Original Article

Presence of Enteric Viruses in Shellfish Samples from the Persian Gulf

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ABSTRACT

Shellfishes are a significant economic and nutritious seafood amongst people in different countries. Seafood products, particularly shellfish, are potential reservoirs of enteric viruses. This research investigated the incidence of rotavirus (RoV), norovirus (NoV) GI and GII, hepatitis A virus (HAV), and hepatitis E virus (HEV) in shellfish samples from the Persian Gulf, Iran. One hundred and fifty shellfish samples were collected. RNA extraction and cDNA synthesis were performed using commercial kits. The real-time polymerase chain reaction assessed the presence of enteric viruses in extracted cDNA samples. Thirty-two out of 150 (21.33%) shellfish samples were contaminated with enteric viruses. Prevalence rates of NoV GI, NoV GII, HAV, and RoV amongst shellfish samples were 8.00%, 11.33%, 1.33%, and 0.66%, respectively. There were no contaminated shellfish samples with HEV. Simultaneous prevalence of HAV and NoV GI, and HAV and NoV GII viruses were 0.66% and 0.66%, respectively. Examined viruses had a higher prevalence in shellfish samples collected in the winter season (P<0.05). Prevalence of HAV, RoV, NoV GI, and NoV GII amongst shellfish samples gathered in the winter season was 2.85%, 9.09%, 11.90%, and 20%, respectively. To the best of our knowledge, this was the first report of the incidence of enteric viruses, particularly HAV, NoV GI, NoV GII, and RoV, in shellfish samples from the Persian Gulf, Iran. Shellfish samples may serve as a potential source of enteric viruses for the human population. Therefore, routine viral assessments should be conducted. The consumption of fully cooked shellfish can significantly reduce the risk of HAV, RoV, NoV GI, and NoV GII infections. Furthermore, given the export value and importance of shellfish samples, their microbial quality and safety should be routinely monitored.

Keywords: Hepatitis A Virus, Noroviruses, Persian Gulf, Rota Virus, Shellfish

1. Introduction

Shellfish is a significant economic and nutritious seafood enjoyed by people in different countries. They are rich sources of proteins and essential amino acids, such as isoleucine, glutamic acid, lysine, methionine, proline, glycine, alanine, leucine, aspartic acid, and arginine. Additionally, they contain lipids and essential fatty acids, including long-chain polyunsaturated fatty acids and monounsaturated fatty acids. Shellfish also provide various minerals, such as calcium, sodium, magnesium, zinc, potassium, copper, selenium, and iron, as well as vitamins A, B, D, E, K, niacin, and folic acid (1). Their consumption is recommended for all people around the world. Despite shellfish's high economic and nutritional importance, they may be the reservoir of diverse kinds of enteric viruses with a high potential for the occurrence of foodborne diseases (2). Enteric viruses are propagated in the human intestine and secreted through stool. They are usually transmitted by the fecal-oral route and by consuming contaminated food and water. Norovirus (NoV), hepatitis A virus (HAV), hepatitis E virus (HEV), and rotavirus (RoV) are the most significant enteric viruses with numerous foodborne aspects (1, 3). Several outbreaks of viral foodborne diseases have been reported due to consuming contaminated seafood products, particularly shellfish (1, 4). Norovirus is a member of the family Caliciviridae and comprises a positive-sense single-stranded RNA genome. It is responsible for acute viral gastroenteritis of which two main genogroups (NoV GI and NoV GII) are found in the classification of NoV viruses. They are mainly accompanied by viral gastroenteritis outbreaks (5). The main transmission route of NoV is fecal-oral by contaminated seafood and water (6). Rotavirus is a double-stranded RNA virus of the Reoviridae family. It is characteristically divided into seven different serogroups (A to G). Rotavirus serogroups A, B, and C mostly accompany viral gastroenteritis outbreaks. Group A RoV is the main reason for diarrhea in young children. It is transmitted from person to person or circuitously by contaminated seafood and water (7). Hepatitis A virus and HEV are other substantial enteric viruses responsible for human hepatitis in developed and developing countries. Hepatitis A virus and HEV are single-stranded RNA viruses belonging to the Picornaviridae and Hepeviridae families, respectively (8). Contaminated seafood and water are the most imperative sources for transmitting HAV and HEV to humans (9). According to the high consumption and exportation rates of shellfish and the unwanted and permanent presence of enteric viruses in these seafood samples, the current survey was performed to evaluate the incidence of NoV, HAV, HEV, and RoV in shellfish samples caught from the Persian Gulf, Iran.

