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Distribution of Human Virus Contamination in Shellfish from Different Growing Areas in Greece, Spain, Sweden, and the United Kingdom

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Viral pollution in shellfish has been analyzed simultaneously across a wide range of geographical regions, with emphasis on the concomitant variations in physicochemical characteristics and social features. The methods for sample treatment and for the detection of human enteric viruses were optimized by the participating laboratories. The second part of this study involves the selection of a protocol for virus detection, which was validated by analyzing the distribution and concentration of human viral pathogens under diverse conditions during an 18-month period in four European countries. Shellfish-growing areas from diverse countries in the north and south of Europe were defined and studied, and the microbiological quality of the shellfish was analyzed. Human adenovirus, Norwalk-like virus, and enterovirus were identified as contaminants of shellfish in all the participating countries. Hepatitis A virus was also isolated in all areas except Sweden. The seasonal distribution of viral contamination was also described. Norwalk-like virus appeared to be the only group of viruses that demonstrated seasonal variation, with lower concentrations occurring during warm months. The depuration treatments currently applied were shown to be adequate for reducing *Escherichia coli* levels but ineffective for the elimination of viral particles. The human adenoviruses detected by PCR correlate with the presence of other human viruses and could be useful as a molecular index of viral contamination in shellfish.

Many viruses transmitted by the fecal-oral route are widely prevalent in the community, and infected individuals can shed millions of viral particles in their feces. Consequently, viruses of many types occur in large numbers in sewage. Sewage treatment processes, when present, are only partially effective at viral removal (41). Coastal discharges constantly release human viruses into the marine environment. Once in the environment, viruses can survive for weeks to months (7, 16, 18) either in the water column or by attaching to particulate matter and accumulating in sediments. Bivalve shellfish, in the process of filter feeding, also concentrate and retain human pathogens derived from sewage contamination in the shellfish-growing water. Epidemiological evidence suggests that human enteric viruses are the most common etiological agents transmitted by bivalve shellfish (26, 29, 45).

Human health problems associated with shellfish consumption are recognized internationally and have been recorded since medieval times (26). Shellfish quality and safety regarding bacterial pathogens have been improved with the implementation of sanitary controls. The scientific community has realized that contaminating viruses are responsible for nearly all episodes of gastroenteritis as well as other outbreaks of illness related to consumption of contaminated shellfish. For example, in 1988 in Shanghai (China), 300,000 cases of hepatitis A were traced to the consumption of clams harvested from a sewage-polluted area (21, 43). There have been several outbreaks of illness due to the consumption of bivalve shellfish within the legal limits of bacterial standards allowed by the current legislation (26).

Analyses of fecal coliforms and *Escherichia coli* are thought to have limited predictive value for viral pathogens such as enteroviruses (EV) (14, 17, 24, 44), Norwalk-like virus (NLV), and hepatitis A virus (HAV) (12, 46). Some studies show different rates of reduction of bacteria and viruses in depurated shellfish (11, 37, 38).

There is a need for indicators of human-specific viral fecal pollution to improve the microbiological control of shellfish. Molecular techniques have been developed for the detection of human viruses with a high level of sensitivity and specificity (1, 25, 33, 34, 36). The detection of human adenovirus (ADV) by PCR has been proposed as a molecular parameter for monitoring the presence of human viruses in the environment.

Therefore, it was necessary to develop a study to define and validate reliable techniques for the detection and quantification of human enteric viruses in shellfish in order to analyze the level of viral pollution in shellfish produced in widely diverse geographical areas and to evaluate potential viral indicators. For these purposes, shellfish samples collected at shell-

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fish-growing areas in the North Sea, the Atlantic Ocean, and the eastern and western Mediterranean Sea were analyzed for the presence of *E. coli*, ADV, EV, HAV, and NLV, including genogroup I (NLVI) and genogroup II (NLVII). Their prevalence and distribution throughout the year were also evaluated.

MATERIALS AND METHODS

Human virus standard suspensions. Isolates of ATCC ADV 5 prototype strain and ADV 41 Tak prototype strain were obtained by inoculation of A549 cells and HEp-2 cells, respectively. Cells were grown in 75-cm^2 plastic flasks containing Dulbecco's minimum essential medium (MEM) supplemented with 2% fetal calf serum. For EV, the vaccine strain of poliovirus type 1 and two different patient strains representing coxsackievirus type B5 and echovirus type 11 were all inoculated on green monkey kidney (GMK) cells under the same conditions as the ADV strains.

HAV vaccine strain HM175 was inoculated on both MRC5 human diploid cells and Chang liver cells. These cells were grown in Dulbecco's MEM supplemented with 5% calf serum, 20 U of penicillin per ml, and 20 μg of streptomycin per ml. During this infection, no cytopathic effect (CPE) was detected. Approximately 12 days postinfection, HAV was harvested and the infected cell suspensions were freeze-thawed four or five times to release the virus particles. Standard suspensions were prepared for each virus and were used as performance assessment controls throughout the study.

NLV cannot be cultured; therefore, to prepare the viral suspension, clinical fecal samples were obtained. The presence of NLVII was confirmed in a fecal sample associated with an outbreak of gastroenteritis. Sequence analysis confirmed the identity of the strain detected as Grimsby virus. Due to the high titer of virus in this particular clinical sample, serial dilutions of the 10% fecal extract were prepared and used as a standard suspension for positive and quality controls.

