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Research Article

Investigation of Rotavirus, Adenovirus and Astrovirus in Mussels and Shrimps Using Multiplex Real-time PCR

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Abstract

Viruses are one of the most common pathogens transmitted via food. Based on epidemiological evidence, human enteric viruses are considered to be as the most commonly transmitted etiological agents by bivalve shellfish. In Istanbul, the most widely consumed sea products after fish are mussels and shrimps. In this study, the presence of rotavirus, adenovirus and astrovirus were investigated in mussel and shrimp samples consumed by hunting on the shores of Istanbul. For this purpose, a total of 28 groups of shrimp and 52 groups of mussel were collected from different places in Istanbul. Each group was analyzed separately as a sample. Viruses were extracted from the digestive tissue by direct elusion method in a glycine/NaCl, pH 9.5 buffer followed by PEG-6000 precipitation. Multiplex Real-time RT-PCR technique was used to analyze the shellfish samples. Astrovirus was found in 63.46% of the total 52 analyzed mussel samples, whereas adenovirus was found in 46.15%. None of the mussel samples was positive for rotavirus. All tested shrimp samples were negative for all three researched viruses. This is the first report on the prevalence of rotavirus, adenovirus and astrovirus in mussels and shrimps in Turkey. The results show that mussels sold in Istanbul are highly contaminated with adenovirus and astrovirus and this could pose a great threat to public health.

Keywords: *Mussel, Shrimp, Rotavirus, Adenovirus, Astrovirus, RT-PCR*

Midye ve Karideslerde Rotavirus, Adenovirus ve Astrovirus Varlığının Multiplex Real-time PCR Kullanılarak Araştırılması

Öz

Viruslar gıda vasıtasıyla en yaygın bulaşan patojenlerden biridir. Epidemiyolojik bulgulara göre insana ait enterik viruslar kabuklu deniz ürünleri vasıtasıyla en çok aktarılan etiyolojik ajan olarak tanımlanmaktadır. İstanbul'da balıktan sonra en çok tüketilen deniz ürünleri midye ve karidestir. Bu çalışmada, İstanbul kıyılarında avlanarak tüketilen midye ve karides numunelerinde rotavirus, adenovirus ve astrovirus varlığı araştırılmıştır. Bu amaçla toplam 28 karides ve 52 midye grubu İstanbul'un farklı yerlerinden toplanmıştır. Her bir grup ayrı bir numune olarak analize alınmıştır. Viruslar glisin/NaCl, pH 9.5 tamponu kullanarak direkt elüsyon metodu ile doğrudan sindirim dokusundan ayrıştırılmış ve PEG-6000 ile konsantre edilmiştir. Alınan midye ve karides numuneleri Multiplex Real-time PCR yöntemi ile analiz edilmiştir. Toplam çalışılan 52 midye numunesinin %46.15'inde adenovirus ve %63.46'sında astrovirus saptanmıştır. Rotavirus hiçbir midye numunesinde tespit edilmemiştir. Karides numunelerinin hiçbirinde araştırılan virusların hiçbiri saptanmamıştır. Bu, Türkiye'deki midyelerde ve karideslerde rotavirus, adenovirus ve astrovirus prevalansı hakkındaki ilk rapordur. Sonuçlar, İstanbul'da satılan midyelerin adenovirus ve astrovirus ile kontamine olduğunu ve bu durumun halk sağlığı için büyük bir tehdit oluşturabileceğini göstermektedir.

Anahtar sözcükler: *Midye, Karides, Rotavirus, Adenovirus, Astrovirus, RT-PCR*

INTRODUCTION

Viral contamination of food and water represents a significant threat to human health [1]. Viruses are now recognized as a major cause of food-borne diseases and in recent years the incidence of such diseases has been increasing worldwide. Over 100 virus species which cause a wide variety of illnesses in humans may be present in sewage

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contaminated waters [2]. Water quality and poor hygiene practices are the key factors having the highest influence on fresh produce contamination [3].

Diarrhea is the second leading cause of death in children under five years old worldwide and is held responsible for around 525000 child deaths annually [4]. Different enteric viruses like group A rotaviruses, adenoviruses, astroviruses and caliciviruses may infect children in the early childhood phase and cause gastroenteritis [5]. Moreover, several studies have described human adenoviruses (HAdV) as the third causative agent of acute gastroenteritis in infants and young children right after rotaviruses and noroviruses [6].

