

## Detection of Multiple Noroviruses Associated with an International Gastroenteritis Outbreak Linked to Oyster Consumption

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**An international outbreak linked to oyster consumption involving a group of over 200 people in Italy and 127 total subjects in 13 smaller clusters in France was analyzed using epidemiological and clinical data and shellfish samples. Environmental information from the oyster-producing area, located in a lagoon in southern France, was collected to investigate the possible events leading to the contamination. Virologic analyses were conducted by reverse transcription-PCR (RT-PCR) using the same primer sets for both clinical and environmental samples. After sequencing, the data were analyzed through the database operated by the scientific network FoodBorne Viruses in Europe. The existence of an international collaboration between laboratories was critical to rapidly connect the data and to fully interpret the results, since it was not obvious that one food could be the link because of the diversity of the several norovirus strains involved in the different cases. It was also demonstrated that heavy rain was responsible for the accidental contamination of seafood, leading to a concentration of up to hundreds of genomic copies per oyster as detected by real-time RT-PCR.**

Noroviruses (NoVs), belonging to the *Norovirus* genus of the *Caliciviridae*, are the predominant agents of nonbacterial gastroenteritis in humans (34). NoVs are small, simple viruses but are highly diverse genetically and antigenically, with five genogroups including 29 genetic clusters (8 in genogroup I [GI], 17 in GII, 2 in GIII, and 1 each in GIV and GV) (14, 36). Infection leads to a brief illness of acute gastroenteritis characterized by vomiting as the predominant symptom (14, 34). During the 2 to 3 days of clinical disease (and probably longer after the disappearance of symptoms), viruses replicate in the intestine, which results in watery diarrhea and the shedding of a large quantity of progeny viruses (14). This, together with the very low infectious dose of NoVs and their high resistance in the environment, explains why more than half of food-borne NoV outbreaks have been associated with food requiring handling but no subsequent heating, with water, or with foods such as raspberries or shellfish (6, 15, 17, 19, 20, 26). In this respect, shellfish are a unique food since they filter large volumes of water for feeding, they grow in coastal areas that may be contaminated by sewage, and they are usually consumed uncooked (8, 18, 31). Therefore, in countries where raw oysters represent a traditional food, a number of food-borne outbreaks of NoV gastroenteritis have been reported (6, 12, 15, 19). However, it is sometimes a challenge in multiple-

strain outbreaks to link cases to the consumption of the food (6, 15, 20).

We describe here an international outbreak linked to oyster consumption analyzed by several approaches. Epidemiological data and clinical samples were collected in the two countries implicated in the outbreak, and in the producing country both oyster samples and environmental data were collected to investigate the possible events that led to the transnational epidemics. The existence of an international collaboration between our laboratories in the framework of the scientific network FoodBorneViruses in Europe (FBVE) was critical in order to rapidly analyze the data and identify an outbreak, since it was not obvious that one food was the source due to the diversity of the NoV strains identified in the different cases. It was also demonstrated that heavy rain was responsible for the accidental contamination of seafood, leading to a concentration of up to hundreds of genomic copies per oyster as detected by real-time reverse transcription-PCR (RT-PCR). We also considered here how food exportation can act as a mechanism for the spread and exchange of viral strains between countries.

### MATERIALS AND METHODS

**Epidemiological data.** In December 2002, the French public health authority received reports of several clusters of acute gastroenteritis from different geographic areas in the country: (i) in the Paris area 58 cases that belonged to 14 households (median age, 44.5 years; range, 3 to 88 years) were reported from December 19 to 24 and (ii) in the region to the east and south several small clusters of cases were reported to local health authorities between 15 December and 15 January.

For all clusters, a standardized questionnaire covering foods consumed, symp-

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toms, and timing of the illness was completed for each participant in the study. All other foods consumed were compared to identify possible sources of contamination, and the origin of the suspected food items was recorded in order to evaluate the potential link between the different clusters. An association between food consumption and illness was estimated by calculating the relative risk and its 95% confidence interval using Epi Info version 6.

