Detection of *Norovirus* in warm water and cold-water fish culture pools

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Abstract

The contamination of fish with enteric pathogens like norovirus are risky to human health. Among 120 enteric viruses have been found in sewage, norovirus are the most common foodborne and waterborne viruses. There is a few information about norovirus contamination in fish culture pools and seafood in Iran. Therefore, the goal of this study was detection of contamination of fish in warm water and cold-water fish pools in Gillan province by using RT-PCR method. A total of 50 fish, were collected from 20 fish pools in Gillan province in Iran, during April to July 2020. Ten samples from ten rainbow trout pools and forty samples from ten warmwater fish pools (grass, bighead, silver and common carps) were collected. Amplification of 320 bp PCR product showed the presence of norovirus in collected samples. Three sample (6%) founded be contaminated with norovirus. One of positive samples were from rainbow trout that is cold water fish and the two other positive samples were from silver carp and common carp that both of them are warm water fish. The two warm water fish were from different pools in which the source of water entered to these pools was different. Although norovirus is the most common and environmentally stable food born viruses and considering low infectious dose of these viruses, detection of norovirus in water and seafood like fish are very important. The primer pair that were used in current study were specific to detect norovirus in human clinical specimens. So, all positive samples that were reported in current study contained strains that can infect human. This fact shows hazards for human health and needs further studies.

Keywords: Detection, Fish pool, Gillan province, Iran, Norovirus

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Introduction

Norovirus was identified from diarrheic people specimens during an out-break of gastroenteritis in Norwalk in 1929. This was the first viral gastroenteritis that established in human that causative agent was virus. Because of unsuccess in culture of virus in cell culture and define animal modeling, the viruses named as small round structured viruses, the shape that viruses showed in immunoelectron microscopy. The virus was named as norwalk virus too (Robilotti *et al.,* 2015). Norovirus is a genus in the family *Caliciviridae*. Norovirus is one of the important foodborne and waterborne human viral gastroenteritis causing agents and they were determined in most of 90% of nonbacterial gastroenteritis outbreak reports in developed countries. Infection by norovirus has symptoms like watery non-bloody diarrhea, vomiting, nausea, headache, general muscle ache and fever or chills in infected person. The illness lasting 1 to 3 days and norovirus infection is self-limiting (Robilotti *et al*., 2015; Hassard *et al.,* 2017). Transmission of infection with norovirus frequently occur through human-to-human contact indirectly, via contact with contaminated surfaces or consumption of infected food or water (Sharma *et al.,* 2020). The genome of norovirus consists of three open reading frames (ORFs), ORF1 that encodes a polyprotein that by the viral protease fragmented to non-structural proteins, while ORF2 encode major capsid protein and ORF3 encode minor capsid protein (Mattison *et al.,* 2018; Niendorf *et al.,* 2020). Based on complete capsid genes analysis, they are seven distinct genogroups between norovirus. Genogroups GI, GII and GIV contain strains that infect human (Hassan and Baldrige 2019; Niendorf *et al.,* 2020). Whiten different norovirus genogroups GII,4 is a predominant genotype that couse gastroenteritis in most outbreaks of norovirus infections worldwide (Lu *et al.,* 2020; Ai *et al.,* 2021).

Consumption of proteins has a direct relationship with health. Fish culture in pools are one of the certain ways to prepare protein source for human consumption. In spite of the fact of seafood consumption have several health benefits to human, there is a possible risk for transmission of some pathogens such as norovirus (Iwamoto *et al.,* 2010). The contamination of fish with enteric pathogens like norovirus has hazard to human health. Risk factors about contamination of pools with norovirus are low water temperatures and high rainfall that lead to contamination of pool with sewage (Lees, 2010; Atmar *et al*., 2014; Campos *et al.,* 2017). Evaluation of contamination of food to enteric pathogens rely upon the presence of *E. coli* as a bacterial indicator of fecal pollution. However, bacterial indicators are a problematic approach as indicator to enteric viral pathogens, such as norovirus and it may fail to detect viral contamination (Lees, 2010; Hassard *et al*., 2017; McKee and Cruz, 2021). Propagation of norovirus cannot be done in tissue culture properly and this fact couse significant obstacle to the

study of this group of viruses. So, due to lack of the cell culture system, detection of viral pathogens in food is based on molecular detection of virus genome using methods such as PCR or RT-PCR (Ghazali *et al.*, 2018). However, currently, there is not any standard procedures to detection of viral contamination in seafood in Iran. There is a few information about norovirus contamination in fish culture pools and seafood in Iran. Therefore, the goal of this study was detection of contamination of fish in warm water and cold-water fish pools in Gillan province by using RT-PCR method. Gillan is the one of the most important area of production of sea food in north of Iran nearby Caspian Sea.