Rotaviruses are found in waste water and can also be concentrated by shellfish [7,8]. Human astrovirus is a significant cause of acute diarrhea among children. Fresh products such as shellfish, infected water, lettuce, green onions and other green vegetables, soft berries such as strawberries and raspberries are among the susceptible foods that can be contaminated with human astrovirus in the pre-harvest stage [9]. Adenoviruses are associated with a variety of clinical diseases involving nearly every organ of the human body. Adenoviruses can be transmitted through the fecal-oral route and infected food and water intake [10]. In a study on the prevalence of multiple viral agents in patients with upper respiratory tract infection, adenovirus was found in 11 samples (6.4%) of the total 171 patients tested [11]. Adenoviruses can also be transmitted during food processing. In a study on the presence of adenovirus genomes, it was reported that 5.8% of 291 swabs contained the adenovirus genome, particularly in restaurants and canteen kitchens. These findings indicate that kitchen surfaces can lead to viral contamination and that food workers should be educated on virus transmission [12]. Enteric HAdV has also been reported to be very common in aquatic environments [13-15].

In spite of the fact that foodborne diseases are a notable problem, testing of foods for viral contamination is done infrequently. The most used methods for clinical diagnosis are electron microscopy, passive particle agglutination tests or enzyme-linked immunosorbent assays (ELISA). Nonetheless, these methods do not possess a sensitivity high enough in order to be used to recognize viral particles usually present in low numbers in environmental samples. Therefore, methods based on the amplification of viral nucleic acids by polymerase chain reaction (PCR) have been applied to an increasing extent for the detection of viruses in water and food samples [16].

Virus detection in shellfish has never been a trouble-free process. It is to be expected that viruses are present in very low numbers in shellfish tissue, which nevertheless are still enough to cause an infection. Special methodologies should be used in order to achieve a highly efficient virus recovery from shellfish tissues. Additionally, when viruses are extracted from shellfish tissues, the resulting extracts are highly cytotoxic because inhibitory substances are concentrated and recovered along with the viruses. Shellfish extracts will often cause inhibition to the molecular assay, especially if PCR based methodologies are to be used. Consequently, the ultimate objective would be the development of new procedures for shellfish analysis which result in a low volume of none-cytotoxic or highly pure nucleic acid preparation with no inhibitory effects to the PCR. Molecular techniques, especially RT-PCR, serve as excellent tools for the detection of health-significant viruses in food and environmental samples [17].

In the Turkish Food Codex Microbiological Criteria Regulation, acceptable limits of *E. coli* and *Salmonella* for live bivalve molluscs; acceptable limits of Histamine, *Salmonella* and *L. monocytogenes* for processed bivalve molluscs and shellfish have been defined. There are no regulations for virus contamination in the Turkish Food Codex [18]. There is also no official system of monitoring of the virus for bivalve molluscs and shellfish; in addition, there are very few scientific studies on this subject in Turkey. Although most cases of foodborne illness caused by enteric viruses, there is a lack of knowledge about sources of enteric viruses and outbreak reports.

The research about the presence of norovirus in shellfish was carried out in Turkey [19]; but there is no study of adenovirus, rotavirus and astrovirus. For this reason, in this study, the prevalence of rotavirus, adenovirus and astrovirus in mussles and shrimps that are frequently consumed in Istanbul has been researched.

MATERIAL and METHODS

Materials

Sampling

A total of 80 shellfish samples (52 Mediterranean black mussels *[Mytilus galloprovincialis]* and 28 deep sea pink shrimps *[Parapenaeus longirostris]*) were collected in April 2017 from three different locations in Istanbul before the end of the fishing season. The shellfish were collected live, fresh and unfrozen. Additionally, it was made sure that no chemical substances nor additives were added to the shellfish. Each sample contained either 400 g shrimp or 25 mussel pieces. Sampling quantity and location is shown in *Table 1*.

Immediately after collection, shellfish were placed in sterile blender bags (Stomacher® bags) and transported under cold conditions to the laboratory.

Methods

Analyses were carried out at Istanbul Aydin University Food Processing Laboratory and Intertek Test Laboratories (Yenibosna/Bahcelievler/Istanbul).