Shellfish samples. Mussels and oysters (*Mytilus galloprovincialis*, *Mytilus edulis*, *Crassostrea gigas*, and *Ostrea edulis*) were obtained over an 18-month period from shellfish-growing areas with different levels of fecal pollution in four European countries. A total of 54 mussel samples from Sweden were collected in the North Sea, and 173 mussel and oyster samples from the United Kingdom were collected in harvesting areas in the Atlantic Ocean; 144 samples of oysters and mussels were harvested from Greece in the eastern Mediterranean Sea, and 84 oyster and mussel samples from Spain were collected in the western Mediterranean. Additionally, 20 oyster samples were collected in the Spanish Atlantic Ocean. After being harvested, shellfish were shipped directly to each laboratory under cold storage and processed within a 24-h period. Half of the oysters and the mussels from B areas (European Union "category B" shellfish-growing areas must contain less than 4,600 *E. coli* cells per 100 g of shellfish flesh in 90% of the samples [3]) from Spain and the United Kingdom were sent to a depuration (purification) plant. They were analyzed before and after the current commercially applied treatment (a tank-based depuration system in which clean seawater from a sea well is flushed continually for 24 to 48 h).

Shellfish processing. Shellfish were washed, scrubbed under clean running water, and opened with a sterile shucking knife. We selected 10 to 12 oysters and 15 to 20 mussels for bacterial analysis. Shellfish flesh and liquor were collected into a sterile beaker and diluted with 0.1% peptone water (1:2, wt/vol). The digestive tract of 20 oysters and 20 to 30 mussels was examined for human enteric viruses (4, 39). The digestive tract of the filter-feeding organisms had shown a higher accumulation and concentration of viruses, causing a reduction of the working volume and a decrease of enzymatic reaction inhibitors. Viral particles were eluted by the methods described below.

***E. coli* analysis.** The procedure used for detection of *E. coli* was, with slight modification, that described by Donovan et al. (13), which consists of a two-stage, five-tube, three-dilution most-probable-number (MPN) method. This technique shows no significant differences in final results compared to the standard procedure and gives results in 48 h (13).

Elution and concentration of viral particles and nucleic acid extraction. Shellfish samples collected in Greece, Spain, and Sweden were processed by elution of 30 g of the digestive gland with 0.25 N glycine buffer (pH 10) (1:5, wt/vol) by the method previously described by Pina et al. (34) and Muniain-Mujika et al. (32), with some modifications (method 1, Fig. 1). The mixture was homogenized by magnetic stirring for 15 min. Once the pH was adjusted to 7 ± 0.2, the treated homogenate was clarified by centrifugation at 2,170 × *g* for 15 min at 4°C. The supernatant was centrifuged at 39,800 × *g* for 45 min at 4°C. To pellet all viral particles, the supernatant was ultracentrifuged at 81,584 × *g* for 1 h at 4°C. The final pellet was resuspended in 200 to 400 μl of phosphate-buffered saline with a maximum volume of viral concentrate of 500 μl. The viral concentrate was stored at −80°C prior to nucleic acid extraction (Fig. 1).

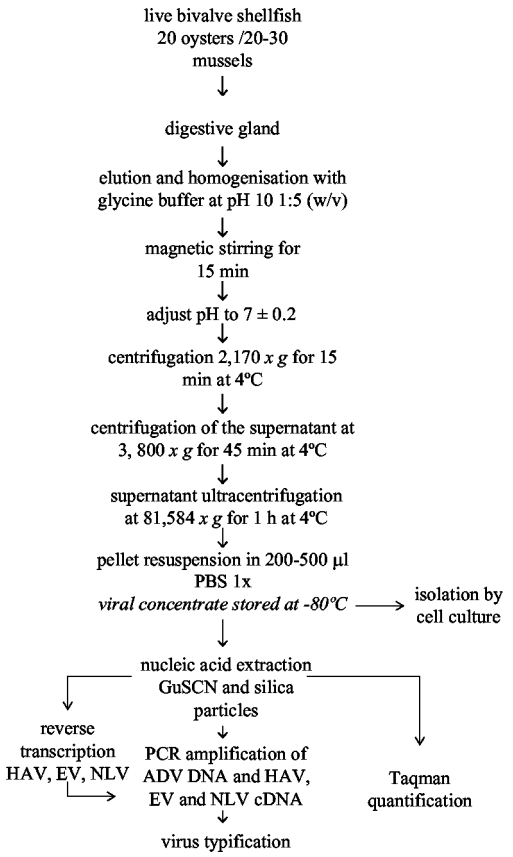

FIG. 1. Method 1 for the processing of shellfish samples and the detection of ADV, EV, HAV, and NLV.

Nucleic acids were extracted by the method described by Boom et al. (5) with minor modifications (36), using guanidinium thiocyanate as the principal component of the lysis buffer and the adsorption of nucleic acids to silica particles. Briefly, 50 μl of viral concentrate was added to a mixture of 50 μl of silica particle suspension and 900 μl of lysis buffer. The mixture was incubated at room temperature for 10 min and washed twice in 1 ml of washing buffer (12 g of guanidine thiocyanate in 10 ml of Tris-EDTA), twice more in 70% ethanol, and once in acetone. The pellet obtained after the complete evaporation of acetone was resuspended with 50 μl of elution buffer (49.4 μl of DTT and 0.6 μl of RNase inhibitor [Perkin-Elmer] for RNA viruses, and 50 μl of Tris-EDTA for DNA viruses). The extracted nucleic acids were then used for cDNA synthesis and nested PCR amplification of HAV, EV, and NLV and for nested PCR amplification of ADV (Fig. 1). This procedure had been previously applied to ADV, enterovirus, and hepatitis A virus detection in shellfish (32, 34) but not to NLV detection.

Oysters and mussels from the United Kingdom were analyzed by a procedure developed by the British participant based on direct nucleic acid extraction (method 2). Briefly, 10 g of digestive glands was weighed, placed in a tube with an equal volume (1:1, wt/vol) of peptone water, shaken on a Glas-Col platform shaker, and centrifuged at 1,000 × *g* for 5 min. The supernatant was removed into a bijou and frozen. Viral nucleic acid was extracted from the oyster digestive concentrates by previously published methods (27).

The laboratories in Greece, Spain, and Sweden selected method 1 after comparing the two procedures (data not shown). Method 1 was considered highly sensitive and more practical for application to the routine analysis of diverse viral parameters in shellfish. A larger number of positive results was found when participant laboratories analyzed two common sets of highly polluted oysters by method 1. Additionally, method 1 was easier to apply to testing a large number of samples for the presence of diverse viruses.

