The lack of virus control in oysters could lead to a norovirus outbreak

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Abstract. Norovirus, a genus in the family *Caliciviridae*, is a leading cause of viral gastroenteritis in humans and responsible for many outbreaks worldwide. Filter-feeding shellfish are important vehicles for transmission of foodborne pathogens, including enteric viruses such as norovirus, when grown in sewage-polluted water. In this study, we investigated a norovirus outbreak linked with consumption of oysters and mussels. In January 2019, a gastroenteritis outbreak was identified involving eight patients with symptoms of gastroenteritis. Norovirus was diagnosed in stool samples using immunochromatographic test RIDA® QUICK Norovirus, and confirmed with real-time PCR. Of four shellfish (oysters, mussels) samples analyzed using real-time PCR, three were norovirus GI-positive and GII-positive, while one sample was only GII-positive. Six stool samples were collected, two of which were norovirus GI-positive and GII-positive, while three were GII-positive only. Following phylogenetic characterization of the human stool viruses, five out of eight belonged to the GII.6 cluster. Shellfish collected during this outbreak investigation contained the same GII.6 sequence. This is the first norovirus outbreak connected with shellfish consumption in Croatia. Understanding the transmission routes and vehicles of norovirus outbreaks is of great public health importance, and these results imply the co-circulation of GII.6 norovirus in people and oysters in Croatia.

1. Introduction

Foodborne viruses are an important and emerging problem for food safety and public health, according to a report by EFSA [9]. In 2014, viruses were, for the first time, the most commonly detected (20.4%) causative agent in foodborne outbreaks. Since the discovery and identification of Norwalk virus in 1972 [16], human noroviruses have been identified as the leading cause of acute viral gastroenteritis worldwide [25]. This genetically diverse group of viruses forms a separate genus in the family *Caliciviridae*, and the noroviruses cluster phylogenetically into at least seven genogroups with more than 30 genotypes located within three human-occurring genogroups (I, II, and IV) [47]. GI and GII viruses are responsible for the majority of disease in humans, whereas GIV viruses are rarely
detected as the cause of epidemic or sporadic disease [2, 45]. It has been estimated that norovirus infections cause 1 million hospitalizations and 200,000 deaths in children under 5 years of age in the developing world [31].

The association between shellfish and viral gastroenteritis has been recognized for a long time [27]. Filter-feeding shellfish, when grown in sewage-polluted water, are important vehicles for transmission of foodborne pathogens, including enteric viruses such as norovirus and hepatitis A virus [23]. Numerous shellfish-associated outbreaks have been attributed to enteric viruses, particularly norovirus [39, 18, 34, 13]. The risks related to consumption of shellfish are greater when these products are eaten raw or lightly cooked, as in some European countries, including the Croatia. European Regulation 2073/2005, and subsequent amendments, defines food safety criteria of shellfish only on the basis of bacterial indicators (e.g., Salmonella and Escherichia coli), which may not be correlated with the presence of viruses [7, 18].

Consequently, foodborne viruses are recognized among the top food safety priorities in a recent report by risk assessment experts who applied the Delphi technique [36]. Thus, over the past few years, foodborne viruses have become a greater concern to both the food industry and regulatory bodies [4]. In countries where rotavirus vaccines are implemented, norovirus has surpassed rotaviruses as the most common cause of childhood gastroenteritis requiring medical attention [32].

Gathering information on virus contamination in shellfish has, therefore, become increasingly important in countries with shellfish production. In 2017, Croatia produced 920 tonnes of Mytilus galloprovincialis and 62 tonnes of Ostrea edulis, according the Croatian competent authority [14].

2. Materials and methods

Epidemiological data: Information concerning sick oyster consumers was provided by medical doctors, who diagnosed norovirus gastroenteritis and informed the veterinary inspection services. When a foodborne illness is suspected, a standardized questionnaire concerning all consumed food is completed by the people who shared the meal. Following the implication of oysters in the outbreak, veterinary inspectors collected two oyster (Ostrea edulis) and two mussel (Mytilus galloprovincialis) samples from two producers that were directly linked to the human illness cases.

