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Prevalence of enterovirus and hepatitis A virus in bivalve molluscs from Galicia (NW Spain): inadequacy of the EU standards of microbiological quality

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Abstract

A study of the presence of hepatitis A virus (HAV) and enterovirus (EV) in shellfish from the northwestern coast of Spain, one of the most important mussel producers in the world, was carried out employing dot-blot hybridization and RT-PCR techniques. In addition, bacterial contamination of the samples was evaluated by *Escherichia coli* (EC) counts, according to the European Union (EU) standards of shellfish microbiological quality. Shellfish samples included raft-cultured and wild mussels, as well as wild clams and cockles. Bacterial counts showed that the majority of samples (40.8%) could be classified as moderately polluted following the EU standards, and therefore should undergo depuration processes. However, differences in bacterial contamination were observed between cultured mussel and wild shellfish. Thus, percentage of clean samples (< 230 EC/100 g shellfish) was clearly higher in cultured mussels (49.1%) than in wild mussels (22.8%) or clams and cockles (10.7%). HAV was detected in 27.4% and EV in 43.9% of the samples that were analyzed. Simultaneous detection of both viral types occurred in 14.1% of the samples. Statistical tests of dependence (chi-square test) showed no relationship either between viral and bacterial contamination, or between the presence of HAV and EV. Comparative analysis of hybridization and RT-PCR for viral detection yielded different results depending on the virus type that was studied, RT-PCR being effective for HAV but not for EV detection. The obtained results reinforce once again the inadequacy of bacteriological standards to assess viral contamination and suggest that although virological analysis of shellfish is possible by molecular techniques, interlaboratory standardization and validation studies are needed before the routine use in monitoring shellfish microbiological safety.

Keywords: Enterovirus; Hepatitis A virus; Shellfish; Detection; Hybridization; RT-PCR

1. Introduction

Bivalve molluscs (including mussels, clams, and oysters) have been implicated as vectors in the transmission of bacterial and viral enteric diseases for many decades (Lindberg-Braman, 1956; Richards, 1985,

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1987). The periodic appearance of outbreaks, mainly of hepatitis A and gastroenteritis with viral etiology, associated with shellfish consumption is an important health problem which results in a lack of public confidence over this marketable product and, therefore, in high economic losses by the seafood industry (Morse et al., 1986). To avoid these health hazards, majority of the countries implemented several control measures for both shellfish and growing waters, based on the levels of fecal coliforms (FC) and/or *Escherichia coli* (EC), such as the European Directive 91/492/EEC (Anonymous, 1991) or the US interstate agreement set out by the Food and Drug Administration (Anonymous, 1993) or the UK Advisory Committee on the Microbiological Safety of Food (Anonymous, 1998). However, the failure of these bacterial indicators in detecting viral contamination (CDC, 1991; Desenclos et al., 1991; Burkhardt et al., 1995) has led to the evaluation of other putative indicators such as bacteriophages, enteroviruses (EV), and adenoviruses (Metcalf et al., 1980; Power and Collins, 1989; De Mesquita et al., 1991; Hsu et al., 1995; Pina et al., 1998).

Biotechnology advances in the last few years allowed the development of methods for the detection of clinically important and nonculturable viral pathogens in shellfish. Hybridization procedures have been described for the direct detection of hepatitis A virus (HAV), other enteroviruses (mainly poliovirus), and rotavirus (Jiang et al., 1986; Rotbart, 1991; Zhou et al., 1991) in shellfish meat and water. However, sensitivity of the method is not always enough to detect the low viral concentration present in this type of environmental samples (Zhou et al., 1991; Kogawa et al., 1996). Today, nucleic acid amplification by PCR constitutes the base for the development of effective tests for HAV and the agents causing viral gastroenteritis in shellfish.

Galicia (NW Spain) (Fig. 1), due to its particular geography presenting a number of big estuaries with high primary productivity, is a natural area specially suited to shellfish production. In fact, it is one of

