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Detection of Hepatitis A Virus, Rotavirus, and Enterovirus in Naturally Contaminated Shellfish and Sediment by Reverse Transcription-Seminested PCR

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A reverse transcription-PCR method was developed to detect enterovirus (EV), hepatitis A virus (HAV), and rotavirus (RV) RNAs in shellfish and sediment. The method was first tested under experimental conditions by using virus-spiked shellfish to evaluate assay sensitivity. The use of CC41 cellulose was found to be efficient for removing inhibitors of RV detection. For sediment samples, a Sephadex column was used to allow the detection of EV and HAV RNAs. The specificity of amplified products was controlled by hybridization with digoxigenin-labeled oligoprobes. The method was then applied to naturally contaminated shellfish and sediments. EV, HAV, and RV RNAs were detected in 22, 14, and 20% of the shellfish samples, respectively. No relationship between viral contamination and bacterial contamination was found. When viral RNAs (HAV or EV) were detected in sediments, they were also detected in shellfish.

Human viruses able to persist in the environment are essentially enteric viruses which are extremely resistant to unfavorable conditions and include adenoviruses, caliciviruses, enteroviruses (EV), and hepatoviruses. After replicating in the gastrointestinal tract, these viruses are excreted into sewage and may be dispersed into the environment if sewage is not adequately treated (21, 29, 34, 48, 49). One of the most important consequences of the contamination of coastal areas is the concentration of viruses by shellfish through filter feeding. Standards based on coliform bacteria and established to protect shellfish consumers are known not to be correlated with the presence of viruses, and little about viral depuration is known. Outbreaks of shellfish-transmitted viral disease occur periodically, causing problems for public health and resulting in economic losses for the seafood industry (12, 17, 23). In many outbreaks of viral gastroenteritis, the causative agent is not identified either because it is not possible to detect some viruses by cell culture or because of methodological problems with traditional techniques (e.g., toxicity for cell culture or low concentrations).

The development of molecular technology has provided sensitive, specific, and rapid tools for viral detection, and the applicability of these methods to environmental samples is beginning to be demonstrated (1, 4, 13, 18, 26, 29, 34, 48). Most of the available literature has reported laboratory studies of improvements of methodology with virus-spiked shellfish or water. This paper describes our first results in detecting viral RNAs in naturally contaminated shellfish and sediment from open-harvesting areas, which is a distinction from other published studies. We used seminested PCR followed by hybridization to detect hepatitis A virus (HAV), rotavirus (RV), and EV. Initially, virus-spiked shellfish allowed us to improve the elution concentration step and the removal of inhibitors of enzymatic reactions; then the method was applied to field samples.

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MATERIALS AND METHODS

Virus strains. HAV strain CF53 adapted to growth in PLC/PRF/5 cells (10) was used in this study. The infectious HAV titer was determined as previously described (38), and the 50% tissue culture infectious dose ($TCID_{50}$) per milliliter was calculated according to the method of Reed and Muench (40). Poliovirus strain type 1 (Sabin strain) was cultivated in HEP-2 cells and titrated by the most probable number cytopathogen unit (MPNCU) method (22). A simian RV strain (SA 11 strain) grown in MA-104 cells was titrated by the immunofluorescence method (foci fluo units [FF]) (5).

Samples. Shellfish were collected from two areas in western France (Fig. 1). For site 1, samples were collected bimonthly from November 1992 to April 1993. At each collection, 2 cockle (*Cerastoderma edule*) samples, 1 mussel (*Mytilus edulis*) sample, and 1 sediment sample were collected (20 cockle samples, 10 mussel samples, and 10 sediment samples were obtained). For site 2, there were four sampling dates, 15 June 1992, 12 October 1992, 21 June 1993, and 14 October 1993. Shellfish were collected at five different points known to have different levels of bacterial contamination: two oyster (*Crassostrea gigas*) beds and one clam (*Ruditapes philippinarum*) bed without contamination (points 2 to 4), one clam bed occasionally contaminated (point 5), and one clam bed always contaminated (point 1). Therefore, three clam beds and two oyster beds were investigated, and 12 clam samples and 8 oyster samples were obtained. Shellfish were shucked and stored at −20°C until used. Sediment samples were frozen immediately (−20°C).