2. Materials and Methods

2.1. Samples

From June 2017 to June 2018, 150 shellfish samples were randomly collected from the local harbors and retail centers in Isfahan, Iran. The number of shellfish samples gathered in the summer, autumn, winter, and spring seasons was 35, 42, 40, and 43, respectively. Species identification of shellfish samples was made by an expert professor in the field of aquaculture. Shellfish samples were identified as two major species of *Cypraea princess* and *Cypraea annulus* caught from the Southern ports, the Persian Gulf, Iran. Samples were placed in separate sterile plastic bags to prevent spilling and cross-contamination. They were immediately transported to the laboratory in a cooler with ice packs and processed within 6 h. All shellfish samples showed typical physical characteristics, including odor, color, and consolidation.

2.2. Sample preparation

A volume of 1.5 g of the shellfish samples was homogenized with 0.25 M glycine and 0.14 M NaCl buffer (pH 7.5) (Sigma, St. Louis, MO, USA). The homogenate sample was transferred to a 50 ml tube and centrifuged at 10,000 × g for 30 min at 4°C (Shimadzu, Japan). The primary supernatant was collected in a new tube. The pellet was resuspended in 0.25 M Threoninee-0.14 M NaCl buffer (pH 7.5), and was then centrifuged at 10,000 × g for 30 min at 4°C (Shimadzu, Japan). The secondary supernatant was subsequently mixed with the primary supernatant. Forty percent polyethylene glycol 6,000 (PEG 6,000) (Sigma, St. Louis, MO, USA) and 3 M NaCl (Amresco, Solon, OH, USA) solution were added to the collected supernatant. The mixture was precipitated at 4°C for 3 h. After precipitation, the pellet was dissolved in 0.2% Tween 80-50 mM Tris-HCl (Merck, Germany), sterilized distilled water, and chloroform: isoamyl alcohol (24:1) (Sigma, St. Louis, MO, USA). The mixture was centrifuged at 10,000 × g for 30 min at 4°C (Shimadzu, Japan). The supernatant was transferred to a new tube. The remaining precipitate was dissolved in sterilized distilled water and chloroform: isoamyl alcohol (24:1). The mixture was then centrifuged another time at 10,000 × g for 30 min at 4°C (Shimadzu, Japan), and the supernatant was combined with the first supernatant. The mixture was precipitated with 40% PEG 6,000 and 3 M NaCl solution at 4°C for 3 h. The pellet was then

suspended with diethylpyrocarbonate (DEPC)-treated deionized water (Sigma, St. Louis, MO, USA).

2.3. RNA extraction and cDNA synthesis

The pellet suspended with DEPC-treated distilled water was used for RNA extraction using the QIAamp viral RNA mini kit (Qiagen, Valencia, CA, and USA). The reverse transcription reaction mixture (Applied Biosystems, USA) consisted of sterile DEPC-treated water, 10X polymerase chain reaction (PCR) Buffer, 25 mM deoxynucleotide triphosphates, 50 µM oligo d (T), RNase inhibitor (20 U/µl), murine leukemia virus reverse transcriptase (50 U/µl), and 5 µl RNA template. Reverse transcription was performed with an MJ mini cycler (Bio-Rad, CA, USA) with the following program: 42°C for 50 min, 94°C for 5 min, and 4°C for 5 min **(10)**. Purity (A260/A280) and concentration of extracted cDNA were then checked (NanoDrop, Thermo Scientific, Waltham, MA, USA). The truth of the cDNA was assessed on a 1.5% agarose gel stained with ethidium bromide (0.5 µg/ml) (Thermo Fisher Scientific, St. Leon-Rot, Germany). Two negative control reactions were performed alongside the main reactions to evaluate the accuracy of the synthesis of cDNA. One of these reactions was a PCR in the presence of extracted RNA as the reaction sample and the other one was a PCR without the presence of any sample and only in the presence of water. As a rule, the lack of amplification in these two reactions and amplification in the presence of cDNA indicated the validity of the synthesis.

2.4. Real-time polymerase chain reaction

Qualitative real-time PCR was used for the detection of pathogenic viruses. Table 1 represents the oligonucleotide primers, PCR conditions, and the volume of each reaction used to detect NoV, HAV, HEV, and RoV in fish and shrimp samples. Primers were designed to detect pathogenic viruses. First, the sequences for the desired viruses were extracted from the National Center for Biotechnology Information database. Afterward, primers were designed based on the conserved regions of the different sequences for each virus using MEGA 7.0 and Oligo 7 software. PCR amplification was performed with a Thermal Cycler Dice Real-Time System (TaKaRa, Japan).

