APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1995, p. 4418–4424 Vol. 61, No. 12
0099-2240/95/$04.00+0

Detection of Small Round Structured Viruses in Shellfish by Reverse Transcription-PCR

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Received 12 July 1995/Accepted 23 September 1995

We describe the application of a previously developed sample extraction procedure to the detection of small round structured viruses (SRSVs) in shellfish. Initial seeding experiments showed that PCR inhibitor removal and virus recoveries were comparable to those in previous studies with poliovirus. Shellfish from a range of sewage-contaminated sites were then tested for the presence of SRSVs by using broadly reactive PCR primers followed by Southern blotting with internal probe sites. Positive results were obtained from 5 of 31 field samples tested. Four of these positive samples were from highly polluted sites. PCR product sequence analysis confirmed their identity as SRSV and showed sequence diversity compared with virus controls, suggesting that the results were not a consequence of PCR cross-contamination. Finally, shellfish associated with four separate outbreaks of viral gastroenteritis were tested by PCR and Southern blot for the presence of SRSVs. All outbreak samples tested gave positive results. As far as we are aware, this is the first demonstration of the detection in environmentally contaminated shellfish of the SRSVs responsible for human gastroenteritis. This development may help contribute to the further development of public health controls for molluscan shellfish.

The Norwalk-like viruses or small round structured viruses (SRSVs) are important human pathogens that are frequently associated with gastroenteritis following consumption of sewage-contaminated molluscan shellfish (4, 5, 22, 29, 30, 32, 33). Public health controls are hampered by the absence of methods for the detection of these viruses in shellfish. Recent advances in genome characterization of Norwalk and related viruses (20, 23) have led to reverse transcription-PCR (RT-PCR)-based assays for detection of these viruses in human stools (6, 13, 19, 28); these assays have proved valuable in the epidemiological investigation of outbreaks (21, 22). However, the application of these techniques to the detection of Norwalk-like viruses in molluscan shellfish associated with such outbreaks has proved more problematic (22) and has not yet been successfully demonstrated. This may be due in part to the presence of potent PCR amplification inhibitors in complex samples such as shellfish. We have previously described the development, using poliovirus, of a sample extraction procedure for shellfish compatible with PCR (25). Initial virus extraction stages based on a modified polyethylene glycol precipitation technique (27) were followed by virus purification with 1,1,2-trichloro-2,2,1-trifluoroethane (Freon TF) and concentration by ultrafiltration. A guanidine isothiocyanate glass powder extraction system was used for sample lysis, RNase protection, and nucleic acid purification. Seeding experiments showed that overall sensitivity limits were <10 PFU of poliovirus in up to 5 g of shellfish. Other workers have reported similar developmental work based on seeding experiments for enteroviruses, hepatitis A virus, and rotavirus (3, 7, 10, 17, 26) and have proposed various sample extraction protocols. Similar approaches have recently been applied to Norwalk virus, with virus being detected in shellfish following laboratory seeding experiments (3, 11). However, application of such methods to detection of Norwalk virus in oysters associated with a large multistate outbreak was not successful (22). We, and others, have demonstrated the successful application of PCR sample extraction protocols to the detection of human enteroviruses in shellfish from polluted field sites (7, 24, 26), but we are not aware of comparable studies for Norwalk virus in either outbreak-associated shellfish or polluted field samples. We describe here the application of our sample extraction protocol to the detection of SRSVs in both environmentally polluted field samples and shellfish associated with outbreaks of human infection.

Several studies have highlighted sequence diversity in the Norwalk virus group (1, 13, 18, 28, 31, 35), which has important consequences for design of PCR primers optimal for detection of unknown strains in environmentally polluted and outbreak samples. Although several suggestions have been made (1, 15, 34), consensus primer sets appropriate for all Norwalk and Norwalk-like virus strains are not yet available. In this study, we used a broadly reactive PCR primer set known to react with approximately 90% of SRSVs currently circulating in the United Kingdom (12).

MATERIALS AND METHODS

Viruses. For seeding experiments, a well-characterized stool sample shown to contain SRSVs by electron microscopy and RT-PCR was used. Analysis of the genomic sequence from a portion of the RNA polymerase of this SRSV showed it to be a genogroup II strain most closely related to Bristol virus (14). All seeding experiments were performed with 10% fecal extracts prepared by diluting stool 1:10 (wt/vol) in phosphate-buffered saline (Dulbecco's formula) (PBS) followed by thorough mixing and centrifugation at 3,000 × *g* for 5 min. Supernatants were stored at 4°C until use. Poliovirus type 1 (VR-192) was grown and subjected to titer determination as previously described (25).