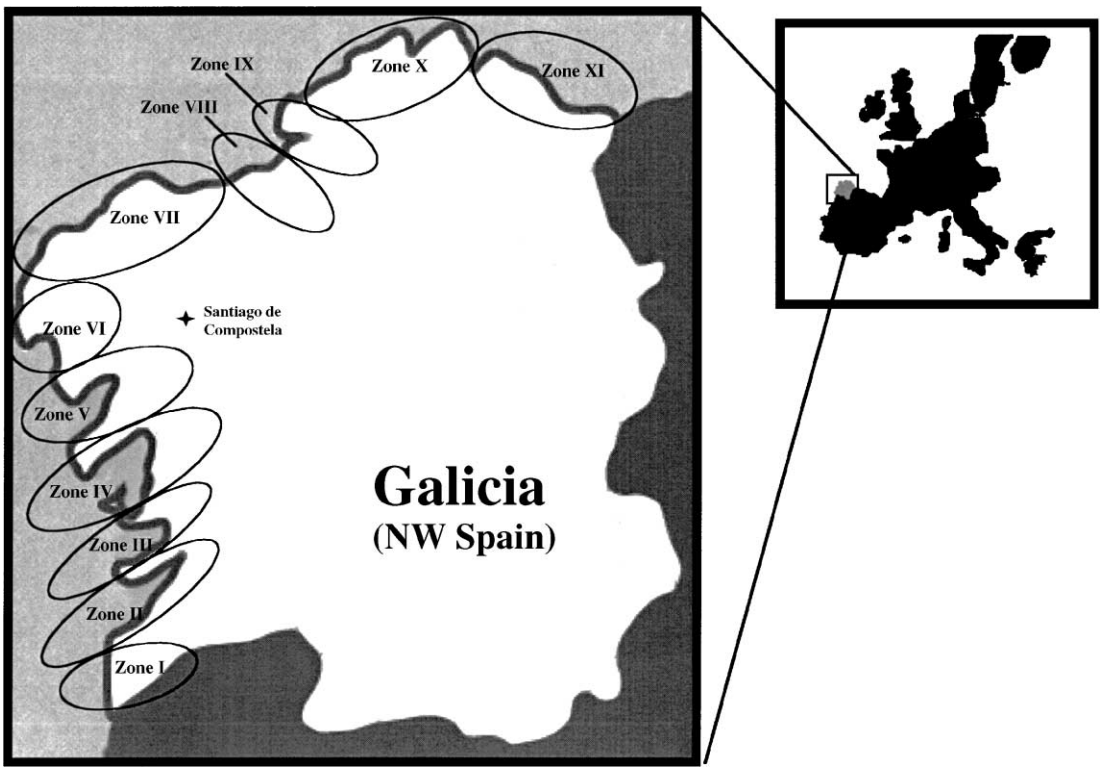

Fig. 1. Sampling areas on the northwestern coast of Spain.

the main world producers of mussels, cultured on floating rafts, with annual productions of approximately 250,000 metric tonnes (mt) (FAO, 1997, 2000). In addition, captures of other bivalve molluscs from natural beds are also important and include several clam species, cockles, and European flat oysters, with captures ranging between 2000 and 3000 mt depending on the species. The bacteriological quality of these shellfish has been monitored for more than 10 years, using the obtained data to classify the harvesting areas according to the EU standards. Such standards are based on the number of FC or EC per 100 g shellfish meat and intervalvar water. Category A has less than 300 FC or 230 EC; Category B has less than 6000 FC or 4600 EC; and Category C has less than 60,000 FC. These categories correspond, respectively, to areas of nontreatment requirement, depuration, and relaying of shellfish before retail sale. However, no studies on the incidence of enteric viruses were performed, probably due to the lack of legal requirements or appropriate methodology.

This work constitutes the first systematic survey of viral contamination of bivalve molluscs from the main-producing region in Europe. Shellfish were examined for the presence of hepatitis A virus and enterovirus by two different detection methods, hybridization with cDNA probes and RT-PCR. In addition, the correlation with bacteriological contamination was assessed by the estimation of the FC and EC counts.

2. Materials and methods

2.1. Sampling

A total of 164 samples, collected in different zones of the Galician coast (NW Spain) (Fig. 1), was employed in this study. Sampling was performed following the procedures recommended by the EU directives (2) on the basis of the size of mollusc populations and the situation of the harvesting areas. Mollusc species included raft-cultured and wild mussels (*Mystilus galloprovincialis*) (57 and 79 samples), clams (*Tapes* sp.) (21 samples), and cockles (*Cerastoderma* sp.) (7 samples).

Shellfish tissues (stomach and hepatopancreas) were mechanically homogenized in an equal volume of sterile artificial seawater and distributed into pools of 50 g each for the subsequent bacteriological and virological analysis.

2.2. Bacteriological analysis

Enumeration of FC and EC was performed following the most-probable-number (MPN) procedure (2, 35) employing Fluorocult® BRILA broth in a 5 tubes–3 dilutions test (Merck, Darmstadt, Germany) and EMB Levine agar plates (Pronadisa, Madrid, Spain). The isolates were biochemically confirmed using the API 20E system (bioMerieux, Madrid, Spain).