Shellfish sample processing. For the sensitivity study, cockles from a farming area were depurated for 5 days, shucked, and seeded with a known concentration of HAV (10-fold dilutions of 10^7 $TCID_{50}$/ml), RV (10-fold dilutions of 6×10^6 FF/ml), or EV (10-fold dilutions of 10^5 MPNCU/ml). Tissues (50 g) were added to 200 ml of 0.2 M glycine–0.15 M sodium chloride buffer (pH 9.5) and homogenized in a Waring blender at high speed for 5 min. The mixture was stirred for 15 min at room temperature and centrifuged for 10 min at 10,000 × *g* and 4°C, and the aqueous phase was harvested. For the first virus concentration study, this phase was divided into two

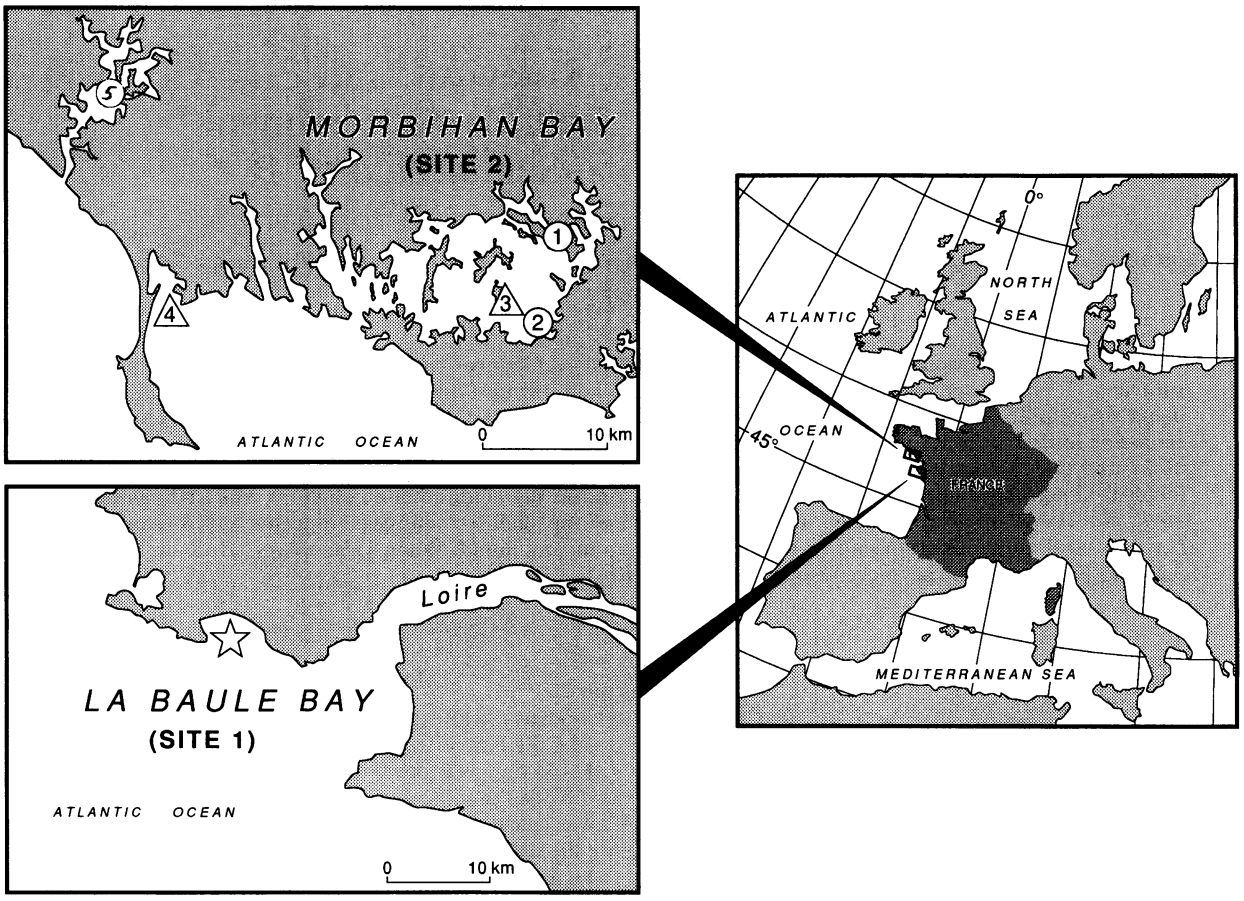

FIG. 1. Sampling locations on the Atlantic coast of France. The star indicates the point for collecting samples of cockle and mussel beds and of sediment at site 1. ○, point for cockle sampling (points 1, 2, and 5); △, point for mussel sampling (points 3 and 4) at site 2.