Shellfish. Commercially depurated oysters (*Crassostrea gigas*) and mussels (*Mytilus edulis*) were obtained from Oakford Oysters, Blandford, England. RT-PCR inhibitor removal was evaluated in depurated and highly polluted (Holes Bay, Poole Harbour) mussels. Depurated oysters and mussels were used for virus recovery seeding experiments. SRSV contamination was evaluated in shellfish obtained from a range of sites subject to high levels of sewage pollution. The sites were Pennington, The Solent (oysters [*Ostrea edulis*]); Holes Bay, Poole Harbour (mussels and oysters [*C. gigas*]); Lympstone, River Exe (mussels and oysters [*C. gigas*]); West Huntspill, Somerset (mussels); Barton-on-Sea, Christchurch Bay (mussels); and Mersey, Liverpool (cockles [*Cerastoderma edule*]). All sites were adjacent to domestic sewage discharges. Shellfish were also obtained from com-

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mercial growing areas in England and Wales subject to various levels of pollution. *Escherichia coli* counts in shellfish were assayed by a multiple-tube most-probable-number (MPN) procedure incorporating a resuscitation stage in mineral-modified glutamate broth (36). Depurated shellfish contained an MPN of less than 230 *E. coli*/100 g of shellfish flesh. *E. coli* counts in highly polluted shellfish generally exceeded an MPN of 60,000 *E. coli*/100 g of shellfish flesh. All environmental samples were stored frozen whole at −20°C prior to use in the RT-PCR. Shellfish associated with human infection were processed either from whole animals or from crude shellfish homogenates diluted to 1:10 in tryptose phosphate broth (Lab M, Bury, United Kingdom)–0.05 M glycine buffer (pH 9.0 to 9.5).

Shellfish processing and virus extraction and purification. The procedure for shellfish processing was a modification of that by Lewis and Metcalf (27) and has been described in full previously (25). Essentially, shellfish were shucked, homogenized, sonicated, and centrifuged and supernatants were precipitated with polyethylene glycol. For seeding experiments and environmental samples, 50 g of shellfish flesh was processed. For shellfish associated with disease outbreaks, up to 50 g was processed. Resuspended pellets were sonicated and centrifuged prior to further virus purification by extraction with Freon TF followed by centrifugal concentration and storage at −20°C. Extracts at this stage were termed purified concentrates.

Extraction of viral RNA from purified concentrates. The RNA extraction procedure has been described in full previously (25). Essentially, a reaction mix of glass powder matrix and guanidine isothiocyanate was used to extract total nucleic acid from purified shellfish concentrates. Shellfish tissue weight equivalents extracted were 7, 2.4, and 0.8 g for all seeding experiments and environmental samples. Nucleic acid was extracted neat and at 1:3 and 1:9 dilutions for purified concentrates from shellfish associated with disease outbreaks. Guanidine isothiocyanate served to lyse samples and protect RNA from enzymatic digestion. RNA bound to glass powder was washed with guanidine isothiocyanate, ethanol, and acetone separately prior to elution in Tris buffer. RNA was then precipitated in ethanol, and pellets were stored at −70°C prior to RT-PCR.

Oligonucleotide primers. The poliovirus primers were directed at the conserved 5′ region of the poliovirus genome and have been described previously (25). SRSV RT-PCR primers were derived from genomic sequences from nine SRSV strains detected in the United Kingdom, representing solid phase immunoelectron microscopy (SPIEM) types UK1 to UK4 and untypeable strains (31). Primer pair NI-E3 (NI, 5′-GAATTCCATCGCCCACTGGCT; E3, 5′-ATCTC ATCATCACCATA) amplifies a 113-bp region of the RNA polymerase gene corresponding to nucleotides 4756 to 4867 of Norwalk virus. This SRSV primer set has been shown to amplify more than 90% of strains circulating in the United Kingdom in 1993 and 1994 (12).

