

# Investigation of parasitic and viral pathogens in mussels (*Mytilus galloprovincialis*) in the Gulf of Izmir, Turkey

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## ABSTRACT

**Objectives:** To investigate *Microsporidia spp.* parasite, hepatitis A virus (HAV), and norovirus (NoV) contamination in mussels collected from 8 stations in the inner, middle, and outer regions of the Gulf of Izmir.

**Methods:** In this cross-sectional study carried out between August 2009 and September 2010 in the Gulf of Izmir, Turkey, 15 mussels collected from each of the stations each season were pooled and homogenized to create a single representative sample. Thirty representative samples were available for analysis. Direct polymerase chain reaction (PCR), RT-nested PCR, and RT-booster PCR were used to investigate the pathogens.

**Results:** The mussels were negative for *Microsporidia spp.*, but 8 (26.7%) samples analyzed were positive for HAV and 9 (30%) were positive for NoV. Excluding Foca and Gediz, viral contamination was detected in all of the stations sampled.

**Conclusion:** Our results suggest that viral contamination is present in mussels in the Gulf of Izmir and may pose a potential threat to human health in the region. Necessary measures should be taken to prevent future illness due to these pathogens.

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In recent years, aquatic organisms including mussels are being increasingly used to measure level of marine pollution. Mussels filter the sea water between their shells and accumulate viral, bacterial, and parasitic pathogens in their tissues.<sup>1</sup> *Microsporidium spp.* and hepatitis A virus (HAV) and norovirus (NoV) are among the most important food-borne pathogens that can be transmitted to humans through consumption of mussels. Hepatitis A virus, classified within the *Picornaviridae* family of viruses, can stay alive in seawater for up to 10 months and it is thought that less than 100 copies of the virus is sufficient to cause illness

in humans.<sup>2</sup> The first HAV infection due to ingestion of shellfish was reported in Sweden in 1955 with 629 confirmed cases due to consumption of raw oysters.<sup>3</sup> In China, over 290,000 cases due to consumption of shellfish grown in water contaminated with sewage have been reported.<sup>4</sup> Dangerously, high levels of viral contamination in mussels have recently been reported in Italy.<sup>5</sup> Noroviruses, classified within the *Caliciviridae* family of viruses, is known to persist and retain their infectivity for months in shellfish tissue samples. Outbreaks of acute gastroenteritis due to NoV infection have been reported in many countries.<sup>6</sup> Currently, 5 genogroups (GI-GV) have been reported. Most human infections are caused by genogroups I and II (GI and GII).<sup>7</sup> *Microsporidia spp.* are intracellular eukaryotic parasites, which form small oval spores. Common infections in fish caused by *Microsporidia spp.* have been reported in Turkey as well as in other countries.<sup>8</sup> Data on investigation of parasitic and viral contamination in mussels in Turkey are very limited in the literature.<sup>9,10</sup> The objective of this study was to investigate the *Microsporidia* parasite and HAV and NoV viruses in the black mussel (*Mytilus galloprovincialis*), the most commonly consumed mussel, in different seasons and breeding stations in the Gulf of Izmir, Turkey, using molecular methods.

**Methods. Samples.** Mussel samples were collected from 8 stations in the inner, middle, and outer areas of the Gulf of Izmir, Turkey between August and November of 2009 and February and May 2010.<sup>10</sup> Gills and digestive tissues from 15 mussels collected from each site in each season were removed and pooled individually. Twenty-three gills and 30 digestive tissue pools created using 795 mussels were used in this study. No samples could be taken from Degaj and Gediz stations in February of 2010. This research was approved by the Research Animals Ethics Committee, Adnan Menderes University, Aydın, Turkey.

**Investigation of *Microsporidium spp.*** Small-subunit ribosomal DNA (SSU-rDNA) gene was investigated by direct PCR method using primers (5'-CACCAGGTTGATTCTGCCTGAC-3'), and (5'-CCTCTCCGGAACCAAACCCTG-3'). Approximately one µL sample was examined by Animal Tissue Direct PCR kit (Thermo Scientific, USA). *Microsporidium spp.* grown in culture was used as positive control.

