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Diversity of enterovirus sequences detected in oysters by RT-heminested PCR

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Abstract: Oysters harvested in western France, from five sites associated with outbreaks of food-borne norovirus gastroenteritis between February 2000 and March 2001, were assayed for enterovirus RNA by reverse transcriptase-heminested polymerase chain reaction (RT-heminested PCR). Forty percent (21/52) of shellfish samples (pool of seven oysters) were contaminated by enteroviruses. Infectious coxsackieviruses serotype A21 were isolated from three of these positive samples. Amplicons corresponding to 65 base sequences in the 5′ untranslated region of the enteroviral genome were analyzed by direct sequencing. Interpretable results were obtained from 18 amplicons, but mixtures of sequences confused the results from 3 samples. Sequences isolated from samples from the different sites were different but similarities were observed between sequences detected in shellfish from two sites at different dates. Sequences were also compared to sequences of human, bovine and porcine enteroviruses. Both human and animal origins of enterovirus contamination of shellfish seemed likely.

Keywords: Enterovirus; Shellfish; Cell culture; RT-PCR; Sequence analyze

1. Introduction

Enteroviruses are non-enveloped enteric RNA viruses belonging to the family *Picornaviridae*. These viruses infect human and a wide range of mammals, including cattle and pigs (Ruecker, 1996). Infected humans or animals excrete feces containing viral particles, which remain infectious for several days in environmental water (Wait and Sobsey, 2001). Human enteroviruses have been detected in water, sewages, sludge and shellfish, and are used, notably in Europe, as indicator of fecal contamination of drinking or bathing waters (Anonymous, 1975, 1980). However, these viruses are not indicators of hazardous viral contamination, because recent studies found that contamination of shellfish with pathogens such as hepatitis A virus (HAV) or Norwalk-like viruses (NLV) was not correlated with the presence of enteroviruses (Le Guyader et al., 1994; Le Guyader et al., 2000, Romalde et al., 2002). Reduction in the frequency of enterovirus detection has probably arisen because attenuated poliovirus vaccine strains are now not excreted by the human population, and because of changes in the epidemiology of enterovirus infections. Nevertheless, non-poliovirus enteroviruses are still associated with acute diseases and, possibly, with chronic disease, and were responsible for large outbreaks of meningitis in France and Europe in 2000-2001 (Chambon et al., 2001). During these outbreaks, as in most enterovirus outbreaks, inter-human transmission seemed likely. However, the mode of transmission is difficult to discern and in a few cases waterborne outbreaks have been described (Melnick, 1996). Epidemiological surveillance plays an essential role in the control of infectious diseases. The detection of viruses in both clinical and environmental samples may help understanding of the mode of transmission of strains responsible for human infections. In France, as recommended by the World Health Organization (WHO) for eradication of wild poliovirus, enteroviruses detected from sewages and/or sludge have to be titrated and characterized (World Health Organization, 2000). However, there has been no systematic identification of enteroviruses found in shellfish.

During our investigation of Norwalk-like gastro-enteritis associated with oyster consumption (Dubois et al., 2001; Le Guyader et al, 2003), we searched for enteroviruses in the shellfish using a novel combination of previously published primers (Abbasadegan et al., 1993; Leparc et al., 1993) for the reverse transcriptase-heminested polymerase chain reaction (RT-heminested PCR). This method was chosen because of its high specificity and sensitivity for viral RNA detection (Dubois et al., 2002). Our objectives were to evaluate the frequency of detection of enteroviruses and to identify the strains origin. This note presents the results of the enterovirus RNA amplification, the amplicon sequencing, and the virus isolation by cell culture.