RT-PCR. Poliovirus RT-PCR was performed as previously described (25). SRSV RT-PCR was performed by resuspending RNA pellets in 6.9 μl of sterile water, adding 20 U of RNase inhibitor (RNasin [Promega]) and 1 μl of 50 mM random hexamers (PdN6 [Pharmacia Biotech]), and overlaying the mixture with 50 μl of mineral oil (400-5 [Sigma]). The mixture was heated at 70°C for 5 min, chilled on ice, and then added to 6.1 μl of reaction mix containing (final concentrations) 10 mM Tris (pH 8.3), 50 mM KCl, 5 mM $MgCl_2$, 1 mM each deoxynucleoside triphosphate (Pharmacia Biotech), and 100 U of Moloney murine leukemia virus reverse transcriptase (fast protein liquid chromatography-pure cloned Moloney murine leukemia virus [Life Technologies]). RT was performed at room temperature for 10 min followed by incubation at 37°C for 1 h. The reaction was terminated by incubation at 95°C for 5 min, the tubes were then chilled on ice, and 35 μl of PCR reaction mix was added (10 mM Tris [pH 8.3], 50 mM KCl, 20 pmol of each primer NI and E3, 1 U of *Taq* polymerase). After an initial denaturation at 94°C for 2 min, 30 amplification cycles of 95°C for 1 min, 40°C for 1 min, and 72°C for 1 min were performed, followed by a final extension of 72°C for 10 min. Amplification products were examined by electrophoresis of 20 μl of reaction mix in an agarose gels (4% composite gel, 3% Nusieve, 1% SeaKem [Flowgen]) at 10 V/cm for 1.5 to 2 h. Primer pair NI-E3 amplifies a 113-bp region of the RNA polymerase gene. Molecular sizes were determined by comparison with a 1-kb DNA ladder (Gibco BRL).

Southern blot hybridization of amplification products. Southern blot hybridization of SRSV RT-PCR amplicons was performed to confirm the specificity of amplification and to detect weakly positive samples where amplicon bands were not visible under UV radiation. A pool of four oligonucleotides was used as a probe. These oligonucleotides were derived from genomic sequences of strains assigned to SPIEM types UK1 to UK4, respectively. The oligonucleotide sequences are as follows: probe 1, 5′-TATGTGCCCTGTCAGAAGT; probe 2, 5′-TATCACCTGATGTTATACAATCC; probe 3, 5′-GTCCCCTGACATCA TACAGGCT; probe 4, 5′-ATCCCCTGACATCGTCCAGGCT. Agarose gels were prepared for the Southern blot procedure by submersion for 30 min in denaturation solution (0.5 M NaOH, 1.5 M NaCl) followed by neutralization (3 M NaCl, 0.5 M Tris [pH 7.0]) for 30 min. Amplification products were transferred onto a positively charged nylon membrane (Boehringer Mannheim) by a standard capillary procedure and cross-linked to the membrane by baking at 120°C for 20 min. Hybridization was performed at 40°C overnight. After two 5-min washes in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate, hybridized probe was detected by chemiluminescence. Hybridization and chemiluminescent detection reagents were as recommended by the manufacturer (Boehringer Mannheim).

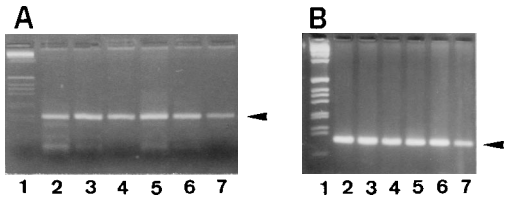

FIG. 1. RT-PCR inhibitor removal in depurated and highly polluted mussels. Shellfish were processed through to the purified concentrate stage, and neat extract or dilutions were seeded with either poliovirus or an SRSV fecal extract before nucleic acid extraction and RT-PCR. Agarose gel electrophoresis of PCR products is shown. Lanes: 1, 1-kb DNA ladder; 2 to 4, 6.2, 2.1, and 0.7 g, respectively, of shellfish tissue equivalents of depurated shellfish extract; 5 to 7, 6.2, 2.1, and 0.7 g, respectively, of shellfish tissue equivalents of polluted shellfish extract. (A) Extracts seeded with 10^5 PFU of poliovirus. (B) Extracts seeded with 50 μl of a 10% SRSV fecal extract. Arrowheads denote the 155-bp poliovirus and the 113-bp SRSV PCR products.

PCR product sequencing. Following RT-PCR, amplification products were separated from unincorporated oligonucleotide primers and nucleotides on a Chromaspin or Centricon 100 +TE column (Cambridge Bioscience). DNA concentrations were determined by A_{260} readings. The DNA sequence was determined with an ABI 373A automated sequencer and a *Taq* DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems) as described previously (13). The RT-PCR products were sequenced in both orientations with the NI and E3 primers.

Sequence data analysis. DNA sequence data obtained were initially analyzed with Seq Ed (version 1.0.3) before being imported into the DNAStar package (DNAStar Ltd., London, United Kingdom) for alignment.