2.3. Concentration of viral particles

The adsorption–elution protocol developed by Sobsey (1987) to concentrate and recover viruses from shellfish was followed with minor modifications. Each 50 g of sample homogenate was diluted (1:7 wt./vol.) in sterile distilled water, to get a salinity lower than 2000 ppm NaCl/l, and adjusted to pH 5. After centrifugation at $1850 \times g$ for 15 min, supernatants were discarded and the pellets were resuspended in 7 volumes of glycine saline buffers (0.37% glycine, 0.85% NaCl). Then, pH was adjusted to a value of 7.5 and the mixtures were gently shaken for 10 min. The supernatants that were obtained after centrifugation at $1850 \times g$ for 15 min were adjusted to a pH of 4.5 and shaken for 20 min. Samples were then centrifuged as before and the pellets were resuspended in 2 ml of 1 N PO_4Na_2H (pH 7.4). To eliminate possible inhibitors, samples were cleaned by filtering through Sephadex G25/G100 (1:1) columns (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Area, 1998).

2.4. Extraction of viral RNA from viral concentrates

Sephadex-cleaned samples (250 μl) were treated with 2.5 ml of proteinase K solution (0.1 mg/ml in Tris–EDTA [TE] buffer; TE is 151 mg Tris–HCl, 46 mg EDTA/l H_2O, pH 8) for 30 min at 65 °C. Then, 250 μl of a solution of formaldehyde (10% wt./vol.) and β-mercaptoethanol (1% vol./vol.) were added, and the samples were incubated as before. RNA was extracted with five changes of phenol/chloroform/isoamyl alcohol (25:24:1), recovered by ethanol precipitation and dissolved in 250 μl of sterile distilled water. RNA solutions were stored at − 80 °C until use.

2.5. Preparation and hybridization with cDNA probes

The HAV probe was a cDNA fragment corresponding to 2078 nucleotides of the 5′ end of the HAV genome (HM175/7 MK-5 strain) (Bosch et al., 1991; Gajardo et al., 1991). For EV, the cDNA probe corresponds with a fragment of 1699 bp (positions 115 to 1814) obtained from poliovirus type 1 (Bosch et al., 1996; Area, 1998). The cDNAs were inserted in the plasmids pGem (Promega, Madison, WI, USA) and pBR322 (Roche Diagnostics, Barcelona, Spain), respectively. After amplification, purification from the bacterial host (*E. coli* HB101) and digestion with *Hin*dIII for HAV cDNA and *Pst*I for EV cDNA, inserts were separated by agarose gel electrophoresis and recovered with a QIAEX II column (Qiagen, West Sussex, UK). These purified cDNA inserts were digoxigenin-labeled with the DNA labeling and detection kit (Roche) following the manufacturer's recommendations.

For hybridization assays, total RNA extracted from mollusc samples (100 μl) was spotted onto nylon membranes (Bio-Rad, Hertfordshire, UK) using a manifold apparatus with vacuum. After 3 min of exposure to UV light, filters were baked for 30 min at 120 °C. Prehybridization and hybridization were carried out at 42 °C in the presence of 50% formamide as previously described (Bosch et al., 1991). Positive signals were detected by the use of a commercial signal generating system (DIG DNA labeling and detection kit; Roche) following the instructions provided by the manufacturer.

2.6. RT-PCR of viral RNA

For EV detection, primers P1 (5′ -CGT TAT CCG CTT ATG TAC TT-3′) (9) and PV444 (5′ -CAT TCA GGG GCC GGA GG-3′) (Shieh et al., 1997) were employed. These primers correspond respectively to positions 225–244 and 444–460 of poliovirus 1, as reported by Racaniello and Baltimore (1981). For HAV detection, primers HAV240 (5′ -GGA GAG CCC TGG AAG AAA GA-3′) and HAV68 (5′ -TCA CCG CCG TTT GCC TAG-3′) were used (Bosch et al., in press).