At the same time, gastroenteritis cases were reported from a public health unit in Italy or were reported by general practitioners or the local hospital emergency unit. A retrospective cohort study was undertaken to investigate the outbreaks, and a questionnaire was administered via personal or telephone interviews to 124 subjects. Questionnaires were analyzed by using SPSS 11.0 (SPSS, Inc., Chicago, IL). The information collected, such as geographic data, clinical information, and food eaten within the 48 h preceding onset, and knowledge of similar cases was used to generate the epidemic curve (not shown) and to describe the clinical presentation of the disease, using chi-square and Fisher exact test statistics. Attack rates and relative risk and 95% confidence interval values were calculated for the foods eaten. Members or guests of the case families who had not eaten oysters and showed no enteric symptoms were considered as case-controls for analysis.

**Clinical sample analysis.** In France, 12 fecal samples were collected from patients from five different clusters (four located in the same district in the south and one in the east). RNA was extracted from 10 to 25% (wt/vol) stool suspensions in phosphate-buffered saline with a QIAmp viral RNA kit (QIAGEN, Hilden, Germany) (3). NoVs were detected by several RT-PCRs allowing the amplification of regions localized in the RNA polymerase and/or in the capsid gene (4, 18, 32).

In Italy, 42 fecal samples were collected from different clusters. Ten percent stool suspensions (wt/vol) were prepared in sterile water, and 100- $\mu$ l portions of the suspension were extracted by using the Nucleospin RNA II kit (Macherey-Nagel, Düren, Germany). NoVs were detected by RT-PCR using primers from the polymerase region (32) in a one-step format using the Access RT-PCR system (Promega, Madison, WI) or using a previously described RT-booster-PCR method. The results obtained from both RT-PCR and RT-booster-PCR analyses were confirmed by Southern hybridization (11).

**Shellfish sample analysis.** Three oyster samples (*Crassostrea gigas*) associated with cases of gastroenteritis came from the same producing area and were packaged between December 17 and 21. The samples analyzed were leftovers from the three French clusters and were collected directly from private refrigerators. Shellfish, kept at 4°C during shipment, were washed and shucked, and the total weight was recorded. The stomach and digestive diverticula were removed by dissection and divided into 1.5-g portions. For analysis, digestive diverticula were homogenized, extracted with chloroform-butanol, and precipitated with Cat-floc (Calgon, Ellwood City, PA), followed by polyethylene glycol 6000 (Sigma, St. Quentin, France) precipitation (1). Viral nucleic acid was extracted and purified as previously described and then suspended in 100  $\mu$ l of RNase-free H<sub>2</sub>O (18).

For NoV detection, six primer sets located in the polymerase gene (18, 32, 35) and three primer sets in the capsid gene (13, 16) were used. RT-PCRs were performed in a two-step format (Applied Biosystems), and PCR products were confirmed by a dot blot hybridization with 10 chemiluminescent probes (Roche, Meylan, France) (13, 18). Samples were considered to contain NoVs only if the amplicons were detected by hybridization using NoV-specific probes. In some cases, a seminested PCR was performed under the same amplification conditions, and all precautions to avoid cross contamination (each sample was amplified alone and with negative controls) were taken.

To estimate the number of RNA copies present in oyster extracts, real-time RT-PCR was performed using primer sets and probes located at the 3' end of ORF2 (15, 22). The  $C_T$  value was compared to a standard curve to estimate the number of genome copies.

**Sequence analysis.** Positive samples were purified and sequenced with a Big-Dye terminator v3.1 cycle sequencing kit (Applied Biosystems). Sequences were analyzed through the FBVE database (<https://hypocrates.rivm.nl/bnwww>; FBVE QLK1-CT-1999-00594) to identify the virus genotype. The sequence homologies obtained from all samples were evaluated by using the BLAST search program.

**Environmental investigations.** Oysters were produced in several sites, all of which were located in a lagoon in southern France. The data for climate events (Meteo France), sewage treatment plant efficiency, and epidemiological status of the population (Reseau Sentinelle; <http://rhone.b3e.jussieu.fr/senti/>) were obtained. At the same time, the data from shellfish producers or the Ifremer surveillance network (REMI; <http://www.ifremer.fr>) for shellfish quality were collected.

## RESULTS

**Epidemiological investigation.** In France, 13 clusters of cases with acute gastroenteritis could be related to oyster consumption (9 in southern France, 2 in the southwest, 1 in the east, and 1 in Paris) between 15 December and 15 January. A total of 127 cases were identified; 47 ill persons had consumed oysters collected from site A, 5 had consumed oysters collected from site B, 72 had consumed oysters collected from site C, and the oyster collection sites could not be identified precisely for 3 cases but were still from the same lagoon.