equal parts. Beef extract (3% [wt/vol]) (Sigma) was added to one part, and the pH was adjusted to 3.5. After being stirred for 30 min at room temperature, the mixture was centrifuged at 10,000 × *g* for 15 min at 4°C. The pellet was suspended in 5 ml of diethyl pyrocarbonate-treated distilled sterile water (DEPC water) and adjusted to pH 7.0. Polyethylene glycol 6000 (PEG 6000 [Sigma] as a 50% [wt/vol] solution in phosphate-buffered saline) was added to a final concentration of 8% to the second aliquot of the aqueous phase and adjusted to pH 7.0. The mixture was stirred for 2 h at 4°C. After 30 min of centrifugation at 10,000 × *g* and 4°C, the pellet was suspended in 5 ml of DEPC water and adjusted to pH 7.0. For the RV sensitivity and field studies, only the PEG 6000 protocol was performed, with 25 g of shellfish tissue added to 100 ml of 0.2 M glycine–0.15 M sodium chloride buffer (pH 9.5).

Extraction of viral RNA from shellfish concentrates and sediments. Viral RNAs were extracted from specimens by pretreatment with proteinase K in a detergent medium: 1/10 of viral concentrate (after beef extract or PEG 6000 treatment) was mixed with 0.1 M Tris-HCl (pH 7.5)–5 mM EDTA–1% sodium dodecyl sulfate (SDS)–proteinase K (200 μg/ml) at 56°C for 1 h. The digest was extracted with phenol (Gibco-BRL)-chloroform (Prolabo)-isoamyl alcohol (Prolabo) (25:24:1). RNAs were recovered by ethanol precipitation and dissolved in 100 μl of DEPC water. For RV detection, nucleic acids were purified by adsorption onto granular cellulose (6). Briefly, samples were mixed with 2× STE (vol/vol) (10× STE is 1 M NaCl, 0.01 M EDTA, and 0.1 M Tris-HCl [pH 6.8]) containing 66% ethanol (vol/vol) and 10 mg of CC41 cellulose (Prolabo). The mixture was agitated for 45 min and then centrifuged for 3 min at 10,000 × *g*. The pellet was washed three times with 1× STE–20% ethanol buffer. Adsorbed RNA was eluted with 50 μl of DEPC water for 10 min at 55°C. After centrifugation for 3 min, the supernatant was used for reverse transcription PCR (RT-PCR).

For sediment samples, nucleic acids were extracted from 1 g of sediment with proteinase K under the same conditions used for shellfish concentrate. After ethanol precipitation, an additional step of purification was performed with Sephadex G150 columns (1).

Primers. We used primers selected from published sequences. For EV, the 5′ noncoding region reported to be highly conserved among picornaviruses has been used in most of the published PCR (reviewed in reference 28) so we selected primers from this region (7, 43). For HAV, primers were chosen to bind the conserved sequences of the VP1 capsid region (8, 42). For RV detection, we used primers from the VP7 gene, an area conserved within group A RV but highly divergent among different RV groups (15, 20). The oligonucleotide primer and probe sequences used in this study are summarized in Table 1.

cDNA synthesis and PCR. RT was performed with a 10-μl mixture containing 2 μl of nucleic acid extract and 1 μM downstream primer (E1 or H1) according to the instructions of

TABLE 1. Sequence and localization of each oligonucleotide and probe used

Virus and oligonucleotide	Sequence (5′–3′)	Localization[a]	Fragment length (bp)
HAV			
H1	GGAAATGTCTCAGGTACTTTCTTTG	2389–2413	
H2	GTTTTGCTCCTCTTTATCATGCTATG	2167–2192	247
H3	TCCTCAATTGTTGTGATAGC	2358–2377	210
Hp	TCAACAACAGTTTCTACAGA	2233–2252	
EV			
E1	TCCGGCCCCTGAATGCGG	446–463	
E2	CACCGGATGGCCAATCCAAT	623–642	196
E3	CTCTCAATTGTCACCATAAG	584–603	157
Ep	ACACGGACACCCAAAGTAGTCGGTTCC	533–559	
RV			
R1	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1–28	
R2	GATCCTGTTGGCCATCC	376–392	392
R3	GTATGGTATTGAATATACCAC	51–71	342
Rp	TCCATTGATCCTGTTATTGG	220–239	