2. Materials and methods

2.1. Viruses and cells

Poliovirus type 1 (Sabin strain) was used in this study as an enterovirus model for evaluating the sensitivity of the reverse transcriptase-polymerase chain reaction (RT-PCR). The virus was grown on Vero cells (Membres de la commission de normalisation, 1989). Poliovirus particles (d = 1.34) were purified by isopycnic centrifugation in a CsCl density gradient. Virus titration was carried out on Vero cells in microtitration plates and expressed as 50%-tissue culture infectious dose ($TCID_{50}$) per volume unit (Membres de la commission de normalisation, 1989). The titer of the poliovirus stock was 1.8×10^8 $TCID_{50}$/ml. Assuming that the Poisson model was applicable to the viral samples analyzed, 1 $TCID_{50}$/ml was equivalent to 0.69 PFU/ml (Maul et al., 1991). Consequently, the titer of poliovirus stock was equivalent at about 1.2×10^8 PFU/ml. The titer of stock was also determined as RT-PCR amplification units (RT-PCRU) by endpoint dilution, heating to release the viral genome from capside, and then performing RNA detection by RT-heminested PCR. Given that the last positive dilution contained one RT-PCRU, the titer was estimate to be about 1.2×10^{10} RT-PCRU/ml (mean of three different experiments). Consequently, 1 $TCID_{50}$ of poliovirus was equivalent to about 67 RT-PCRU.

2.2. Shellfish samples

Oysters (*Crassostrea gigas*) associated with gastro-enteritis outbreaks (Haeghebaert et al., 2002) were collected by veterinarian inspectors. Five different sites were targeted. Site A (South Brittany, Atlantic Ocean) was sampled between February and May 2000; site B (North Brittany, English Channel) between March and May 2000; site C (North Brittany, English Channel) between January 2001 and March 2001; site D (Normandy, English Channel) in December 2000; and site E (Normandy, English Channel) during January and February 2001. Shellfish were kept frozen or refrigerated during shipment. The samples reached the laboratory one day after collection. On arrival, all shellfish were stored at –20°C.

2.3. Viral concentrations in oyster digestive glands

Viral concentrations were determined by a procedure adapted from the protocol previously described by Atmar et al. (1995). Briefly, 3 g of digestive gland removed by dissection from 7 shellfish were homogenized in 15 ml of phosphate-buffered saline solution pH 7.4 (PBS). After addition of one volume of chloroform-butanol (1:1 v/v), the mixture was shaken vigorously for 1 min, then 150 µl of Cat-Floc 20% in water (Sigma-Aldrich, Saint-Quentin-Fallavier, France) was added. After rocking for 5 min and settling for 15 min at room temperature, the suspension was centrifuged at 10,000 x *g* for 15 min at 4°C. The aqueous solution was recovered and supplemented with polyethylene glycol 6,000 (Promega, Lyon, France) and NaCl (Sigma-Aldrich) to obtain final concentrations of 10% and 0.3 M, respectively. After homogenization and settling overnight at 4°C, the mixture was centrifuged at 10,000 x *g* for 1 h at 4°C. The pellet was then suspended in 2.5 ml of water and stored at -80°C until use.

2.4. Identification of infectious enterovirus

One ml of each viral concentrate positive for enterovirus RNA was stored at –20°C and forwarded to the National Reference Center for Enterovirus for enterovirus isolation. Sample was diluted in culture medium (1/100) and inoculated on five different confluent mono-layers cell lines (BGM, MRC5, Hep2, HRT, and L20B). Three consecutive, 8-days subcultures were used to allow detection of cythopatic effects. Identification of virus was carried out by neutralization test using the "LBM" WHO intersecting pools of hyperimmune antisera against enteroviruses (Schnurr, 1992).

2.5. RNA extraction

RNAs were purified using the RNA-Plus purification kit (QBiogène, Illkirch, France) according to the manufacturer's instructions. Briefly, 500 µl of viral concentrate were mixed with two volumes of guanidinium thiocyanate-phenol-chloroform solution. The aqueous phase was separated from the organic phase by centrifugation. The RNAs were recovered by isopropanol precipitation, washed with 75% ethanol, and dissolved in 50 µl of sterile water.

2.6. Oligonucleotides

The primers used for the amplification of poliovirus RNA were selected in the 5' noncoding region of the enteroviruses (Abbaszadegan et al., 1993; Leparc et al., 1993). The antisense primer (5' ATTGTCACCATAAGCAGCCA 3', base 596 to 577) was designated primer 3, the sense primer (5' TCCGGCCCCTGAATGCGGCT 3', base 446 to 465) was designated primer A1, and the internal antisense primer was designated primer A2 (5' CCCAAAGTAGTCGGTTCCGC 3', base 550 to 531). All the map positions refer to poliovirus type 1 Sabin strain (accession number: V01150).