PCR amplification. The sequence, specificity, and sensitivity of the oligonucleotide primers used were described in previous studies (19, 20, 30, 34, 36). The reaction mixture for reverse transcription (RT) had a total volume of 8 μl and contained 5 μl of the extracted nucleic acids, 1 μl of 10 × PCR Buffer II (Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3 at 25°C), 0.6 μl of 25 mM $MgCl_2$, 200 μM each deoxynucleoside triphosphate (Amersham Pharmacia Biotech), and 20 μM corresponding external primer for EV, HAV, or NLV (19, 20, 34, 36). The reaction mixture was incubated at 95°C for 5 min before the addition of 50 U of Moloney murine leukemia virus reverse transcriptase (Applied Biosystems), 10 U of RNase inhibitor (Applied Biosystems), and 0.5 μl of 0.2 M dithiothreitol. The temperature was cycled as follows: 30 min at 42°C and 5 min at 95°C.

For the first PCR amplification, 10 μl of cDNA (corresponding to 0.1 to 0.2 g of shellfish digestive gland or approximately 1 to 2 g of whole-animal flesh) was used for EV, HAV, and NLV analysis. For ADV analysis, 10 μl of extracted nucleic acids (corresponding to 0.2 to 0.4 g of shellfish digestive gland or approximately 2 to 4 g of whole animal), was used. Amplification was carried out with 50 μl of reaction mixture with 1 μl of 10 × PCR buffer II containing 10 mM Tris-HCl (pH 8.3 at 25°C), 1.5 mM of $MgCl_2$, 1 μl of primer left for EV (Ent 1), HAV (HAV1), and NLV (SM31) and 1 μl of each primer for ADV, and 2 U of AmpliTaq polymerase (Applied Biosystems). The first cycle of denaturation was carried out for 2 min at 95°C. For HAV, EV, and ADV, the conditions during the 30 cycles of amplification were denaturation at 95°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 1 min. For NLV, the conditions for the 35 cycles were 1 min at 94°C, 1 min at 37°C, and 1 min at 72°C.

For the nested PCR amplification, 1 μl of the amplified DNA from the first PCR was added to a new batch of 50 μl of PCR mixture containing 9 μM each inner primer (nHAV1 plus nHAV2 for HAV, nEnt1 plus nEnt2 for EV, and nehexAA1893 plus nehexAA1905 for ADV) or 0.4 μM NLV inner primers (GA plus E3 for genogroup I and NI plus E3 for genogroup II) (20, 30). The amplification cycles were as described above. Thermal cycling was carried out in a programmable heat block (Gene Amp PCR System 2400; Applied Biosystems). The results were analyzed by gel electrophoresis on a 3% agarose gel stained with ethidium bromide.

Standard precautions were applied in all the manipulations to reduce the probability of sample contamination. Separate areas were used for reagents, treatment of samples, and manipulation of amplified samples. Undiluted samples and 10-fold dilutions of nucleic acid extracts were analyzed twice in independent experiments. A negative control was added to each sample. Viral particle suspensions of low concentration were used as positive controls to limit the use of modified nucleic acids.

Evaluation of the sensitivity of the method. Tenfold dilution series of viral standards were analyzed in limiting-dilution experiments to establish and compare the sensitivity of the PCR-based detection of human viruses. For ADV 5 and ADV 41, the results could be confirmed by real-time TaqMan PCR. All viral dilution suspensions were divided into 60-μl batches to be used only once, since repeated freeze-thawing can reduce viral content up to 50 to 80% (data not shown).

Digestive gland samples (10 g) from each of three commercial samples (two sets of oysters and one set of mussels) were supplemented with 5×10^7 PFU of poliovirus type 1 to evaluate the sensitivity of method 1 and to analyze the presence of inhibitors of the RT amplification. These artificially seeded samples, together with a positive control which had been previously assayed as PFU in monolayers of buffalo green monkey (BGM) cells, were studied in a limiting-dilution experiment.

Evaluation of possible false-negative results. To evaluate potential false-negative results of viruses in shellfish by PCR due to the presence of inhibitors, six shellfish samples containing *E. coli* but with negative results for human viruses were inoculated with 5×10^3 PFU of poliovirus type 1 and analyzed by method 1. These seeded samples were studied in limiting-dilution experiments by PCR in parallel with a positive control that has an equivalent titer of PFU.

Typing of human viruses. Nested HAV and ADV amplicons were sequenced to evaluate the variability of the detected HAV and ADV strains. Briefly, nested PCR products were purified by using the QIAquick PCR purification protocol as specified by the manufacturer (Qiagen). Both strands of the purified DNA were sequenced with the ABI Prism Big Dye Terminator cycle-sequencing kit (Applied Biosystems) as specified by the manufacturer. The results were checked with the ABI Prism 3700 DNA analyzer (Applied Biosystems). The obtained sequences were compared with all sequences in the GenBank and EMBL databases by the PubMed NCBI BLAST program.

EV typing via nested RT-PCR was performed using the degenerate primers described by Casas et al. (8) that allow the amplification of the VP1 region. These primers were designed for clinical samples, and direct PCR was very inefficient and produced negative results due to lower sensitivity in comparison to the 5′ NTR primers used for the detection.

For NLV, a minimum of five clones from each genogroup were sequenced to facilitate the identification of more than one target sequence (23). All clones were sequenced in both orientations. Nucleotide ambiguities were resolved by comparison of ABI 310 chromatograms. NLVI contained 78 bp, and NLVII sequence contained 76 bp. A consensus sequence was generated, if all clones from the nested RT-PCR product had the same sequence following alignment.

Sequences used to generate the phylogenetic tree were obtained from the GenBank sequence database. The sequence accession numbers used to generate the NLV phylogenetic tree were Mexico U22498, Lorsdale X86557, Murl1-1997-JP AB019269, Richmond AF414419, Hawaii UO7611, Haytread S71765, Jena AJ011099 (bovine enteric calici-like virus), Na2-contig AF097917 (bovine enteric calici-like virus), Grimsby/1995/UK AJ004864, Valletta/1995/Malta AJ277616, and a clinical strain from the United Kingdom, Harrow.