Reverse transcription reactions were carried out in a volume of 25 μl. Ten microliters of each sample was mixed, after a thermic shock of 99 °C for 5 min, with 7 μl of bidistilled water and 8 μl of RT mix containing RT buffer (Tris–HCl, 10 mM; KCl, 50 mM; $MgCl_2$, 2 mM), dideoxynucleoside triphosphates (dNTP) (200 μM), downstream primer (0.5 μM), and RT enzyme (2.5 U; Promega). Mixtures were then incubated for 1 h at 42 °C. After incubation, 10 μl of the RT product were mixed with 27.5 μl of bidistilled water and 12.5 μl of a PCR mix containing PCR buffer (Tris–HCl, 10 mM; KCl, 50 mM; $MgCl_2$, 1.5 mM), dideoxynucleoside triphosphates (dNTP) (200 μM), upstream and downstream primers (0.5 μM each) and *Taq* polymerase (2.5 U; BioLine, London, UK). Amplification was carried out in a Perkin Elmer 2400 thermocycler (Perkin Elmer, Madrid, Spain), employing the following cycling conditions for both EV and HAV: initial heat denaturation at 94 °C for 4 min; 40 cycles of template denaturation at 94 °C for 1 min; primer annealing at 55 °C for 1 min, and primer extension at 72 °C for 90 s; and a final extension at 72 °C for 10 min. Amplified products were 236 and 174 bp in length for EV and HAV, respectively, and were detected by agarose gel electrophoresis (FMC BioProducts, Rockland, USA) and ethidium bromide straining. A negative control consisting of sterile water was included in each RT-PCR setup.

2.7. Southern blot

DNA separated by electrophoresis was transferred to nylon membranes (0.45 μm pore size; Roche) after denaturation by treatment for 15 min in a 150 mM NaOH solution and neutralization with 0.5 × TBE. After transfer, DNA was fixed with UV light for 3 min, and vacuum (30 min at 120 °C) and membranes were kept desiccated 4 °C until use.

Prehybridization (3–4 h) and hybridization (overnight) were performed, at 50 °C for EV and 40 °C for HAV, in hybridization solution containing 5 × SSC, blocking reagent (1% wt./vol.; Roche), 0.1% (wt./vol.) sarcosyl (Sigma Química, Madrid, Spain), and 0.02% (wt./vol.) SDS. For the hybridization, 5 μl of the cDNA probe solution were added to the hybridization buffer, giving a final concentration of 8.5 and 3.3 pmol/μl for HAV and EV, respectively. The membranes were washed once at room temperature in 2 × SSC, 0.1% (wt./vol.) SDS and once in 0.5 × SSC, 0.1% SDS at room temperature or 56 °C for HAV or ENV, respectively. After washing, membranes were blocked and incubated with the anti-digoxigenin–alkaline

Table 1
Escherichia coli counts and detection of HAV and EV in the mollusc samples[a]

Sample	EC ($\times 10^2$)	HAV	EV
Zone I			
7w	>240	–	+
56w	160	–	+
107w	>240	+	–
146w	160	–	–
159c	>240	–	–
Zone II			
1w	92	+	+
2w	35	+	+
3w	54	+	+
4w	7.9	+	+
5w	24	–	+
6w	0.5	+	+
8w	35	+	+
9w	13	–	+
16w	>240	–	+
39c	0.2	–	–
40c	92	–	–
41c	11	–	–
54w	54	–	+
55w	160	–	+
59w	>240	–	–
60w	35	–	–
88w	>240	+	–
106r	>240	–	–
108r	54	–	+
111w	22	–	+
112w	>240	+	+
113r	35	–	–
115w	1.4	–	–
141w	0.6	–	–
156c	28	–	–
158c	35	–	+
Zone III			
30r	7	–	–
34c	3.4	+	+
35c	13	+	+
36c	13	–	–
71r	0.8	–	–
72r	0.5	–	–
89w	>240	+	–
94r	2.1	–	–
95r	35	–	–
97r	7	–	–
101r	28	+	–
102r	3.3	–	–
103r	28	+	+
104r	>240	+	–
105r	14	–	–
126r	3.4	–	–

Table 1 (*continued*)

Sample	EC ($\times 10^2$)	HAV	EV
Zone III			
127r	4.9	+	–
128r	3.3	–	–
132r	1.3	–	+
133r	3.4	–	–
134r	54	+	+
135r	2.6	+	–
136r	3.3	+	+
138r	14	–	–
140r	160	+	–
142r	22	–	–
143r	92	–	–
144r	160	–	–
145w	160	–	–
147ck	35	–	–
148w	160	–	+
149w	>240	–	+
154c	24	–	+
Zone IV			
17r	0.2	–	–
18r	0.2	–	+
19r	0.6	–	+
20r	0.9	–	–
21r	<0.1	–	+
22r	0.2	–	+
23r	0.2	–	+
24r	<0.1	–	+
37c	2.2	–	+
38c	160	–	+
45ck	9.4	+	–
61r	4.9	–	–
62w	2.3	–	–
63w	7.9	+	–
64w	0.5	–	–
65w	54	–	–
66w	17	–	+
67w	7.9	–	–
68w	92	+	–
69w	1.3	–	–
78r	2.3	–	+
79r	0.7	–	–
80r	0.8	–	–
81w	160	–	–
82w	0.2	–	+
91w	7.9	+	–
92w	54	+	+
118r	1.1	–	–
119w	1.4	+	–
120w	0.7	–	+
121w	0.4	–	–
129r	1.4	–	–
130r	2.7	+	–