Material and methods

Sample collection

A total of 50 fish, were collected from 20 fish pools in Gillan province in Iran, during April to July 2020. Ten sample were collected from ten rainbow trout (*Oncorhynchus mykiss*) culture pool, one sample from each pool. Forty sample were collected from ten warm water culture pools. Four sample were selected from each pool including grass, bighead, silver and common carps *(Ctenopharyngodon idella*, *Hypophthalmichthys nobilis*, *Hypophthalmichthys molitrix* and, *Cyprinus carpio)*. Each fish that selected as sample had body weight 1000±300 gr. Each sample, placed in sterile zip lock and transferred to the laboratory while kept them on ice box.

Sample preparation and genome extraction

In laboratory the muscles of abdominal cavity and stomach were dissected and separated and homogenized using sterile equipment. For this purpose, 5-10 gr of separated tissues were homogenized then 5-10 ml sterile PBS were added to achieve intracellular liquid. To remove tissue debrides the achieved liquid were centrifuged at 8000 g for 5 minutes. Then, the supernatant was used as possible source of norovirus genome. The genome of virus was extracted by following instruction of DNA – RNA extraction Mini Kit (Dyna Bio viral, Cat#KI0025, Takapouzist, Iran).

RT-PCR and PCR

To detection norovirus two step RT-PCR were used. First, the cDNA were synthesized from RNA by using random hexamer primers and following protocol of cDNA Synthesis Kit (Cat No: YT4500, Yektayajhiz, Iran). Briefly, the reverse transcription mixture contained 1-5 µg extracted genome, mixed with 1 µL random hexamer primer (50µM) and to 13.4 µL DEPC-treated water. The mixture mixed gently, centrifuge briefly and incubate at 70°C for 5 min. Then chilled on ice, spined down and then the vial back placed on ice. Then 4 µL first-strand buffer (5x), 1 µL dNTPs (10 mM each), 0.5 µL RNasin (40U/µL) and 1 µL M-MLV were mixed gently and centrifuged and incubated for 60 min at 37°C. The reaction was terminated the by heating at 70°C for 5 min. The

cDNA was kept at -70°C till all expected samples were collected. PCR was performed in 20 µL of reaction mixture containing 2 µL of P290 forward and P289 reverse primer pair, 5 µL cDNA, 2.5 µL of 10X PCR buffer, 0.5 µL dNTPs (10 mM each), 0.2 µL Taq DNA Polymerase (5U/µL), and to 20 µL DW.

Confirmed norovirus genome that were kindly prepared from Razi vaccine and serum Institute were used as positive control and Distilled Water as negative control. The thermocycler program included 95°C for 5 min, 40 cycles at 95°C for 30 s, 49°C for 1 min 20 s and 72°C for 1 min and final extension at 72°C for 10 min. (Hassanpour *et al.*, 2021). The amplified products were visualized by standard gel electrophoresis using 8 µL of the PCR products in 2% agarose gel in TBE buffer (89 mM Tris, 89 mM boric acid, 2.5 mM EDTA) and the specific DNA bands were visualized using ethidium bromide staining under UV illumination.

Results

Agarose gel electrophoresis of the amplification product showed the presence of bands of 320 bp fragment for norovirus (Figs. 1 to 3). Therefore, of the total 50 fish samples that were collected from 20 fish pools in Gillan province, Iran, three sample (6%) founded be contaminated with norovirus. One of positive samples were from rainbow trout that is cold water fish and the two other positive samples were from silver carp and common carp that both of them are warm water fish. The two warm water fish were from different pools in which the source of water entered to these pools was different. Table 1 show the samples that collected from 20 fish culture pools.

