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Possibilities of using mussels (*Mytilus galloprovincialis*) to predict rotavirus contamination in Albania

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Abstract

Introduction: Rotaviruses are non-enveloped viruses that each consist of 11 double-stranded RNA molecules. These viruses are able to persist in the environment, and therefore play a fundamental role in the epidemiology of gastroenteritis and severe diarrhoea in children worldwide. While mussels have been primarily used as indicators of chemical pollution, they can also be used to monitor viral contamination. The purpose of this study was to demonstrate that the *Mytilus galloprovincialis* mussel can also be used to detect microbial contamination, owing to its tendency to naturally concentrate viruses and other pathogens. **Material and Methods:** A total of 102 *Mytilus galloprovincialis* mussel samples from Albania were collected over a three-year period: 37 samples off the Cape of Stillo in 2015, 39 samples from Butrinti Lake in 2019 and 26 samples from Butrinti Lake in 2021. **Results:** The presence of rotavirus in the Cape of Stillo samples in 2015 was noted in 47% of samples from site 1, 33% from site 2, and 52% from site 3. In Butrinti Lake the percentage of infected individuals in 2019 was 33% from site 1, 41% from site 2, and 33% from site 3, whereas in 2021, it was 50% from site 1, 19% from site 2, and 0% from site 3. In total the percentage of infected individuals off the Cape of Stillo in 2015 was 44%, in Butrinti Lake in 2019 it was 36%, and in Butrinti Lake in 2021 it was 23 %. **Conclusion:** These results indicate the presence of rotavirus in the shellfish specimens tested, and further analysis is needed to assess the potential health risks associated with consuming these shellfish. This study also indicates that mussels can be used in marine virological surveillance programmes.

Keywords: rotaviruses, *Mytilus galloprovincialis*, shellfish, Butrinti Lake, Cape of Stillo.

Introduction

Rotavirus is a highly contagious virus that can spread easily through contaminated food and water. It is a major cause of severe diarrhoea in children worldwide, as rotaviruses are a major cause of acute gastroenteritis and can lead to dehydration, malnutrition, and even death. The virus is able to persist in the environment because of its stability and resistance to disinfection, making it a persistent threat to public health (15). Rotavirus is a double-stranded RNA virus that is non-enveloped, consists of 11 double-stranded RNA molecules, and belongs to the *Reoviridae* family. The virus has three layers: the central core, inner capsid, and outer capsid.

The outer capsid contains two proteins, VP7 and VP4, which are used to classify rotavirus strains into different serotypes (8). These proteins are also targeted by neutralising antibodies produced by the immune system following a natural infection. Group A rotaviruses are responsible for most cases of severe disease and deaths, particularly in low-income countries with poor sanitation and limited access to healthcare (5). Vaccination is an effective strategy to protect against rotavirus and other enteric viruses, especially in areas where the disease is endemic. Rotavirus vaccines have been shown to be highly effective in preventing severe rotavirus disease and have been approved by the WHO for global use. RotaTeq, a pentavalent vaccine, and Rotarix,

a monovalent vaccine, are currently the two vaccines that are widely used.

The widespread use of these vaccines has led to a significant reduction in the burden of rotavirus disease globally (2, 29). Nevertheless, previous studies have demonstrated the presence of rotavirus in mussels from various locations. Since shellfish are widely consumed all over the world, this raises concerns about the potential for human exposure to this and other pathogens (20). Shellfish, particularly mussels, are a common source of foodborne illnesses caused by viral pathogens, including rotavirus. Mussels are particularly resistant to chemical and bacteriological pollution and can accumulate xenobiotics in their tissues in proportion to the environmental concentration. They can also trap and accumulate bacteria and viruses present in seawater and act as passive carriers of human pathogens. Mussels use their ciliated gill epithelia and mucous membranes to filter and sieve food particles from the water (10, 24, 28). This ability to filter water and concentrate pathogens makes mussels a useful bioindicator for detecting microbial contamination in seawater (24, 27). While mussels have been primarily used as indicators of chemical pollution, they can also be used to monitor viral contamination. Regular testing of water and shellfish can help to prevent outbreaks of foodborne illness and ensure the safety of seafood for human consumption. Typically, the concentration of viruses in seawater is very low, which makes their detection challenging and requires the concentration of large volumes of water (4, 18, 28). Overall, the prevention and control of viral contamination of food and water requires a multi-faceted approach, including proper waste water treatment, improved hygiene practices, and vaccination programmes. By implementing these measures, we can reduce the incidence of viral infections and protect public health.