The biggest cluster of cases was in Paris and was related to oysters collected from site C. Fifty-eight persons (29 male and 29 female) from 14 households, with a median age of 44.5 years (range, 3 to 88 years), participated in meals in which oysters were served between 19 and 24 December. All subjects answered the questionnaire. Thirty-six persons had eaten oysters, and twenty-one of them from 11 households became ill (an attack rate of 58%); 22 persons did not eat oysters, and none of these subjects was ill. The risk of being ill was significantly higher for oyster consumers ( $P = 2 \times 10^{-5}$ ). Other than oysters, no other food was common to the 14 households. Illness generally began 4 to 58 h after oyster consumption (mean incubation, 34 h). Abdominal pain was reported in 76% of cases, diarrhea in 76%, nausea in 62%, vomiting in 43%, and fever in 9.5%. The mean duration of illness was 36 h (range, 24 to 72). The 12 other clusters included 106 cases. Diarrhea was reported in 78% of cases, and vomiting was reported in 64% of cases. The mean incubation time was 34 h (range, 1.5 to 68 h).

In Italy, 202 subjects with acute gastroenteritis were reported to the public health service between 25 and 27 December. The retrospective cohort study showed that all cases had eaten raw oysters for dinner (24 December) or lunch (25 December) either at a restaurant or at home. A strong association ( $z = 2.88$ ) was found between oyster consumption and disease and not with other food items. The mean incubation time was 36 h (range, 24 to 48 h). The symptoms were vomiting (100%) with diarrhea (92%), whereas fever was rare. Two patients were admitted to the hospital and were dismissed within 24 h. The mean age of patients was 44 years (range, 6 to 68 years). Attack rates within families ranged between 92 and 100%.

**Stool sample results.** Of the 12 French stool samples analyzed, 7 were determined to be positive for NoVs, and 6 of these could be typed. Bristol-like genotype (GII-4) viruses were detected in four stool samples, the European-variant GII-b genotype viruses were detected in two stool samples, and Chiba-like genotype (GI-4) viruses were detected in one stool sample. One stool contained two strains (a Bristol and a Chiba strain). Of 41 stool samples from symptomatic patients in Italy, 22 were found to be positive for NoV, and 20 of these could be sequenced. Seven sequences were belonged to the Bristol-like genotype (GII-4), nine belonged to the Amsterdam-like genotype (GII-8), three belonged to the Sindlesham-like genotype (GI-6), and one belonged to the Chiba genotype (GI-4) (Table 1).

**Shellfish results.** The three oyster samples analyzed were NoV-positive; however, one sample yielded amplicons too weak for confirmation by sequencing. Several sequences were obtained from the two other samples (Table 1).

Using different combinations of primer sets either in the

TABLE 1. Norovirus-specific nucleotide sequences obtained from clinical and shellfish samples

Sample	No. of sequences obtained	No. of sequences with genotype (% sequence identity) <sup>a</sup> :				
		GI-4 (98.55)	GI-6	GII-4 (98.55)	GII-8 (98.59)	GII-b <sup>b</sup>
Italian patient stools	20	1	3	7	9	
French patient stools	6	2		6		2
Oysters	4	2		1 <sup>c</sup>	1, 1 <sup>d</sup>	

<sup>a</sup> The sequence identity is given as a percentage in parentheses as determined by a BLAST search program.

<sup>b</sup> New variant GIIb.

<sup>c</sup> Sequence obtained after seminested PCR.

<sup>d</sup> Sequence obtained using capsid primers.

polymerase or in the capsid coding region, a Chiba-like genotype (GI-4) sequence was detected in both samples, and an Amsterdam-like genotype (GII-8) was detected in one sample. After a seminested PCR, a Bristol-like genotype (GII-4) sequence could also be detected in one sample. The same sample was also determined to be positive by amplification in the capsid region. After sequencing, 99% identity was observed with the strain Saitama U25 (genotype GII-8). Despite several attempts, this strain could not be amplified using primers targeting the polymerase region.