Table 1. *Sampling quantity and location*

Sample Type	Quantity	Groups Analyzed (n)	Sampling Location
Shrimp	8 kg	20	Beylikduzu - Gurpinar Seafood market
Shrimp	3.2 kg	8	Buyukcekmece - Mimarsinan Fishers port
Mussel	500 pieces	20	Beylikduzu - Gurpinar Seafood market
Mussel	350 pieces	13	Rumeli Kavagi - Fresh fish selling shops
Mussel	500 pieces	19	Beyoglu - Restaurants

Shellfish Processing for Virus Concentration

After arrival at the laboratory, shellfish were washed with sterile distilled water and shucked with a sterile knife. It has been reported that most viruses are found in the highest concentrations in the stomach and digestive diverticula of shellfish [17]. Using these organs for virus analysis makes the isolation of viral nucleic acids easier and faster and also increases the sensitivity of the molecular test by increasing the number of shellfish analyzed [17,20]. Therefore, the digestive tissues were dissected for analysis under aseptic conditions and used for virus isolation. For each sample 18-22 g of shellfish digestive tissue were extracted. For each 18-22 g sample a total of 400 g of shrimp or 25 mussel pieces were processed. Viral particles were eluted by the methods described below.

Virus Elusion and Concentration

Direct glycine elution and virus concentration was applied according to the method by Kingsley and Richards [21] with minor modifications. First, viral particles were eluted from shellfish tissue using glycine buffer (0.1 M glycine and 0.3 M NaCl, pH 9.5) (Glycine GR for analysis-Merck Millipore-Germany). Later, PEG-6000 (Polyethylene Glycol 6000-Merck Millipore-Germany) was used for the concentration of viruses.

For direct elusion, 18-22 g of shellfish digestive tissue sample was homogenized with 175 mL of glycine buffer (0.1 M glycine and 0.3 M NaCl, pH 9.5) in a blender for 3 min at maximum speed. Then, 40 mL of the homogenate were centrifuged at 7000 x g for 30 min at 4°C.

Virus particles present in the supernatant were concentrated using PEG-6000. A 16% PEG-6000 and 0.525 M NaCl solution was added to an equal volume of the supernatant and left for one hour on ice for precipitation. After precipitation, samples were centrifuged at 7000 x g for 10 min at 4°C. Resulting pellets were diluted in 400 µL of distilled nuclease free water and stored at -30°C to be used later for virus nucleic acid isolation.

Isolation of Viral DNA/RNA

Viral nucleic acid extraction was performed using a commercial DNA/RNA kit (InnuPrep Virus DNA/RNA Kit-AnalytikJena-Germany) which is an extraction kit for isolating viral DNA and RNA at the same time and from the same sample. The isolation procedure was carried out based on the manufacturer's instructions. For isolation 400 µL of sample solutions containing the concentrated viruses prepared in the previous step were used. Standard precautions were applied during handling of samples in order to reduce the probability of contamination. The isolation procedure combines lysis of starting material with subsequent binding of viral nucleic acids onto the surface of a spin filter membrane. After several washing steps the viral nucleic acids are eluted from the membrane using RNase-free water. The extracted viral nucleic acids are suitable for use with applications like PCR, real-time PCR or any kind of enzymatic reaction.

Before the molecular analysis, the resulting nucleic acid quantity was measured for each sample using a spectrophotometer (Spectrophotometer for Life Science, Shimadzu Corporation Analytical & Measuring Instruments Division, Japan). In order to prevent PCR inhibition and to acquire accurate results, a dilution step was applied to samples with a DNA/RNA concentration higher than 40 ng/µL.

Multiplex Real-time PCR

Real-time PCR assay was performed using the PowerChek Adeno/Astro/Rota Real-time PCR Kit (Kogene Biotech-Korea) according to the manufacturer's instructions. The kit contains the primer/probe mix (Adenovirus specific primer and probe, Astrovirus specific primer and probe, Rotavirus specific primer and probe, IC specific primer and probe and DNA for IC), RT-PCR Reaction Buffer (containing dNTPs and MgCl2), RT Enzyme Mix (One-step RT-PCR Enzyme Mix), Exogenous Positive Control (EPC) and Adeno/Astro/Rota Virus positive control. The Internal Control (IC) allows the user to determine and control possible PCR inhibition. The IC reagents are built in the Primer/Probe Mix. Also, it needs not to be run separately. The IC is introduced into each amplification reaction and is co-amplified with target DNA from specimen. The Real-time RT-PCR test includes cDNA synthesis by reverse transcriptase and PCR amplification using *Taq.* DNA polymerase and specific primer and probe labeled with the fluorescent dye in a single tube. The kit is suitable for detection of the target gene region without requirement for any additional reagents between the reverse transcription and PCR amplification steps, which minimizes contamination risk for the samples.