Quantification of human ADV. A total of 18 of 19 Swedish and 8 Spanish samples positive for ADV were quantified by real-time TaqMan PCR (Fig. 1). A pair of degenerate PCR primers was selected from the conserved region of the ADV hexon gene (2). The upstream and downstream primer sequences were 5′-C(AT)T ACA TGC ACA TC(GT) C(CG)G G-3′ and 5′-C(AG)C GGG C(GA)A A(CT)T GCA CCA G-3′, respectively. Two fluorogenic probes, Ad: ACDEF [5′-(6FAM) CCG GGC TCA GGT ACT CCG AGG CGT CCT (TAMRA)-3′] and Ad:B [5′-(6FAM) CCG GAC TCA GGT ACT CCG AAG CAT CCT (TAMRA)-3′], with sequences located between the PCR primers were synthesized by Applied Biosystems. The amplification was performed in a 25-μl reaction mixture with a PCR core reagent (Applied Biosystems). The reaction mixture contained 5 μl of silica-purified DNA or 10 μl of titrated QIAgen extracted standard DNA, 1× TaqMan core buffer, 5 mM $MgCl_2$, 200 μM each dATP, dCTP, and dGTP, 400 μM dUTP, 900 nM each primer, 225 nM probe, 0.25 U of AmpErase uracil *N*-glycosylase, and 1U of AmpliTaq Gold polymerase. Following activation of the uracil *N*-glycosylase (2 min 50°C) and activation of the AmpliTaq Gold for 10 min at 95°C, 40 cycles (15 s at 95°C and 1 min at 60°C) were performed with an ABI 7700 sequence detector system (Applied Biosystems). The principle of the real-time PCR is described elsewhere (22).

Viral quantification and isolation in cell culture. Forty-six EV-positive samples were selected for infecting BGM cells, and some of them were used with A549 and HEp-2 cells. The viral concentrates were filtered through a 0.22-μm-pore-size low-binding protein filter (Millipore) and assayed in cell culture. Ten samples were tested for PFU on BGM cells by using the MPN culture unit method including three flasks per dilution (6) with minor modifications. Cell monolayers were grown on MEM supplemented with 3% sodium bicarbonate, 1% L-glutamine, 1% penicillin-streptomycin at 10,000 IU/ml (GIBCO, Invitrogen Corporation), and 5% fetal bovine serum; (BioWhittaker Europe). The monolayers were inoculated with three 50-μl replicas of the neat virus and 1:10 dilution and incubated at 37°C for 1 h. After virus adsorption, the inocula were removed and the monolayers were overlaid with MEM supplemented with 1% fetal bovine serum and returned to incubation at 37°C. The flasks were examined daily for 14 days for CPE. Regardless of the appearance of the monolayers, cells were frozen and thawed, and 50 μl of each culture was submitted to a second passage (Fig. 1).

Quality assurance. Interlaboratory studies based on the testing of common samples and reference materials to validate the defined protocols and to determine the sensitivity and efficiency of the method were applied in this study. Standard suspensions of viruses and *E. coli* were distributed among all the laboratories to be used as performance controls. Highly polluted oysters (*C. gigas*) from shellfish-harvesting areas in the United Kingdom were collected and distributed among the four laboratories to assess the ease and reproducibility of the described procedures for the detection human enteric viruses. Live oysters were transported at 4°C and delivered to the laboratories within 48 to 72 h and were processed immediately.

Statistical analysis. All the statistical tests were computed using the statistical package Spss 10.0.7 on a Pentium III machine running MS-Windows 2000 Professional. The dependence of every pair of viruses in the entire sample with or without country distinction was tested. The Fisher exact test was used to evaluate significant dependencies between all the possible pairs of viruses.

RESULTS

Analysis of standard suspensions of human viruses. We determined the sensitivity of the assay by limiting-dilution analysis by nested PCR. ADV 5 and ADV 41 were detected up to a 10^{-7} to 10^{-8} dilution, which corresponds to 1 to 10 viral

TABLE 1. Microbiological parameters tested during the two oyster trials[a]

Date of trial	Laboratory	Detected virus[b]				
		ADV	EV	HAV	NLVI	NLVII
November 1999	1	+	+	+	+	−
	2	+	+	−	−	−
	3	+	−	−	−	+
	4	+	+	+ (weak)	+	+
January 2000	1	+	+	+	+	+ (weak)
	2	+	−	NT	+	−
	3	−	−	−	+	+
	4	−	+	−	+	−

[a] Laboratories 1, 2, and 4 applied method 1 and laboratory 3 applied method 2 as described in Materials and Methods.
[b] NT, not tested; +, positive results by PCR; −, negative result by PCR.

particles according to the quantification by real-time TaqMan PCR. For the different types of EV, the limit of detection was estimated to be within 1 to 10 viral particles. Previous studies have shown a sensitivity to the PCR and RT-PCR assays of 1 to 10 viral particles for ADV and EV (36). ADV particles were quantified by spectroscopy. EV were analyzed by nested RT-PCR after total-RNA extraction of 1 PFU so that 1 PFU was found to be equivalent to 10^3 viral particles for poliovirus type 1 (36). For HAV, a limit of 1 to 100 genome equivalents was estimated with method 1. From 5 to 50 viral particles of poliovirus type 1 (5×10^{-2} to 5×10^{-1} PFU) per of shellfish flesh were detected in the inoculated shellfish samples. The nested endpoint experiments carried out monthly remained stable for the tested viruses (ADV 5, ADV 41, and echovirus 11).

