Molecular Detection of Norwalk Virus in Carp Fish and Shrimp Ponds in Khuzestan Province, Iran by RT-PCR Method

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Abstract
Background: Norwalk virus is one of the most common causes of viral gastroenteritis. The aquatic products are potential sources of contamination with this virus.

Objectives: The main objective of the study was to investigate the presence of the Norwalk virus in different aquatic animals in Khuzestan provinces, Iran.

Materials and Methods: A total of 40 pieces of fish (silver carp, common carp, big head, and grass carp species) and 10 pieces of shrimps were caught from ponds, and the samples were transferred to the laboratory in ice bags. After the separation of the intestine, the content of the intestine was extracted using two sterile filters. Then, the supernatant was used for reverse transcription polymerase chain reaction (RT-PCR) using Calicivirus-specific primers (p289/p290). Then, Norwalk virus-specific primers (NVp36/NVp35) were detected in Calicivirus positive samples.

Results: The results showed 8% (4 samples) and 6% (3 samples) of the samples were infected with Calicivirus (p289/p290 genes) and Norwalk virus (NVp36/NVp35 genes), respectively. Calicivirus positive samples included 2 common carp, 1 silver carp, and 1 shrimp. Norwalk virus-positive samples included 2 common carp and 1 shrimp. In other words, the highest prevalence of virus was observed in aquatic fish feeding from the bottom of the pool. Due to the fact that this species is bred with other species and considering that this virus lives in the gastrointestinal tract, the ingestion of feces of other infected organisms can lead to the increase of this virus in the digestive system of carp.

Conclusion: Therefore, due to the importance of Norwalk as a zoonotic agent and the possibility of human infection through consumption of aquatic products, preventive measures such as not using animal manure for fertilization and preventing the growth of phytoplankton in aquaculture ponds and cooking meat properly are suggested.

Background
One of the main problems of food hygiene is the transmission of common diseases between humans and animals or zoonotic diseases. The World Health Organization identifies these diseases as infections that are naturally transmitted between humans and vertebrates. Viruses are zoonotic agents which can be transmitted via foods. Each year, at least 5 million cases of food-borne gastroenteritis are reported in the world. Approximately one-third of gastrointestinal illnesses due to diarrhea and vomiting are caused by eating food contaminated with foodborne viruses. The source of infection can be found in closed food establishments such as dormitories, dining halls, canteens, camps, prisons, cruise ships, etc.

Norovirus or Norwalk virus, which is a species of the Calicivirus family, is one of the most common causes of viral gastroenteritis in humans that can affect people of all ages. Norovirus infection is characterized by gastroenteritis (nausea, vomiting, and diarrhea), lethargy, weakness, muscle aches, headache, fever, loss of appetite, and dehydration. Generally, the disease is self-limiting with complete recovery within 2-3 days; however it may cause death in some cases (especially children and the elderly). Most of these diseases occur where healthy people live or work in the vicinity of sick people in nursing homes, hospitals, and child care centers.

Norovirus outbreaks have often been reported to be associated with the consumption of bivalve mollusks such as oysters and other sea products. The rate of infection with the virus through food consumption especially aquatic products is about 11%-25%. This high rate indicates the importance of conducting research on the causes of food contamination.

Many species of cold-water fish, warm-water fish, and sea fish as well as shrimps are bred in Iran. Among these species, four Cyprinidae family species including...
silver carp (*Hypophthalmichthys molitrix*), common carp (*Cyprinus carpio*), big head (*Hypophthalmichthys nobilis*) and grass carp (*Ctenopharyngodon idella*) are the main carp fish species in Iran.\(^{19,20}\) White leg shrimp (*Litopenaeus vannamei*) is also the main species of farmed shrimp. Due to their rapid growth and disease resistance, these species are considered as the dominant aquatic species in Iran and many other countries.\(^{21,22}\)

Animal manure fertilizers are used in the aquatic species ponds with the aim of producing an effective food chain for the desired aquatic feeding.\(^{23}\) Given the contamination of these animals with a variety of viruses, including Norwalk virus, the survey of these contaminated foods is of great importance.\(^{1,22,24}\) Due to human infection through consumption of aquatic products, investigation of the presence of Norwalk virus in various aquatic animals (farmed shrimp and carp) in Khuzestan province using molecular reverse transcription polymerase chain reaction (RT-PCR) method was the main aim of this study.