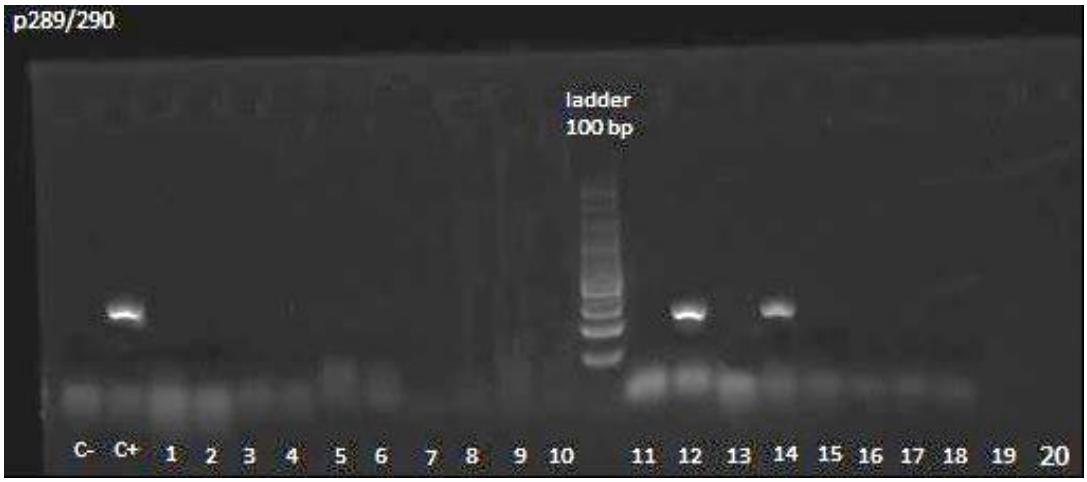

Figure 1: Agarose (1%) gel electrophoresis of PCR product 320 bp for detection polymerase gene of norovirus. C-: negative control, C+: positive control, lanes 12: positive isolates from a warm water fish culture pool and 14: positive isolates from a cold-water fish culture pool. DNA ladder 100 bp were used between lanes 10 and 11.

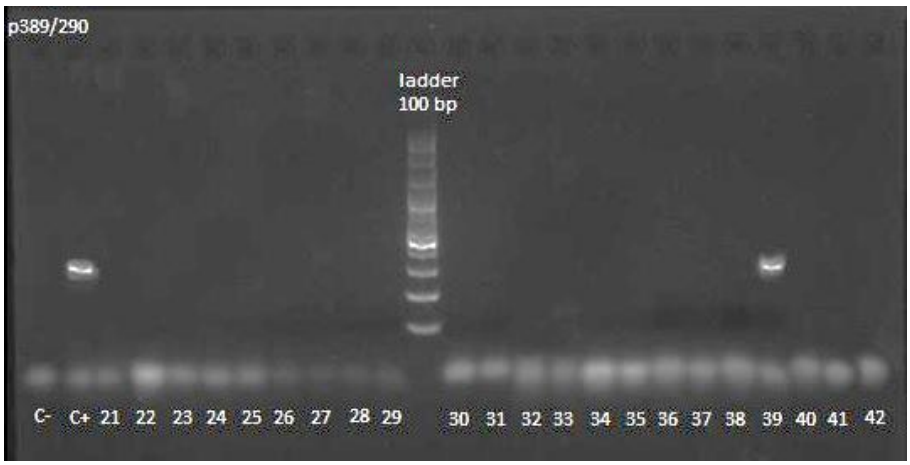

Figure 2: Agarose (1%) gel electrophoresis of PCR product 320 bp for detection polymerase gene of norovirus. C-: negative control, C+: positive control, lanes 39: positive isolates from a warm water fish culture pool. DNA ladder 100 bp were used between lanes 29 and 30.

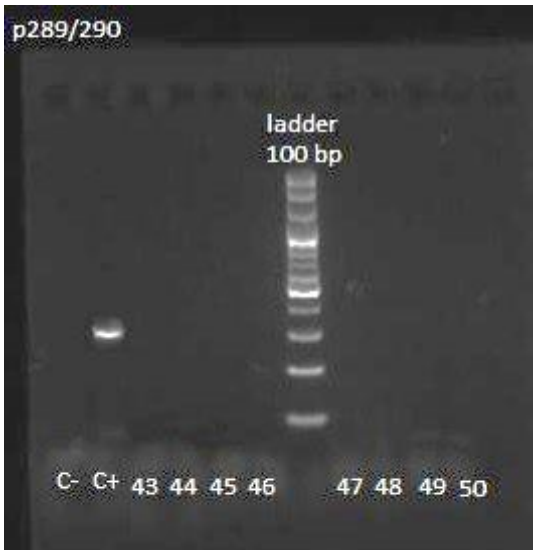

Figure 3: Agarose (1%) gel electrophoresis of PCR product 320 bp for detection polymerase gene of norovirus. C-: negative control, C+: positive control. DNA ladder 100 bp were used between lanes 46 and 47.

Table 1: Samples that collected from 20 fish culture pools.

Samples	Fish culture pool	Number and type of infected fish
1, 2, 3, 4	Warm water fish culture	-
5, 6, 7, 8	Warm water fish culture	-
9, 10, 11, 12	Warm water fish culture	Silver carp (1)
13	Cold water fish culture	-
14	Cold water fish culture	Rainbow trout (1)
15	Cold water fish culture	-
16	Cold water fish culture	-
17, 18, 19, 20	Warm water fish culture	-
21, 22, 23, 24	Warm water fish culture	-
25, 26, 27, 28	Warm water fish culture	-
29, 30, 31, 32	Warm water fish culture	-
33	Cold water fish culture	-
34	Cold water fish culture	-
35	Cold water fish culture	-
36	Cold water fish culture	-

Table 1 (continued):

