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Use of Genomic Probes To Detect Hepatitis A Virus and Enterovirus RNAs in Wild Shellfish and Relationship of Viral Contamination to Bacterial Contamination

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Genomic probes were used to investigate hepatitis A virus (HAV) and enterovirus RNAs in two types of shellfish from natural beds (Atlantic coast, France). After elution concentration, nucleic acid extracted by proteinase K and purified by phenol-chloroform and ethanol precipitation was assayed by dot blot hybridization. The probes used were a specific HAV probe corresponding to the 3′ end (3D polymerase coding region) and an enterovirus probe corresponding to the 5′ noncoding region. The method was first tested under experimental conditions by using virus-spiked shellfish before being applied under field conditions. Our results show that shellfish were highly contaminated: enterovirus and HAV RNAs were found in 63 and 67%, respectively, of samples examined with the riboprobes. On the same site, viral (HAV and enterovirus) RNAs were found in a larger fraction of cockles than mussels. Statistical tests of dependence showed no relationship between viral contamination and bacterial contamination (evaluated by fecal coliform counts).

Outbreaks of bacterial and viral enteric diseases after consumption of shellfish are an important public health problem. Effective control of enteric bacterial disease has resulted from establishment of regulations (total and fecal coliform [FC] indexes); however, viruses such as hepatitis A virus (HAV), Norwalk virus, rotavirus, and enterovirus (EV) are still often incriminated in disease outbreaks linked to shellfish consumption (6, 11, 12, 16, 20, 36, 48). Methodological problems (e.g., toxicity for cell culture and low concentrations) and problems in cultivating some strains (e.g., HAV, Norwalk virus, and coxsackievirus) have made the study of these viruses in the natural environment difficult. Development of rapid, sensitive, and specific methods, such as hybridization, for their detection is of great interest. This technique has been successfully applied by some workers to environmental (14, 23, 29, 31) or experimentally contaminated samples (24, 42, 51).

The Atlantic coast near the Loire River estuary is a recreational area for water sports and also has numerous natural shellfish beds. About 1,000 metric tons of shellfish are harvested annually, in addition to the large quantity gathered by tourists for their own consumption. Since 1984, the Hygiene Service of the Direction Départementale des Affaires Sanitaires et Sociales has monitored the bacteriological quality of these shellfish. For example, in 1986 and 1987, about 60% of the natural beds were classified as unfit for harvesting because of contamination by sewage. For our study, we selected four sites with mussel (*Mytilus edulis*) or cockle (*Cerastoderma edule*) beds known to be contaminated by FCs since the probability of contamination by viruses would also be high under these conditions (7, 26). Moreover, as these shellfish grow in different habitats (sediment and water), it was possible to evaluate the influence of environment on contamination. Bacteriological contamination was evaluated by FC counts. Viral RNA (vRNA) was searched for by using HAV and EV probes after elution by glycine buffer and concentration by beef extract (28, 39, 51). Few studies have used viral genomic probes and bacterial counts to investigate contamination of environmental samples by enteric viruses and bacteria over a relatively long period. This report presents results from our study.

Shellfish were collected from four polluted areas near the Loire River estuary in western France (Fig. 1). We studied three mussel beds (no. 1, 2, and 3) and one cockle-and-mussel bed (no. 4). Eighty-three samples were obtained between January 1990 and September 1991 (64 mussel and 19 cockle samples). Shellfish were shucked and stored at −20°C until use. Tissue and intervalvular water (100 g) were homogenized in a Waring blender for 5 min at high speed. Extraction and concentration of viral particles were performed as described by several researchers (4, 19, 32, 41). For sensitivity studies, shellfish (with no vRNA) were shucked and seeded with known concentrations of HAV (10-fold dilutions of 10^7 50% tissue culture infective doses [$TCID_{50}$]) and treated as natural contaminated samples.

The shellfish concentrates (3 ml) were treated with proteinase K (200 μg/ml) (Sigma) in 0.1 M Tris hydrochloride (pH 7.5) (Sigma)–1.25 mM EDTA (Prolabo)–0.15 M NaCl–1% sodium dodecyl sulfate (SDS) (Prolabo) for 1 h at 56°C and extracted with phenol (GIBCO-BRL)-chloroform (Prolabo) (vol/vol) one to three times. The RNAs were recovered by ethanol precipitation and dissolved in 400 μl of sterile water. An estimate of nucleic acid purity was provided by the ratio of the optical density at 260 nm to that at 280 nm. If the ratio was too low (<1.7), another phenol-chloroform extraction was performed.

