Molecular diagnostic methods for detection and investigation of human Noroviruses- *Norwalk* virus from *Callista umbonella* (Bivalvia) in the Northern Persian Gulf (Iran)

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Abstract

*Norwalk* virus is the major cause of food borne gastroenteritis worldwide with the highest infections by GII genotype. The burden of *Norwalk* virus related disease is considerable, affecting all age groups around the world. *Norwalk* virus is extremely contagious pathogen which can simply transmitted by consuming contaminated food and lead to serious outbreak gastroenteritis disease. The aim of this study was to investigate the incidence of *Norwalk* virus contamination in Bivalvia in the Northern Persian Gulf (southwest of Iran). All samples which examined for *Norwalk* virus (norovirus) contaminations were negative as investigated and verified by reverse transcriptase- polymerase chain reaction (RT-PCR) tests. The results of PCR products on gel electrophoresis indicated that there were no norovirus contaminations in tested samples. Our investigation indicated that molluscs from southwest of Iran are not contaminated with norovirus, however, it would be wisely to eat molluscs with adequate cooked to avoid any contamination including norovirus.

**Keywords:** Norwalk Virus, Gastroenteritis outbreak, Seafood, Norovirus

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Introduction
The Norwalk virus, recently known as norovirus, is a small RNA virus and the most common cause of viral gastroenteritis. (Lopman et al., 2016). Norovirus is one of the pathogenic agents that can be related to food borne disease (Iwamoto et al., 2010). Norovirus is associated with approximately fifty percent of the gastroenteritis outbreaks because of its extremely transmittable nature which can result in serious illness such as diarrhea, nausea and vomiting (Sharma et al., 2020). It has been reported that norovirus is the main cause of outbreaks worldwide with 18% of diarrheal illnesses along with 200,000 deceases per year (Lopman et al., 2016, Sharma et al., 2020). Apart from its periodical incidence, norovirus infectious is more widespread in colder months, autumn, and winter.

Norovirus is belong to a group of virus family named Caliciviridae (Katayama et al., 2006). Based on molecular investigation, seven genogroups have been recognized which among them GI, GII and GIV are known to infect humans (Vinjé, 2015). One of which, GII genogroup has a predominant genotype namely GII.4 which has identified as an aetiology of global epidemics of gastroenteritis (Siebenga et al., 2007, Siebenga et al., 2009). It has been stated that norovirus related disease by GII.4 genotype has caused around 80% of all reported outbreak over the last two decades (Lu et al., 2020). The genome of norovirus consists of three open reading frames (ORFs), of which ORF1 encodes a high polyprotein that is fragmented by the viral protease in mature non-structural proteins while ORF2 and ORF3 encode the major and minor capsid protein respectively (Thorne and Goodfellow, 2014; Mattison et al., 2018). Norovirus may be recognised by reverse transcriptase PCR (RT-PCR) or electron microscopy of contaminated samples or by enzyme immunoassay for the discovery of viral antigen in serum samples.

Previous investigations have reported that gastroenteritis outbreak mostly occurred in crowded communities such as health care facilities, schools, hotels and restaurants (Girish et al., 2002; Meakins et al., 2003; Lu et al., 2020). Numerous studies have demonstrated that norovirus can be transmitted via different routes such as person to person, through the fecal-oral path, or by digestion of contaminated water and food (Lopman et al., 2006; Hewitt et al., 2007; Wolf et al., 2009). As a result, consumption of non-hygiene food and drink which are prepared by infected persons can cause gastroenteritis disease. Reported norovirus associated outbreaks are mostly due to contaminated vegetables, fruits, cereals and raw food (Sharma et al., 2020). It is worthy to mention that besides humans, norovirus can contaminate a wide range of hosts including aquatic animals such as mollusc (Le Guyader et al., 2009; Iwamoto et al., 2010). Callista umbonella (Lamarck, 1818) classified to the Veneridae or venerids,
common name, venus clams, are a very large family of minute to large, saltwater clams, marine bivalve molluscs (Carpenter, 1997; Jousseaume, 1888). Nevertheless, along with the advantages obtained from seafood consumption, there is a possible risk for transmission of norovirus-contaminated seafood to humans (Iwamoto et al., 2010). Norovirus is extremely contagious pathogens which can simply transmitted by consuming contaminated food and lead to serious outbreak gastroenteritis disease. Due to its minuscule infectious dose and its high outbreak rates, norovirus can persevere in the environment and contaminate huge numbers of people (Iwamoto et al., 2010). Therefore, this study aimed to investigate the contamination of molluscs to norovirus in Ahvaz. Ahvaz is a capital city of Khuzestan province and is in the southwest of Iran nearby Persian Gulf. Bivalve mollusc is one of the most favoured food in Khuzestan accompanying with other Iranian cuisine.