The PCR test was performed in optical grade 96-well plates. The required PCR reaction mix was prepared based on the number of wells available in each PCR cycle. Each well was filled with 15 µL of PCR reaction mix and 5 µL of extracted sample (total 20 µL). For the positive control (C+), 5 µL of the positive control available in the kit was used instead of the sample and 5 µL of nuclease-free water was used for the negative control (NTC). RT-PCR runs were performed on a real-time thermocycler (Agilent Technologies-USA, Mx3005P). Reaction conditions were as follows: 30 min at 50°C, 10 min at 95°C, followed by 45 cycles of 15 seconds at 94°C and 60 seconds at 55°C as recommended by the manufacturer. According to the information given by the kits manufacturer company, test sensitivity is 100-1000 copies limit of detection (LOD) and specificity is 100% exclusivity for about 35 non-target strains.

After preparation of the reaction mix and addition of isolated viral nucleic acids to the reaction tubes, the target sequences of adenovirus, astrovirus and rotavirus specific genes and the Internal Control (IC) were detected through the FAM, VIC (HEX), ROX and Cy5 channels respectively.

RESULTS

Of the total 52 tested mussel sample groups (1350 pieces), 24 groups (46.15%) contained adenovirus and 33 groups (63.46%) contained astrovirus while rotavirus was not detected in any of the tested mussel samples. Additionally, it was determined that 40.38% of analyzed mussels groups (21 groups) contained both astrovirus and adenovirus. Rotavirus, adenovirus and astrovirus were not detected in any of the analyzed shrimp samples.

The distribution of adenovirus (AdV) and astrovirus (AsV) in mussel samples based on sampling location is shown in *Table 2*.

The obtained Ct values during the PCR run were used to establish a standard curve. A different color was specified

Table 2. *Adenovirus (AdV) and astrovirus (AsV) in mussel samples based on sampling location*

Mussel Samples		**AdV Positive**		**AsV Positive**		**AdV & AsV Positive**	
Sampling Location	**No. of Groups**	**N***	**%**	**N**	**%**	**N**	**%**
Gurpinar Seafood market	20	17	85	19	95	16	80
Rumeli Kavagi	13	5	38.46	12	92.31	4	30.77
Beyoglu	19	2	10.53	2	10.53	1	5.26
Total	52	24	46.15	33	63.46	21	40.38