The following strains were used: (i) HAV strain CF53 (donated by R. Deloince, CCRSA, Grenoble, France); (ii) poliovirus types 1, 2, and 3 (Sabin strains); (iii) coxsackieviruses B1 to B6 (provided by the Laboratoire National de Référence des Enterovirus, Lyon, France); (iv) echoviruses 7, 11, and 22 (isolated at the virology laboratory, CHR,

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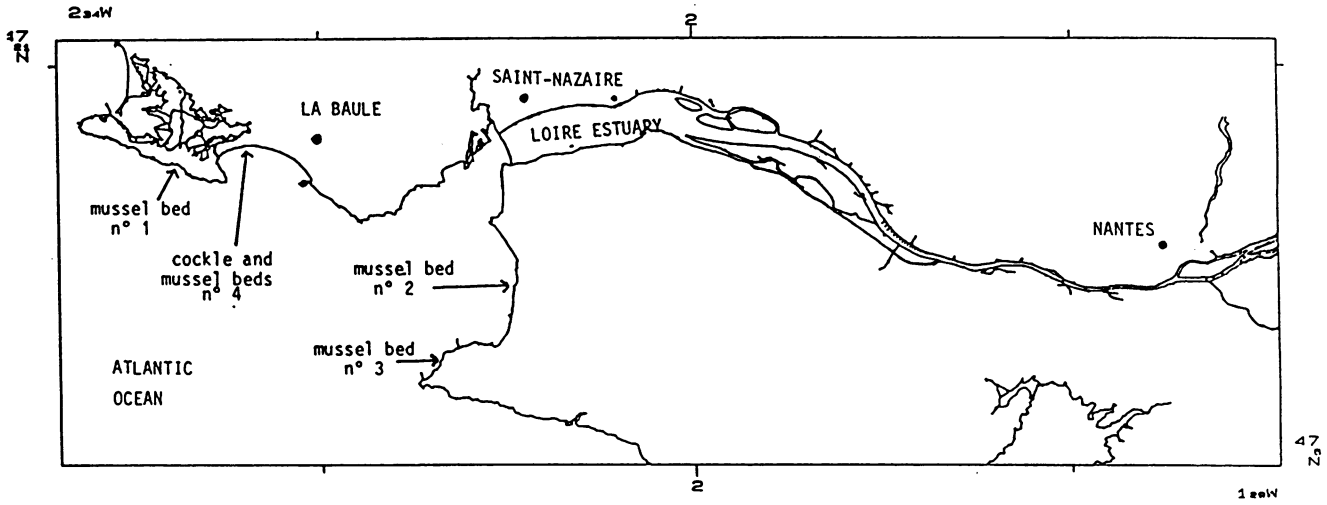

FIG. 1. Sampling locations on the Atlantic coast of France.

Nantes, France). Nucleic acids were extracted with proteinase K (100 μg/ml) for 1 h at 56°C and then purified as for shellfish samples.

The HAV probe was a cDNA fragment (1,466 bp) of the HAV genome (HAS15 strain) subcloned into plasmid pBR322 (*Pst*I site). The clone cDNA was prepared from vRNA, with oligo(dT) as a primer, in the laboratory of W. Robinson (Stanford University, Palo Alto, Calif.). This sequence, provided by B. Robertson (Centers for Disease Control and Prevention, Atlanta, Ga.), corresponds to the 3′ end that codes for the 3D polymerase (nucleotides 5949 to 7449). For the EV probe, the cDNA sequence obtained from poliovirus type 1 (Mahoney strain) corresponds to the 5′ noncoding region (nucleotides 221 to 670) (9), which is well conserved among EVs (38). This cDNA (450 bp) was inserted into transcription vector pGEM1 (*Bam*HI site) (kindly provided by H. Kopecka, Institut Pasteur, Paris, France).

After amplification, purification from the bacterial host, and digestion with *Pst*I for HAV cDNA or *Bam*HI for EV cDNA, inserts were separated from vectors by agarose gel electrophoresis and recovered with a Geneclean kit (Bio 101). These purified cDNA inserts were labeled with [α-^{32}P]dCTP (410 Ci/mmol; Amersham) by random multipriming. The labeled probes were purified from unincorporated deoxynucleoside triphosphate by running the reaction product over Sephadex G50 minicolumns (Pharmacia). Incorporation of radioactivity was determined by analyzing 1 μl of the purified labeled products in a scintillation counter.