2.7. Amplification of RNAs

An RT-heminested PCR method for detection of enteroviruses contaminating vegetal surfaces was previously described (Dubois et al., 2002). This method was adapted for shellfish analysis, by doubling the reaction volume of RT-PCR to increase the proportion of analyzed sample. RNA extract (6 µl) was analyzed by RT-PCR in a single tube. The 20-µl RT mixture was made of 1X PCR Gold Buffer (Applied Biosystems, Courtaboeuf, France), 2.5 mM $MgCl_2$, 500 µM of four deoxynucleoside triphosphates, 1.25 µM of downstream primer (primer 3), 5 U of RNase Inhibitor (Applied Biosystems), and 12.5 U (Applied Biosystems) of MuLV reverse transcriptase (Applied Biosystems). The mixture was incubated for 1 h at 42°C, and the enzymes were then inactivated at 95°C for 5 min. PCR was carried out in a 100 µl reaction mixture containing 1X PCR Gold Buffer, 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphates, 0.25 µM of downstream and upstream primers (primer 3 and primer A1), and 1.5 U of AmpliTaq Gold (Applied Biosystems). Heminested PCR was performed with 2 µl of the amplified product used as a template, in a 25-µl reaction mixture of the same composition as that used for PCR, except that the primer set was composed of the internal and the upstream primers (primer A2 and primer A1). A negative

amplification control (water) was included in each amplification series. The cycling conditions were as follows: initial preincubation at 93°C for 9 min (12 min for heminested PCR); 40 cycles (30 cycles for heminested PCR) with denaturation at 94°C for 30 s and annealing/extending at 60°C for 1 min; and a final extension at 60°C for 10 min. Amplified products, 151 bp and 105 bp in length after RT-PCR and heminested PCR, respectively, were detected by polyacrylamide gel electrophoresis and ethidium bromide staining. To avoid false-positive results associated with heminested-PCR, separate rooms were used for RT-PCR and heminested-PCR master-mix preparation, sample processing, DNA amplification, and amplicon detection.

2.9. Sequence analysis

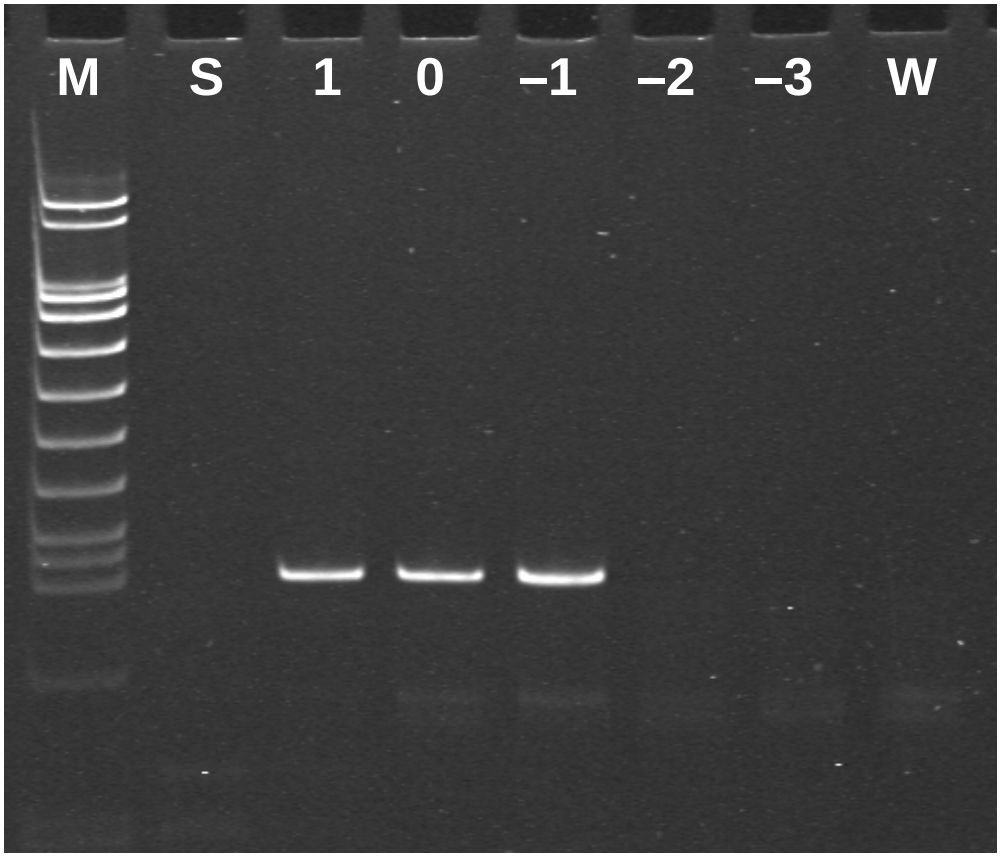
Both strands of amplicons were sequenced by GenomeExpress (Grenoble, France), directly from heminested-PCR reactions with Taq DyeDeoxy Terminator cycle-sequencing kit and ABI 373XL automate sequencer (Applied Biosystems). Sequences of 65 bases (corresponding to nucleotides 466 to 530 of poliovirus genome) were analyzed with Lasergene software (DNAStar, Madison, Wisconsin).