[a] Nucleotide positions for HAV, EV, and RV are in reference to the genomes of HM 175 HAV, poliovirus 1 (Mahoney), and Wa (serotype 1) RV, respectively.

the Moloney murine leukemia virus reverse transcriptase manufacturer (Stratagene). The RT mix was incubated for 45 min at 37°C, heated to 95°C for 5 min, and then placed on ice. The conditions for RT of RV were slightly different: 2 μl of nucleic acid extract was denatured with 10 μM of downstream primer (R1)–10 mM of methylmercuric hydroxide (STREM Chemicals) for 5 min at room temperature in accordance with conventional procedure (44). Then the mix was completed to obtain the same concentrations of other reagents as for EV and HAV RT except for dithiothreitol (60 mM) and the primer R1 (5 μM).

For EV and HAV amplification, 2 μl of RT mixture was added to 23 μl of PCR mixture according to the instructions of the *Taq* polymerase manufacturer (Perkin-Elmer Cetus). For RV amplification, PCR mix (40 μl) was added to RT mix (10 μl) to yield the same mixture as described above except the $MgCl_2$ concentration was 2.5 mM. Amplifications were performed with a 9600 Cetus cycler (Perkin-Elmer Cetus). The first 3 cycles (94°C for 30 s, 50°C for 30 s, and 72°C for 30 s) were followed by 27 cycles (94°C for 15 s, 50°C for 15 s, and 72°C for 20 s). A final extension period of 2 min at 72°C was performed, and then the temperature was decreased to 4°C. For seminested PCR, 1 μl of the first amplification reaction was further amplified with internal (E3, H3, or R3) and upstream primers under the same conditions of amplification as for the first PCR. Negative controls (extraction and RT-PCR) and a positive control (viral RNA) were included in each amplification series.

Gel electrophoresis and Southern blot hybridization. PCR products were electrophoresed on 9% polyacrylamide gels, stained with ethidium bromide, and visualized by UV light. The products obtained were compared with a molecular weight marker (Marker V or VI; Boehringer), and positive and negative controls were verified. A sample was suspected to be positive if the PCR product comigrated with the amplified product of the control virus RNA. DNA was transferred by electrotransference (Fast Blot; Eurogentec) onto a positively charged nylon membrane (Boehringer) according to the manufacturer's protocol. Oligoprobes were 3′ end labeled with digoxigenin-dUTP by using terminal transferase according to the instructions of the manufacturer (DIG oligonucleotide tailing kit; Boehringer). Nylon membranes were prehybridized for at least 30 min at 37°C (or 42°C for RV) in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.2% (wt/vol) SDS–5× Denhardt solution–1% (wt/vol) proprietary blocking reagent (Boehringer)–0.1% (wt/vol) N-lauroylsarcosine. Probe (Hp, Ep, or Rp) was added to a final concentration of 10 ng/ml, and hybridization was continued for a further 4 h at 37°C (or 42°C for RV). Membranes were washed two times sequentially in 2× SSC, 1× SSC–1% (wt/vol) SDS, and 0.2× SSC–0.1% SDS at 40°C. PCR product–digoxigenin-labeled probe hybrids were detected with an anti-digoxigenin antibody conjugated with alkaline phosphatase which dephosphorylates the chemiluminescent substrate Lumigen PPD (nucleic acid detection kit; Boehringer). Membranes were exposed to X-ray film for 1 h at 37°C, and the film was developed. The specificity of positive results after ethidium bromide visualization was confirmed by hybridization with the corresponding probe.

Bacteriological studies. Bacteriological studies were done on shellfish; tissue and liquor were homogenized in a Waring blender with 1 volume of 10% (wt/vol) NaCl–water. Fecal coliform (FC) counts were determined by the most probable number method in liquid broth (2), and values were obtained from published tables (11). Values above acceptable levels (3×10^2 FCs/100 g [fresh weight]) were considered too contaminated for human consumption (3).