HAV cDNA was ligated in an in vitro transcription vector (pBlueScript SK+) containing promoter sequences for T3 or T7 RNA polymerase. After amplification and purification from the bacterial host, linearized plasmid DNAs made by cutting with *Bam*HI (Boehringer) or *Eco*RI (Appligene) were used as templates in the transcription reactions to obtain cRNA (cRNA probe) and vRNA (vRNA probe), which were used as internal controls. For the EV probes, the transcription vector (pGEM1) was linearized with *Hin*dIII (Boehringer) or *Sma*I (Boehringer) to obtain cRNA or vRNA.

These linearized plasmids were used as templates in in vitro transcription reactions with SP6, T7, or T3 polymerase and [α-^{32}P]UTP in accordance with the supplier's (Pharmacia) recommendations. Purification and quantification of incorporation of radioactivity were performed as described for cDNA probes.

Nucleic acid extracted from shellfish was assayed by dot blot hybridization. The extract was denaturated at 65°C for 5 min in 3 volumes of the following solution: 500 μl of formamide (Sigma), 162 μl of formaldehyde (Sigma), 100 μl of 10× morpholinepropanesulfonic acid (MOPS) buffer (10× MOPS buffer is 0.2 M MOPS [Sigma] plus 0.05 M sodium acetate [Sigma] plus 0.01 M EDTA [Prolabo]). Dilutions were done by using the same solution. After being chilled on ice, cold 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate, pH 7) (1 volume) was added. Denatured nucleic acid samples (100 μl), as well as positive (HAV or poliovirus RNA) and negative controls, were spotted onto a nylon membrane (Hybond N+; Amersham) prewetted in 10× SSC by using a vacuum manifold apparatus (Touzard et Matignon). The filters were air dried and then baked for 2 h at 80°C.

For hybridization with cDNA probes, filters were prehybridized in fast hybridization buffer (Amersham) at 65°C for at least 1 h. Hybridizations were performed overnight at the same temperature and in the same buffer but with addition of a ^{32}P-labeled cDNA probe (10^6 cpm/ml). After hybridization, the filters were washed twice for 10 min each time in 2× SSC–0.1% SDS at room temperature, once for 15 min in 1× SSC–0.1% SDS at 65°C, and twice for 15 min each time in 0.5× SSC–0.1% SDS at 65°C.

For single-stranded RNA (ssRNA) probes, prehybridization and hybridization buffer contained 0.9 M NaCl, 2× Denhardt solution (Sigma), 0.2 mM EDTA, 0.2 g of herring sperm DNA (Sigma), 5% dextran sulfate (Sigma), 0.5% phosphate buffer (pH 6.5), and 1% SDS. Only the formamide concentration differed in accordance with the probe used (40% for the EV ssRNA probes and 50% for the HAV ssRNA probes). Prehybridization was performed at 42°C for at least 1 h, and hybridization was performed overnight with addition of a ^{32}P-labeled ssRNA probe (10^6 cpm/ml). After hybridization, the filters were washed twice for 10 min each time in 2× SSC–0.2% SDS at room temperature and twice for 15 min each time in 2× SSC at 50°C for HAV ssRNA probes. For EV ssRNA probes, a series of four washes of 20 min each at 50°C were done in 2× SSC–0.1% SDS, 1×

SSC–0.1% SDS, 0.5× SSC–0.1% SDS, and 0.1× SSC–0.1% SDS.

The filters were exposed to MP film (Amersham) with intensifying screens and developed after 24 h of exposure at −70°C.

The bacteriological studies were done on shellfish collected from sites 2, 3, and 4. Tissue and liquor, obtained as described above, were homogeneized in a Waring blender with 1 volume of 10% (wt/vol) NaCl water. FC counts were determined by a most-probable-number method in liquid broth (1). Most probable numbers were computed by using published tables. Values above 300 FCs/100 g (fresh weight) were considered too contaminated for human consumption.

A statistical test of dependence (chi-square test) between categorized FC concentrations (<300 or >300 FCs/100 ml) and virological results (positive or negative) was performed.