The primer application was evaluated in three different stages. In the first step, the gradient PCR reaction was performed at six different temperatures for each primer pair, and the optimum binding temperature for each primer pair was selected. Secondly, PCR reactions were performed at the optimum temperature for each primer pair in the presence of positive samples (sample infected with the target virus) and negative samples (sample that did not contain the desired virus or was infected with another virus). In the third step, a standard curve was drawn for each primer pair in the presence of a positive sample.

2.5. Statistical analysis

Fisher's exact test was used to analyze the correlation of virus detection rate with shellfish samples. Analyses were conducted using R (Institute for Statistics and Mathematics of the WU Wien). Statistical difference was determined by a *p*-value of < 0.05.

3. Results

3.1. Analysis of real-time polymerase chain reaction

One hundred fifty shellfish samples were studied for the presence of NoV, HAV, HEV, and RoV. The applied method failed to amplify HEV in examined shellfish samples. Real-time PCR amplification curves are displayed in figure 1. Additionally, real-time PCR melting curves are illustrated in figure 2.

3.2. Incidence of enteric viruses

Table 2 represents the distribution of NoV, HAV, HEV, and RoV amongst the shellfish samples. Thirty-two out of 150 (21.33%) shellfish samples were contaminated with enteric viruses. Incidences of HAV, NoV GI, NoV GII, and RoV amongst all examined samples were 1.33%, 8.00%, 11.33%, and 0.66%, respectively. The applied method failed to detect HEV in the studied samples. The simultaneous incidence of HAV and NoV GI viruses was 0.66%. Additionally, the simultaneous incidence of HAV and NoV GII viruses was 0.66%. A statistically significant difference was found between the incidences of enteric viruses ($P<0.05$).

3.3. Seasonal distribution of enteric viruses

Figure 3 shows the seasonal incidence of enteric viruses amongst the shellfish samples. Findings revealed that all detected enteric viruses had a higher incidence rate on samples collected throughout the winter. However, those collected throughout the summer season had the lowest incidence of all examined viruses.

Table 1. Oligonucleotide primers and probes used for detection of HAV, HEV, NoV GI, NoV GII and RoV in fish and shrimp samples

Target viruses	Oligonucleotide primers (5'-3')	PCR volume (25 µL)	PCR programs
HAV	F: GGTAGGCTACGGGTGAAAC R: TTGCCCTAAGCACAGAGAGGT	cDNA: 100 ng Primers (10 pmol): 0.3 µM of each primer 2X Master Mix Syder-Green: 12.5 µL	1 cycle: 95°C -- 10 min. 45 cycle: 95°C ----- 15 s 58°C ----- 35 s 72°C ----- 25 s
HEV	F: GGTGGTTTCTGGGGTGAC R: AGGGGTTGGTTGGATGAA	cDNA: 100 ng Primers (10 pmol): 0.3 µM of each primer 2X Master Mix Syder-Green: 12.5 µL	1 cycle: 95°C -- 10 min. 45 cycle: 95°C ----- 15 s 55°C ----- 35 s 72°C ----- 25 s
NoV GI	F: CGTCCTTAGACGCCATCATCATT R: CCAGAGGAAAGTTCAGCTTATATCC	cDNA: 100 ng Primers (10 pmol): 0.3 µM of each primer 2X Master Mix Syder-Green: 12.5 µL	1 cycle: 95°C -- 10 min. 45 cycle: 95°C ----- 15 s 58°C ----- 35 s 72°C ----- 25 s
NoV GII	F: CTCGACGCCATCTTCATTCAC R: GAAACAATGATACCACACTCCCAA	cDNA: 100 ng Primers (10 pmol): 0.3 µM of each primer 2X Master Mix Syder-Green: 12.5 µL	1 cycle: 95°C -- 10 min. 45 cycle: 95°C ----- 15 s 56°C ----- 35 s 72°C ----- 25 s
RoV	F: CAGTGGTTGATGCTCAAGATGGA R: TCATTGTAATCATATTGAATACCCA	cDNA: 100 ng Primers (10 pmol): 0.3 µM of each primer 2X Master Mix Syder-Green: 12.5 µL	1 cycle: 95°C -- 10 min. 45 cycle: 95°C ----- 15 s 56°C ----- 35 s 72°C ----- 25 s