RESULTS

RT-PCR inhibition. Previous studies by an enterovirus PCR have documented the potent inhibitory potential of shellfish, shown that this may be exacerbated in heavily polluted samples, and developed shellfish-processing and nucleic acid extraction methods to overcome it (25). The application of these sample extraction procedures to the SRSV PCR was studied by comparing shellfish-mediated amplification inhibition in both the enterovirus and SRSV PCRs. Commercially depurated and environmentally polluted mussels were processed through to the purified concentrate stage and seeded with either poliovirus or an SRSV fecal extract. Nucleic acid was then extracted, and the RT-PCR was performed. There was no discernible amplification inhibition in either PCR assay for both commercially depurated and environmentally polluted shellfish (Fig. 1). Sample tolerances exceeded 6 g (equivalent weight) of shellfish in all cases. These results are comparable to those previously obtained (25) and demonstrate that the shellfish-processing procedures developed for removal of enterovirus PCR amplification inhibitors are also applicable to the SRSV PCR.

Virus recovery. SRSV recovery efficiencies were determined for both oysters (*C. gigas*) and mussels by seeding crude shellfish homogenates with various concentrations of SRSV and determining subsequent recoveries after sample processing, RT-PCR, and Southern blot hybridization. Initially, a PCR titer was established for a 10% fecal extract by serial dilution in PBS, extraction of RNA as described in Materials and Methods, RT-PCR, and Southern blotting. One PCR unit was defined as the highest dilution giving a band by Southern blot. PCR titers of 10 U/μl were determined for 10% fecal extracts prepared on two separate occasions. Mussel and oyster crude homogenates containing 50 g of shellfish flesh were seeded with various titers (1,250, 2,500, 5,000, and 10,000 PCR units) of SRSV and processed to the purified concentrate stage prior to serial dilution in PBS, nucleic acid extraction, RT-PCR, and Southern blot hybridization. Figure 2 illustrates representative

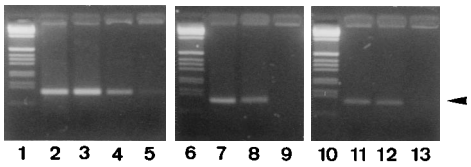

FIG. 2. SRSV recovery from seeded oysters (*C. gigas*) and mussels. Crude shellfish homogenates were seeded with various doses of a titrated SRSV fecal extract and processed to the purified concentrate stage, and a dilution series made prior to nucleic acid extraction and RT-PCR. Agarose gel electrophoresis of PCR products for a representative dilution series is shown. Lanes: 1, 6, and 10, 1-kb DNA ladder; 2 to 5, 100, 10, 1, and 0.1 μl, respectively, of a 10% SRSV fecal extract; 7 to 9, oyster extracts seeded with 36, 12, and 4 μl, respectively, of a 10% SRSV fecal extract; 11 to 13, mussel extracts seeded with 0.6, 0.22, and 0.06 μl, respectively, of a 10% SRSV fecal extract. The arrowhead denotes the 113-bp SRSV PCR product.

PCR results for a 10% fecal extract titer determination and serially diluted oyster and mussel concentrates. Shellfish tissue equivalent weight analyzed did not exceed 7 g in any experiment to avoid shellfish-mediated amplification inhibition. End point determination was by the highest dilution giving a band by Southern blot. It was noticeable for both the fecal extract and shellfish seeding titer determinations that Southern blot hybridization gave a higher level of sensitivity than that achievable by gel electrophoresis alone (data not shown). Three independent experiments with oysters gave quantifiable results of 5, 18, and 45% (average, 23%) recovery from initial seeding doses. Results with mussels were more variable, giving recoveries of 5 and 187% (average, 96%). These recoveries, although variable, are similar to previous average recoveries for poliovirus of 17 to 31% by the same sample extraction methods (25).