Probe specificity was determined by hybridization with homologous and heterologous nucleic acids. The HAV probes (cDNA and cRNA) showed specific hybridization with purified HAV RNA and did not react with any heterologous vRNAs (poliovirus, coxsackievirus, or echovirus). The EV probes (cDNA and cRNA) detected all of the viral strains tested (poliovirus types 1, 2, and 3; coxsackieviruses B1, B2, B3, B4, B5, and B6; and echoviruses 7 and 11), except echovirus 22 and HAV strains. By taking advantage of the two different promoter sequences flanking each side of the HAV or EV insert in transcription vectors, we were able to generate separately two strands of HAV and EV ssRNAs with opposite polarities. One strand of the RNA transcripts was complementary to the viral genome (negative sense, cRNA probe), and the other strand was identical to the viral genome (positive sense, vRNA probe). The vRNA probe hybridized not with vRNA but with double-stranded cDNA. This difference in specificity was useful for environmental samples: we never observed hybridization with the vRNA probe.

Sensitivity was evaluated by determining the detection limits of vRNA extracted from titrated HAV ($10^{6.5}$ $TCID_{50}$/ml) and poliovirus type 1 (10^7 $TCID_{50}$/ml). Nucleic acid serial dilutions were hybridized with either the cDNA probes or the RNA probes. The sensitivity of cRNA probes was much higher (10^4 $TCID_{50}$/ml) than that of cDNA probes (10^5 $TCID_{50}$/ml). Sensitivity was studied after inoculation of known concentrations of HAV into shellfish. After elution concentration and nucleic acid purification, hybridization with ssRNA probes showed a positive result with shellfish seeded with 10^7 and 10^6 $TCID_{50}$.

Field samples collected from January 1990 to September 1991 were prepared and tested in triplicate with cDNA, cRNA, and vRNA probes. Representative dot blot results obtained with filter paper are shown in Fig. 2.

With the EV cDNA probe, 34% (28 of 83) of the samples were positive, whereas 63% (52 of 83) scored positive with the cRNA probe. The difference was greater with the HAV probes: 22% (18 of 83) of the samples hybridized with the cDNA probe versus 67% (56 of 83) with the cRNA probe (Table 1). Some samples were positive with both the HAV and EV probes: 52% (43 of 83) hybridized with cRNA probes, and 20% (17 of 83) hybridized with cDNA probes. We found 12 samples positive for only one type of RNA (i.e., HAV or EV) with cDNA probes versus 22 samples with cRNA probes (Table 1).

At site 1, EV RNA was detected in 12 of 19 mussel samples (Table 2). In 1990, contamination occurred mainly from July to November, and in 1991 it occurred from April to September. Fewer samples (9) were found to be positive with the HAV cRNA probe. The June, July, September, October, November, and December samples in 1990 and the January, May, and September samples in 1991 were found to be positive. No bacteriological data were collected from this site.

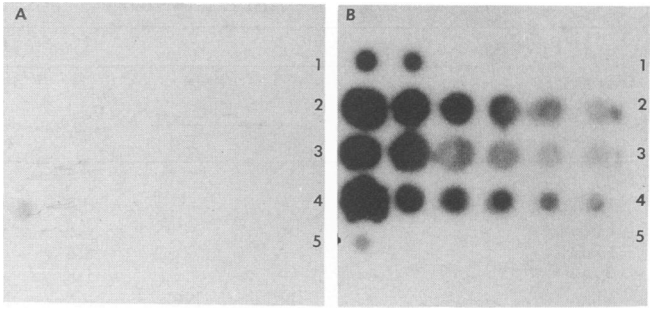

FIG. 2. Dot blot hybridization of nucleic acid extracts with HAV ssRNA probes. (A) Hybridization with a vRNA probe. (B) Hybridization with a cRNA probe. Rows: 1, positive control; 2, 3, 4, and 5, twofold dilutions of nucleic acid extracts of samples collected at site 4 during January (row 2), March (row 3), April (row 4), and June (row 5).

Among the 17 mussel samples from site 2, 9 hybridized with the EV RNA probe (Table 2). Samples positive by the EV RNA probe were obtained from June to December 1990, whereas only June and July samples were found to be positive in 1991. Contamination by HAV RNA was found more frequently (13 samples hybridized to the HAV cRNA probe), mainly from June 1990 to July 1991. Bacteriological data indicated that only the March, September, and November 1990 samples were contaminated above the acceptable level.

Among the 18 mussel samples collected at site 3, 9 were positive by hybridization with the EV cRNA probe (Table 2) (January, February, March, July, November, and December 1990 and March, June, and August 1991). HAV RNA was detected in 12 samples (February, March, June, July, September, October, November, and December 1990 and January, March, April, and August 1991). Only four samples were classified as unacceptable on the basis of bacteriological data (January, March, and July 1990 and March 1991).