The study aimed to investigate the presence of rotavirus in *Mytilus galloprovincialis* mussels, the most common bivalve species in the Mediterranean Sea, Black Sea, and eastern Atlantic Ocean and one able to survive in a range of temperatures and salinities (11). The species is used for aquaculture, and sites of this activity in Albania include Butrinti Lake in Saranda, which was a sampling location. Wild populations of *Mytilus galloprovincialis* were sampled from the sea off the Cape of Stillo, the southernmost point of Albania. The purpose of the study was to demonstrate that *Mytilus galloprovincialis* can be used to detect microbial contamination in seawater. Polymerase chain reaction results obtained from analysing the *Mytilus galloprovincialis* species in Butrinti Lake and off the Cape of Stillo in 2015, 2019, and 2021 were desirable to collect, in order to provide valuable information about the presence and prevalence of enteric viruses in these study areas.

Mytilus galloprovincialis was chosen as the test organism because it is one of the most widely consumed shellfish worldwide and can adapt easily to various environmental conditions, making it a suitable allochthonous organism (12). Prevalence data for rotavirus in this species may highlight the need for continued monitoring and surveillance of shellfish as potential sources of viral contamination.

Material and Methods

Sample collection. Butrinti Lake, one of the study areas, is 16.3 km^2 in size, has been intensively used for aquaculture of mussels (*Mytilus galloprovincialis*) and has had approximately 80 mussel cultivation facilities over the last few decades, with an annual gross production of 2,000 to 4,500 tons (9). However, due to restrictive export rules to Europe, the production rate has decreased to approximately 1,500 tons per year. The other study area was the sea off the Cape of Stillo, which has the same name as the island of Stillo and is located near the border with Greece. The Cape of Stillo is part of the strict marine and terrestrial nature reserve of Cape and Pagane-Stillo Island (IUCN Category I), has a surface area of 5 km^2, and is characterised by a Mediterranean climate with dry summers and mild winters (28). Specimens were collected from different sampling sites at Butrinti Lake and off the Cape of Stillo (Fig. 1 and Table 1) in June, August, and September and transported in a portable freezer to the laboratory of Environmental Physiology at the Department of Biology of the University of Padua. A total of 102 *Mytilus galloprovincialis* mussel samples were collected over the three-year period: approximately 37 samples off the Cape of Stillo in 2015 (3), 39 samples in Butrinti Lake in 2019, and 26 samples in Butrinti Lake in 2021.

Rotavirus detection methods. Reverse transcription polymerase chain reaction (RT-PCR) was used to detect enteric viruses in the *Mytilus galloprovincialis* mussel samples (7), because it is a successful method for detecting viruses in shellfish (21). The mussels, collected from each sampling location in spring and autumn of each year, had lengths ranging between 50 and 80 mm in spring and 40–70 mm in autumn. In addition to being measured, the mussels were washed, scrubbed under clean running water, and opened with a sterile shucking knife. From these samples, individuals with lengths between 50 and 70 mm were selected for rotavirus analysis from all sampling sites, as shown in Fig. 1.