3. Results and discussion

3.1. Sensitivity of shellfish analysis evaluated by RT-heminested PCR

Four experiments were performed with oysters negative for enterovirus RNA detection. Digestive glands (3 g) were inoculated with 4.6×10^{1} to 4.6×10^{-3} $TCID_{50}$ of poliovirus and processed for viral detection. In three experiments, viral RNA was detected in digestive glands inoculated with 4.6×10^{-1} $TCID_{50}$/3 g (about 3.2×10^{-1} PFU/3 g or 3.1×10^{1} RT-PCRU/3g) (Fig. 1). In the other experiment viral RNA was detected at a 10-fold higher concentration. Given the proportion of samples analyzed (2.4%), these results suggested that RT-heminested PCR reaction was likely to detect 1.1×10^{-1} to 1.1×10^{-2} $TCID_{50}$ (about 7.6×10^{-2} to 7.6×10^{-3} PFU or 7.4×10^{0} to 7.4×10^{-1} RT-PCRU) as enumerated by cell culture assay. According to these results, the efficiency of the virus concentration and RNA purification process was estimated to be about 105% (mean of three experiments in which efficiencies ranged from 13.5 to 135%). The detection method was at least as sensitive as previously reported methods that used poliovirus for their evaluation. For example, De Medici et al. (1998) detected 1 $TCID_{50}$ per 10 g of mussels, Häfliger et al. (1997) detected 3 to 30 $TCID_{50}$ per 1.25 g of mussel or oyster, and Dix and Jaykus (1998) detected 10^{3} PFU per 50 g of hard-shelled clams. Our results were also comparable to those of a method recently evaluated with HAV and NLV, with which sensitivities were about 0.015 PFU and 22.4 RT-PCRU per 3.75 g of shellfish, respectively (Kingsley and Richards, 2001). As compared with the original method (Atmar et al., 1995), the increased sensitivity seemed to be associated with improvements of RNA purification and viral RNA detection by RT-heminested PCR.

Fig. 1. Detection sensitivity of poliovirus inoculated in oyster digestive glands, by RT-heminested PCR. Digestive glands were inoculated with poliovirus (Sabin strain) and processed for viral RNA detection by RT-heminested PCR. Lanes 1 to -3: 4.6 x 10^{1} to 4.6 x 10^{-3} poliovirus $TCID_{50}$ seeded in 3 g of tissus, respectively. Lanes S: non-seeded shellfish digestive glands. Lanes W: negative control for RT-heminested PCR (water). Lanes M: DNA molecular weight marker VIII (Boehringer Mannheim, Meylan, France).



3.2. Analysis of oyster samples

Of the 52 shellfish samples that were processed, 21 (40.4%) were positive for enterovirus RNA (Table 1). Infectious viruses were isolated from three samples, which were collected in April 2000 from site B, and were positive by RT-heminested PCR. For each sample, cythopatic effects were observed after three 8-days subcultures on MRC5 cells, and coxsackieviruses type A21 were identified by neutralization assay. The laboratory was also involved in the investigation of the association between oyster consumption and the gastro-enteritis outbreaks. When we compared the results obtained from the same samples, no correlation was found between the presence of the aetiological agent of diarrhea and the presence of enteroviruses. About 24 oyster samples previously found contaminated by noroviruses, 8 (33%) were not contaminated by enteroviruses (data not shown).