(continued on next page)

Table 1 (*continued*)

Sample	EC ($\times 10^2$)	HAV	EV
Zone IV			
131r	1.7	+	–
153ck	33	+	+
155c	54	–	–
157ck	24	+	+
160r	2.2	–	–
162r	1.1	+	+
163r	0.7	+	–
Zone V			
25r	1.3	–	+
26r	0.2	–	+
27r	2.7	–	–
28r	<0.1	–	–
29r	<0.1	+	+
70w	1.1	–	–
93w	160	–	+
96r	0.7	–	+
98r	13	+	+
99r	24	–	–
109ck	160	–	–
137r	7.9	+	–
139r	11	–	–
161r	2.3	–	+
164r	1.1	–	–
Zone VI			
48w	4.9	+	–
49w	18	+	+
50w	1.7	–	+
100w	4.9	–	+
110w	22	–	–
Zone VII			
46w	92	–	+
47w	0.8	–	+
51w	35	+	+
83w	>240	+	–
150ck	11	–	–
151c	14	+	+
152ck	4.9	–	–
Zone VIII			
10w	54	–	+
12w	54	+	+
14w	>240	–	+
15w	3.3	–	–
42c	54	–	–
43c	1.7	–	+
44c	2.7	–	–
52w	7.9	+	–
53w	2.3	–	–
84w	1.7	–	–
85w	11	–	+

Table 1 (*continued*)

Sample	EC ($\times 10^2$)	HAV	EV
Zone VIII			
86w	9.4	–	+
122c	>240	–	+
Zone IX			
11w	17	–	+
13w	1.1	–	–
57w	>240	–	+
58w	17	–	+
87w	>240	–	–
90w	14	–	+
Zone X			
31w	92	–	+
33w	>240	–	+
76w	<0	+	–
114w	92	–	–
125w	24	–	–
Zone XI			
32w	92	–	–
73w	92	–	–
74w	0.2	–	+
75c	11	–	–
77w	54	–	+
116w	28	+	–
117w	11	–	–
123c	7.9	–	–
124c	11	+	+

w, wild mussel; r, raft cultured mussel; c, clam; ck, cockle; +, positive result by hybridization with cDNA probe; –, negative result by hybridization with cDNA probe.

[a] EC, *E. coli* counts for 100 g shellfish.

phosphatase conjugate, and the bands were colorimetrically visualized using the DIG DNA labeling and detection kit (Roche) following the manufacturer's instructions.

The digoxigenin-labeled probes employed were complementary to internal fragments of the correspondent amplification product, having sequences of 5′ -GGC TGC GTT GGC GGC CTA CCT-3′ for EV (unpublished data) and 5′ -TTA ATT CCT GCA GGT TCA GG-3′ for HAV (Bosch et al., 2000).

2.8. Statistical analysis

A statistical test of dependence (chi-square test) between the different categories of samples according

Table 2
Bacteriological and virological results for the different areas included in the study (percentage of positive samples)

Sampling zone	Number of samples	*E. coli*			Hybridization with cDNA probe for:		
		<230	230–4600	>4600	HAV	EV	HAV + EV
Zone I	5	0	0	100	20	40	0
Zone II	26	15.4	42.3	42.3	30.7	57.69	26.9
Zone III	33	12.1	60.6	27.3	33.3	27.3	15.1
Zone IV	40	57.5	27.5	15	30	40	10
Zone V	15	46.7	40	13.3	20	46.7	13.3
Zone VI	5	20	80	0	40	60	20
Zone VII	7	14.3	57.1	28.6	42.8	57.1	28.5
Zone VIII	13	15.4	46.1	38.5	15.4	53.8	7.6
Zone IX	6	16.7	50	33.3	0	66.7	0
Zone X	5	20	20	60	20	40	0
Zone XI	9	11.1	55.6	33.3	22.2	33.3	11.1
Total	164	29.9	40.8	29.3	27.4	43.9	14.1

to the numbers of EC or FC and the virological results was performed employing the software SPSS (version 6.1 for Windows). In addition, the same test was applied to study the correlation for the presence of different viral types (HAV and EV).