2.1. Environmental Data and Sampling

The shellfish growing area is in Maloston Bay, located between the southeast-northwest oriented coast and Pelješac Peninsula. The majority of oyster production in that area is consumed locally, so the main source of contamination is not identified. According to European regulation 54/2004/EC, the production area is classified as a Category A area. Oysters were collected from two producers at two production sites, of which one site is regularly surveilled for Escherichia coli levels. That site is considered to be representative of the microbiological contamination for that part of production area. Each shellfish sample comprised at least 15 individual oysters or mussels. On the first sampling day (1 February, 2019), four samples were collected and on the second sampling (20 February, 2019), ten samples were collected. All shellfish samples were transported in refrigerated boxes and were subjected to viral analysis for determination of norovirus GI and GII using the molecular method described by international standard EN ISO 15216-1:2017 (International Organization for Standardization).

2.2. Shellfish processing

All the shellfish were shucked, weighed, and their digestive tissues (DTs) dissected and homogenized. For analysis, Mengovirus (MgV) was added to each DT sample (2 g), before incubation with 2 ml of proteinase K solution (EN ISO 15216-1 2017).

2.3. Stool processing

Stool samples from patients with viral diarrhea were collected and transported to the laboratory for testing. RNA in the stools was extracted from 500 µL of a 10% stool suspension in phosphate buffered
saline (PBS, pH 7.4). One human stool sample was tested using a commercial immunochromatographic test RIDA® QUICK Norovirus (31 January, 2019).

2.4. Nucleic Acid (NA) Extraction and Purification
Amounts of 2.0 g DT, spiked with 10 µl of process control MgV, were digested with 2 ml of proteinase K (0.1 mg/ml) at 37°C for 60 min with shaking, and then placed at 60°C for 15 min to inactivate the enzyme. Finally, the samples were centrifuged at 3000g for 5 min, and the supernatant was collected. Volumes (100 µl) of each stool sample was suspended in 1 ml of phosphate buffered saline (PBS, pH 7.4) and spiked with 10 µl of MgV. Nucleic acid extraction and purification were performed using the Nuclisens extraction kit (BioMerieux, Paris, France) according to the manufacturer’s instructions, and the eluted RNA (100 µl) was stored at -80°C until real-time PCR (RT-PCR) analysis.

2.5. RT-PCR and genotyping
RT-PCR for norovirus detection was carried out on a Mastercycler EP realplex (Eppendorf, Germany) using amplification conditions, primers and probes reported in EN ISO 15216-1:2017. RT-PCR reagents QuantiTect Virus + ROX Vial kit (Qiagen, Germany) were prepared according to the manufacturer’s instructions. The presence of PCR inhibitors was evaluated by testing samples along with an external control RNA (EC-RNA of target sequence) and amplification efficiency.

All GII norovirus-positive samples detected by RT-PCR were further amplified by conventional nested RT-PCR, using the primer pairs COG2F/G2SKR and G2SKF/G2SKR that targets the partial capsid region C [15, 17]. The PCR amplicons were purified and subjected to direct sequencing on both strands (Macrogen). Typing of norovirus sequences was performed using the Norovirus Automated Genotyping Tool (http://www.rivm.nl/mpf/norovirus/typingtool). The phylogenetic analysis of aligned partial capsid sequences (300 nt) was carried out using MEGA 7. The reliability of the phylogenetic tree was assessed by bootstrap sampling of 1,000 replicates, and genetic distances were calculated by Tamura-3 parameter method.

3. Results
In January 2019, a gastroenteritis outbreak was identified and linked with consumption of oysters and mussels sourced from the Maloston Bay production area. Eight patients presented with symptoms of gastroenteritis (vomiting and/or diarrhea), and one patient was diagnosed as having norovirus by a medical doctor using immunochromatographic test RIDA® QUICK Norovirus on 31 January. The epidemiological investigation implicated oysters and mussels from two producers. Four shellfish samples were collected on 1 February and tested using RT-PCR. Three shellfish samples were positive to both norovirus GI and GII, and one shellfish sample was GII-positive. Stool samples from six patients were collected on 12 February, two samples of which were positive to both norovirus GI and GII, and three samples of which were GII-positive. Norovirus was not detected in one stool sample. On 20 February, ten more shellfish samples were tested, four of which were GII-positive (Table 1). The production area was closed until the confirmation of negative RT-PCR results on 5 March, 2019.

