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Full Length Research Paper

Detection of *Norwalk virus* in marine foods in Iran

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The objective of this study was to determine the prevalence of *Norwalk virus* in marine foods samples in Isfahan and Shahrekord townships of Iran. From September 2010 to April 2011, a total of 300 samples of fresh fish, shrimp, crab and lobster samples were obtained from randomly selected retail stores in Isfahan and Shahrekord townships of Iran. The samples were tested for the presence of *Norwalk virus* using a Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) method. In this study, out of the total of 300 samples examined, 46 (15.33%) were found to be positive for *Norwalk virus*. This virus was detected in 25, 8.33, 10 and 10% of fresh fish, shrimp, crab, and lobster samples, respectively. This study shows the importance of marine foods as potential sources of *Norwalk virus* infection in people in Iran.

Key words: Iran, *Norwalk virus*, RT-PCR, marine foods.

INTRODUCTION

Viruses, bacteria and parasites can cause acute gastrointestinal infections in humans. One type of virus that causes acute gastroenteritis is a norovirus, previously called a 'Norwalk-like virus (NLV)' or small round structured virus (Hutson et al., 2004).

Norovirus (NoV), a member of the *Caliciviridae* family, is considered the major cause of acute gastroenteritis in adults (Fankhauser et al., 2002; Roche et al., 2002; Meakins et al., 2003) and is estimated to be responsible for 93% of food-related outbreaks of gastroenteritis in the United States (Fankhauser et al., 2002). NoV is associated with sporadic and outbreak cases of gastroenteritis in individuals of all ages, with a distinct seasonality linked to the winter months (O'neill et al., 2002).

Infection is characterized by the acute onset of nausea, vomiting, abdominal cramps, and diarrhea, which generally last for about 48 h (Bull et al., 2006). Transmission occurs predominantly through ingestion of contaminated water; food (particularly oysters); and person to person by the fecal-oral route, airborne transmission, and contact with contaminated surfaces (Iversen et al., 1987; Lahti and Hiisvirta, 1995; Girish et al., 2002; Meakins et al., 2003).

The ease with which NoV is transmitted and the low infectious dose required to establish an infection results in extensive outbreaks in numerous environments, including hospitals, hotels, schools, nursing homes, and cruise ships (Cheesbrough et al., 2000; Marks et al., 2000, 2003; Widdowson et al., 2004).

Food and waterborne NLV outbreaks are common and have been linked to exposure to contaminated vehicles of infection such as salads, celery, shellfish (e.g. raw oysters, clams), sandwiches, swimming water, drinking water and ice (Cannon et al., 1991; Morgan et al., 1994).

Because NLVs cannot be propagated in cell culture or animal models, diagnostic methods have relied on the examination of fecal specimens to visualize viral particles by electron microscopy (EM), detection of the viral genome by using reverse transcriptase-polymerase chain

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reaction (RT-PCR), and measurement of antibody responses in infected individuals (Ando et al., 1995; Lewis et al., 1995; Brown et al., 2001).

PCR assay has become a useful tool for the detection of *Norwalk virus* in food samples because of the low detection limit and high sensitivity. Currently, there is limited information regarding the prevalence of food borne viruses in sea food in Iran, so the objective of the present study was to determine the prevalence rate of *Norwalk virus* in marine foods samples obtained from Isfahan and Shahrekord townships of Iran using a RT-PCR assay.

MATERIALS AND METHODS

Samples

A total of 300 marine foods samples were collected from September 2010 to April 2011 from supermarket and retail outlets in Isfahan and Shahrekord townships of Iran. The marine foods samples analysis comprised samples of fresh fish, crab, lobster and shrimp (Table 1). The samples were transferred to the Food Microbiology Laboratory at the Islamic Azad University of Shahrekord Branch in portable insulated cold-boxes. Samples were analyzed on the day they were collected.