Table 1. Coordinates of the investigated sampling sites

	Off the Cape of Stillo	Butrinti Lake
Sampling 2015	39°41'19.7"N	39°45'09.3"N
	19°59'28.1"E	20°01'35.3"E
Sampling 2019	39°41'16.6"N	39°45'22.6"N
	19°59'05.8"E	20°01'53.6"E
Sampling 2021	39°41'26.5"N	39°45'18.1"N
	19°59'49.4"E	20°02'22.6"E

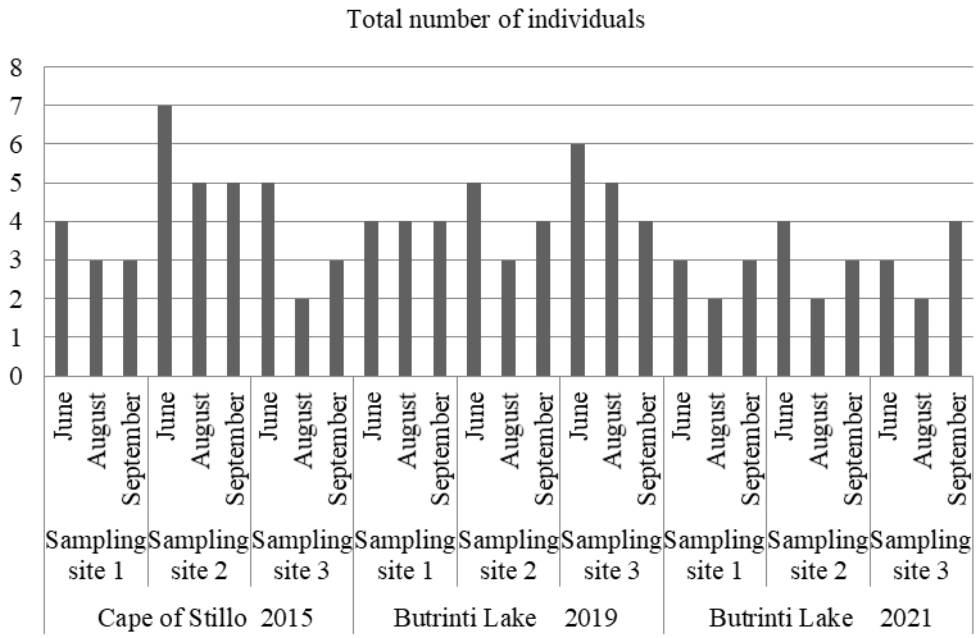

Fig. 1. Total numbers of collected *Mytilus galloprovincialis* mussels from Butrinti Lake and off the Cape of Stillo

The digestive glands of these selected individuals were removed, frozen in liquid nitrogen, and stored at −80°C until they were tested by molecular biology techniques at the Laboratory of Environmental Physiology at the Department of Biology of the University of Padua and at the Laboratory of Wildlife Diseases at the Faculty of Veterinary Medicine, of the Agricultural University of Tirana.

The methodology used to concentrate viruses and purify viral RNA from digestive gland tissue was adopted from a previously published paper (23). The tissues were homogenised in MilliQ water supplemented with Proteinase K (Sigma-Aldrich, St. Louis, MO, USA), and the resulting homogenate was incubated at 37°C for 1 h. After Proteinase K was inactivated at 65°C for 15 min and centrifuged at 3,000 rpm for 5 min, the supernatant was used for viral RNA purification and was purified using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and 8 M LiCl to remove glucidic contaminants, according to the manufacturer's protocol (14). The purified RNA was then quantified using a spectrophotometer and assessed for integrity by capillary electrophoresis. An ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) was used for quantification, and an Agilent 2100 Bioanalyzer was used for capillary electrophoresis with an RNA 6000 Nano kit (Agilent Technologies, Palo Alto, CA, USA). The first strand of cDNA was reverse transcribed at 42°C for 1 h from 1 µg of total RNA in a 20 µL reaction mixture containing 1 µL of ImProm-II Reverse Transcriptase (Promega, Madison, WI, USA) and 0.5 µg of Random Primers (Promega). For generic detection of Group A rotaviruses, an RT-PCR method based on amplification of a VP7 fragment was used. The primers selected were VP7-fw (5′-TAAATGAAT GGTTATGTAACCCAAT-3′; position 527–551 of human wild-type strain) and VP7-re (5′- AATCCG CTACTTTTCTCTTGG-3′; position 829–808 of human wild-type strain). The PCR programme consisted of 30 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 2 min, followed by a final elongation step at 72°C for 10 min. To verify the successful amplification of viral genetic material, the PCR amplicons were purified using the NucleoSpin gel extraction and PCR clean-up 2-in-1 kit (Macherey-Nagel, Düren, Germany), ligated into the pGEM-T Easy Vector (Promega), and cloned in XL1-Blue *E. coli* cells (Invitrogen). The positively screened clones were then sequenced at BMR Genomics (University of Padua) on an ABI PRISM 3700 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) and at the Wildlife Diseases Laboratory in the Faculty of Veterinary Medicine of the Agricultural University of Tirana.