Table 2. Distribution of HAV, HEV, NoV GI, NoV GII and RoV in shellfish samples

Type of samples	N. samples collected	Incidence of enteric viruses (%)						
		HAV	HEV	NoV GI	NoV GII	RoV	HAV+NoV GI	HAV+NoV GII
Shellfish	150	2 (1.33)	-	12 (8.00)	17 (11.33)	1 (0.66)	1 (0.66)	1 (0.66)

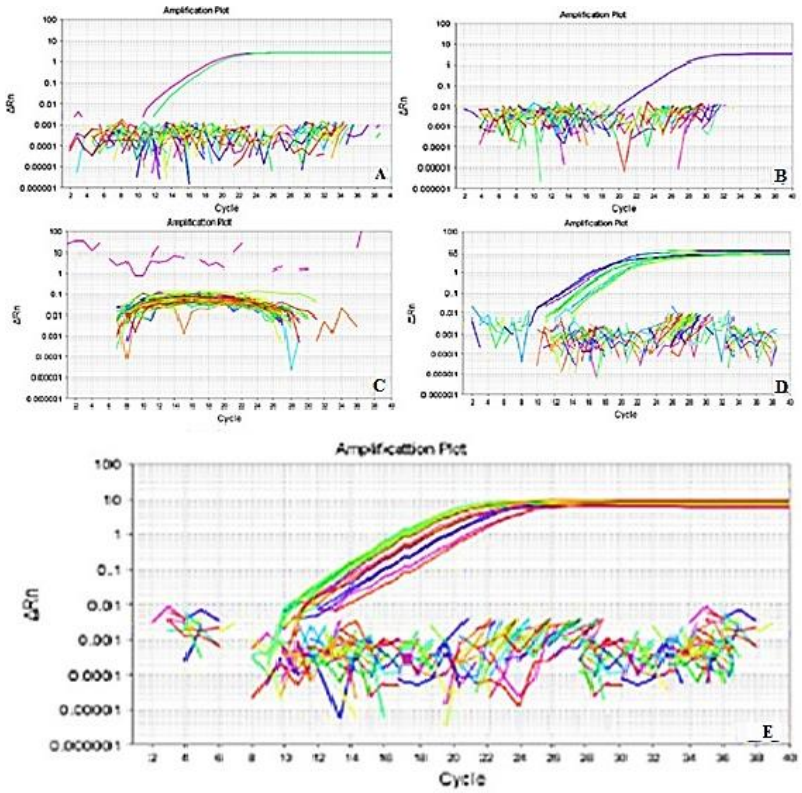

Figure 1. Samples of amplification cycles of studied enteric viruses in shellfish samples. A: HAV, B: RoV, C: HEV, D: NoV GI, E: NoV GII.