| Samples     | Date      | Quantity | Norovirus GI+ | Norovirus GII+ | Norovirus GI and GII+ | Genotyped as GII.6 |
|-------------|-----------|----------|---------------|----------------|-----------------------|---------------------|
| Oysters     | 01 February | 2        | 2             | 2              | 2                     | 1                   |
| Mussels     | 01 February | 2        | 1             | 2              | 1                     | 1                   |
| Stool       | 12 February| 6        | 2             | 5              | 2                     | 5                   |
| Oysters     | 20 February| 5        | 0             | 1              | 0                     | 0                   |
| Mussels     | 20 February| 5        | 0             | 3              | 0                     | 3                   |
| Oysters     | 05 March  | 5        | 0             | 0              | 0                     | 0                   |
Of the 11 GII-positive samples further amplified by nested RT-PCR, 10 were characterized by direct sequencing of the amplicons, identifying a single norovirus genotype, GII.6 (Figure 1). Phylogenetic analysis confirmed clustering of our human and shellfish sequences into a single cluster.

![Phylogenetic tree](image.png)

**Figure 1.** Neighbour-joining phylogenetic tree based on partial capsid viral protein 1 norovirus sequences. The GII.6 genotype is indicated with the square.

### 4. Discussion

The current European legislation classifies molluscan shellfish harvesting areas into A, B or C categories. Regulations based on measurement of the levels of *E. coli* in shellfish tissues (European regulation 54/2004/EC) or fecal coliforms in shellfish-growing waters (United States National Shellfish Sanitation Program) have been instituted to protect consumers. Viral contamination decreases very slowly within shellfish tissues, and although the use of depuration tanks is efficient in the case of bacterial contamination, it has little utility in addressing viral contamination [20,24,29,22].

In this study, we investigated a norovirus outbreak linked with consumption of oysters and mussels. One of the production sites implicated in the outbreak is considered to be representative for microbiological contamination, and the Maloston Bay production area is classified as A category.
Sanitary controls in Croatia are based on *Escherichia coli* counts in shellfish flesh and liquor as an indicator of fecal (sewage) contamination in shellfish [1], as in many other countries. However, this approach has been repeatedly demonstrated to inadequately indicate the risk from human enteric viruses [5,12]. In our case, patients who had consumed A category oysters and mussels developed gastroenteritis due to viral contamination. In one patient, norovirus was diagnosed using the immunochromatographic test, RIDA® QUICK. The outbreak investigation confirmed (using RT-PCR) the presence of norovirus GI and GII in oysters and mussels from two producers implicated in outbreak.

Human stool samples collected 12 days after the onset of symptoms tested norovirus GI-positive and/or GII-positive, using RT-PCR. According to Plantenga et al [33], norovirus can be readily confirmed as the cause of an outbreak of acute gastroenteritis if specimens are collected at any time during the first seven days after onset of diarrhea and (although the period is somewhat variable) for almost that long after symptoms resolve. No apparent decline in sensitivity occurs for specimens collected up to 6 days after onset of diarrhea. Sensitivity drops during days 7-14, but remains substantial for up to 2 weeks [33]. In our case, we positively detected norovirus in ill patients’ stools collected on day 12, but probably with higher Ct values than would have been measured if we collected samples during gastroenteritis.

Genotyping of PCR-amplified partial capsid sequences obtained from human stools and from shellfish revealed a single norovirus genotype, GII.6. Phylogenetic analysis of shellfish and stool sequences confirmed the same epidemiological strain of GII.6 and provided a proven link of the outbreak with consumption of oysters and mussels. Sequences are difficult to obtain from oyster samples with low levels of norovirus [22] such as those implicated in this outbreak, and stools were collected only 12 days after the symptoms, not earlier. Despite these difficulties, we were still able to obtain partial capsid sequences. For many years, oysters have been known as concentrators of virus particles. Among shellfish, oysters are the most common vector of foodborne illness, and the pathogens most frequently involved in these outbreaks are noroviruses, responsible for acute gastroenteritis in humans [21]. The patients involved in this outbreak eat raw oysters and lightly cooked mussels. Consumption of either raw or undercooked shellfish can lead to transmission of disease, as human pathogens can be accumulated during the shellfishes’ filter-feeding activity [38].