RNA extraction

Two grams of inoculated mussel samples was rocked for 10 min with 1 ml of TRIzol® Reagent. The TRIzol® (Roche applied sciences) Reagent solution that reacted with the sample was kept separately. Another 1 ml TRIzol® Reagent was added to the sample, rocked again for 10 min and was brought together with the previously collected TRIzol® Reagent. The aqueous phase was taken after centrifugation (8000 g, 20 min, and 4°C) and stored by freezing. A volume of 100 µl was purified by the use of a RNeasy Mini kit (Qiagen) according to the manufacturer's instructions (Baert et al., 2007).

Primers

Oligonucleotides were purchased from MWG-Biotech AG (Ebersberg, Germany). Sequences of oligonucleotides for amplifying a fragment of 224, respectively, from the NV (Matsui et al., 1991) were as follows:

M5 (5'- CACCACCATAAACAGGCTG -3') and M3 (5'-AGCCTGATAGAGCATTCTTT -3').

RT-PCR

Synthesis of cDNA was carried out using moloney murine leukemia virus reverse transcriptase (MMLV-RT, Fermentas) and random hexamer primers (Fermentas). Reverse transcription of heat-denatured RNA (5 min at 70°C in 32 µl of reaction buffer for MMLV-RT in the presence of 0.1 mM of each dATP, dCTP, dGTP and dTTP) was performed after addition of 8 µl of reaction mixture (10 mM dithiothreitol, 0.4 µg of random hexamer, 5 U of RNase inhibitor (Fermentas) and 400 U of MMLV-RT) for 5 min at 22°C, 15 min at 37°C and 30 min at 42°C. After reverse transcription, the reactions were heated to 99°C for 5 min in order to inactivate MMLV-RT. Amplification of cDNA by PCR was carried out in a total volume of 50 µl in the reaction buffer for Taq DNA polymerase containing 1.5 U of Taq DNA polymerase (Fermentas), 100 pmol of each primer (M5 and M3), 1.25 mM $MgCl_2$, 0.2 mM dNTP, and 4 µl of cDNA. Amplification was performed by 30 cycles of denaturation at 95°C for 30 s, annealing at 56°C for 1 min and extension at 72°C for 1 min. After amplification, the PCR products were characterized by 1.5% agarose gel electrophoresis in Tris–borate–EDTA buffer (Kingsley and Richards, 2001).

Statistical analysis

Data were analysed using SPSS ver. 16.0 statistical software, a Chi-square test and fisher's exact two-tailled test analysis was performed and differences were considered significant at values of $p<0.05$.

RESULTS

According to results 46 samples of total 300 studied samples found to be infected with Norwalk virus, respectively. The product of 224 bp was obtained, as expected, from RT-PCR amplification of the amplicon encoded portions of *Norwalk virus* strain 8F11a. Number of infected samples and percent of infection are shown in Table 1. A high infection rate to *Norwalk Virus* was found in samples ranging from 8.33% in shrimp to 25% in fresh fish.

DISCUSSION

The term “seafood products” or "fish" includes all fresh or saltwater finfish, molluscan shellfish, crustaceans, and other forms of aquatic animal life. Fish and shellfish are an important part of a healthful diet. They contain high quality protein and other essential nutrients which can be low in saturated fat and may contain omega-3 fatty acids. In fact, a well-balanced diet that includes a variety of fish and shellfish can contribute to heart health and children's growth and development. But, as with any type of food, it's important to handle seafood safely in order to reduce the risk of food borne illness.

Following these basic food safety tips for buying, preparing, and storing fish and shellfish ensures that consumers enjoy fine taste and good nutrition of seafood (FDA, 2007; FSIS, 2007). It is well known that humans can catch illness as a consequence of the exposure to risk factors occurring in water (Hoxie et al., 1997; Proctor et al., 1998; Hunter and Syed, 2001; Fong and Lipp, 2005). Several outbreaks have been documented with reference to the exposure to these risk factors through ingestion of contaminated drinking waters, shellfish and sea foods (Bean et al., 1996; Hlady and Klontz, 1996; CDC, 1998) and crabs (Shuval et al., 1986). Moreover, recreational activities in bathing waters have been associated with some human pathology, as gastrointes-

Table 1. Prevalence of *Norwalk virus* isolated from marine foods in Iran.