N: Number of positive samples

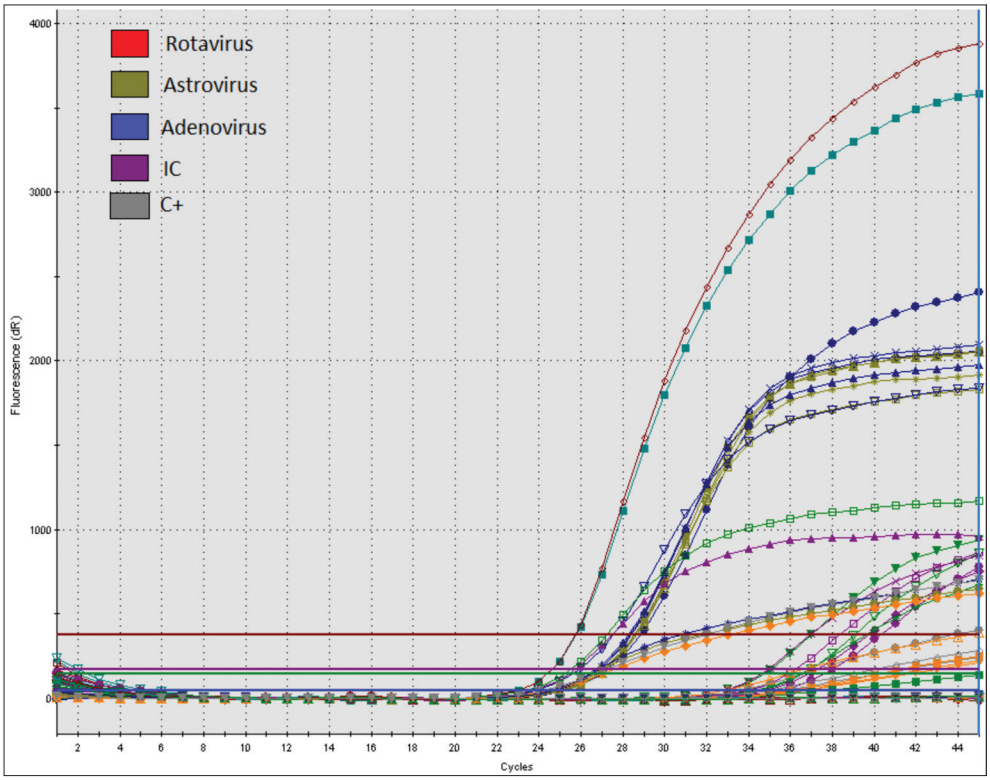

Fig 1. PCR amplification plots

for each of the researched viruses as well as for the internal control (IC) and positive control (C+). The resulting PCR amplification plots were depicted on a diagram as shown in *Fig.1*.

DISCUSSION

As a result of eating raw or undercooked fish and other seafoods, it is possible for consumers to become infected with zoonotic diseases (parasitic, bacterial and viral). Extracting more than 100 types of enteric viruses in their feces, seafoods, especially shellfish, are considered to be the main contamination source of enteric viruses in humans. Fish, crabs, clams, mussels, shrimps and oysters living in sewage contaminated waters have been shown to carry bacteria and viruses of enteric origin. Based on experimental studies, enteric viruses were present in the structures of fish and mussels feeding on lobster, sand worms and other residuals. Especially oysters and mussels are of big importance because these types of seafoods can be consumed raw or half-cooked [22].

Enteric viral pathogens like adenovirus, astrovirus, norovirus and hepatitis E virus (HEV) are health significant viruses and may be associated with the consumption of contaminated water or shellfish. During their filter feeding process, shellfish tend to concentrate viruses and bacteria in their edible tissues and the concentrations on these microorganisms are expected to be much higher in shellfish than in the surrounding waters [2].

In the study by Le Guyader et al.[23], astrovirus was detected in 50% of the analyzed mussel samples. Elamri et al.[24] tested the prevalence of multiple enteric viruses in shellfish samples collected from different locations in Tunisia. Astrovirus was found in 61% of the total analyzed mussel and clam samples. In a study in south Italy by Fusco et al.[25], the prevalence of different enteric viruses in Mediterranean mussels *(Mytilus galloprovincialis)* has been researched. In the course of two years, 108 mussel groups (25 groups in 2014 and 83 groups in 2015) were collected and analyzed using Real-time RT-PCR. Among the researched viruses, astrovirus was found in 16% of the first mussel group collected in 2014 and in 32.53% of the second group collected in 2015. In present study, astrovirus was found in 63.46% of the total 52 tested mussel samples which is higher than the results of Le Guyader et al.[23], Elamri et al.[24] and Fusco et al.[25].

Adenovirus was found positive in 18.6% of the total 86 mussel samples collected from the Norwegian coast [26]. Formiga-Cruz et al.[27] tested the prevalence of enteric viruses in *(Mytilus edulis)* mussel samples in a wide range study conducted in many geographical regions throughout Europe. Adenovirus was found in 33% of 144 samples collected from Greece, in 33% of 54 samples collected from Sweden and in 36% of 104 samples collected from Spain. These ratios are relatively low when compared to the results of this study. In this study adenovirus was found in 46.15%.

For the 173 samples collected from England adenovirus was found positive in 46% which matches the results of this study. Muniain-Mujika et al.[28]. researched the prevalence of human pathogenic viruses in Mediterranean black mussels *(Mytilus galloprovincialis)* and clams *(Crassostrea gigas)*. While hepatitis A virus was found in 24% of the analyzed samples, adenovirus was found in 47%. This result is almost the same with the result of this study in which adenovirus was found in 46.15%.

The results of this study show that as high as 85% of mussel samples collected from Gurpinar seafood market in Beylikduzu were positive for adenovirus, 95% were positive for astrovirus and 80% contained both adenovirus and astrovirus at the same time. In addition, while 38.46% of mussel samples collected from Rumeli Kavagi contained adenovirus, as high as 92.31% contained astrovirus and 30.77% contained both adenovirus and astrovirus. This is an indicator of the high levels of contamination of mussels collected and sold in Beylikduzu and Rumeli Kavagi counties. On the other hand, only 10.53% of mussel samples collected from Beyoglu county contained adenovirus and 10.53% as well contained astrovirus which is relatively low compared to other sampling locations.