Using real-time PCR, a  $C_T$  value of 33 was obtained with the genogroup II primer set and probe. Taking into account the standard curve and the volume of nucleic acid used for the amplification, about 50 to 250 RNA copies of genogroup II RNA were present in the 1.5 g of digestive tissues, corresponding to about two oysters. Thus, the amount of genogroup II virus was estimated to be about 25 to 125 RNA copies per oyster. No  $C_T$  value was obtained with the genogroup I primer set and probe, showing either a lower concentration or a lower detection sensitivity.

**Environmental data.** All of the oysters consumed either in France or in Italy were produced from the same harvesting area in the south of France. For some French case clusters, the exact point of production could be traced to three distinct places in the lagoon and for the oysters consumed in Italy it was just noted that it was a mixture of two producers from the same French pond. Figure 1 reports the different events observed during the December month in this area. Heavy rain and wind had been reported in this geographic area between 9

and 12 December. Up to 150 mm of water rain had been monitored (Meteo France data), and the ground was already water saturated, resulting in water runoff and river overflowing. Sewage treatment failures were noticed on 10 and 11 December, with an increase in *Escherichia coli* counts in treated sewage (up to  $4 \times 10^6$  CFU/100 ml). The IFREMER surveillance network (REMI) detected bacterial contamination in shellfish: five samples of nine analyzed were contaminated by more than 230 *E. coli* organisms/100 g (the concentrations were about 1,000 to 1,200 *E. coli* organisms/100 g of shellfish meat for four points) on 16 December, and three of nine samples analyzed on 19 December were contaminated by more than 230 *E. coli* organisms/100 g (the concentrations were 550, 750, and 900 *E. coli* organisms/100g of shellfish meat, respectively). Producers in this area were asked to subject shellfish to depuration for 2 days before marketing, and all shellfish put onto the market was in accordance with European regulation. For example, samples sent to Italy contained 120 *E. coli* organisms/100 g for one producer (analysis made on 17 December) and 180 *E. coli* organisms/100 g for the other one (analysis made on 20 December). On 29 December, all concentrations were below 230 *E. coli*/100 g of shellfish meat.

## DISCUSSION

Shellfish are well recognized to be a source of NoV infections, and sensitive methods have been described for characterizing viral contamination in shellfish. The large outbreak described here involving two European countries could be related to one oyster production area, and the causal link between oyster consumption and illness in patients was demonstrated by both epidemiological investigation and virologic analysis. Moreover, these data were also combined with environmental investigations and with the use of real-time RT-PCR to assess the viral concentration in oyster samples.

The clustering of cases both in France and in Italy was compatible with a common source of infection, although different families were exposed to the contaminated food in distinct places. However, based on clinical data it was confusing to find such a diversity of strains (up to five different NoV strains). The involvement of four distinct strains of NoV was misleading in addressing the cause of infection in the Italian outbreak and did not promptly support the involvement of a food-borne norovirus, although oysters had clearly emerged as the vehicle. In fact, NoVs are widely spread through the population during winter months, and the occurrence of distinct

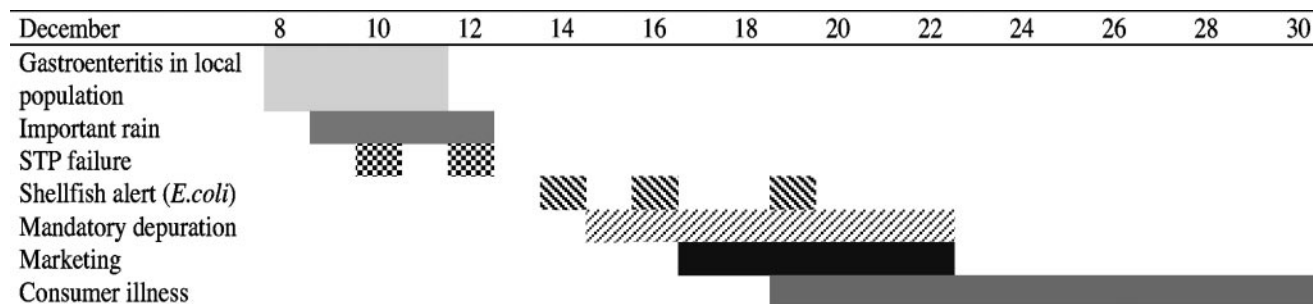


FIG. 1. Schematic representation of environmental events that lead to oyster contamination and outbreaks.