3. Results

3.1. Bacterial indicators

The numbers of *E. coli* cells per 100 g of shellfish were determined for all of the 164 samples by the MPN method (Table 1). To facilitate the analysis, three groups were established that correspond with the three categories in the EU legislation: clean shellfish that go directly to the consumer (equivalent to Category A), with less than 230 EC/100 g shellfish; moderately polluted (equivalent to Category B) with number of EC ranging from 230 to 4600 per 100 g shellfish; and heavily polluted (equivalent to Category C) with more than 4600 EC/100 g shellfish. Most samples (40.8%) fall in the moderately polluted shellfish group. The percentages of samples obtained for the other two groups were 29.9% and 29.3% for clean and heavily polluted samples, respectively (Table 2). No significant differences were observed when the FC counts were analyzed since only 15.2% would be grouped in a different category on the basis of FC or EC numbers (data not shown).

To perform a more exhaustive study and determine the role of several environmental factors on the shellfish contamination, samples were geographically grouped in 11 zones (Table 1 and Fig. 1). The number of samples in each zone was dependent on the density of the wild shellfish populations and the presence of cultures in floating rafts. The percentage of samples within the different contamination groups of each zone is shown in Table 2. In general, the percentage of clean samples ranged from 10% to 20%, being much higher in zones IV and V with 57.5% and 46%, respectively (Table 2). Interestingly, zone IV corresponds with the area of highest density of cultured mussels. However, other important harvesting areas, such as zones II and III, showed a great percentage of samples within the categories of moderately and/or heavily polluted (Table 2).

On the other hand, differences were observed depending on the mollusc species studies. Clams, cockles, and wild mussels, harvested in sediments or rock shore, were more polluted from the bacteriological point of view, with percentages of contaminated samples (moderately and heavily polluted) ranging from 75.2% to 89.3%, than cultured mussels which showed 49.1% clean samples (Table 3).

3.2. Virological analysis

Table 1 shows the results obtained for the presence of HAV and EV in the individual samples by hybrid-

Table 3
Bacteriological and virological results for the different mollusc species included in the study (percentage of positive samples)

Mollusc	Number of samples	*E. coli*			Hybridization with cDNA probe for:		
		<230	230–4600	>4600	HAV	EV	HAV + EV
Cultured mussel	57	49.1	38.6	12.3	26.3	33.3	7.0
Wild mussel	79	22.8	34.2	43.0	29.1	51.9	13.9
Others: clams, cockles	28	10.7	64.3	25.0	25.0	42.8	21.4
Total	164	29.9	40.8	29.3	27.4	43.9	14.1

ization with the respective specific cDNA probes. HAV was detected in 45 samples (27.4%) while the presence of EV was confirmed in 72 samples (43.9%). Both viral types were detected in 23 samples (14.1%) (Table 2). A lack of correlation between bacteriological and virological contamination was observed. The two viral types were detected in samples with less than 10 EC/100 g shellfish meat and, in contrast, samples that were heavily polluted were free of virus (Tables 1 and 4).

When the analysis was performed for the different geographical zones, in the majority of cases, the prevalence of HAV was lower than that of EV (Table 2). The percentage of samples positive for HAV ranged from 0% in zone IX to 42.8% in zone VII, whereas the percentage of samples containing EV ranged between 27.3% and 66.7%. The minimum EV percentage (27.3%) was observed in zone III that, strikingly, was the only area where incidence of HAV was greater than that of EV (Table 2).

With respect to the type of sample (Table 3), similar percentages of HAV positive samples (from 25% to 29%) were detected for cultured and wild mussels, as well as for other mollusc species. However, for EV, the number of positive samples was higher in wild mussels (51.9%) and the group of clams and cockles (42.8%) than in cultured mussels (33.3%). Simultaneous detection of the two viruses was markedly lower in cultured mussels, with only 7% samples positive for the two viral types.

No correlations could be established based on statistical analysis by the chi-square test, neither among the different categories of bacterial contamination and the presence of HAV or EV, nor between the presence of the two viruses, since the values obtained with both Pearson index or likelihood ratio were not significant at a confidence level of 95%. Statistical analysis for the same correlation by individual geographic zones showed that a link between the presence of HAV and EV existed only in zone II. In fact, as shown in Tables 1 and 4, the majority of samples with HAV were also positive for EV. In the other zones, a significant statistical association could not be established between the presence of the two viral types.