RESULTS

Sensitivity study. Sensitivity was evaluated after the addition of known concentrations of HAV (10^7 to 10 $TCID_{50}$), EV (10^5 to 1 MPNCU), and RV (6×10^6 to 6 FF) into shellfish. For HAV and EV detection, two methods (beef extract and PEG 6000) were compared. In samples seeded with HAV, we detected 10^6 $TCID_{50}$ after seminested PCR by the beef extract technique. When the PEG 6000 technique was used, the sensitivity was 10^6 $TCID_{50}$ after the initial PCR and 10^4 $TCID_{50}$ after seminested PCR. After hybridization, the sample seeded with 10^3 $TCID_{50}$ gave a positive result (Fig. 2). For EV, the highest concentration used to inoculate shellfish was 10^5 MPNCU and no PCR products were detected by the beef extract method. By the PEG 6000 method, we detected the appropriate band for shellfish inoculated with 10^5 MPNCU after the first PCR and a very weak band after seminested PCR for shellfish inoculated with 10^3 MPNCU. This PCR product was confirmed by hybridization (data not shown). For RV, only the PEG 6000 technique was tested. An appropriately sized

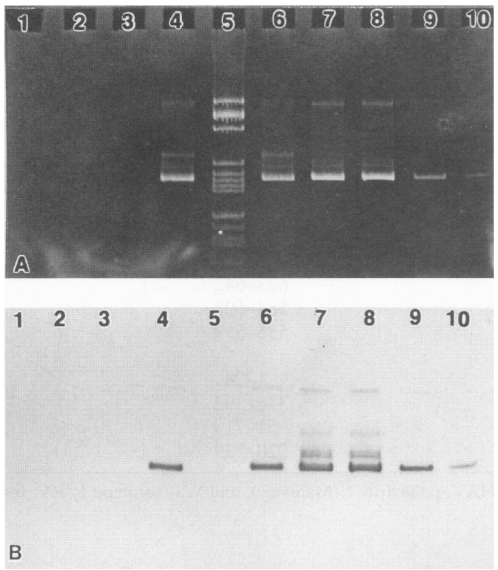

FIG. 2. Sensitivity study of HAV in shellfish. Serial dilutions of HAV were seeded into whole shellfish. Viral concentration, nucleic acid extraction, RT-seminested PCR, and hybridization were performed as described in Materials and Methods. RT-seminested PCR products were visualized by polyacrylamide gel analysis and ethidium bromide staining (A) and by Southern blot hybridization (B). Lanes: 1, negative control for elution concentration; 2, negative control for nucleic acid extraction; 3, negative control for RT-seminested PCR reagent; 4, RT-seminested PCR positive control (viral RNA); 5, DNA molecular weight marker (Marker V; Boehringer); 6 to 10, serial 10-fold dilutions of HAV (10^7 $TCID_{50}$ to 10^3 $TCID_{50}$, respectively).

band was observed only after purification with CC41 cellulose and after seminested PCR for the sample seeded with 6×10^3 FF (data not shown). In assessing sensitivity, we estimated that since only 1/25 of the sample was analyzed and 2 μl of nucleic acid extract (100 μl) was used as the template for RT, the detection limit was one infectious particle, providing that there was no loss of either viral or nucleic material during the concentration and purification procedures. This would represent a sensitivity of about 20 $TCID_{50}$/g of shellfish tissue for HAV, 20 MPNCU/g of shellfish tissue for EV, and 100 FF/g of shellfish tissue for RV. Because the total particle-to-infectious particle ratio is about 100/1 for EV (43), 60/1 for HAV (24), and 50/1 for RV (14), this method would allow the detection of about 1,200 viral particles per gram of shellfish for HAV, 2,000 viral particles per gram for EV, and 5,000 viral particles per gram for RV.

Field study of site 1 samples. Among the 20 cockle samples, 3 were positive for EV RNA (February, March, and April 1993) and only 1 was positive for HAV RNA (January 1993) (Table 2). Among the 10 mussel samples, 2 were positive for EV RNA (November 1992 and February 1993) and none were positive for HAV RNA. Bacteriological data indicated that almost all the shellfish samples were contaminated above the acceptable level; only the samples collected in March 1993 were acceptable. EV RNA was amplified in 4 sediment samples (among 10), and HAV RNA was amplified in 2 samples. In February, one sediment sample (22 February 1993) was positive for both RNAs.