The experimental data were analysed using SPSS version 20.0 (IBM, Armonk, NY, USA). StatGraphics Plus software (StatGraphics Technologies, The Plains, VA, USA) calculated the descriptive statistics as mean, standard deviation, and minimum and maximum values for the studied parameters at each sampling point.

Results

The PCR results and descriptive statistics obtained from analysing *Mytilus galloprovincialis* species from Butrinti Lake and off the Cape of Stillo in 2015, 2019, and 2021 are shown below. As shown in Fig. 2, there was a presence of rotavirus in the sea off the Cape of

Stillo in 2015. The average positivity was 44% over three sampling sites, with 47% in sampling site 1, 33.3% in site 2, and 52% in site 3. In Butrinti Lake the percentages of infected individuals in 2019 were 33% in sampling site 1, 41% in site 2 and 33% in site 3, whereas in 2021 the percentages were 50% in site 1, 19% in site 2, and 0% in site 3, as shown in Figs 3 and 4.

There were no specimens collected from Butrinti Lake in the autumn because of the high water temperatures, which may have impacted the presence of rotavirus.

In total, the percentage of infected individuals in the sea off the Cape of Stillo in 2015 was 44%, in Butrinti Lake in 2019 it was 36%, and in 2021 it was 23%.

SPSS version 20.0 (IBM, Armonk, NY, USA) was used to calculate the descriptive statistics values at each sampling point at Cape of Stillo and Butrinti Lake, as shown in Table 2. According to these summary statistics, we can emphasize that a higher variance in Cape of Stillo suggests greater dispersion of data points, whereas in Butrinti Lake, the degree of variability in 2021 is higher than in 2019.

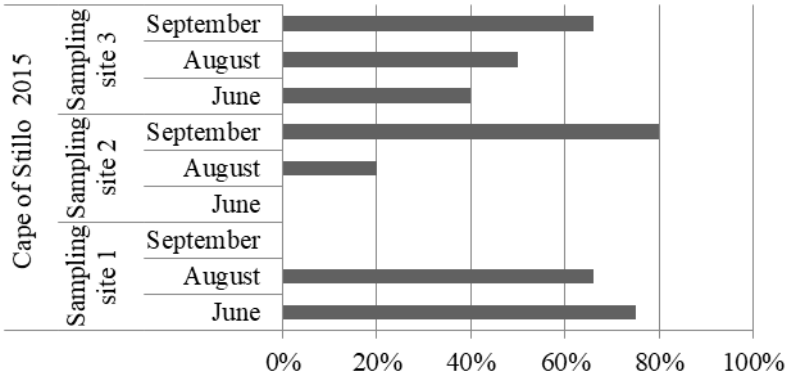

Fig. 2. Percentages of rotavirus-infected *Mytilus galloprovincialis* from the sea off the Cape of Stillo, Albania in 2015