3.3. Hybridization versus RT-PCR

A representative number of the samples included in our study were randomly selected and subjected to amplification by RT-PCR using specific primers, to compare the efficacy of hybridization and RT-PCR, combined with Southern blot, methods in the detection of HAV and EV.

For HAV, 35 samples (17 positive and 18 negative samples by hybridization with cDNA probes) were selected for the RT-PCR analysis. The RT-PCR/Southern blot method showed a higher sensitivity in the detection of HAV (69% positive results) than hybridization (49%) (Table 5). Therefore, nine samples that were considered negative by hybridization yielded positive results after the analysis by RNA amplification and Southern blot. On the other hand, two samples giving positive results by hybridization were negative by RT-PCR procedure (Table 4). Sensitivity of the RT-PCR method was determined in vitro by testing serial dilutions of a viral stock containing 1.2×10^6 infectious particles/ml. The detection limit was of 100 infectious particles/ml ($4-6 \times 10^3$ physical particles/ml) (data not shown).

Forty-two samples were analyzed by RT-PCR/Southern blot for the presence of EV. Curiously, a lower sensitivity of RT-PCR with respect to hybridization was achieved, the number of positive samples

Table 4
Distribution of HAV and EV detections in molluscs according to the categories of the EU standards

Sampling zone	Number of samples	HAV detection only				EV detection only				HAV + EV detection			
		Number of positive	Percentage in category			Number of positive	Percentage in category			Number of positive	Percentage in category		
			A	B	C		A	B	C		A	B	C
Zone II	26	1	0	0	100	8	0	50	50	7	14.4	42.8	42.8
Zone III	33	6	16.7	33.3	50	4	25	25	50	5	0	80	20
Zone IV	40	8	50	37.5	12.5	12	83.4	8.3	8.3	4	25	50	25
Zone V	15	1	0	100	0	5	80	0	20	2	50	50	0

Results are from the main production zones.

Table 5
Comparative results of hybridization and RT-PCR/Southern blot for detection of HAV and EV in mollusc samples

	Number of samples	Hybridization (number of +)	RT-PCR (number of +)	Both methods (number of +)	Both methods (number of −)	+ Hybridization/ − RT-PCR (number)	− Hybridization/ + RT-PCR (number)
HAV	35	17	24	15	9	2	9
EV	42	22	6	4	18	18	2

decreasing from 22 (52.4%) by hybridization to 6 (14.3%) by RT-PCR (Table 5). It is interesting to point out that of the six PCR positive samples, two gave negative detection by hybridization. These results are in clear contradiction to those obtained for HAV. Moreover, the sensitivity that was obtained in vitro for the EV detection by RT-PCR/Southern blot was higher than for HAV, with a detection limit of 4 infectious particles/ml (400 physical particles) (data not shown).

4. Discussion

A number of procedures have been reported for the detection of enteric viruses in shellfish (Lees et al., 1994; Atmar et al., 1995; Cromeans et al., 1997; De Medici et al., 1998) and many of them have been applied to the study of viral contamination of molluscs from harvesting areas in different countries (Le Guyader et al., 1998, 2000; Croci et al., 1999; Lee et al., 1999). Despite the importance of Galicia (NW Spain) as mussel producer in Europe (FAO, 1997, 2000), no data are available about the prevalence of enteric viruses in shellfish or shellfish-growing waters of this geographic area. Therefore, the main purpose of the present study was to determine the sanitary conditions of the Galician molluscs from a virological standpoint, evaluating the incidence of HAV and enterovirus in both cultured and wild bivalve molluscs. In addition, the relationship between viral and bacterial contamination was assessed.

The EC counts indicate that the majority of samples would be considered as included in Category B (less than 4600 EC), which correlates with the official classification of most of the Galician harvesting areas as zone B, according to the Center for Quality Control of Marine Environment, a branch of the regional government. The numbers of FC were very similar to those of EC, the samples being grouped in the same contamination category. This finding corroborates the results obtained by other authors who found that approximately 93% of the FC are, in fact, EC (Martínez-Manzanares et al., 1992). As expected, within each area, the human population density showed a clear influence in the contamination level of the samples. In general, the more polluted sampling sites were close to main towns and villages, whereas the clean sites are located in less populous areas.

Previous studies of bioaccumulation in oyster showed that most of HAV and enteroviruses were localized in stomach and hepatopancreas (Romalde et al., 1994; Metcalf et al., 1995). Therefore, we employed these organs for the virus extraction instead of the whole mollusc which, in addition, yielded not only an increase of test sensitivity but also a decrease in the processing time and in the concentration of possible sample-associated inhibitors (Atmar et al., 1995; Le Guyader et al., 2000).