Field study of site 2 samples. Samples were collected at five points for 2 years before and after the summer tourist season (Table 3). Among the 12 clam samples, 3 were positive for HAV RNA, 3 were positive for EV RNA, and 2 were positive for RV RNA. Among these, one was positive for both HAV and RV RNAs (point 1 in June 1993), one was positive for both HAV and EV RNAs (point 2 in June 1993), and one was positive for both EV and RV RNAs (point 5 in October 1993). In October 1992, the samples collected at points 1 and 5 contained only EV RNA. Bacteriological data for the three points for clam samples showed that point 1 samples were always contaminated above the acceptable level, the point 5 sample of June 1993 was not contaminated, and point 2 samples showed no contamination. Among the eight oyster samples collected, three were positive for HAV RNA, three were positive for EV RNA, and two were positive for RV RNA. Two oyster samples were positive for both HAV and EV RNAs, one sample (point 4 in October 1992) contained all three viral RNAs, and one sample was positive only for RV RNA (point 4 in June 1993). Bacteriological data indicated very low levels of contamination at these two points at all times.

DISCUSSION

This study was undertaken to evaluate an RT-seminested PCR method for the detection of viral RNAs in naturally contaminated shellfish from open-harvesting areas. We chose to use seminested PCR because of its sensitivity, as low levels

TABLE 2. Virological and bacteriological results for site 1 samples

Date (mo/day/yr)	RT-PCR result										
	Sediment		Cockle 1			Cockle 2			Mussel		
	EV	HAV	EV	HAV	FCs (10^2)[a]	EV	HAV	FCs (10^2)[a]	EV	HAV	FCs (10^2)[a]
11/13/92	+	−	−	−	ND[b]	−	−	ND	+	−	ND
12/14/92	−	−	−	−	**98**	−	−	**26**	−	−	**7**
12/24/92	−	−	−	−	ND	−	−	ND	−	−	ND
1/11/93	−	+	−	+	**22**	−	−	**12**	−	−	**16**
1/24/93	−	−	−	−	**18**	−	−	6	−	−	**14**
2/9/93	+	−	−	−	**27**	−	−	**14**	+	−	2
2/22/93	+	+	−	−	**14**	+	−	4	−	−	**12**
3/9/93	−	−	−	−	3	−	−	3	−	−	2
3/22/93	−	−	+	−	3	−	−	1	−	−	0.4
4/7/93	+	−	−	−	**42**	+	−	**54**	−	−	**76**

[a] Per 100 g (fresh weight). Concentrations higher than the standard level (3×10^2 FCs/100 g) appear in boldface.
[b] ND, not done.

TABLE 3. Virological and bacteriological results for site 2 samples

Date (mo/yr)	RT-PCR result																			
	Clams												Oysters							
	Point 1				Point 2				Point 5				Point 3				Point 4			
	HV	EV	RV	FCs[a]	HV	EV	RV	FCs[a]	HV	EV	RV	FCs[a]	HV	EV	RV	FCs[a]	HV	EV	RV	FCs[a]
6/92	−	−	−	**7.2×10^5**	−	−	−	$<3 \times 10^1$	−	−	−	**6×10^2**	−	−	−	**5.5×10^2**	+	+	−	$<9 \times 10^1$
10/92	−	+	−	**3.8×10^6**	−	−	−	**1.3×10^2**	−	+	−	1.3×10^3	+	+	−	$<3 \times 10^1$	+	+	+	$<3 \times 10^1$
6/93	+	−	+	**1.1×10^5**	+	+	−	$<3 \times 10^1$	−	−	−	$<3 \times 10^1$	−	−	−	$<3 \times 10^1$	−	−	+	$<3 \times 10^1$
10/93	−	−	−	**2.5×10^5**	−	−	−	2.7×10^2	+	−	+	**4.9×10^2**	−	−	−	5×10^1	−	−	−	$<3 \times 10^1$

[a] See Table 2, footnote *a*.

of virus concentration in shellfish were anticipated, as well as its specificity. Severini et al. (46) predicted that viral isolates detectable by nested PCR would not always be recognized by PCR plus hybridization if they carried point mutations in the region complementary to the internal primer. We believe that, with environmental samples, it is important to confirm the specificity of the amplified product even after nested PCR since a number of different genomic fragments can be present. We never observed discordant results.

A variety of methods have been used to recover virus from shellfish (36, 52, 54). We compared two methods, beef extract flocculation and PEG 6000 precipitation, usually used to recover virus from shellfish. The higher sensitivity observed with PEG 6000 precipitation is in agreement with the results obtained by Lewis and Metcalf (33). The sensitivity of our procedure is better than (4, 26) or about the same (18) as those previously described for HAV detection and is the same as that previously described for EV detection (4, 26). For RV detection in shellfish, no sensitivity study has been reported previously.