Cockles and mussels were present at site 4. Among the 10 mussel samples analyzed for the 2 years, EV RNA was detected during 5 months from October to December 1990 and in August and September 1991 (Table 2). These samples, as well as the January 1991 sample, were also contaminated by both HAV and EV RNAs. In the cockle bed, all samples collected during 1990 were found to be contaminated by both HAV and EV RNAs. The January, March, April, and September 1991 samples were also found to be positive with

TABLE 1. Results obtained by hybridization with a DNA or RNA probe

Probe(s)[a]	No. of samples for:	
	cDNA	cRNA
+HAV/+EV	17	43
+HAV/−EV	1	13
−HAV/+EV	11	9
−HAV/−EV	54	18

[a] +HAV or +EV, positive by hybridization with the probe; −HAV or −EV, negative by hybridization with the probe.

TABLE 2. Virological and bacteriological results for the four sites[a]

Date sample collected	Mussels										RNA detection in cockles (site 4)	
	Site 1 RNA detection		Site 2 RNA detection		Site 2 FCs (10^2)	Site 3 RNA detection		Site 3 FCs (10^2)	Site 4 RNA detection		Site 4 FCs (10^2)	
	HAV	EV	HAV	EV		HAV	EV		HAV	EV		HAV	EV
1990													
January	−	−	−	−	0.8	−	+	**4.6**	+	+	**4.6**	NC	NC
February	−	−	+	+	1.8	+	+	0.8	+	+	<0.6	NC	NC
March	−	−	−	−	**8.6**	+	+	**4.6**	+	+	1.8	NC	NC
April	−	−	−	−	1.8	−	−	<0.6	+	+	<0.6	NC	NC
May	−	+	NC	NC	NC	NC	NC	NC	+	+	<0.6	NC	NC
June	+	−	+	+	<0.6	+	−	<0.6	+	+	0.6	NC	NC
July	+	+	+	+	0.8	+	+	1.8	+	+	3	NC	NC
August	−	+	−	−	0.8	−	−	<0.6	+	+	**4.6**	NC	NC
September	+	+	+	+	**8.6**	+	−	0.8	+	+	**8.6**	NC	NC
October	+	+	+	+	<0.6	+	−	1.8	+	+	1.8	+	+
November	+	+	+	+	**8.6**	+	+	<0.6	+	+	0.8	+	+
December	+	−	+	+	1.8	+	+	<0.6	+	+	<0.6	+	+
1991													
January	+	+	+	−	1.8	+	−	<0.6	+	+	1.8	+	−
February	NC	NC	+	−	0.8	NC	NC	NC	NC	NC	NC	NC	NC
March	−	−	+	−	0.6	+	+	**8.6**	+	+	**4.6**	−	−
April	−	+	+	−	0.6	+	−	0.8	+	−	0.8	−	−
May	+	+	NC	NC	NC	−	−	<0.6	NC	NC	NC	NC	NC
June	−	+	+	+	1.8	−	+	<0.6	−	−	<0.6	−	−
July	NC	NC	+	+	0.8	−	−	0.8	−	+	0.8	−	−
August	−	+	NC	NC	NC	+	+	NC	−	+	<0.6	+	+
September	+	+	NC	NC	NC	NC	NC	NC	+	+	0.8	+	+

[a] +, positive result by hybridization with cRNA probe; −, negative result by hybridization with cRNA probe; FC, FC count for 100 g (fresh weight); boldface numbers, concentrations higher than the standard level (3.10^2 FC/100 ml); NC, sample not collected.

the HAV cRNA probe. For EV, RNA was detected in all samples except those collected in April and June 1991. Bacterial contamination was present in January, July, August, and September 1990 and March 1991.

The existence of a wide variety of viral pathogens in seawater, sediment, and shellfish has been previously shown (3, 10, 43, 44, 49). The purpose of this study was to evaluate the use of genomic probes to characterize viral contamination in shellfish beds. The genetic relatedness among different EVs allowed us to design a hybridization test for detection of EV genomes using a probe corresponding to the 5′ noncoding region (38). The probe used in this study detected all EVs except echovirus 22 (9, 25, 34) and was successfully used to detect EVs in environmental samples (14). HAV genome sequences are quite different from those of other EVs, and specific probes are needed (8, 40, 46). The pHAV fragment used corresponded to the 3D polymerase coding region, which is a well-preserved part of the genome within the different HAV strains (37). This probe has been used to detect HAV nucleic acid within infected cell cultures (36a) or in shellfish (2). The specificity of the purified cDNA and ssRNA probes was as previously described (23, 25). The sensitivity studies realized with seeded shellfish showed that the method described here detected approximately 10^4 $TCID_{50}$. This is a little less sensitive than the test of Zhou et al. (51), which was able to detect 10^3 infectious particles.