**Materials and methods**

**Specimens collection and processing**

A total of 110 molluscs, were collected from main fish market in Ahvaz and Mahshahr during the colder months of autumn to winter 2019. After collecting samples, placed them on sterile containers and transferred to the workplace while kept them on ice box. In laboratory, all samples were washed by deionised water, then dissected by sterile scalpel and their digestive tissues separated for further usage. Later, digestive tissues from all molluscs were separately homogenized, then PBS buffer with an equal volume of each sample added and centrifuged for 10 min at 5,000 rpm at 4°C and the supernatant were used as a possible source of norovirus. Figure 1 shows the processing of molluscs in the laboratory.

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**Figure 1:** processing of molluscs in the laboratory.
Nucleic acid extraction and purification
The possible RNAs of norovirus were extracted and purified using RNA extraction Mini Kit (Dyna Bio viral, Cat #K10025, Takapouzist, Iran) according to the manufacture’s guidelines. The purified RNA then dissolved in RNAase free water and kept at -80°C till further use.

Primers
Two oligonucleotides, both for GII genogroup of norovirus, were purchased from the Takapouzist Company, Iran, and used for amplification in RT-PCR assays as follow:
P290 (5’-GATTACTCCAAGTGGAAGCCAC-3’) and P289 (5’-TGACAATGTAATCATCACCATA-3’)

Reverse Transcription- Polymerase Chain Reaction (RT-PCR)
For reverse transcription (RT) reaction, in a total volume of 10 µL, added the subsequent components into an Eppendorf tube. A volume of 2 µL 10 x PCR buffer, 0.5 µL random hexamer primers, 1 µL dNTPs, 0.5 µL Super Script II reverse transcriptase, 3 µL extracted RNA, 0.25 µL RNase inhibitor, and 2.25 µL water. Then all samples were incubated at 42°C for 1 hour and after that the cDNA denatured at 95°C and kept it on ice.

For PCR reaction, firstly prepared and mixed the following components in a mini centrifuge PCR tube for each sample. A volume of 2.5 µL 10 x PCR buffer, 1 µL primer 1 (P289), 1 µL primer 2 (P290), 0.2 µL Taq DNA polymerase, 2 µL cDNA product, 17.8 µL water. Secondly, performed PCR starting at 94°C for 3 minutes, 40 cycles of denaturation for 30 seconds at 94°C, then annealing for 80 seconds at 53°C followed by 60 seconds at 72°C and 10 minutes at 72°C for final extension. All materials were from Gen Fanavaran company, Iran. The PCR products were then demonstrated by 1% agarose gel electrophoresis and visualised by Gel Documentation (Vilber Lourma).
We used confirmed norovirus that were prepared from Razi vaccine and serum Institute as positive control and DW as negative control in all test stage of study, including extraction of genomes, cDNA synthesis and PCR.

Statistical analysis
A Chi-square test and fisher’s exact test with two-tailed analysis were done using SPSS version 16.0 statistical software. All differences were considered significant when the values were p<0.05.