False-negative PCR results have been reported as a result of the presence of inhibitors either in samples or introduced during the concentration procedure (26, 29, 45, 49). To limit these negative effects and to avoid the introduction of too much salt in RT-PCR, we suspended the viral pellet in DEPC water instead of phosphate buffer; a comparison of the two suspensions showed more positive samples when water was used (32). A variety of extraction procedures have been devised to eliminate inhibitors (1, 4, 10, 16, 19, 25, 51, 53). In our first procedure, no additional purification step was added because too many extraction steps might increase the loss of genetic material or the likelihood of viral RNA damage (25, 35). Unfortunately, without further purification, we were unable to detect RV double-stranded RNA in shellfish or viral RNAs in sediment samples. For RV double-stranded RNA purification, different methods have been published (16, 19, 51, 53). We chose CC41 cellulose, which is quite similar to CF11 and allows good detection of double-stranded RNA (6). All the purification steps were performed in the same tube, thereby avoiding excessive manipulation and risk of contamination. With sediment samples, humic substances have been reported to inhibit PCR (47, 49). The procedure with Sephadex columns developed by Abbaszadegan et al. (1) was found to be very effective in removing inhibitory factors for the detection of EV in groundwater. A preliminary study in which humic acids were added to sediment samples allowed us to adapt this protocol to our technique and to verify that inhibitors were effectively removed.

To our knowledge, no previous reports describing the use of seminested PCR for the detection of viral contamination in shellfish from open-harvesting areas have been published, and reports about RV in shellfish are very rare. However, this study is a preliminary report and relatively few samples were tested. At both sites chosen for our investigation, we detected viral RNAs. Because RNAs are easily degraded in seawater (39) and no viral amplification occurred when proteinase K digestion was omitted, it is likely that the amplification products resulted from encapsidated viral RNA. Some studies have shown a correlation between the detection of viral RNA by PCR and the detection of infectious particles (21, 29, 31). Crance et al. (9) detected HAV infectious particles in 25% of the shellfish extracts positive by RT-seminested PCR.

HAV RNA detection was always correlated with detection of EV and/or RV RNAs, whereas EV RNA alone was detected in several samples. Because the primers currently used to detect EV allow the detection of a wide range of strains, the probability of detecting EV RNA may be greater than those for RNAs of the two other viruses. These results also suggest that EV may serve as an indicator of the possible presence of other human pathogenic viruses, as suggested by some authors (41, 52). In a previous study, we found that cockles were more contaminated than mussels from the same site but we couldn't determine whether differences in living conditions or in the natures of these two bivalves were responsible (30). Therefore, in this study, in the first area where the two types of shellfish were growing, we also analyzed sediment samples. The results showed that whenever viral RNA (EV or HAV) was detected in sediments, it was also detected in shellfish. There was almost a twofold-higher incidence of EV and HAV in sediments than in shellfish. Although this may not be significant, this point needs further elaboration and these results suggest a relationship between sediment contamination and shellfish viral contamination.

At the second site, no difference in viral contamination was found among shellfish from the five points with different levels of bacterial contamination. This lack of correlation has been described previously (30, 50, 53). Samples with FC counts as high as 10^5 FCs/100 g were negative for all three viral RNAs. These negative results were not false negatives caused by the presence of inhibitors in the samples, because the samples were tested for the presence of inhibitors by the addition of EV RNA to shellfish extracts and by amplification (data not shown).

This study showed that about 14% of shellfish samples were contaminated with HAV RNA. As an outbreak (about 660 cases presumably linked to shellfish consumption) occurred during the winter of 1992 (37), this contamination may reflect residual virus. In a study of the same area, infectious particles of HAV were found 3 months after the outbreak (9). This contamination may also be a consequence of the presence of virus in sewage despite the decline of seropositivity for HAV in France (27).

In conclusion, this study demonstrates that we have developed a method of virus detection in shellfish that can be used for the detection of different types of virus in several species of shellfish. Detection of virus in the environment could prevent outbreaks, and molecular techniques such as PCR are particularly promising procedures for monitoring viral contamination of the environment. Even though these techniques cannot discriminate between infectious and noninfectious particles, they offer a rapid way for screening and possibly detecting viruses not detectable by other methods. Epidemiological studies could provide information on the real impact of the detection of viral RNA in shellfish.

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