Study of potential false-negative results. Poliovirus type 1 was detected in all human virus-negative, *E. coli*-positive samples at the expected dilution, confirming the absence of detectable false-negative results for RT-PCR associated with the presence of inhibitory substances in the extracted nucleic acids. Poliovirus type 1 was detected down to the $1:10^5$ dilution in the positive control and in three of the analyzed samples. In addition, it was detected down to the $1:10^4$ dilution in one sample and to the $1:10^3$ dilution in three samples. These results were consistent with the estimated sensitivity of the assay described above.

Intercalibration studies testing highly polluted oysters. Before the beginning of the 18-month sampling, the analysis of two common shellfish samples was carried out by all laboratories. Table 1 shows the results.

Depuration. Paired samples collected from the B shellfish-harvesting areas were analyzed before and after the purification treatment by nested PCR. Table 2 summarizes the percentage of positive samples. In all tested samples, depuration effectively reduced the *E. coli* counts but was not effective at eliminating viral contaminants (Table 2).

Microbiological contamination of shellfish. Only in the analyzed shellfish production areas in Spain were the levels of *E. coli* compatible with those used to classify the different areas according to the European directive (3). The studied A area showed levels of *E. coli* lower than 230 MPN/100 g of shellfish meat. In the studied B area, all values were within 230 to 4,600 MPN/100 g. In the other countries, the *E. coli* values in some areas were higher than expected on the basis of the original classification. These data are complied in Fig. 2. Besides, an eventual increase of *E. coli* numbers occurred in the three Swedish areas due to several natural phenomena such as flooding and thawing of water on the ground (Fig. 2).

Regarding the presence of human enteric viruses, ADV was the most prevalent group of viruses detected, followed by EV and NLV. HAV was detected in five samples from the areas of endemic infection (Greece and Spain) and in one sample from the United Kingdom (Table 3). From Table 4, it is concluded that 89% of samples positive for any virus type was positive for human ADV. Overall, it was observed that the detection of contaminating viruses was, as expected, higher in the more heavily fecally polluted areas on the basis of *E. coli* levels (Table 5).

The data obtained were analyzed to determine whether there was any seasonal effect on the detected viral contamination. Figure 3 suggests a clear seasonal distribution in the number of presumptive NLV-positive shellfish detected in the four geographical areas, with the largest number of positive samples in the cooler months. Human ADV and EV had similar patterns of distribution throughout the monitoring period, but they were not affected by any seasonal effect (Fig. 3). The apparent seasonal trend of NLV was supported by the statistically significant correlation found between the two groups of NLV and the temperature when a logistic regression model was applied. In contrast, the other analyzed virus did not have any significant relationship to temperature (data not shown). As mentioned above, in Sweden the water quality of the studied shellfish-growing areas was influenced by several natural phenomena that resulted in high levels of the tested viruses and *E. coli*.

TABLE 2. Percentage of human enteric viruses before and after depuration of 18 oyster and 18 mussel samples[a]

Shellfish	% of samples with contamination[a]:											
	E. coli[b]		ADV		HAV		EV		NLVI		NLVII	
	ND	D	ND	D	ND	D	ND	D	ND	D	ND	D
C. gigas	61	6	28	25	6	6	28	25	17	25	22	19
M. galloprovincialis	61	6	39	39	0	6	44	31	6	0	6	6
Total	61	6	33	31	3	6	36	28	11	13	14	13

[a] ND, nondepurated shellfish; D, depurated shellfish.
[b] *E. coli* values were within the limits allowed by current legislation for human consumption in all depurated samples (3).

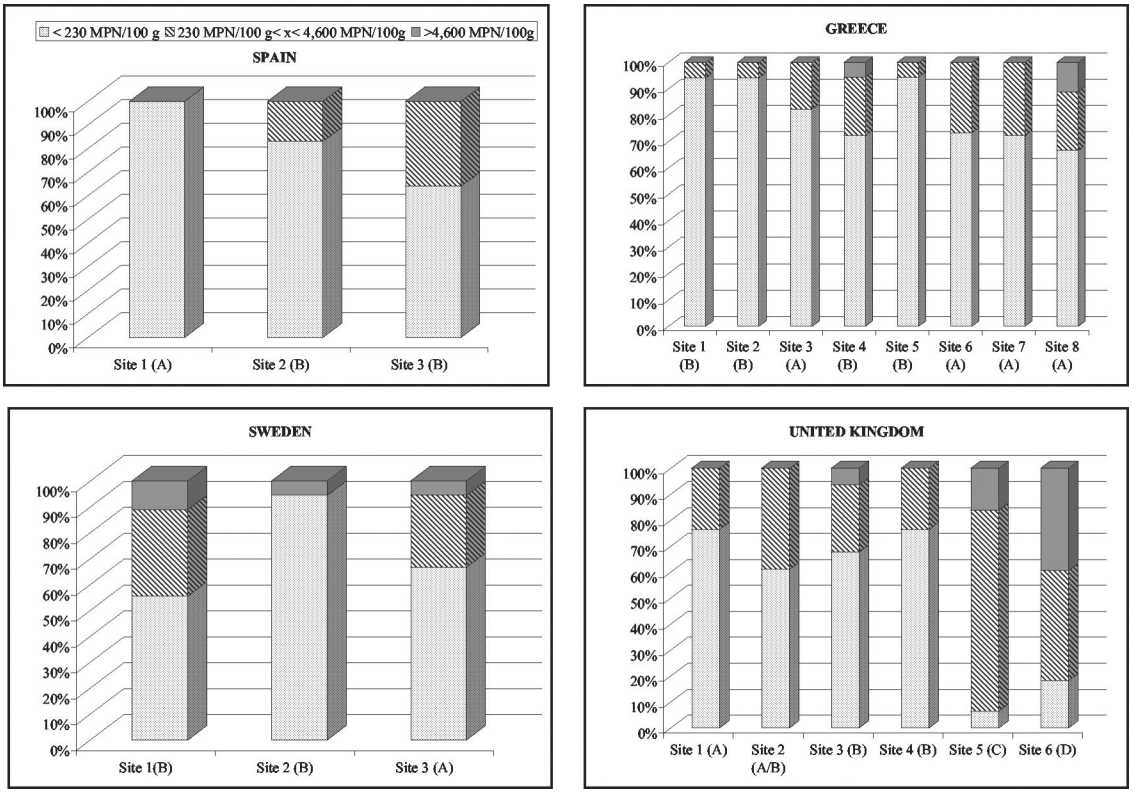

FIG. 2. *E. coli* results at the different sampling sites in the four countries. Area classifications according to current European directives are shown in parentheses.