**Sample preparation and RNA extraction for viral analyses.** Sample suspensions were frozen in liquid nitrogen and thawed at 37°C before centrifugation at 10,000 x g for 5 minutes. Viral RNA was extracted

from 140 µL of the supernatant using QiaAmp Viral RNA mini kit (Qiagen, USA). The RNA isolated from stool and serum samples taken from infected patients were used as positive controls, stool for NoV and serum for HAV. No template controls were included in all experiments.

**Investigation of HAV using RT-nested PCR.** The HAV was investigated using a slightly modified version of the RT-nested PCR protocol previously described by De Medici et al.<sup>11</sup> The reaction mixture for reverse transcription included 5 µL RNA, 17 pmol antisense HAV primer (5'-CATATGTATGGTATCTCAACAA-3'), 1 x reaction buffer, 10 mM dNTP, 20 U RNase inhibitor, and 20 U M-MuLV reverse transcriptase. In the first round of PCR, the reaction mixture included 1x reaction buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, one µM HAV-sense (5'-CAGGGGCATTTAGGTTT-3') primer, one µM HAV-antisense (5'-CATATGTATGGTATCTCAACAA-3') primer, and 2.5 U Taq DNA polymerase. A 5 µL of the first PCR amplicons were used as template in nested PCR, which included 1 x reaction buffer, 2 mM MgCl<sub>2</sub>, 0.2 mM dNTPs, 0.4 pmol HAV-nested-sense (5'-TGATAGGACTGCAGTGACT-3') primer, 0.4 pmol HAV-nested-anti-sense (5'-CCAATTTTGCAACTTCATG-3') primer, and 1.5 U Taq DNA polymerase. Amplicon length was 211 bp.<sup>11</sup>

**Investigation of NoV using RT-booster PCR.** Norovirus was investigated using a slightly modified version of the RT-booster PCR protocol previously described by De Medici et al.<sup>12</sup> Access RT-PCR system kit (Promega, Madison, Wisconsin, USA) was used to perform RT-booster PCR. Amplicon length was 326 bp.<sup>12</sup> Reaction mixture for reverse transcription included 5 µL RNA, 5 U avian myeloblastosis virus reverse transcriptase (AMV-RT), 1 x AMV/Tfl buffer, 2 mM MgSO<sub>4</sub>, 0.2 mM dNTPs, 4 µL of 5 µM antisense primer

JV13 (5'-TCATCATCACCATAGAAAGAG-3'), and RNase-free water in a total volume of 45 µL. In the first round of PCR, the reaction mixture included 5 U Taq DNA polymerase, one µL of 5 µM JV13 primer, and one µL of 5 µM JV12 primer (5'-ATACCACTATGATGCAGATTA-3'). The reaction mixture for the booster PCR in the second round included 1 x AMV/Tfl buffer, 2 mM MgSO<sub>4</sub>, 0.2 mM dNTP, 5 U Tfl polymerase, and 2 µL of each of the 5 µM JV13 and JV12 primers in a total reaction volume of 50 µL. The PCR conditions consisted of an initial denaturation at 94°C for 2 minutes followed by 40 cycles at 94°C for one minute, 37°C for 90 seconds, and 68°C for 2 minutes. A final extension step was carried out at 68°C for 7 minutes.

**Results.** All of the mussel samples investigated in this study were negative for *Microsporidium spp.* using direct PCR. Of the 30 samples analyzed, 8 (26.7%) were positive for HAV and 9 (30%) were positive for NoV. Only one of the 23 gill samples (4.3%) analyzed in this study was positive for NoV. All of the remaining gill samples were negative for both of the viruses. Presence of viral contamination varied among the stations (Table 1). Excluding Foca and Gediz stations, all of the sites were contaminated with at least one of the viruses except that the Gediz station was not sampled in February of 2010. Viral contamination was more frequent in the winter. Excluding HAV contamination detected in February 2010 in Inciralti station, HAV was found exclusively in the inner regions of the Gulf whereas NoV contamination was found in the middle and outer regions. Hepatitis A virus was detected in Bayrakli, Bostanli, and Inciralti stations whereas NoV was detected in Degaj, Inciralti, Mordogan, and Gulf of Mersin. Only samples collected in February of 2010 in Inciralti were contaminated with both HAV and NoV. The remaining stations were infected with either HAV

**Table 1** - RT-PCR results from mussel samples collected from 8 stations in 4 seasons and investigated for HAV and NoV. Blank cells represent negative test results.