**Materials and Methods**

**Sampling**

A total of 40 pieces of carp fish (common carp, silver carp, big head, and grass carp species (with a mean weight of 1000 ± 320 g) and 10 pieces of white-leg shrimps (with a mean weight of 32 ± 7.5 g) were caught from the ponds in Khuzestan province and transferred to the laboratory of the Faculty of Veterinary Medicine.

**RT-PCR steps**

**RNA Extraction**

Because Norovirus accumulates in the gastrointestinal tract and this part has the least amount of PCR inhibitors, after disinfecting the abdominal surface of fish and the lumbar surface of shrimp with 70% alcohol, the whole gastrointestinal tract was dissected and isolated using sterile instruments. For this purpose, the intestine was separated and placed separately in a homogenizer under sterile conditions. Then, the same volume of phosphate buffer solution (PBS) was added to the sample to obtain homogeneity. Then, the contents were transferred to numbered sterile test tubes using a Whatman filtration paper, cellulose syringe filters, and membrane filters. Afterwards, 30 mL of the resulting solution was centrifuged at 5000 rpm for 5 minutes (twice) until a completely clear and transparent liquid was obtained, and then, the samples were kept at -80°C. In the next step, RNA extraction from the samples was performed using DynaBio™ Viral Nucleic Acid (DNA/RNA) Extraction Mini Kit according to the manufacturer's protocol and the samples were kept at -80°C until cDNA synthesis.

**cDNA Synthesis**

A master mix containing 2.25 μL of distilled water, 2 μL of RT Buffer, 1 μL of dNTPs, 0.25 μL of RNA inhibitor and 1 μL of Random Hexamer Primer was prepared. Then, 7 μL of this master mix was poured into 0.5 mL tubes and 1 μg of each sample was added. Each tube was numbered and the tubes were transferred to a thermocycler. The thermocycler for cDNA synthesis was programmed as: 37°C for 5 minutes, 42°C for 60 seconds, and 94°C for 2 minutes. The synthesized cDNAs were transferred to a -20 freezer until the amplification of relevant genes by the thermocycler.

**Polymerase Chain Reaction**

A master mix containing 17.8 μL of distilled water, 2.25 μL of PCR Buffer, 2 μL of Calicivirus-specific primers (P290 and P289), 0.5 μL of dNTPs, and 0.2 μL of Taq DNA polymerase was prepared (Table 1). Then, 23 μL of the master mix was transferred to 0.5 mL tubes and 2 μL of the related cDNA sample was added, and it was transferred to a thermocycler. The thermocycler temperature program consisted of initial denaturation at 94°C for 3 minutes, 40 cycles of PCR at 94°C for 3 seconds, 49°C for 1 minute and 20 seconds, 72°C for 1 minute, and a final extension at 72°C for 10 minutes.

In the next stage, the Norwalk virus was investigated in Calicivirus positive samples using specific primers. For this purpose, specific primers of Norwalk virus (NVP35 and NVP36) published in 1997 by Atmar and Estes were used (Table 2).\(^{25}\) The thermocycler temperature program used was as follows: initial denaturation at 94°C for 4 minutes and 40 cycles of PCR at 94°C for 1 minute, 55°C for 90 seconds and 72°C for 1 minute and final extension at 72°C for 2 minutes. The band size of 470 bp confirmed the presence of Norwalk virus in the sample.

**Electrophoresis**

Visualization was performed by means of electrophoresis on agarose gel 1% in 1× TBE buffer stained with safe stain (Thermo Fisher Scientific, Germany). Negative control (PCR grade water (Thermo Fisher Scientific, Germany)) and positive control (positive cDNA gene obtained from Razi Vaccine and Serum Research Institute, Iran) were

| Primer Name | Sequence | Product Size |
|-------------|----------|-------------|
| P290        | 5’-GATTACCTCCAAGTGGGACTCCAC | 470 bp |
| P289        | 5’-TGCAATGTAATCATCACCATA | 319 bp |
| P289        | 5’-ATAAAAGTTGGCATGAACA | 319 bp |
| NVp36       | 5’-CTGTGTGGTTTGGAGGCCATAT-3’ | 470 bp |
| NVp35       | 5’-CTGTGTGGTTTGGAGGCCATAT-3’ | 470 bp |

**Table 1.** List of Primers Used to Detect Genes Encoding the Presence of Calicivirus (P) Isolated from Samples of Fish and Farmed Shrimp in Khuzestan Province, Iran.