Typing of human enteric viruses detected in shellfish. Human ADV were typed by direct sequencing of the nested-PCR amplified product. In Sweden, 17 samples positive for ADV were identified as six different serotypes and one genome variant, together representing subgenus A, B, C, and F. In Spain, six ADV-positive samples were typed as ADV 2 and two were typed as ADV 41. Eleven samples from the United Kingdom were identified as ADV 41, and one was identified as ADV 31.

All the identified types represent ADV types excreted in human fecal material for a long time, i.e. ADV 1, 2, 5, and 7. ADV 31 causes gastrointestinal problems, and ADV 41 is a well-known cause of severe gastrointestinal infections in children and is excreted in large amounts in feces. All types represent common virus types that normally circulate in the population and so are expected to be found in water contaminated with sewage and in wastewater (15, 47). In two samples, two different types of human ADV contaminating the same batch were detected by repeated assays.

Nested PCR products from two of the three Spanish samples positive for HAV were typed as genotype IB strains. One British strain belonged to genotype IA.

EV-positive samples were typed by direct sequencing of a 609-bp PCR fragment generated from the VP1 gene. Two Swedish samples were identified as echovirus type 6. Due to the low sensitivity of the primers, EV-positive viral concentrate was previously inoculated on BGM cells to increase the virus titer. After that, three of the EV-positive Spanish samples could be typed as poliovirus type 1.

With regard to the strains of NLVI, the results from the sequence divergence/similarity plot showed a positive strain

TABLE 3. Percentage of positive samples for human enteric viruses in four European countries

Country (no. of samples tested)	% of samples positive for:				
	ADV	EV	HAV	NLVI	NLVII
Greece (144)	33	15	4	0[a]	2[a]
Spain (104)	36	26	3	12	14
Sweden (54)	33	24	0	17	24
United Kingdom (173)	46	14	1	5[a]	5[a]

[a] Data confirmed by sequencing. The results for NLV from the other countries have been only partially confirmed by sequencing some of the positive samples.

TABLE 4. Relationship between ADV results with other virus type results in the four countries

Country	% of samples[a]			
	+ ADV, + other virus	+ ADV, − other virus	− ADV, − other virus	− ADV, + other virus
Greece	15	19	63	3
Spain	23	13	47	17
Sweden	13	20	41	26
United Kingdom	15	30	44	10
Total	16	22	50	11

[a] +, positive result; −, negative result.

TABLE 5. Relationship between virus results and the degree of site contamination

Country	Sampling site	Category[a]	% of samples positive for::				
			ADV	EV	HAV	NLVI	NLVII
Spain	1	A	31	0	0	6	6
	2	B	35	32	4	12	13
	3	NC	40	20	0	15	20
Greece	1	B	33	28	0	6	6
	2	B	22	17	0	0	0
	3	A	17	6	0	0	0
	4	B	50	22	11	11	0
	5	B	44	17	0	0	0
	6	A	33	6	0	0	0
	7	A	22	17	11	0	6
	8	A	39	11	11	0	0
Sweden	1	B	37	16	0	8	0
	2	NC	20	15	0	11	15
	3	A	17	13	0	10	10
United Kingdom	1	A	28	0	0	0	16
	2	A/B	28	12	0	0	0
	3	B	39	19	0	16	0
	4	B	38	8	0	0	2
	5	C	54	14	0	7	7
	6	D	78	28	3	13	13

[a] A area, all samples had <230 *E. coli*/100 g of shellfish. B area, 90% samples had <4,600 *E.coli*/100 g; C area, A and B levels exceeded; NC, nonclassified area. The degree of contamination is represented by European Union classification of shellfish-growing areas.

identification; i.e., >95% was found for all samples. Most of the samples were identified as the Valletta strain, which has lately become the most common circulating strain in Europe.

Concerning the strains of NLVII, the results from the sequence divergence plot showed a positive strain identification for all samples. A number of NLVII strains were identified, including Harrow, a newly emergent European NLVII strain (Fig. 4).

Quantification of the detected ADV. Eighteen Swedish and eight Spanish ADV-positive samples were quantified by real-time TaqMan PCR. The levels were quite low, and we do not know if these quantities are sufficient to infect humans. The analyzed Spanish samples were shown to have 2 to 32 ADV particles per g of digestive gland. The Swedish samples were shown to have 11 to 1,994 ADV particles per g, with the higher values obtained in May to July 2001, after the water had thawed from the ground.

Infectivity of EV detected in shellfish. Human ADV were not isolated in cell culture because of the difficulties in culturing these slow- growing viruses. Additionally, the lack of an efficient cell culture system for isolating wild-type HAV strains and the fact that NLV is uncultivable made EV the object for isolation from shellfish viral concentrates (Fig. 1). Thus, 46 positive samples for EV were selected for infecting BGM cells. Some samples showed cytoxicity problems and others did not grow, probably because of the lower sensitivity of cell culture. Only six samples showed CPE on BGM cells.

Statistical analysis. To examine the capacity of *E. coli* and other parameters to predict virus presence, a logistic regression model was generated. In this model, *E. coli* did not present any significant relationship to human viruses (data not shown). The relationship between every pair of viruses was also tested by considering the entire sample without country distinction in a Fisher exact test. This test showed a significant dependence ($\alpha = 0.05$ level) between all the possible pairs of viruses, except for the pairs NLVI-ADV and NLVI-HAV (Table 6). Thus, there was no significant relationship between ADV and NLVI and HAV. However, the small number of shellfish samples positive for NLVI and for HAV might affect this analysis. It must be taken into account that the larger the number of positive samples included in the analysis, the stronger is the significance of the relationship between the parameters.