Field samples. Environmentally contaminated oysters (*O. edulis* and *C. gigas*), mussels, and cockles were obtained from several highly polluted field sites and processed by the virus and nucleic acid extraction procedures, and the SRSV PCR and Southern blot hybridization were performed. Positive results were obtained for several samples, indicating the presence of naturally occurring virus acquired through filter feeding in sewage-polluted waters (Table 1). One sample (Lympstone 3) was positive by both gel electrophoresis of PCR products and Southern blot at all shellfish concentrations tested. For other samples (Pennington 1 and Holes Bay 4), specific PCR product was discernible only by Southern blot hybridization and only when the highest concentration (7 g equivalent weight) of shellfish was tested. These results are consistent with those obtained in the seeding studies at low virus levels and probably indicate low virus titers in field samples. One sample (Holes Bay 6) was positive by Southern blot only at the lowest concentration (0.8 g equivalent weight) of shellfish tested. The Southern blot band for this sample was faint and was not apparent in a duplicate extraction (Fig. 3). It is not clear why higher shellfish concentrations were negative for this sample. The previous seeding experiments suggest that RT-PCR inhibition is unlikely within this range of shellfish concentrations. Electrophoresis and Southern blot results for shellfish samples Lympstone 1 (negative), Lympstone 3 (positive), and Holes Bay 6 (positive/negative) are shown in Fig. 3. Southern blot results show clearly the differing PCR product yields of the two positive samples, which is probably a reflection of initial virus titers.

TABLE 1. Detection of SRSVs in shellfish from highly polluted sites[a]

Sample(s)	Shellfish species	Detection by:					
		PCR			Southern blot		
		7 g[b]	2.4 g[b]	0.8 g[b]	7 g	2.4 g	0.8 g
Pennington 1	*O. edulis*	−	−	−	+	−	−
Pennington 2, 3, 4, and 5	*O. edulis*	−	−	−	−	−	−
Holes Bay 1, 5, and 7	Mussels	−	−	−	−	−	−
Holes Bay 2 and 3	*C. gigas*	−	−	−	−	−	−
Holes Bay 4	Mussels	−	−	−	+	−	−
Holes Bay 6	Mussels	−	−	−	−	−	±
Lympstone 1 and 2	Mussels	−	−	−	−	−	−
Lympstone 3	*C. gigas*	+	+	+	+	+	+
West Huntspill 1 and 2	Mussels	−	−	−	−	−	−
Barton	Mussels	−	−	−	−	−	−
Mersey	Cockles	−	−	−	−	−	−

[a] All sites were adjacent to sewage discharges and exceeded contamination levels of an MPN of 60,000 *E. coli*/100 g of shellfish flesh, i.e., prohibited for human consumption under European Community Directive 91/492.

[b] Shellfish tissue equivalent weight tested in grams.

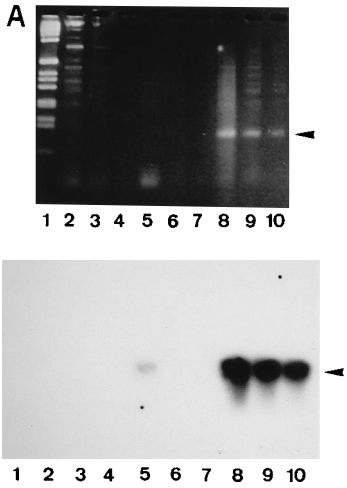

FIG. 3. Application of RT-PCR to detection of SRSVs in polluted field samples. Lanes: 1, 1-kb DNA ladder; 2 to 5, sample Holes Bay 6 containing 7, 2.4, 0.8, and 0.8 g, respectively, of shellfish tissue equivalents; 6 and 7, sample Lympstone 1 containing 7 and 2.4 g, respectively, of shellfish tissue equivalents; 8 to 10, sample Lympstone 3 containing 7, 2.4, and 0.8 g, respectively, of shellfish tissue equivalents. (A) Agarose gel electrophoresis. (B) Southern blot hybridization. The arrowhead denotes the 113-bp SRSV PCR product. Sample details and results are summarized in Table 1.

Similar experiments were then performed on shellfish obtained from commercial growing areas subject to various levels of pollution (Table 2). Only one positive sample (Colwyn Bay 2) was obtained from this limited survey. This sample was positive by both gel electrophoresis and Southern blot but again only at the lowest concentration of shellfish tested. This SRSV PCR-positive sample did not correlate with an elevated *E. coli* titer (Table 2).

Outbreak samples. Following successful demonstration of SRSVs both in shellfish from heavily polluted field sites and in shellfish from commercial growing areas, shellfish associated with incidents of human gastroenteritis were tested for the presence of SRSVs. Shellfish associated with four separate food-poisoning incidents were kindly provided by T. Humphrey, Exeter Public Health Laboratory. Clinical symptoms for all cases associated with these outbreaks were consistent with viral gastroenteritis caused by SRSVs; however, laboratory confirmation by stool analysis was not available. Epidemiolog-