**Table 2.** List of Primers Used to Detect Genes Encoding the Presence of Norovirus (P) Isolated from Samples of Fish and Farmed Shrimp in Khuzestan Province, Iran.
applied in PCR reactions. Additionally, a 100 bp Ladder was put in a separate well. The gel was electrophoresed for 35 minutes at a voltage of 120 V. Then, the gel was placed inside a gel docking system with a UV lamp. The images of the bands were captured by a camera located in the device.

**Statistical Analysis**
Finally, for statistical analysis, the results of molecular studies related to Norwalk virus infection and the type of fish were transferred to the SPSS version 23.0 and analyzed. The chi-square test was used to compare frequency among negative and positive groups. The chart was plotted using Microsoft Excel 2016 software for positive and negative groups.

**Results**
The results of this study showed that among the different experimental groups, a total of 4 out of 50 samples (8%) of the samples were infected with Calicivirus (Figures 1 and 2). Two common carp, one silver carp, and one white leg shrimp contained p289/p290 genes. In other words, the highest prevalence of Calicivirus was observed in common carp (20%), followed by silver carp (10%) and white-leg shrimp species (10%). Norwalk virus infection rate was investigated in positive samples of Calicivirus family using Norwalk virus-specific primers. The results showed that 3 of the 4 samples infected with Calicivirus were in fact infected with Norwalk virus, and there was no statistically significant difference between different groups ($P > 0.05$) (Table 3 and Figure 3).

**Discussion**
Viruses, bacteria, and parasites, as zoonotic agents could cause acute infections in humans. Foodborne viruses, especially Norwalk virus, have been the second most commonly reported cause of food-related problems in the European Union and the most common cause of non-bacterial gastroenteritis in the United States.

![Table 3. Norwalk Virus in Various Samples of Farmed Fish and Shrimp Detected by RT-PCR in Khuzestan Province, Iran](image)

| Aquatic Animal        | Positive/Negative | Number (%) |
|-----------------------|-------------------|------------|
| Common carp           | Positive          | 2 (20)     |
|                       | Negative          | 8 (80)     |
| Silver carp           | Positive          | 0 (0)      |
|                       | Negative          | 10 (100)   |
| Bighead carp          | Positive          | 0 (0)      |
|                       | Negative          | 10 (100)   |
| Grass carp            | Positive          | 0 (0)      |
|                       | Negative          | 10 (100)   |
| Withe leg shrimp      | Positive          | 1 (10)     |
|                       | Negative          | 9 (90)     |
| Total                 | Positive          | 3 (6)      |
|                       | Negative          | 47 (94)    |

$P$ Value=0.225

![Figure 1. Detection of Calicivirus by Gel Electrophoresis in Different Aquatic Species Using p289/p290 Gene. (A) Samples 22-1, (B) Samples 23-23, (C) Samples 50-45. Marker or leader: 100 pairs, C-: Negative control sample (PCR-grade water), C+: Positive control sample (diarrheal stool sample prepared by Razi Vaccine and Serum Research Institute).](image)
High concentrations of the virus are excreted in the feces of infected people (with or without symptoms) and transmitted through contaminated food and water.\textsuperscript{29} In fact, the infection occurs mainly through the consumption of contaminated water, and partially treated or untreated wastewater and sewage overflow from urban areas are the main sources of environmental pollution caused by human intestinal viruses.\textsuperscript{18} These pathogens can be transferred via animal manures used in fish and shrimp farms, accumulate in aquatic mollusks and be transmitted to other aquatic animals through contaminated water.\textsuperscript{3,30} Due to the use of fertilizers to complete food chain in the aquatic animal ponds, contamination of these resources and transmission of viruses are possible through their consumption by humans. Therefore, the fish and shrimp groups were selected and examined for the presence of the virus in this study. In the present study, 3 out of the 50 samples were infected with Norwalk virus, including 2 common carp and 1 shrimp.