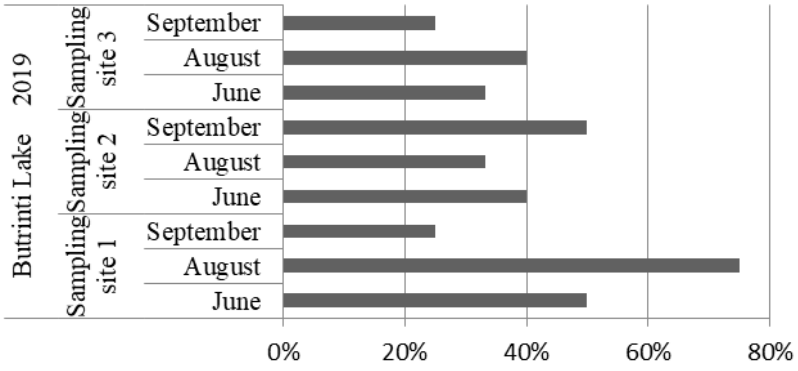

Fig. 3. Percentages of rotavirus-infected *Mytilus galloprovincialis* from Butrinti Lake, Albania in 2019

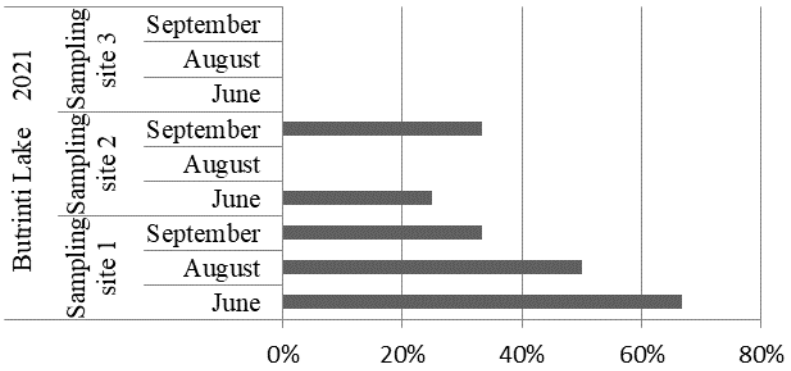

Fig. 4. Percentages of rotavirus-infected *Mytilus galloprovincialis* from Butrinti Lake, Albania in 2021

Table 2. Descriptive statistics from Butrinti Lake and Cape of Stillo in 2015, 2019 and 2021

Study area	N	Range	Mean	Std. deviation	Variance
Cape of Stillo (2015)	9	80	44.111	31.0743	965.611
Butrinti lake (2019)	9	25	35.733	10.00587	100.118
Butrinti lake (2021)	9	66.7	23.144	24.9266	621.335

Discussion

We used a successful molecular method, namely reverse transcription polymerase chain reaction (RT-PCR), for the detection of enteric viruses in shellfish from *Mytilus galloprovincialis* in Butrinti Lake and off the Cape of Stillo. It is worth noting that the method used has a sensitivity of 33 viral particles per gram of digestive tissue, meaning that it can detect even small amounts of the virus in the tissue samples. Overall, this study and the analysis of the collected samples provided insights into the presence and prevalence of rotavirus in *Mytilus galloprovincialis* mussels from Butrinti Lake and off the Cape of Stillo.

According to the results, rotavirus was present in wild Mediterranean mussels from three sampling sites off the Cape of Stillo, three sampling sites from Butrinti Lake in 2019, and two sampling sites 1 and 2 from Butrinti Lake in 2021, but not in sampling site 3 from Butrinti Lake in 2021. This information suggests that there is a presence of rotavirus in certain areas and years within the aquaculture system in Albania. Further investigation and monitoring will be necesary in the future in order to gain a better understanding of mussel cultivation in the aquaculture system in Albania. This can help determine the extent of rotavirus contamination, its potential impact on mussel populations, and the measures that may need to be taken to manage the presence of rotavirus in the future.