The percentages of positive samples for HAV and enterovirus were similar to those obtained in other studies employing molecular detection procedures (Le Guyader et al., 1998, 2000; Lee et al., 1999). The higher incidence of enterovirus was expected since the probe is detecting diverse species within the genus *Enterovirus*. In fact, the same genomic region was employed by other authors who have reported the detection of Coxsackie A and B strains (Abbaszadegan et al., 1993; Le Guyader et al., 1994). On the other hand, we must have in mind the possible deviation of the EV results caused by the detection of the poliovirus vaccine strains excreted in faeces of the infant population subjected to immunization programs. An interesting finding is that while the numbers of samples positive for HAV were similar regardless of the type of mollusc analyzed, the numbers of EV positive samples were higher for wild molluscs (including mussels, clams and cockles) than for raft-cultured mussels. This fact can be explained by a greater survival in the marine environment of HAV, which makes possible the movement of the viral particles in the water stream

to reach the floating rafts, whereas the EV, more labile, are more influenced by the proximity of contamination sources to the sampling site. Other explanations, suggested elsewhere (Le Guyader et al., 1993), are based on a different depuration rate or habitat of the different molluscs (i.e. clams and cockles live in a sediment which is known to accumulate viruses).

No statistical correlations were found, using the chi-square tests including all the samples, between bacterial and viral contamination or between detection of HAV and EV. When the same test was separately applied to each sampling zone, we encountered a significant relation between these parameters only in zone II, which corresponds to the most populated geographic area. These findings, similar to those reported by other workers (Power and Collins, 1989; Le Guyader et al., 1993, 2000; Pina et al., 1998), indicate that a good correlation between presence of bacterial indicators and enteric viruses is possible only in sites that are highly impacted by human activities, mainly sewage discharges, whereas in those sites lightly polluted or only occasionally contaminated, such correlation rarely occurs. This fact raises once more the important question of the appropriateness of choosing a concrete microorganism as indicator of sanitary quality or, on the contrary, if direct tests for the most important viral pathogens should be performed to ensure the public health safety of shellfish (Metcalf et al., 1995; Lees, 2000).

Another important point is the election of the appropriate method for virus detection. A number of recent works strongly support the RT-PCR as the most reliable and sensitive procedure to detect virus in environmental samples, including shellfish (Lees et al., 1994; Le Guyader et al., 1994, 2000; Atmar et al., 1995; Metcalf et al., 1995; Romalde, 1996; Croci et al., 1999; Lees, 2000). Results of the present study for HAV are in accordance with the data in the literature indicating a much higher sensitivity of RT-PCR versus hybridization. However, a different picture emerged in the EV detection where the number of positive samples was 3.5-fold less by RT-PCR than by hybridization (14.3% versus 52.4%). This unexpected result may be explained by the fact that the RT-PCR reactions were not performed simultaneously with the hybridization experiments. These latter assays were performed on freshly obtained RNA while the RT-PCR reactions were assayed on frozen (-70 °C) RNA. Although the preservation of the RNA should be adequate at this low temperature, the possibility of degradation cannot be ruled out, considering that the stability of the HAV RNA is higher than that of the EV RNA (unpublished observation). Another plausible explanation could be the inefficacy of the primers employed to detect some groups within EV, although theoretically, primers and probe are universal for the genus *Enterovirus*. However, the existence of false positive reactions in the hybridization method cannot be excluded. Therefore, caution must be taken when designing primers and probes in order to obtain an accurate detection of the target virus, selecting conserved regions for those agents that vary among strains.

The results that were obtained in this work clearly indicate, once again, the lack of relationship between bacterial and viral contamination, and strongly support the decision of the European authorities about the need of inclusion of parameters of viral contamination in the microbiological quality standards of shellfish (Decision 1999/313/EC) (Anonymous, 1999). In this sense, and despite their limitations (i.e. impossibility of viral quantification or infectiveness), it seems clear that molecular methods, RT-PCR in particular, are the more suitable tools to be employed for virus detection in this type of samples. Therefore, it is imperative to carry out interlaboratory standardization studies for the molecular procedures developed in the last few years for viral detection in natural samples. Finally, the necessity of systematic surveys of shellfish from different areas is also undoubted, in order to evaluate the true magnitude of environmental viral contamination, as well as to determine if a particular virus could be considered as a reliable indicator of fecal pollution. All these data will be helpful not only to understand the epidemiology of enteric diseases with viral etiology, but also to improve shellfish sanitation and, consequently, public health.

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