In a study, a total of 46 out of 300 aquatic food samples (15.33\%) were infected with the Norwalk virus. In the investigation, the highest prevalence of the Norwalk virus was observed in fish (25\%), followed by crabs (10\%), lobster (10\%), and shrimp (8.33\%).\textsuperscript{31}

In fact, in the study, different fish samples were tested from the artificial ecosystem of the fish pool. Contemporary breeding of these species is due to differences in nutrition and diet behavior in the water body. Silver carp and bighead carp are two fish species that feed on plankton from the water body by gill rays, while grass carp feed on macroalgae or other plants in the pond.\textsuperscript{32} All of these species produce feces, which are ingested by common carp. This investigation revealed that most viruses were distinguished in common carp.\textsuperscript{33-36} Due to the fact that this species is bred with other species and considering that this virus lives in the gastrointestinal tract, the ingestion of feces of other infected organisms can lead to the increase of this virus in the digestive system of carp. Therefore, the results of this study, which showed a higher prevalence of this virus in common carp compared to other aquatic animals, can be justified.\textsuperscript{33-36}

Shrimp is a benthic species that are bred in high numbers in shrimp fields and high-density conditions provide the basis for the transmission of different diseases.\textsuperscript{37,38} The entry of a pathogen can affect the entire group of shrimps, and according to the basic immune system of this species, only the prevention of the entry of various pathogens can be effective in preventing the spread of a virus such as white spot virus. Moreover, because white leg shrimp is a type of saline water species, its breeding needs the use of seawater.\textsuperscript{10,39,40} For this purpose, coastal waters are used, which are likely to be contaminated, especially with biological agents in the human digestive system; therefore, lack of proper treatment of these waters can lead to the outbreak of this viral disease and its transmission to the final consumer.\textsuperscript{41,42} Considering all the above-mentioned facts, the presence of Norwalk virus in a shrimp sample was not far from expected.

Norwalk virus detection was performed by analyzing contaminated samples by SEM electron microscopy, viral genome detection by reverse-transcriptase PCR (RT-PCR), and antibody response measurement.\textsuperscript{43,44} In the study conducted by Schwab et al, methods were...
designed to detect Norwalk-like viruses in clinical and environmental samples including water, oysters, and feces based on the amplification of a small fragment of the virus genome. Viral pathogens such as hepatitis A virus and Norwalk virus are transferred through the consumption of raw or semi-raw aquatic products (fish and shellfish). In the study conducted by Momtaz et al, the Norwalk virus was investigated in 300 samples of fresh fish, shrimp, crab, and lobster using RT-PCR method. The investigation showed that 46 samples (15.33%) were positive for Norwalk virus. The positive samples belonged to 25, 8.33, 10 and 10% of fresh fish, shrimp, crab, and lobster samples, respectively. In the current study, the virus was detected in fresh carp fish and shrimp.

In the study conducted by Baert et al, three Norwalk virus detection methods, including virus and RNA extraction, real-time RT-PCR, and quality controls were compared among France, Belgium, and Canada, which were used in the present study. Waste water is the principal cause of food source in the water. Ideally, there should be no sewage outflow to coastal waters and rivers, and viruses from the collected sewage should not enter the groundwater and pollute water sources. The control of foodborne viral disease depends on particular consideration of hygienic practices in the kitchen. Cross-contamination from poorly cooked and raw aquatic resources should be considered as a potential hazard. Given the pervasiveness of food and waterborne illnesses compared with intestinal viruses, a greater knowledge of the fate and transmission of these viruses is required.

**Conclusion**

This study shows the importance of different species of fish and shrimp meat as potential sources of Norwalk virus infection in Khuzestan province. Considering the importance of Norwalk virus as a zoonotic agent and the possibility of human infection with it through consumption of aquatic products, preventive measures such as not using animal manure for fertilization and preventing the growth of phytoplankton in aquaculture ponds and cooking aquatic products properly are suggested.

**Authors’ Contributions**

All authors participated in conducting the project and approval of the final manuscript.

**Ethical Approval**

The authors of this study have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations.

**Conflict of Interest Disclosures**

The authors declare that they have no conflict of interests.

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