However, it is important to note that there were no specimens collected in autumn from Butrinti Lake because of mussel mortality caused by increased water temperature. It is also worth noting that shellfish tend to accumulate microorganisms during periods of low water temperatures, which can result in a higher incidence of viral gastroenteritis through shellfish consumption during these periods (26). This may explain the seasonality of shellfish-borne viral diseases. Additionally, rotavirus is heat-stable and more resistant to chlorine disinfection than bacteria, which is important to consider for food safety measures. Based on the results of the Cape of Stillo sampling sites, it appears that the percentage of rotavirus-infected mussels was higher in June and September than in August, which was likely because of the lower water temperatures during these periods. Sampling sites 1 and 3 showed higher percentages of infected mussels than site 2, which may indicate site-specific factors contributing to the presence of rotavirus. The results obtained from the Cape of Stillo samples had a wide distribution, which may be linked to the presence of areas used for marine aquaculture in the Greek part (at least 2 km from the Cape of Stillo) of the Greek–Albanian maritime boundary region. Coastal and estuarine areas with marginal domestic pollution can also create ideal conditions for viral transmission through shellfish like Mediterranean mussels. The overall 44% presence of rotavirus in the mussels collected from the sea off the Cape of Stillo is a significant finding and highlights the importance of monitoring and assessing the potential risks associated with consuming these shellfish.

It is important to note that rotavirus is a significant cause of gastroenteritis, particularly in children under the age of 5. An outbreak of acute gastroenteritis was reported by the Public Health Institute in Tirana in 2014. Rotavirus was detected in 21% of samples, more frequently in children under 2 years of age, who accounted for 80.8% of all positive cases (19). More recently, a study conducted in Albania, the Salento peninsula in southern Italy, and different hospitals in Rome found that 31.3% of stool samples collected in Tirana were positive for rotavirus, while the positivity rate was 78.3% in the Salento peninsula and 40.3% in Rome (1). These findings suggest that rotavirus remains an important cause of gastroenteritis in the region and highlight the need for continued monitoring and control measures to reduce the incidence of infections caused by this virus.

It is important to note that the detection of rotaviruses in shellfish should be considered an indicator of human faecal pollution and the possible presence of other human viruses. Therefore, it is recommended to conduct further investigations in Albanian coastal areas to determine the extent of the contamination and the potential risk to public health. Additionally, the current regulations should be revised to include the detection of other pathogens, including viruses, in order to ensure the safety of shellfish and protect public health. It is important to prioritise public health concerns in the management of aquaculture and the regulation of shellfish harvesting and consumption.

This study found that mussels (*M. galloprovincialis*) could potentially transmit viruses. Studies on enteric virus retention by shellfish have shown that viruses can persist for periods significantly longer than bacterial indicator organisms, like *Escherichia coli* and faecal coliforms. If these molluscs are consumed raw or

inadequately cooked, these pathogens can be ingested and cause illnesses in humans (23). This study detected the presence of rotovirus in shellfish, indicating that viruses were present in the seawater. It suggests the potential of mussels to serve as natural accumulators of viral pollution and be used as bio-monitor organisms to test viral marine pollution, even when they are transplanted into contaminated sites for monitoring purposes.

These findings are consistent with those of a previous study conducted by different authors that evaluated the use of mussels in biomonitoring marine environments. We proved the presence of rotavirus in *Mytilus galloprovincialis* and found the viral contamination rate of the mussel samples to be relatively high (37.5%), even though the observed rate of prevalence was not as high as that reported by a follow-up of mussels in France (21). Congruent results have been reported by a variety of similar studies (16, 27). It is concerning that rotavirus may be present in wild mussels in Albania, and it is important to conduct further investigations to determine the extent of the problem in other coastal areas. The requirement of the current regulations of the Agricultural Ministry of Albania for the detection of only traditional faecal contamination indicators is outdated and there needs to be added to it the requirement for the detection of other pathogens, such as viruses. It is also important to note that the detection of rotaviruses in shellfish may indicate the presence of other human viruses and faecal pollution. Therefore, it is recommended that future investigations also include the detection of other human viruses. This will help to ensure that the seafood consumed in Albania is safe and free from harmful pathogens that can cause illness in humans.

Conflict of Interests Statement: The authors declare that there is no conflict of interests regarding the publication of this article.

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Animal Rights Statement: None required.

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