DISCUSSION

Viral pollution was studied in important commercial shellfish-harvesting areas representative of a range of geographical locations throughout northern and southern Europe. Over an 18-month period, regular monthly samples of oysters (*C. gigas* and *O. edulis*) and mussels (*M. edulis* and *M. galloprovincialis*) were collected from each area and analyzed for NLV, HAV, EV, ADV, and *E. coli*. This collaborative study is the first to address issues associated with human pathogenic viral contamination of shellfish across a wide range of geographical regions, with the concomitant variations in physicochemical characteristics and social trends. Furthermore, validated procedures for the direct detection of human viral pathogens in bivalve molluscan shellfish have been developed. The method applied by three of the four laboratories has been effectively standardized, and quality assurance has been undertaken to guarantee a satisfactory relative performance.

Equivalent levels of efficiency were presented by all laboratories when analyzing standard viral suspensions. Some degree of heterogeneity in the results was observed when all laboratories analyzed two common samples of live oysters. This heterogeneity could be explained by the inherent variability within natural samples and by the low levels of viruses, which could be below the detection limit of the technique. However, we cannot rule out inter- and intralaboratory variation.

The method applied by the laboratories in Greece, Spain, and Sweden to analyze viral pollution of shellfish has been developed and improved from previously described procedures (23, 32, 34) and was selected after diverse procedures were compared for the highest sensitivity and applicability. The results of viral detection by PCR presented in this study have been carefully confirmed by independent assays and by sequencing some of the amplified viral genomes. This selected procedure is based on extraction and homogenization of the digestive gland and the elution of viral particles from shellfish using glycine buffer (pH 10) and ultracentrifugation for the study of human viral pathogens. This method has been proven to be effective for the removal of RT-PCR inhibitors present in the shellfish extracts. The sensitivity studies performed have shown a detection limit of 5 to 50 viral particles of poliovirus type 1 per g of shellfish tissue, which is one of the highest sensitivities described to date (9, 28, 31, 40, 42). The method applied routinely in the laboratory in the United Kingdom presented an equivalent sensitivity in the viral standard suspensions at the PCR level and also produced a large number of positive results for human viruses in shellfish.

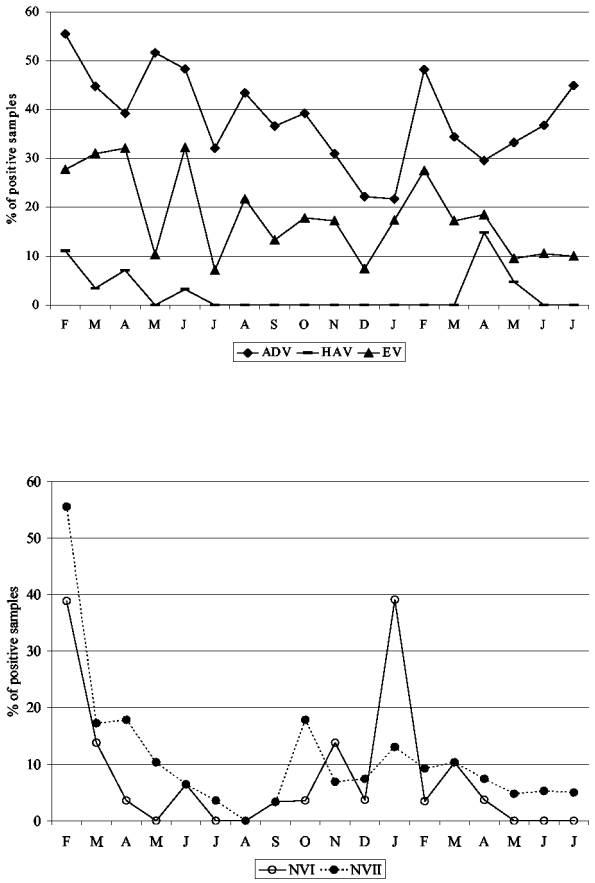

FIG. 3. Seasonal distribution of human enteric viruses in shellfish.

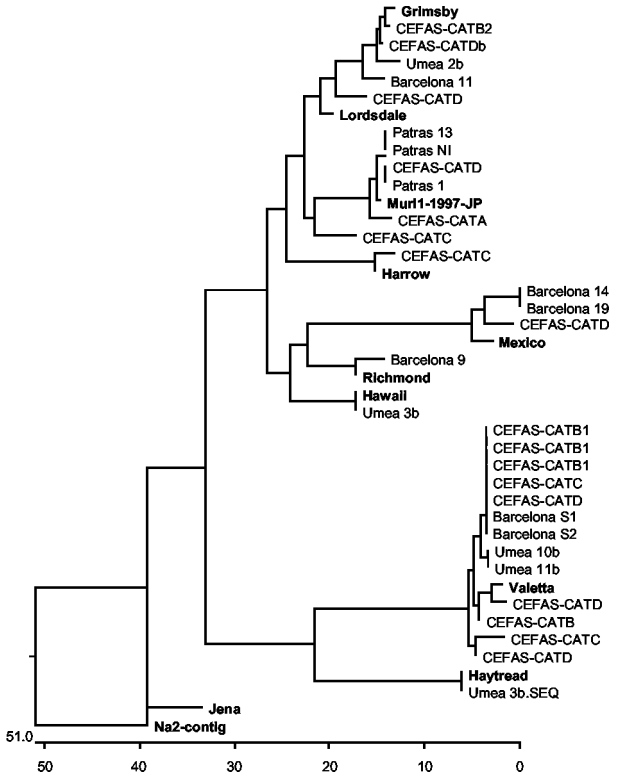

FIG. 4. Phylogenetic tree showing the genetic relationship between NLVI and NLVII detected in shellfish in comparison with representative clinical and published NLV strains at the nucleotide level. The phylogenetic tree was generated using the Clustal V algorithm within MegAlign (DNA Star Inc). The tree is based on approximately 80 nucleotides within the RNA polymerase excluding primers. Reference strains are in bold type.