Lobster	Crab	Shrimp	Fish
Infected samples (n=40)	**Infected samples (n=20)**	**Infected samples (n=120)**	**Infected samples (n=120)**
4 (10%)	2 (10%)	10 (8.33%)	30 (25%)

tinal and dermatologic infections (Montanari et al., 1999; Henrickson et al., 2001).

In this study, 46 of 300 meat samples (15.33%) were found to be contaminated with Norwalk virus. The highest prevalence of *Norwalk virus* was found in fish (30/120; 25.0%), followed by crob (2/20; 10.0%), lobster (4/40; 10.0%), and shrimp (10/120; 8.33%). There were significant differences ($P < 0.05$) in the level of contamination with *Norwalk virus* between different samples; however, no significant differences ($P > 0.05$) were found between shrimp, lobster and crab. No previous report could be found on the occurrence of *Norwalk virus* on the crab, fish, lobster and shrimp.

Viral gastroenteritis is most usually transmitted directly from person-to-person, and food- or water-borne transmission appears to be responsible for only a small proportion of incidents. Unlike bacteria, viruses do not multiply or produce toxins in foods. Food or water merely acts as vehicles for their passive transfer. The true incidence of food-borne viral transmission is undetermined, but probably grossly under-reported. In a study in Italy by Blasi et al., 2008, most of water related disease outbreaks were described with the general term “shellfish” (53.2%). Actually, out of the 710 notified cases, where the vehicle of infection was precisely defined, 39.2% were attributed to “oysters”, 23.5% to “sea salad”, 13.4% to “clams”, 12.1% to “mussels”, 4.5% to “clams and mussels”, 2.5% to “sponge”, 1.7% to “oysters and clams”, 1.0% to “octopus”, 1.0% to “oysters and warty venus”, 0.7% as “sea urchins” and 0.4% as “warty venus”.

Viruses account for 6% of food-borne outbreaks and 5% of water-borne outbreaks occurring in England and Wales and reported to CDSC. Secondary person-to-person transmission to close contacts is a characteristic feature of food-borne and water-borne virus outbreaks. Several different types of viruses cause gastroenteritis: the most important include rota virus, SRSV or Norwalk group viruses, astrovirus and adenovirus types 40 and 41. However, in almost all food-borne outbreaks, where a virus is identified it is an SRSV. Rotavirus and astrovirus are only rarely implicated. Adenovirus has not been associated with food or water-borne transmission (Appleton, 2000).

Foods may be contaminated with viruses in two main ways. Firstly, they may be contaminated at source in their growing and harvesting areas, usually by coming into contact with polluted water. Shellfish have been a particular problem and have been implicated in many outbreaks world-wide. Secondly, foods may be contaminated during handling and preparation, often from infected food-handlers.

A major factor in the contamination of food and water is sewage pollution. Ideally, there would be no sewage discharge into the coastal waters and rivers, thus preventing contamination of shellfish and fish growing areas. Sewage sludge is applied to agricultural land, with the benefit that useful plant nutrients and organic matter are recycled to the soil. Viruses from sewage deposited on land do not bind with sod particles and can enter ground waters, leading to contamination of water sources.

Also, control of food-borne viral illness largely depends on strict attention to normal good hygienic practice in the kitchen and serving areas. Salad items, fruits and raw vegetables should be washed thoroughly. Cross contamination from uncooked shellfish should be regarded as a potential hazard.

This study shows the importance of fish meat as potential sources of *Norwalk virus* infection in people in Iran.

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