Several studies have examined the duration of norovirus excretion and found the average period of shedding is ≈28 days (range 13-56 days), well after the resolution of symptoms [44,3]. However, viral infectivity cannot be inferred from these findings. Considering the number of outbreaks linked to oyster consumption that have occurred in France, the French competent authority has developed a protocol that results in the growing area being closed for at least 28 days following an outbreak [22]. In our case, negative results were obtained on the 33rd day from the start of the outbreak, a temporal frame that is similar to the French experience. Consequently, the production area was closed for 33 days, since we did not have information about the status of the shellfish on the 28th day.

Overall, contamination by multiple norovirus strains has been reported in 65% of reported outbreaks, with GI and GII noroviruses detected, respectively, in 71% and 88% of stool samples and in 75% and 92% of shellfish samples [21]. In the current study, we also detected noroviruses GI and GII co-existing in some of our shellfish samples.

Shellfish are a high-risk food for viral outbreaks, but clear strain identification in shellfish is still often difficult [21], since obtaining a useful sequence from positive RT-PCR foods is problematic [37]. Specific binding of Norwalk virus in *Crassostrea gigas* oysters via a carbohydrate structure very similar to human histo-blood group A antigen was demonstrated and subsequently confirmed to occur in another oyster species (*Crassostrea virginica*) [43]. A simple depuration process should be sufficient for virus removal from oysters, as observed for bacteria [36]. However, long-term virus persistence in shellfish is a serious public health issue, and depuration or relaying is known to be inefficient [28,35,24]. Ligands that facilitate bioaccumulation (the A-like antigen) or that contribute to the elimination of the virus (the sialic acid-containing ligand) could both influence norovirus accumulation and survival in oysters [21].
One characteristic of shellfish-related outbreaks is their frequent association with multiple virus strains observed both in infected patients and in the involved shellfish [21]. In this study, we obtained one sequence of GII.6 in shellfish and stool samples. During the 2014 summer season in Croatia, a norovirus strain genetically related to Hu/GII.4/sydney/NSW05 in human samples, and simultaneously, GII.4 in shellfish originating from Croatian production areas were detected [40]. In humans, the genetic diversity of noroviruses is reflected in their binding capacity to various HBGA structures [42], and such differences also occur in oyster tissues [43,26]. When a number of different virus strains are detected in patients, association of the infection with shellfish consumption can be difficult if only a few stools from an outbreak are collected. Thus, it is essential to collect as many stool samples as possible from affected individuals, so that all strains that are present can be identified. It is also important to rapidly identify the outbreak in order to trace the oyster production and to quickly collect suitable samples related to the outbreak. These data can be used with collected epidemiological data to fully understand the role played by shellfish in the outbreak.

To assess risks associated with viruses and other hazards in the food chain and put in place appropriate control measures, the use of risk assessment techniques has been suggested by international bodies [6,46] and increasingly accepted by governments around the world as a basis for national legislation in relation to food safety [11,8]. Implementing raw material/food production controls (oysters, berries, leafy greens) e.g. harvesting oysters and other shellfish from non-contaminated areas, establishing an acceptable limit for norovirus in oysters to be harvested and placed on the market, and testing of products for compliance to this acceptable limit [9] are examples of these controls. The periodic emergence of viral outbreaks associated with shellfish consumption continues to pose a real public health concern. Although outbreaks related to consumption of shellfish in Croatia have not been previously reported, it is well known that human sewage is a possible source of shellfish contamination. This is the first reported case of a norovirus outbreak related to shellfish consumption in Croatia. Understanding the transmission routes and vehicles of infection for norovirus outbreaks is of great public health importance, and suitable control measures for viruses should be implemented, especially for oysters since these are eaten raw.

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