viral strains might also be interpreted as an NoV superinfection of only some of the patients otherwise linked to all others by a different (undetected) enteric pathogen transmitted through oysters or a common vector other than shellfish. The comparison of genotyping and sequencing data from France and Italy was indeed critical to resolve any doubt about the actual occurrence of an international food-borne NoV epidemic, particularly because relatively rare NoV types (i.e., Amsterdam GII-8) were also found to be involved. The results obtained from shellfish samples confirm that a multiple-NoV contamination was present in the oysters and that some strains were identical to the ones detected in French and/or Italian consumers. Multiple-NoV contamination of food is often suspected to be linked to sewage contamination, and shellfish have been shown to be one of the most common vehicles of outbreaks involving multiple norovirus strains (6, 15, 19, 31). No other enteric viruses or bacteria were detected in stools or shellfish samples, and oysters met the European regulation stipulating fewer than 230 *E. coli* organisms per 100 g of shellfish meat. However, controls performed a few days earlier suggested that some fecal contamination had indeed occurred, as demonstrated by high levels of *E. coli* in shellfish. Environmental investigations showed that important rain with storm and wind occurred 2 weeks previously, with river overflowing and sewage treatment plant failure. At the same time, gastroenteritis outbreaks in the population were already important in this area of France with a consequent relevant amount of viral particles expected to be present in sewage (29). According to European community regulation, advice was given to oyster producers to reinforce control and subject oysters to depuration. The detection of NoV alone in shellfish meat is compatible with a point contamination due to a sporadic event rather than being related to continuous sewage exposure, as previously detected in class B areas where multiple infectious agents can be present at any time (18). Understanding shellfish contamination is important for preventing these types of accidents. The regulations are based on *E. coli* counts, and the failure of bacterial indicators to identify virus contamination in shellfish-associated outbreaks has been noted numerous times previously (5, 8, 19). However, in the present case a punctual bacterial contamination was identified one week before the outbreaks, showing that regular monitoring may be able to identify problems. Nonetheless, it must be kept in mind that whereas *E. coli* may disappear rather quickly either by depuration practices or by natural cleansing, recent data show that viral depuration is difficult and, thus, 2-day depurations as stipulated by the European Community regulations is not adequate to eliminate viral contamination (9, 23, 28, 30). Virus particles may persist for months in shellfish tissues either via ionic binding or specific attachment (7, 21). Flooding has previously been shown to be responsible for virus contamination in other outbreaks and is congruent with the abrupt introduction of multiple NoV strains into the oyster breeding site (25, 27). The data presented here demonstrate that it is important to manage and avoid the risk of fecal discharges into harvesting areas. Setup warning systems could prevent the introduction of contaminated shellfish into the market (29).

Noroviruses are very variable viruses, and no single primer set is able to amplify all strains (2, 33). This is of primary importance for shellfish analysis since sensitivity and specificity

are the only conditions allowing the detection of multiple contaminations. Due to the low concentration and persistence of some inhibitors in food, the use of several primer sets is essential (6, 18, 19, 20), and in some cases methods based on the use of a double-round PCR are needed (10). Using real-time RT-PCR, each oyster was estimated to be contaminated by about 100 copies of the NoV genome. In a previous outbreak, using most-probable-number RT-PCR, we found approximately the same amount of virus per oyster (19). These data are not an exact quantification of virus since the efficiency of the extraction step was not taken into account, but this approach will help risk analysis and is also important for designing reference methods that need to be sensitive enough to detect such low levels of contamination. Since NoV cannot yet be grown in cell culture, semiquantitative data are very important for interpreting molecular detection, and this approach in conjunction with volunteer studies will be helpful to set up viral regulation methods to monitor shellfish or other food.

International outbreaks linked to shellfish consumption have been described for hepatitis A virus (5), but the present study is one of the first to show that oysters are clearly a vehicle for NoV strain transmission into another country. Food was suspected to be the mode of introduction of a novel NoV variant strain into several European countries (24), but no direct evidence could be found. Laboratory networks such as the FBVE are essential in order to get and circulate information rapidly and to share data useful to track the international spread of viruses, especially via food.

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