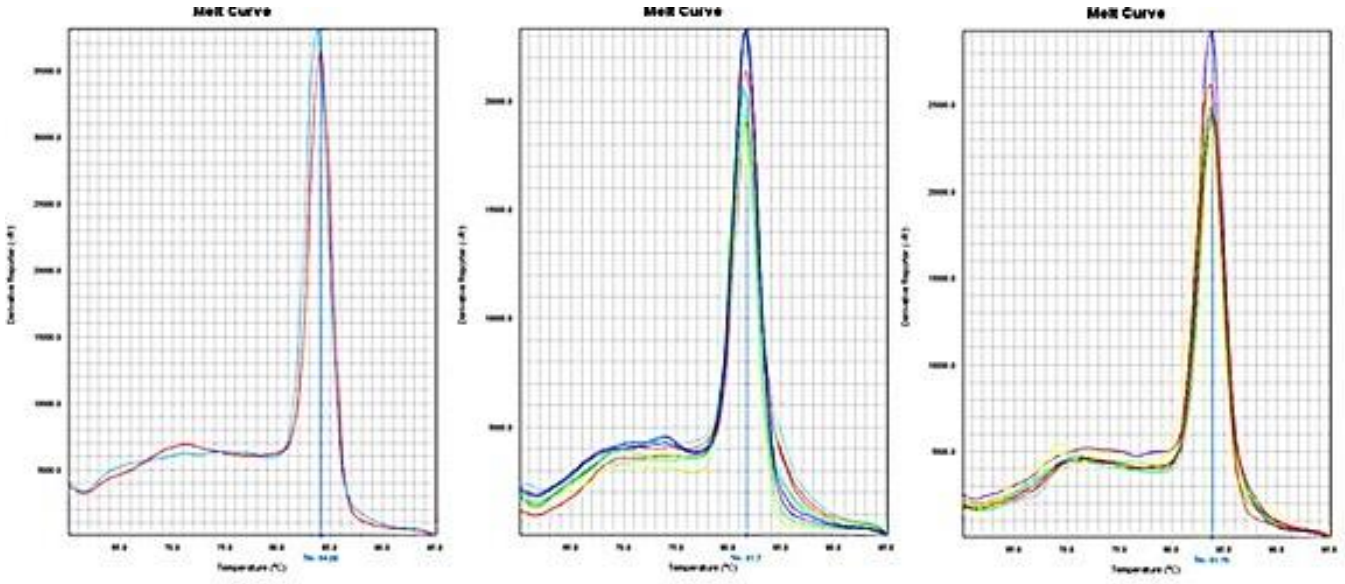

Figure 2. Samples of melting cycles of studied pathogenic viruses in shellfish samples.

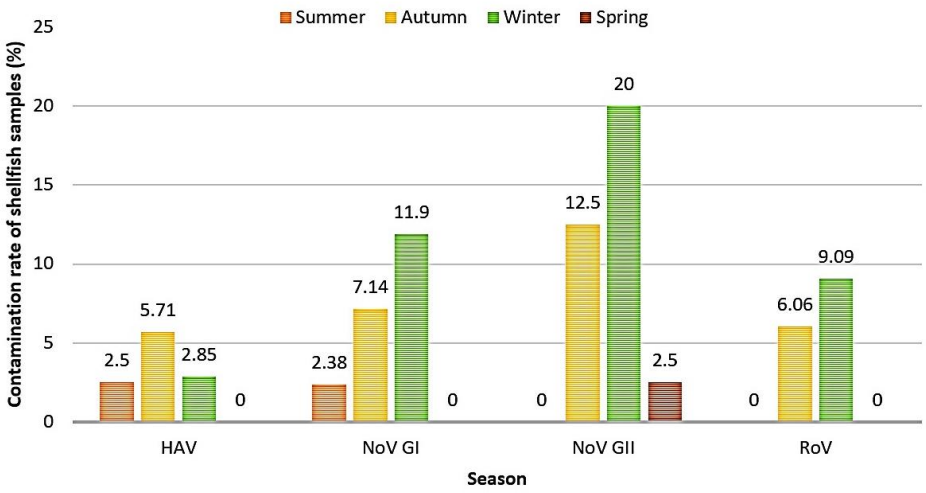

Figure 3. Seasonal incidence of HAV, NoV GI, NoV GII and RoV enteric viruses amongst the shellfish samples.

The total incidence of HAV, NoV GI, NoV GII, and RoV amongst the examined shellfish samples collected through the winter season were 2.85%, 11.90%, 20%, and 9.09%, respectively. Statistically significant differences were found between the season of sampling and the incidence of enteric viruses (P<0.05). Shellfish are an imperative source of transmitting HAV, NoV GI, NoV GII, and RoV enteric viruses. The risks associated with shellfish consumption are more severe if these products are consumed raw or half-cooked. As shellfish are filter-feeding, they can concentrate pathogens dispersed in the water, particularly viruses. Additionally, they can contaminate in the post-harvesting stages, mainly by human manipulation. Considering these, information on viral contamination of shellfish is valuable to attain contextual data on the presence of contamination in the environment, predominantly in shellfish production areas, and to make a clear picture of the epidemiology of enteric viruses in local populations.