Results
After biometry (Ave. L. 5.8 cm; weight 64.5 g), all mollusc samples which examined for norovirus contaminations were negative as investigated and verified by RT-PCR tests. The results of PCR products on gel electrophoresis indicated that there were no norovirus contaminations in our samples. The
Figures 2 and 3 shows DNA bands of all samples together with DNA ladder which 319 bp band determined the presence of norovirus.

Table 1: Shows the results of PCR products on gel electrophoresis and their corresponding figures.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Presence of Norovirus</th>
<th>Figure</th>
</tr>
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<tbody>
<tr>
<td>S1, S2, S3, S4</td>
<td>----\</td>
<td>2A</td>
</tr>
<tr>
<td>C1, C2, C3, C4, C5, C6, C7</td>
<td>Only controls 4 and 6 (C4, C6)</td>
<td>2B</td>
</tr>
<tr>
<td>S5, S6, S7, S8, S9, S10, S11, S12, S13, S14</td>
<td>----\</td>
<td>3A</td>
</tr>
<tr>
<td>S15, S16, S17, S18, S19, S20, S21, S22</td>
<td>----\</td>
<td>3B</td>
</tr>
</tbody>
</table>

Figure 2: (A-B) indicate no DNA bands of four samples S1, S2, S3, S4 (Fig 2A) while DNA ladder with 319 bp band determined the presence of norovirus in controls C4 and C6 (Fig 2B).
Discussion

Norovirus was reported as the third dominant cause of seafood outbreaks and the most frequent viral pathogen with two-third of all outbreaks viral disease associated with seafood (Iwamoto et al., 2010). Up to date, many investigations have been done regarding norovirus transmission across person to person, through the fecal-oral route as well as contact with infected surfaces (Kapikian et al., 1972; Meakins et al., 2003; Lu et al., 2020). However, little is known about transmission of norovirus contamination in seafood, particularly bivalve mollusc, to human. For instance, Mcleod et al, found that
molluscs contaminated with norovirus could be a high threat for human health even following cleaning in hygienic water (McLeod et al., 2009). Another study, was reported the association between molluscs with norovirus contamination and possible hazard indicators, such as environmental temperatures and collecting region classifications to human health risk (Lowther et al., 2012). It has been documented that molluscs with insufficient cooked could not inactivated norovirus and resulted human’s health in high risk (Alfano-Sobsey et al., 2012). Results from two outbreaks investigations, using radioimmunoassay were identified norovirus as a main cause of gastroenteritis which related to consuming raw or inadequate cooked molluscs (Morse et al., 1986).

Seafood is an important cause of outbreak food borne disease around the world which mostly attributed to consumption of raw bivalve molluscs. Among aquatic food, molluscs are in higher risk to be contaminated with various pollution in their surrounding environment compare to other seafoods, because they have inherently a filter feeding motion which lead to accumulation of pathogens such as norovirus (Doré et al., 2010). Therefore, eating molluscs with improper cook may result in infection by this virus that can remain in the guts of poisoned living bivalve molluscs for long time (Le Guyader et al., 2006). Because of this, we particularly target digestive tissue for investigation about norovirus.

In this study, samples were collected during cold months, as outbreaks of norovirus are more common, and performed RT-PCR as the most reliable method for norovirus detection. In addition, digestive tissues of bivalve molluscs were separated and used as the most sources of norovirus. Our investigation indicated that molluscs from southwest of Iran are not contaminated with norovirus, however, it would be wisely to eat molluscs with adequate cooked to avoid any contamination including norovirus.

The best management to avoid norovirus contamination is to consider all associated aspects, including serve qualified people who are involved in preparing, cooking and handling of food on strict hand / environment sanitation, as well as hold updated on hygienic codes. Moreover, avoid consuming raw or inadequate cooked seafood, particularly molluscs, in case of some limitations to perform assays for norovirus detection.

Conflict of Interest: None.

References


Jousséaume, F., 1888. Description des mollusques recueillis par M. le Dr. Faurot dans la Mer Rouge et le Golfe d’Aden, Société zoologique de France.