The molecular epidemiology of the studied viruses is diverse in the geographical areas studied. Infection by HAV is considered endemic in the Mediterranean region, and although the prevalence of antibodies against HAV is diminishing in the population (10), it is still frequently detected in sewage from this region (35). Outbreaks of NLV infection related to shellfish consumption are frequently described (26). Epidemiological studies for EV and ADV are difficult, since persons infected by the virus may act as carriers but show no symptoms. The disease may become apparent only after another person has become infected, which may occur at a distance from the original source. Further studies are needed to define the relationship between the level of viral contamination detected by PCR in shellfish and the potential pathogenic effect after consumption.

The results of this study demonstrated that human ADV were the most prevalent human enteric viruses in shellfish from the areas studied. The most important human pathogenic viruses, NLV and HAV, were present in shellfish available for immediate human consumption (i.e., those taken from category A areas and those sampled following commercial depuration). The effectiveness of commercial depuration in reducing *E. coli* levels to below those required by European legislation (Council Directive 91/492/EEC) (3), i.e., <230 *E. coli* organisms per 100 g of mollusk flesh and intravalvular fluid, was confirmed. However, depuration as currently commercially practiced did not significantly affect the occurrence of human pathogenic viruses in any of the partner countries where this was examined. The data obtained concur with evidence from many outbreaks associated with the consumption of shellfish within legal parameters (26). Because of these findings, it is suggested that the current legislative standards for *E. coli* in postpurification bivalve mollusks do not effectively protect the consumer from the risk of exposure to the pathogenic viruses associated with fecal contamination. It is important to reexamine the protection afforded to shellfish consumers by the current requirements for depuration. A particular issue for examination is the reliance on the removal of *E. coli* and fecal coliforms to determine the duration and conditions of the depuration treatment. It is well established

TABLE 6. General *P* value of independent Fisher exact test

Virus	*P* with respect to:			
	HAV	EV	NLVI	NLVII
ADV	<0.027[a]	<0.001[a]	0.719	0.001[a]
HAV		0.007[a]	0.195	0.013[a]
EV			<0.001[a]	0.001[a]
NLVI				0.002[a]

[a] Significant dependence.

from this study, and from the literature, that this is grossly misleading with regard to removal of human enteric viruses.

Human enteric viruses were detected at higher levels in heavily polluted areas in the *E. coli* classification. In dirty B and C areas, *E. coli* is significantly related to the presence of human viruses, and it is therefore concluded that in areas significantly impacted by human sewage pollution, *E. coli* is an appropriate and adequate indicator of the risk of viral contamination. However, it is important to note that this depends on the sensitivity of the method used for *E. coli* and fecal coliform quantification and the sampling frequency (monthly monitoring was used in this study). This aspect is not currently standardized among European Member States. This study demonstrates that there is a good case for introducing standardized methods for *E. coli* and a standardized sampling frequency and protocol for use in all European Member States. This would maximize the public health protection afforded to shellfish consumers by Directive 91/492/EEC (3). It should also be noted that in cleaner shellfish areas (such as class A and cleaner class B), where titers of *E. coli* are low or absent, human enteric viruses may still be isolated.

In previous studies on viral contamination of the environment (34), human ADV were generally detected when EV and HAV were also present in the sample. An earlier analysis of shellfish flesh in the western Mediterranean found ADV when EV and HAV were also detected (29). These samples presented higher levels of fecal contamination. In the present study, more samples from different areas were analyzed. As shown in Table 4, ADV-positive samples included 89% of the samples positive for any virus type. Human ADV was the most commonly isolated human virus in shellfish and presented a clear significant relationship to the presence of other viruses. These findings and the specific human origin of the ADV detected in the PCR assay indicated that human ADV detected by PCR might be a useful molecular indicator of viral contamination of human origin in the environment. The absence of ADV may be a parameter that could improve the protection of the consumer against contamination with human enteric viruses. The method for ADV detection depends on PCR, and this molecular technique is not currently standardized and controlled enough for adoption in routine food control laboratories. However, this approach may have benefits for the future, particularly in specialist reference laboratories, which would be required to implement molecular techniques for the detection of viral pathogens. The primers used in this study detect the human ADV considered enteric and strains of respiratory origin that are excreted in feces. The infective capability of these strains with respect to shellfish consumption needs to be resolved.

With regard to detection of the most common pathogens causing illness in shellfish consumers, HAV was isolated at a low frequency in samples from Spain, Greece, and the United Kingdom, but not Sweden. This correlates well with our current understanding of the epidemiological presence of HAV in the community in these countries. This is the first time that HAV has been identified in shellfish from the United Kingdom. It further demonstrates that the methods used for HAV detection in shellfish in this study are effective and valuable for future public health controls. NLV was successfully detected in samples from all four countries, demonstrating that this method can be successfully implemented in reference facilities. It is important to indicate that primers used for the detection of HAV, EV, and ADV are specific for these viruses. However, since the detection of NLV requires highly degenerate primers, a significant proportion of presumptive NLV-positive results, as determined by the nested RT-PCR amplicon size, were not confirmed as NLV following sequencing. This highlighted the importance of authentication of positive viral detection using additional typing methods. However, within the scope of this study, confirmation methods were reliant on sequencing, a process that is laborious, time-consuming, expensive, and not suitable for nonspecialist laboratories. It is clear that simplification of viral confirmation is a priority for future work. This study has important implications for the future development of official controls. It is clear that molecular assays for the detection of human viruses in shellfish can be implemented in diverse laboratories throughout Europe providing the sufficient resources that are available to implement the assays. At present, because of the cost and complexity of the assays, this is likely to be restricted to specialist facilities. It is still an important advance in the availability of methods to provide better consumer protection against viral illness occurring following shellfish consumption.

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