Table 1
Detection of enterovirus RNA in harvested shellfish

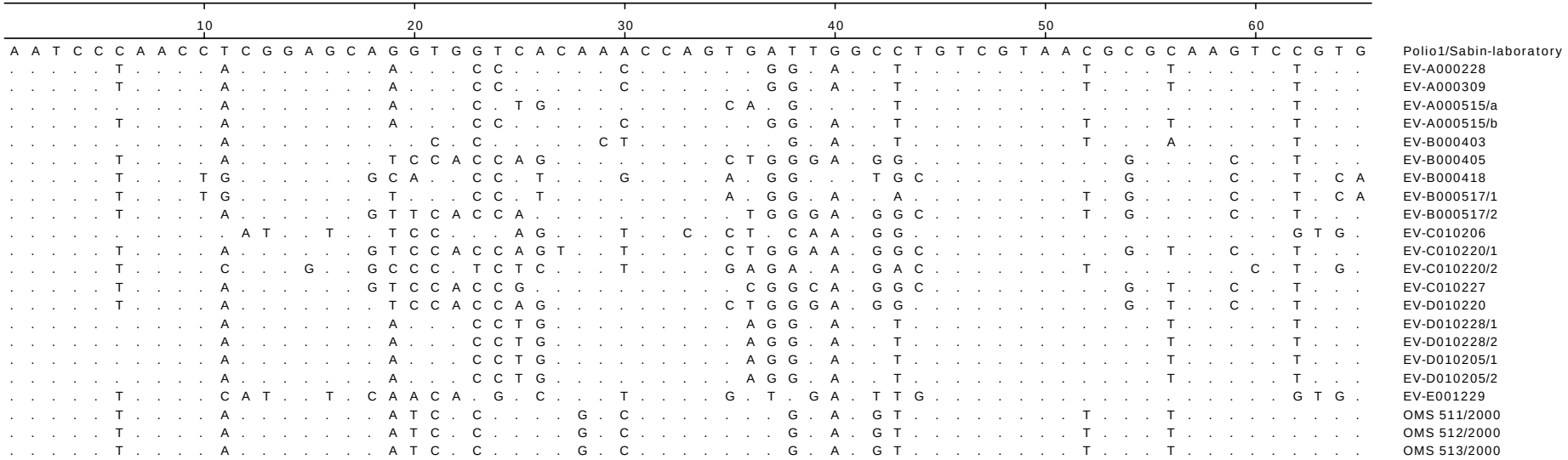
Date	No. of positive samples/total no. of samples[a]				
	Site A	Site B	Site C	Site D	Site E
2000					
Feb.	1/1				
Mar.	1/2	0/3			
Apr.	0/7	6/8			
May.	1/1	2/4			
Dec.					1/6
2001					
Jan.			0/3	0/1	
Feb.			4/8	5/6	
Mar.			0/2		

[a] Positive samples (pool of 7 oysters) refer to a positive result by RT-heminested PCR.

3.3. Analysis of the sequences detected in oyster samples

For the 21 positive samples, chromatogram results indicated that mixtures of at least 2 different viral sequences was present in 3 amplicons, while 18 samples gave interpretable sequencing results. These sequences were compared together and with the sequences of poliovirus strains grown in the laboratory (Fig. 2). No sequences were similar to those of poliovirus. Similarities were observed between sequences detected in shellfish harvested from the same sampling site on the same day and also on different days (Site A: EV-A000228, EV-A000309 and EV-A000515/b; Site D: EV-D010228/1 or 2, and EV-D010205/1). Cross contaminations between shellfish samples during experiments could be excluded because the RT-heminested PCR control and others oyster samples were negative. That is, no inhibition was detected in these samples by adding exogenous viral RNA and performing RT-heminested PCR. Moreover, no similarity was observed between the sequences of viruses isolated from shellfish from different sites. Distinct viral sequences were obtained from different shellfish batches harvested the same day from the same site (Site B : EV-B000517/1 and 2; Site C: EV-C010220/1 and 2; Site D: EV-D010205/1 and 2). One sample analyzed in duplicate gave different enterovirus RNA sequences (EV-A000515/a and b). The three coxsackieviruses A21 isolated by cell culture were also sequenced (OMS 511/2000: Accession no. AY205253; OMS 512/2000: Accession no. AY205254; OMS 513/2000: Accession no. AY205255) and provided the same sequence of 65 bases (Fig. 2). However, the sequence was different from those obtained from the same sample by direct sequencing of the amplicons (nine bases different for EV-B000403 and 18 for EV-B000405).