Bay region	Station	August 2009		November 2009		February 2010		May 2010	
		HAV	NoV	HAV	NoV	HAV	NoV	HAV	NoV
Inner regions	Bayrakli			Positive		Positive		Positive	
	Bostanli	Positive		Positive		Positive		Positive	
Middle regions	Degaj		Positive		Positive	Not Tested	Not tested		Positive
	İnciralti					Positive	Positive		Positive
Outer regions	Foca								
	Mordogan		Positive				Positive		Positive
	Gediz					Not Tested	Not tested		
	Gulf of Mersin						Positive		

RT-PCR - Reverse transcription polymerase chain reaction. HAV - Hepatitis A. NoV - noroviruses

or NoV, but not both. Norovirus was found in Degaj in all seasons except that it was not tested in February of 2010. Samples from the Mordogan station were positive for NoV in all seasons except November of 2009.

**Discussion.** In this study, we investigated HAV and NoV viruses and *Microsporidia spp.* parasites in *Mytilus galloprovincialis*, the most commonly consumed mussels in Turkish coasts, in the Gulf of Izmir, the largest bay in Turkey, in 4 seasons. All samples analyzed in this study were negative for *Microsporidia spp.* Future studies with larger sample size are needed to detect possible parasitic contamination in the Gulf of Izmir. However, viral contamination was detected in all seasons and in all except 2 of the stations sampled. Interestingly, viral contamination was particularly more frequent in the winter season. We detected HAV contamination in 26.7% of the digestive system tissues. These rates are higher than the 3.3% contamination rate detected by Terzi et al<sup>9</sup> using the RT-PCR samples collected from 6 different areas of the coastal Middle Black Sea region in Turkey. Hepatitis A virus infection was frequent in Bostanlı and Bayraklı located in the inner region of the Gulf. It was detected in all seasons in both of the stations except that it was not detected in Bayraklı in August of 2009. No NoV was detected in Bayraklı and Bostanlı regions that were heavily contaminated with HAV whereas it was found frequently in Degaj and Mordogan located at the outer regions of the Gulf.

In our study, 9 of the 30 (30%) digestive tissue samples were positive for NoV by RT-booster PCR. All PCR positive specimens were considered positive for NoV regardless of their genogroups due to genotyping was not performed in our study. Future studies should focus on genetic characterization of NoV in the Gulf to better investigate the source(s) of contamination. A previous study conducted by Yilmaz et al,<sup>13</sup> in Turkey detected 4.5% NoV positivity by RT-PCR in samples collected in Bosphorus in Istanbul. Although based on a limited number of samples, NoV contamination in mussels in the Inciralti and Mordogan stations in our study is interesting. Inciralti is one of the main mussel processing sites in Izmir and Mordogan is a popular resort visited by many tourists in the summer. The area sampled in Mordogan was in only 5 minutes driving distance to the city center. Food and waterborne transmission of NoV in humans is common and different epidemiological studies have implicated NoV in outbreaks linked to shellfish consumption.<sup>6</sup> In our study, both viruses were detected only in digestive tissues except that a gill sample was positive for NoV in February of 2010. Digestive tissue sample from

that mussel with the positive gill sample was also contaminated with the virus. Norovirus and HAV infections after consumption of raw, or undercooked seafood have been reported.<sup>14</sup> Even cooking seafood may not be sufficient to prevent disease. Seafood cooked using the standard cooking techniques may still be infectious. Therefore, the mussels must be subjected to methods such as depuration known to be effective in removing viral contaminants.<sup>15</sup>

In conclusion, mussels in the Gulf of Izmir in Turkey are heavily contaminated with HAV and NoV. Viral contamination was detectable in every season and in all but 2 of the stations sampled. Therefore, consuming raw or undercooked mussels from the Gulf of Izmir can result in illness. Necessary measures need to be taken to prevent this potentially serious public health risk.

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