Meriwether, R.A., Glass, R.I., 1995. A multistate outbreak of oyster-associated gastroenteritis: implications for interstate tracing of contaminated shellfish. J. Infect. Dis. 171, 1497–1503.

Gilgen, M., Wegmüller, B., Burkhalter, P., Bühler, H.P., Müller, U., Lüthy, J., Candrian, U., 1995. Reverse transcription PCR to detect enteroviruses in surface water. Appl. Environ. Microbiol. 61,(4) 1226–1231.

Goswami, B.B., Koch, W.H., Cebula, T.A., 1993. Detection of hepatitis A virus in *Mercenaria mercenaria* by coupled reverse transcription and polymerase chain reaction. Appl. Environ. Microbiol. 59,(9) 2765–2770.

Gouvea, V., Glass, R.I., Woods, P., Taniguchi, K., Clark, H.F., Forrester, B., Fang, Z.-Y., 1990. Polymerase chain reaction amplification and typing of rotavirus nucleic acid from stool samples. J. Clin. Microbiol. 28,(2) 276–282.

Green, J., Norcott, J.P., Lewis, D., Arnold, C., Brown, D.W., 1993. Norwalk-like viruses: demonstration of genomic diversity by polymerase chain reaction. J. Clin. Microbiol. 31,(11) 3007–3012.

Green, S.M., Dingle, K.E., Lambden, P.R., Caul, E.O., Ashley, C.R., Clarke, I.N., 1994. Human enteric Caliciviridae: a new prevalent small round-structured virus group defined by RNA-dependent RNA polymerase and capsid diversity. J. Gen. Virol. 75, 1883–1888.

Guyer, S. (1989) Viruskontamination der Aare im Zusammenhang mit der Wirksamkeit von Kläranlagen. Dissertation, University of Berne.

Hedberg, C.W., Osterholm, M.T., 1993. Outbreaks of food-borne and waterborne viral gastroenteritis. Clin. Microbiol. Rev. 6,(3) 199–210.

Hyypiä, T., Auvinen, P., Maaronen, M., 1989. Polymerase chain reaction for human picornaviruses. J. Gen. Virol. 70, 3261–3268.

Jaykus, L.A., De Leon, R., Sobsey, M.D., 1993. Application of RT-PCR for the detection of enteric viruses in oysters. Wat. Sci. Technol. 27, 49–53.

Jiang, X., Wang, M., Wang, K., Estes, M.K., 1993. Sequence and genomic organization of Norwalk virus. Virology 195, 51–61.

Kapikian, A.Z., Chanock, R.M., 1990. Norwalk group of viruses. In: Fields, B.N. and Knipe, D.M., (Eds), Virology, 2nd ed, Vol. 1, Raven Press, New York, pp. 671–693.

Kohn, M.A., Farley, T.A., Ando, T., Curtis, M., Wilson, S.A., Jin, Q., Monroe, S.S., Baron, R.C., McFarland, L.M., Glass, R.I., 1995. An outbreak of Norwalk virus gastroenteritis associated with eating raw oysters. Implications for maintaining safe oyster beds. J. Am. Med. Assoc. 273, 466–471.

Lambden, P.R., Caul, E.O., Ashley, C.R., Clarke, I.N., 1993. Sequence and genome organization of a human small round-structured (Norwalk-like) virus. Science 259, 516–519.

Larkin, E.P., Hunt, D.A., 1982. Bivalve mollusks: control of microbiological contaminants. Bioscience 32, 193–197.

Le Guyader, F., Dubois, E., Menard, D., Pommepuy, M., 1994. Detection of hepatitis A virus, rotavirus, and enterovirus in naturally contaminated shellfish and sediment by reverse transcription-seminested PCR. Appl. Environ. Microbiol. 60, 3665–3671.

Lees, D.N., Henshilwood, K., Green, J., Gallimore, C.I., Brown, D.W., 1995. Detection of small round structured viruses in shellfish by reverse transcription-PCR. Appl. Environ. Microbiol. 61,(12) 4418–4424.

Lew, J.F., Kapikian, A.Z., Valdesuso, J., Green, K.Y., 1994. Molecular characterization of Hawaii virus and other Norwalk-like viruses: evidence for genetic polymorphism among human caliciviruses. J. Infect. Dis. 170, 535–542.

Moe, C.L., Gentsch, J., Ando, T., Grohmann, G., Monroe, S.S., Jiang, X., Wang, J., Estes, M.K., Seto, Y., Humphrey, C., Stine, S., Glass, R.I., 1994. Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis. J. Clin. Microbiol. 32,(3) 642–648.

Monceyron, C., Grinde, B., 1994. Detection of hepatitis A virus in clinical and environmental samples by immunomagnetic separation and PCR. J. Virol. Methods 46,(2) 157–166.

Pontefract, R.D., Bishai, F.R., Hockin, J., Bergeron, G., Parent, R., 1993. Norwalk-like viruses associated with a gastroenteritis outbreak following oyster consumption. J. Food Prot. 56,(7) 604–607.

Richards, G.P., 1987. Shellfish-associated enteric virus illness in the United States, 1934–1984. Estuaries 10, 84–85.

Rotbart, H.A., 1990. Enzymatic RNA amplification of the enteroviruses. J. Clin. Microbiol. 28,(3) 438–442.

Shieh, Y.-S.C., Wait, D., Tai, L., Sobsey, M.D., 1995. Methods to remove inhibitors in sewage and other fecal wastes for enterovirus detection by polymerase chain reaction. J. Virol. Methods 54, 51–66.

Wang, J., Jiang, X., Madore, H.P., Gray, J., Desselberger, U., Ando, T., Seto, Y., Oishi, I., Lew, J.F., Green, K.Y., Estes, M.K., 1994. Sequence diversity of small, round-structured viruses in the Norwalk virus group. J. Virol. 68,(9) 5982–5990.

Zoll, G.J., Melchers, W.J.G., Kopecka, H., Jambroes, G., van der Poel, H.J.A., 1992. General primer-mediated PCR for detection of enteroviruses: application for diagnostic routine and persistent infections. J. Clin. Microbiol. 30,(1) 160–165.