TABLE 2. Detection of SRSVs in shellfish from commercial growing sites

Sample(s)	Date sampled (day/mo/yr)	Shellfish species	*E. coli* counts[a]	Detection by:					
				PCR			Southern blot		
				7 g[b]	2.4 g[b]	0.8 g[b]	7 g	2.4 g	0.8 g
Torridge 1	21/04/93	Mussels	22,000	−	−	−	−	−	−
Torridge 2	21/04/93	Mussels	11,000	−	−	−	−	−	−
Torridge 3	22/04/93	Mussels	7,500	−	−	−	−	−	−
Fowey	21/04/93	Mussels	7,000	−	−	−	−	−	−
Conwy Bridge	15/07/93	Mussels	54,000	−	−	−	−	−	−
Ynys-mon	21/07/92	Mussels	200	−	−	−	−	−	−
Arfon	01/04/92	Mussels	2,400	−	−	−	−	−	−
Colwyn Bay 1	22/04/92	Mussels	5,400	−	−	−	−	−	−
Colwyn Bay 2	01/07/92	Mussels	500	−	−	+	−	−	+
Colwyn Bay 3	22/04/92	Mussels	5,400	−	−	−	−	−	−
Aberconwyn	22/05/92	Mussels	24,000	−	−	−	−	−	−
Poole Bay	09/03/93	*O. edulis*	16,000	−	−	−	−	−	−

[a] *E. coli* MPN/100 g of shellfish flesh.
[b] Shellfish tissue equivalent weight tested in grams.

ical analysis for outbreak 3 implicated oysters as the vehicle of infection. Epidemiological analysis was not available for the other incidents. Shellfish associated with outbreaks 2, 3, and 4 had been commercially depurated in approved tanks. *E. coli* titers from these shellfish batches (Table 3) complied with the bacteriological end product standard required by European Community legislation (2). The shellfish samples associated with outbreak 1 were taken prior to purification and had *E. coli* counts within the permitted prepurification range. Nine shellfish samples associated with these outbreaks were extracted and tested by the methods described above. PCR products were not demonstrable for any sample by gel electrophoresis; however, Southern blot hybridization gave positive results for all samples tested (Table 3). Most of the samples were positive either neat or at the 1:3 dilution (or at both concentrations) but not at the 1:9 dilution. Sample 321/94 was weakly positive only at the neat concentration. Only two samples (509/90 and 301/93) were positive at all shellfish concentrations tested. The absence of PCR product observable by gel electrophoresis and the titrating out of the majority of these samples by Southern blot at a 1:9 dilution are similar to results obtained for polluted field samples. This again probably suggests that SRSV titers in such outbreak-associated shellfish are generally low.

Overall. Table 4 summarizes results from both the field and outbreak samples. Although sample numbers are small, it is interesting that the incidence of SRSV-positive results was greater in highly polluted field sites than in commercial growing areas.

PCR product sequence analysis. All PCR results in this study were confirmed by Southern blot hybridization. Additional information was generated by sequence analysis of PCR products. Unfortunately, PCR products from most of the positive samples contained insufficient DNA for sequence analysis by the methods described. Attempts at reamplification with the NI-E3 primer pair were unsuccessful, although this approach worked with positive control material. It is not clear why weakly positive signal obtained from shellfish samples is refractory to reamplification, but similar problems have been experienced previously when the enterovirus PCR model was used (data not shown). In practice, sequence determination was possible only in PCR products containing enough DNA to be visible by gel electrophoresis. Samples Lympstone 3 (Table

TABLE 3. Detection of SRSVs in shellfish associated with incidents of human gastroenteritis

Outbreak	Date (day/mo/yr)	Time sampled	*E. coli* counts[a]	Sample identification no.	Detection by:					
					PCR			Southern blot		
					Neat[b]	1:3[b]	1:9[b]	Neat	1:3	1:9
1	17/12/90	Before purification	1,100	506/90 - *C.gigas* hom[c]	−	ND[d]	ND	+	ND	ND
				507/90 - *C.gigas* hom	−	−	−	−	+	−
				508/90 - *C.gigas* hom	−	−	−	−	+	−
				509/90 - *C.gigas* hom	−	−	−	+	+	+
2	12/02/93	After purification	20	301/93 - *C.gigas* hom	−	−	−	+	+	−
				301/93 - *C.gigas*	−	−	−	+	+	+w[e]
3	26/12/93	After purification	<20	321/94 - *C.gigas* hom	−	−	−	+w	−	−
4	06/01/94	After purification	20	322/94 - *C.gigas* hom	−	−	−	+	+	−
				323/94 - *C.gigas* hom	−	−	−	+	−	−

[a] *E. coli* MPN/100 g of shellfish flesh in shellfish batch. Counts for outbreaks 1, 2, and 4 were kindly supplied by T. Humphrey, Exeter Public Health Laboratory.
[b] Dilution of final purified shellfish extract tested. Equivalent weight of shellfish flesh in the neat sample was up to 7 g.
[c] hom, sample received as crude shellfish homogenate.
[d] ND, not done.
[e] +w, weakly positive.