4. Discussion

To the best of our knowledge, this is the first report of the incidence of enteric viruses, particularly HAV, NoV GI, NoV GII, and RoV, in shellfish samples from the Persian Gulf, Iran. The total incidence of HAV amongst shellfish samples was 1.33%. Lower incidence rates of HAV in shellfish samples were reported in Italy (0.9%) (11) and South Korea (0.7%) (12), while higher incidence rates were observed in Iran (2.70%) (13) and Portugal (33%) (14). The total incidence of NoV GI amongst shellfish samples was 8.00%. Italy (1.60%) (3) and Thailand (7.70%) (15) accounted for lower incidence rates of NoV GI in shellfish samples, whereas China (21.48%) (16), India (16%) (17), France (8.40%) (18), and Spain (13%) (19) were responsible for higher incidence rates. The total incidence of NoV GII amongst shellfish samples was obtained at 11.33%. Lower incidence rates of NoV GII in shellfish samples were reported in France (1.60%) (20), United States (3.90%) (21), and Australia (2%) (22), while higher incidence rates were observed in China (20.71%) (16) and Vietnam (79.30%) (23). The total incidence of RoV amongst shellfish samples was 0.66%. Italy (0.50%) (24) contributed to a lower incidence rate of RoV in shellfish samples, whereas India (2.50%) (25) and France (27%) (26) formed higher incidence rates. The findings of the present survey revealed a higher incidence of NoV GII amongst shellfish samples. Similarly, a high incidence of NoV GII was also reported in different previously published investigations conducted in South Korea (27) and Italy (3). In the same vein, the results of research conducted by Fusco et al. (2019) [28] revealed that the incidence of HAV, NoV GI, NoV GII, RoV, and HEV amongst the shellfish samples collected from the Gulf of Naples, Italy, through 2015 to 2017 years were 8.90%, 10.80%, 39.70%, 9.00%, and 0%, respectively. Purpari et al. (2019) [29] reported that the incidence rates of HAV, NoV GI, NoV GII, RoV, and HEV in seafood samples gathered from Italy were 13.00%, 10.20%, 5.60%, 0%, and 0.90%, respectively. High differences found in the incidence of enteric viruses amongst seafood samples of various studies may be due to the variances in the types of examined seafood samples, method of sampling, season of sampling, place of sampling (geographic location), and type of method used for the detection of viruses. Because HAV and RoV were detected in only 1.33% and 0.66% of examined samples,

respectively, it is suggested that there might be a very low possibility of transmission of these two enteric viruses via the consumption of shellfish in Iran. However, a low infective dose of RoV (10-100 virus particles) should be considered an imperative factor that increases the emergence of RoV even with a low incidence rate (25). An important probable reason for the incidence of enteric viruses in examined shellfish samples is the depletion of sewage into the seawater. Additionally, septic tank leakages, boat discharges, overflows and spills from sewage treatment plants, seepage from sewage reticulation networks, and accidental contamination after heavy rainfall are other probable reasons for the occurrence of enteric viruses amongst examined samples.
Seasonal variation in the incidence of enteric viruses was observed in the current survey with a severe increase in the prevalence of viruses in shellfish samples collected through the winter season. Seasonal variation of enteric viruses has been reported in diverse pieces of research (27) and attributed to the fact that viruses are less effectively removed from seafood in winter and could survive better in colder than warmer seasons (3). Seasonality in the incidence of enteric viruses in shellfish samples could be related to numerous factors, including increased stability of viruses at low water temperatures, reduced solar inactivation, and selective bioaccumulation of these pathogens by seafood. A higher incidence of enteric viruses in cold months of the year was also reported by Seo et al. (2014) (12) and Fusco et al. (2019) (28); nevertheless, further studies are obligatory to find more epidemiological properties, particularly investigating the reason of seasonal distribution of enteric viruses in shellfish samples. To the author's knowledge, this existing survey represented the first report on the detection and seasonal variation of HAV, NoV GI, NoV GII, and RoV among shellfish samples from the Persian Gulf, Iran. Norovirus, particularly NoV GII, emerged as the most prevalent enteric virus in the examined shellfish samples. Furthermore, considering this, NoV GI, HAV, and RoV should also be regarded as other important enteric viruses in shellfish samples from the Persian Gulf, Iran. The winter season exhibited a higher contamination rate of shellfish samples. Although the viability of enteric viruses could not be confirmed due to the limitations of real-time RT-PCR and the lack of cultivation techniques, monitoring these viruses in shellfish samples may significantly contribute to the prevention of viral food poisoning and the promotion of public health. Additionally, given the export value and importance of shellfish samples, routine measurement of their microbial quality and safety is imperative.

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Authors' Contribution

ER, and MA carried out the molecular genetic studies, and drafted the manuscript. MA and ER carried out the sampling and method. ER and AS participated in the design of the study, performed the statistical analysis and writing the manuscript. All authors read and approved the final manuscript.

Ethics

The Ethical Council approved the study of Research of the Faculty of Veterinary Medicine, Shahrekord Branch, Islamic Azad University, Shahrekord, Iran.

Conflict of Interest

The authors declare that they have no competing interests.

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