Samples	Fish culture pool	Number and type of infected fish
37, 38, 39, 40	Warm water fish culture	Common carp (1)
41, 42, 43, 44	Warm water fish culture	-
45, 46, 47, 48	Warm water fish culture	-
49	Cold water fish culture	-
50	Cold water fish culture	-

Discussion

Although 120 enteric viruses have been found in sewage, norovirus are the most common food born virus in the US. Norovirus is environmentally stable viruses and very resistant to inactivation. Norovirus transmitted via the fecal-oral rout to human (Le Guyader *et al*., 2009; Robilotti *et al.,* 2015). Enteropathogenic viruses that present in seafood are at low levels to very low levels compared with clinical samples (Lees, 2010; McKee and Cruz 2021). But, about ten particles of norovirus are enough to causes infection in human. So, regarded to low infectious dose of norovirus, a few particles that presence in fish pools and seafood may have health hazard to human. An infected person or animal may shed virus in environment for three weeks or more and about 30% of carriers may not be symptomatic. Viral shedding may be $>10^7$/g feces (Lees, 2010; Robilotti *et al.,* 2015). At the global scale, food and water related transmission of norovirus is approximately between 11% to 25% (Hassard *et al.* 2017). There is a few information about norovirus contamination in fish pools and seafood in Iran. According to the results of current study total rate of contamination to norovirus in fishes that were cultured in warm water and cold-water fish pools in Gillan province were 6% that the contamination rate was lower than previous study that conducted in Boushehr port (15.6%) and higher than study conducted in Khuzestan province (0%) (Amroabadi *et al.,* 2021; Hassanpour *et al.,* 2021). The reasons of this wide variation between countries may be related to different contamination rates of water in different geographic places, type of sample and sample size and the approach and the season of samples collection and approaches of data analysis, and different laboratory detection methods that used to detect the contamination (Lees, 2010; Hassard *et al.,* 2017).

Detection of contamination of water and food to enteropathogenic agents are based on the detection of *E. coli* as enteric indicator. But there is not direct relation between enteropathogenic bacteria like *E. coli* and enteropathogenic virus like norovirus (Lees, 2010; Hassard *et al*., 2017; McKee and Cruz 2021). Detection of contamination of seafood to viral enteric pathogens needs to develop new standard method. In this standard all steps of detection procedure, should be well defined (Robilotti *et al*., 2015; Hassard *et al.*, 2017). This fact will help to uniformity in detection of

contamination of fish and seafood to norovirus. A number of procedures have been reported for the detection of norovirus in seafood that most of them have been applied to the study of viral contamination of shellfish (Le Guyader *et al.*, 2009; Lees, 2010; Robilotti *et al.*, 2015). But there are a few studies about detection norovirus in fish that are an important portion of seafood. The reason of this fact maybe related to most contamination to norovirus which were reported among shellfish. In England, during 1992 to 2014, among 289 norovirus outbreaks by seafood, out of 120 outbreaks were linked to oysters (Hassard *et al.*, 2017). Shellfish such as oyster due to biologic behavior, filtrate a lot of water to feed and this activity can concentrate a lot of pathogenic agents specially from human fecal source (Le Guyader *et al*., 2009; Robilotti *et al.*, 2015). But there is limited information regarding the prevalence of norovirus in fish culture pools and seafood in Iran. To our knowledge this study is on of pioneer evaluation of contamination of fish and fish culture pools to norovirus in north of Iran. Regarding to this fact that the contamination to human enteric pathogens in fish are lower than shellfish, the current contamination rate (6%) indicates that water that used to aqua culture may be contaminated by waste water or swage and this fact needs to further investigations. There were three positive samples in current study, one in cold-water fish pool and two in warm water fish pools. The two positive samples in warm water fish were from two different pools. So, this result indicated broad distribution of contamination to norovirus in Gillan province aquaculture pools maybe existence.

They are seven distinct genogroups between norovirus. Genogroups GI, GII and GIV contain strains that infect human (Campos *et al*., 2017; Hassan and Baldrige 2019; Niendorf *et al.*, 2020). The primer pair that were used in current study were P290 and P289 that amplified a product about 320 bp in RNA polymerase gene that located in ORF1. This primer pair were designed based on human caliciviruses and were specific to detect norovirus in human (Jiang *et al.*, 1999; Hassanpour *et al.*, 2021). So, all positive samples that were reported in current study contained strains that can infect human.

Although norovirus is the most common and environmentally stable food born viruses and considering low infectious dose of these viruses (Lees, 2010; Robilotti *et al.*, 2015), detection of norovirus in water and seafood like fish are very important. Detection of norovirus in some cold and warm water fish culture pools has health hazard about human health and needs further studies. Prevention of contamination of fish culture pools to swage may be primary way to limit infection by norovirus.

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authors declare that they have no conflicts of interest.

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