In present study, all of the tested shrimp samples were negative for all researched viruses. In a study on shrimps, the biological accumulation of adenovirus in pink shrimps collected from south Brazil was researched by Luz et al.[29]. One hundred pink shrimp samples *(Farfantepenaeus paulensis)* were collected between April 2012 and May 2013 and examined using Real-time qPCR for the prevalence of multiple types of adenovirus. 35% of the total analyzed samples contained various types of adenovirus (Avian adenovirus %17, Bovine adenovirus %13, Canine adenovirus 7% and Porcine adenovirus %2) [29]. However, and in a similar way to this study, none of the tested shrimp samples contained human adenovirus. In a different case, 20 shrimp samples *(Penaeus monodon)* were collected from the southwestern shores of India and analyzed using RT-PCR method for the prevalence of enterovirus, adenovirus, norovirus and hepatitis A virus. While only 15% of the tested samples contained enterovirus, none of them contained neither adenovirus nor any of the other researched viruses [30].

Differently than mussels, shrimps are usually collected from deep places in the sea. Mussels normally live close to rocky seashores and are collected from such locations. Wastewater, which is the main contamination source of enteric viruses, is discharged close to seashores. Therefore, it is to be expected that mussels will be more contaminated than shrimps. In the highly populated city of Istanbul, the amount of sewage discharge is expected to be extremely

high. Because Istanbul is one of the most crowded cities in the world, high contamination of seashores can be the reason for the high contamination ratios of mussels that were found in present study.

Foodborne viruses continuously show high resistance to environmental conditions and are always posing a threat to our health. Such viruses are transmitted mainly through food and the fecal-oral route and can cause different diseases such as gastroenteritis and diarrhea. The prevalence of enteric viruses has been detected in seafoods in different studies around the world. On the other hand, there are few studies related to enteric viruses in mussels and shrimps in Turkey. Erol et al.[31] investigated hepatitis A virus (HAV) and norovirus (NoV) contamination in mussels collected from 8 stations in the gulf of Izmir between August 2009 and September 2010. In this study, it was determined that mussels in the Gulf of Izmir in Turkey were heavily contaminated with HAV and NoV; 9 of the 30 digestive tissue samples (30%) were positive for NoV and 8 samples (26.7%) were positive for HAV by direct PCR, RT-nested PCR and RT-booster PCR [31]. These rates are higher than the contamination rate detected in the study by Terzi et al.[32]. In the study by Terzi et al.[32], RT-PCR was used to analyze 60 mussel samples collected from the middle Black Sea region in Turkey for the prevalence of HAV which was detected only in two samples [32]. A previous study conducted by Yilmaz et al.[19] in Turkey researched NoV (Genogroup I and II) positivity by RT-PCR in mussel samples. For this study, a total of 320 mussels were collected from fish distributors and samples were pooled. NoV Genogroup II was detected in 5 (4.5%) of 110 mussel pools collected from the Bosphorus, Istanbul, Turkey [19]. There is no research on other enteric viruses in mussels and shrimps in Turkey. Since there is limited scientific data on the prevalence of enteric viruses in shellfish in Turkey, further research is needed.

Mytilus galloprovincialis (Mediterranean black mussel) is widely available in Turkey and mostly consumed as stuffed and fried mussels. Stuffed mussels are sold as ready-to-eat foods by most street sellers in Istanbul. It is known that hygienic and storage conditions are not sufficiently taken into account during their production and/or sale and that microbiological qualities are generally low. Therefore, consuming raw, undercooked and cross contaminated mussels can result in illness. In this sense, taking necessary measures during obtaining, processing and selling of raw black mussels is very important in terms of public health and food safety.

In the present study, adenovirus and astrovirus were detected in high ratios in mussels. This is an indicator of the high level of contamination of mussels sold in Istanbul with these viruses and possibly other enteric viruses. This study and a few other studies aforementioned in Istanbul show that increasing the awareness of food workers and restaurants about the correct handling and cooking of shellfish is of great importance from the perspective of food safety.

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