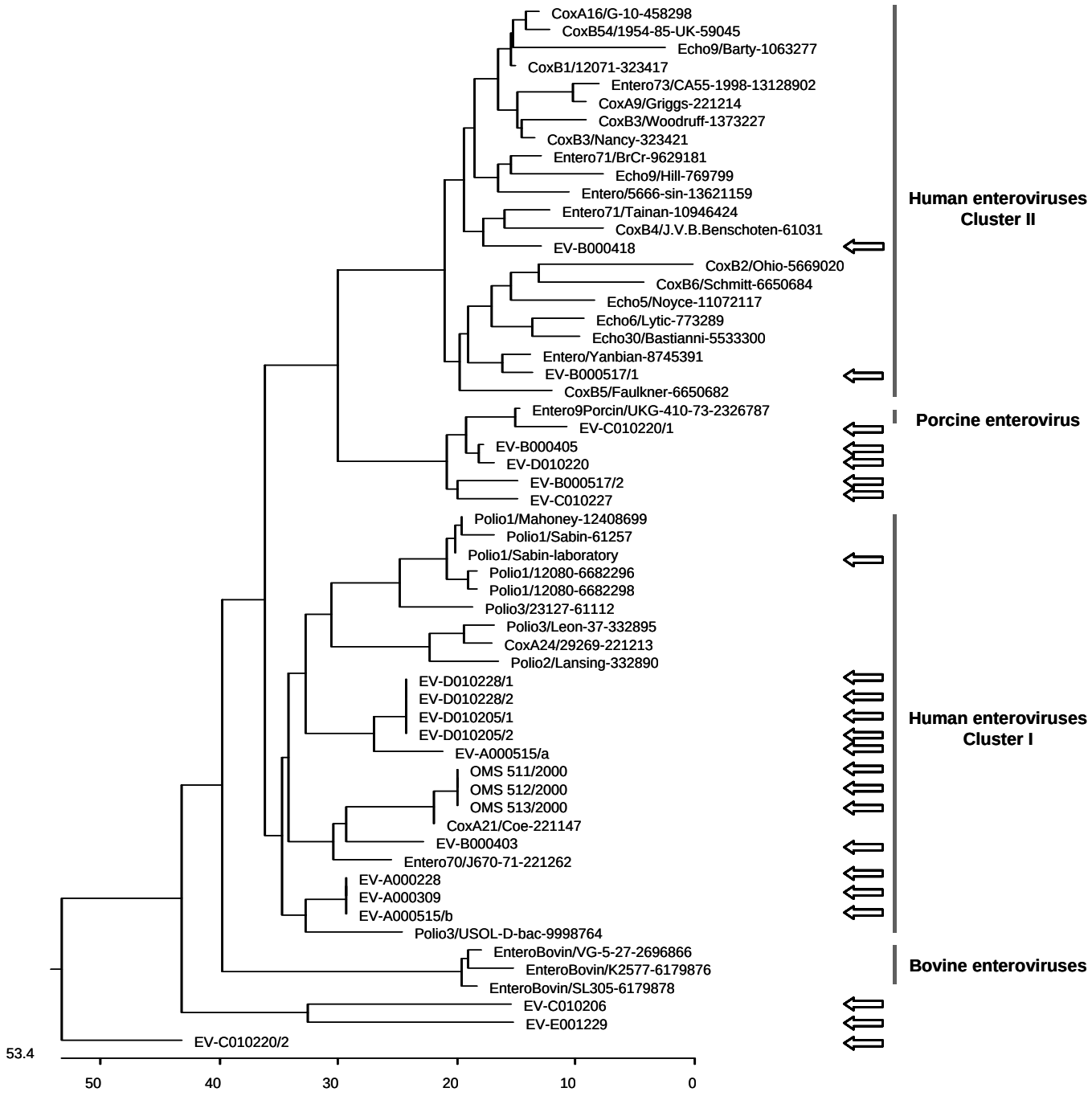
Fig. 2. Alignment of the nucleotide sequences obtained from the 5' non-coding region of the enteroviruses detected in shellfish. The sequences are designated by the letter of the sampling site and a number matching to the year, the month and the day of sample collection. The number or latter after the slash indicates different sample harvested the same day or duplicate of one sample, respectively. The sequences of the viruses isolated by cell culture began by OMS according references providing by the National Reference Center for Enteroviruses.