TABLE 4. Summary of SRSV detection in shellfish

Sample origin	No. tested	No. (%) positive[a]
Highly polluted field site	19	4 (21)
Commercial growing area	12	1 (8)
Associated with disease incident[b]	9	9 (100)

[a] Number positive as judged by Southern blot hybridization.
[b] Samples were obtained from four separate outbreaks.

1; Fig. 3) and Colwyn Bay 2 (Table 2) could be analyzed, and sequence data for these PCR products and for the fecal sample used for both seeding experiments and positive control are shown in Fig. 4. Nucleotide sequence alignment confirmed that both shellfish-derived PCR products had the SRSV sequence but with a degree of sequence diversity. Lympstone 3 contained two nucleotide changes, coding for one amino acid substitution, compared with the positive control SRSV. Colwyn Bay 2 contained five nucleotide changes, but these coded for only one amino acid substitution in comparison with the positive control.

DISCUSSION

In this paper, we report the successful application of a previously developed sample extraction procedure to the detection of SRSVs in molluscan shellfish by PCR. Seeding experiments with fecal extract containing SRSVs showed that PCR inhibitor removal and virus recovery by this sample extraction procedure were comparable to those in previous studies with poliovirus (25) and within a satisfactory range for further evaluation. PCR inhibitor removal was satisfactory in both commercially depurated and highly polluted shellfish, confirming the validity of the technique (25). These results appear to be similar to SRSV-seeding studies by other workers (3, 11); however, detailed comparison is complicated by the different sample extraction protocols employed.

Application of these procedures to environmentally polluted samples required the selection of an appropriate SRSV PCR primer set. The NI-E3 set chosen has been demonstrated to react with most SRSV strains currently circulating in the United Kingdom (12). This primer set is directed at partially conserved regions of the well-studied RNA polymerase region of the SRSV genome and was derived by sequence analysis of PCR products from a variety of SRSV strains amplified with primers based on published sequence data as previously described (31). Fecal samples representing all United Kingdom SPIEM groups were tested by PCR, with a 90% overall success rate. The positivity rate, as confirmed by Southern blot hybridization, for different SPIEM groups with this primer set varied from 50 to 100%. Although coverage was not complete, it was clear that this primer set would react with most SRSV strains presenting in clinical samples and therefore was appropriate to use for this environmental study. Initial studies targeted at highly polluted field sites demonstrated the utility of this approach, with 21% of shellfish samples positive. To our knowledge, this is the first PCR demonstration of SRSV contamination of environmentally contaminated molluscan shellfish. A further limited survey in less polluted commercial growing areas showed a lower contamination rate, with only 1 of 12 samples positive. This is encouraging for legislative controls, such as the Food and Drug Administration shellfish sanitation program (8) and the European Community Directive 91/492 (2), based on control of sewage pollution levels in molluscan shellfish prior to the market. It should, however, be noted that the single positive sample in this limited survey did not correlate with an elevated *E. coli* titer. Further, more extensive surveys are necessary to establish the adequacy of the current legislative controls, which are based largely on fecal coliform determinants, in preventing SRSV-contaminated shellfish reaching the consumer.

Application of the sample extraction procedure and the NI-E3 SRSV primer set to shellfish associated with four separate outbreaks of human gastroenteritis was successful, with positive results obtained from all samples tested. It is noteworthy that all shellfish were purified in licensed tanks prior to consumption and, where data were available, complied with the relevant bacteriological standards. These results help confirm previous suggestions that compliance with *E. coli* criteria at the point of sale does not guarantee consumer safety (9, 16, 29). The procedures described in this report should prove valuable in the investigation of disease incidents associated with molluscan shellfish. It is frequently difficult or impossible to identify the vehicle of infection in food-poisoning incidents involving individual or small numbers of patients. Consequently, virtually all isolated cases of shellfish-associated infection go unreported (33). Laboratory confirmation of SRSV contamination in shellfish will aid and encourage outbreak investigation and help to obtain a more accurate determination of the disease burden associated with sewage-polluted molluscan shellfish. The positive results in both field and disease-associated shellfish confirm the choice of the NI-E3 primer set in this study. However, the definition of universally applicable SRSV PCR primers, or primer sets, will be necessary before