Precautions need to be taken when RNA is detected in environmental samples. (i) RNase-free equipment and reagents are required for all procedures. High concentrations of buffer and salt with EDTA, proteinase K, and SDS inhibit RNases while minimizing sample-to-sample variations in digestion conditions (45). For hybridization procedures, RNase inhibitor is added and dextran sulfate in the hybridization solution also reduces degradation of the probe (23). In our study, we used these conditions for RNA extraction and hybridization. (ii) To avoid false-positive results, good purification of nucleic acid is required but too much extraction increases the loss of genetic material (21). The purity of our extracts was determined by measuring optical density. Several researchers have emphasized the advantage of ssRNA probes over cDNA probes (9, 15, 23, 38, 40). (i) RNA-RNA hybrids are more stable than DNA-DNA or DNA-RNA hybrids, thus allowing a higher stringency of hybridization with a concomitant reduction of background reactivity. (ii) ssRNA avoids complementary probe sequence competition (a problem with double-stranded cDNA probes). No vector sequences are present to cause nonspecific hybridization, and there is no self-annealing among probe strands. (iii) De novo probe synthesis results in a higher specific activity of ssRNA probes, and a large amount of an ssRNA probe can easily be synthesized. (iv) The existence of two independent promoter sequences flanking each side of the inserted DNA in the same transcription vectors makes it possible to generate two separate single-stranded probes. The one complementary to the viral genome (cRNA) hybridizes to the viral nucleic acid, and the RNA made from the other strand of the vector (vRNA) is identical to the viral nucleic acid and thus does not react with the vRNA. This vRNA probe can be used as an internal control to distinguish a positive reaction (cRNA) from nonspecific hybridization reactions (vRNA). Therefore, only results obtained with riboprobes are discussed.

The shellfish were highly contaminated (EV and HAV RNAs were found in 63 and 67% of the samples examined). These results are reliable because cRNA and vRNA probes gave clear positive and negative reactions, and some sam-

ples were found positive by hybridization with only one (HAV or EV) probe. Our results show that cockles were more contaminated than mussels from the same site. However, we could not determine whether a difference in living conditions or a difference in the natures of these two animals was responsible. Mussels may have more efficient depuration due to tidal action in the water column, or there may simply be an inherent difference between the two species. For example, during winter and early spring, mussels have a pumping rate three times as high as that of cockles (13). This difference in contamination might also be explained by a morphological difference in the shellfish (cockles have a very long digestive tube compared with mussels [5]) or by the fact that cockles live in sediment, which is known to concentrate viruses (4, 27, 35). Samples from all sites were found to be contaminated by vRNA from September to December. This probably reflects an increase in the number of people in this region during the summer. Unfortunately, this study was not long enough to evaluate seasonal influences.

Enteric bacteria from three sites were counted. Statistical tests of dependence showed no relationship between viral contamination and bacterial contamination. Bacteria and vRNA were not detected in 15.1% (8 of 53) of the samples, and bacterial contamination without vRNA was detected in 1.9% (1 of 53), whereas 20.7% (11 of 53) of the samples had viral and bacterial contamination and 62.2% (33 of 53) had viral contamination but acceptable levels of bacterial contamination. In the literature, studies show no consistent correlation between FC levels and the presence of viruses in shellfish (7, 18, 22, 47). In fact, viruses have sometimes been detected in oysters without coliform contamination (17, 50). These results are not surprising, because viruses are known to survive longer than bacteria in the marine environment. In vitro studies indicate that HAV and EVs remain viable for long periods of time in water (30, 33, 42). Goyal et al. (17) suggested that EVs survive for at least 17 months under field conditions. Hence, the presence of viral nucleic acids in shellfish suggests a health risk after consumption, which may partly account for about 600 cases of hepatitis A reported in this area between December 1991 and April 1992 (13a).

Evaluation of viral contamination is important to monitor the microbiological quality of the marine environment. It will be essential to monitor viral contamination in shellfish, particularly in view of the decreased prevalence of HAV antibodies in the population. Dot blot hybridization is a sensitive screening technique which can easily be used with the development of nonradioactive labels. As more viral gene clones become available, it will be possible to extend the survey to a large series of viruses. The development of polymerase chain reaction methods combined with hybridization will provide lower sensitivity thresholds compatible with environmental levels.

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