3.4. Comparisons against the sequences in databases

Similarities were observed between shellfish enteroviral RNA sequences and RNA sequences of reference strains of human and animal enteroviruses described in databases (Fig. 3). The phylogenetic tree, based on the similarities of sequences of 65 bases, showed different clusters. Within these clusters were grouped strains belonging to the same cluster according to a classification based on nucleotide identity of larger sequences also in the 5' untranslated region (Muir et al., 1998). Therefore, the analysis of short sequences might allow rapidly identification of strains. Among the three samples containing infectious coxsackieviruses serotype A21, two allowed the identification of viral sequences. One was closely related to a coxsackieviruses A21 (EV-B000403) the other to a porcine enterovirus (EV-B000405). The differences observed between the sequence EV-B000403 and the sequences of isolated coxsackieviruses A21 (OMS 512/2000) from the same oyster sample might be a result of mutations during viral replication in cell culture. Nevertheless, higher discordances observed between molecular (EV-B000405) and neutralization assays (OMS 513/2000) from the same sample might be associated with the occurrence of two distinct viruses in the samples, as was shown for other samples; with one strain being more efficiency detected by RT-heminested PCR than the other, which was detected by cell culture only. The sequence analysis indicated also that 5 sequences among 19 characterized were probably close to porcine enteroviruses sequences and 3 were phylogeneticaly distant from the others strains (Fig. 3). The sequences were too short for assessing their relationship with the sequences of animal viruses, but the occurrence of porcine enterovirus in the environment could be possible as swine production is extensive in western France. Moreover, a high frequency of bovine enterovirus in stool from cattle, with the virus being in oysters collected from a river downstream from the farm has recently been reported (Ley et al., 2002). Others animal enteric viruses have also been detected in environmental samples. In particular, we previously characterized by restriction fragment length polymorphism assay rotavirus sequences in wastewater, and observed similarities with animal rotavirus profiles (Dubois et al., 1997). At the present time, there is no evidence that primers selected to evaluate the viral quality of shellfish and of water do not detect animal viruses, and the impact on human health of these animal viruses is still unclear.

Fig. 3. Phylogenetic analysis of enterovirus sequences obtained from shellfish samples. The phylogenetic analysis was performed on 65-base nucleotide sequences from the 5' non-coding region of enterovirus genome (sequence between primer 3 and primer A1), with Lasergene software (DNAStar) by using clustal method. For reference strains, the last number indicates the identification number in GenBank. The scale represents the number of nucleotide substitution between the sequences. The arrows locate the viral sequences identified in this study. For human enteroviruses, cluster I and II was described by Muir et al (1998).



3.5. Epidemiological background

Most of the strains identified from shellfish were probably enterovirus strains that circulate in the human population, as has been previously demonstrated for rotavirus strains in sewage (Dubois et al., 1997). The frequency of detection of the enteroviruses found in this study was higher than that found for sites occasionally contaminated by fecal coliforms (Le Guyader et al., 2000). The percentage of positive samples was similar to that for enterovirus RNAs detected in shellfish collected from moderately polluted areas on the northwestern coast of Spain (Romalde et al., 2002). The shellfish analyzed in the present study came from probably polluted sites; so the results likely do not reflect the usual prevalence of viruses in French shellfish. The contamination of sites probably arose because of unusually heavy rains and runoff (Miossec et al., 1998), and of outbreaks of enterovirus infections (Lina et al., 2001).

4. Conclusion

The present study describes an important frequency (40%) of enterovirus sequences detection, by RT-heminested PCR, in oysters exposed to fecal contamination. This result was probably associated with exceptional climatic (heavy winter rains) and epidemiological (large enteroviral outbreaks) factors. The characterization of viral sequences suggested that animal strains were also associated with the contamination of shellfish samples. These results highlight the usefulness of studies focused simultaneously on clinical, veterinarian and environmental samples for clarifying enterovirus ecology.

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