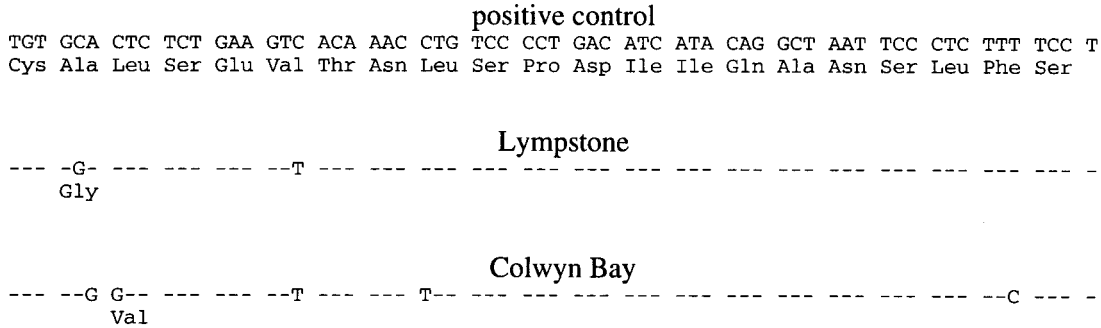

FIG. 4. SRSV PCR product sequence comparison. PCR products visible by gel electrophoresis were sequenced in both directions, and the consensus sequence was determined. Samples are the SRSV-positive control fecal extract and environmentally contaminated shellfish samples Lympstone 3 (Table 1; Fig. 3) and Colwyn Bay 2 (Table 2). Nucleotide and amino acid substitutions, in comparison with the positive control, are shown.

these methods can be fully exploited for outbreak investigation (18, 28).

A feature of the results in this study was the low incidence of positive results containing enough amplified product to be visible by agarose electrophoresis alone. Most field results and all outbreak-associated results required visualization through the added sensitivity of Southern blot hybridization. This was not a consequence of the method used, because results were clearly visible by agarose gel electrophoresis during seeding experiments. These results, and the tendency for samples to titrate out at a 1:9 dilution, suggest that SRSV titers in polluted shellfish are generally very low and that the current method is operating close to its limits of sensitivity. It is possible that other methods for improving PCR sensitivity, such as nested primers, will prove useful but are constrained by the extreme sequence diversity seen in SRSVs. Kohn et al. (22), using PCR, were unable to demonstrate SRSV contamination in shellfish associated with a major disease outbreak. Sensitivity limitations were proposed as a possible explanation; however, it is not clear whether Southern blot hybridization was used to enhance sensitivity. It was also suggested that shellfish-mediated RT-PCR inhibition may have been a factor (22). The sample extraction protocols successfully used here were based on a comprehensive study in which poliovirus was used to determine PCR inhibitor removal during shellfish processing (25). These extraction procedures were successfully applied to the detection of enteroviruses in polluted field samples (24) and have been shown in this study to also be appropriate for PCR inhibitor removal prior to SRSV detection.

All PCR results in this study were confirmed by Southern blot hybridization. The use of internal sites for blot probes confers additional specificity over that obtained by gel electrophoresis alone but does not guarantee authenticity of the results. PCR is known to be susceptible to laboratory cross-contamination, particularly from previously amplified PCR products. In this study, precautions taken were the use of comprehensive negative controls and geographical separation of sample extraction and PCR product analysis. Additional information was generated by sequence analysis of PCR products. Unfortunately, PCR products from most of the positive samples contained insufficient DNA for sequence analysis. However, data were generated for two field samples and the virus control used in seeding experiments. PCR product sequence analysis confirmed the identity of the field isolates as SRSVs and showed sequence diversity compared with the virus control but with overall amino acid conservation. These data strongly suggest that the results are authentic. It would be anticipated that PCR cross-contamination would be manifest by the generation of identical sequence data in positive samples.

In summary, the results of this study show that SRSVs can be detected in molluscan shellfish by PCR. Positive results were obtained in polluted field samples and in all gastroenteritis outbreak-associated samples tested. As far as we are aware, this is the first demonstration of SRSV detection in environmentally contaminated molluscan shellfish. This is significant because these viruses are a major cause of human gastroenteritis following consumption of sewage-contaminated shellfish (4, 5, 22, 29, 30, 32, 33). This development may ultimately contribute to the further development of public health controls for molluscan shellfish for better consumer protection.

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