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Original Article
Özgün Araştırma

Detection of rotavirus in raw and ready-to-eat food samples

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Abstract: Rotaviruses are the common cause of acute gastroenteritis. Since the transmission is generally occurs person-to-person by fecal-oral route, the importance of foodborne transmissions can be underestimated. Food can be contaminated with rotavirus at any stage of food process, from farm to fork. In this study, 105 samples were collected from seafood (mussels, fish, etc.), red meat (sausage, meatballs, etc.) and poultry meat products (turkey ham, wings, etc.). The samples were tested for the presence of rotavirus by RT-PCR. Cell culture based virus isolation methods carried out for the positive samples. DNA sequencing and phylogenetic analysis were performed for the genotyping of positive samples. Rotavirus was detected in 2 mussel sample groups by RT-PCR. DNA sequence analysis was carried out of both VP4 and VP7 gene regions of the rotavirus for one sample and determined that it was a group A human rotavirus G1P[8]. The rotavirus isolation did not occur after the inoculation the samples to cell culture. This study demonstrates the presence of rotavirus in raw mussel samples on market.

Keywords: Foodborne diseases, Genotype, MA-104, Rotavirus, RT-PCR,

Çiğ ve tüketime hazır gıda örneklerinde rotavirusun tespiti

Özet: Rotaviruslar, akut gastroenterit olgularının yaygın bir nedenidir. Rotavirus infeksiyonlarında bulaşmanın genellikle insandan insana fekal-oral yolla gerçekleşmesi sebebiyle gıda kaynaklı bulaşmaların önemi gözardı edilebilmektedir. Gıdalar, çiftlikten sofraya kadar geçen sürecin herhangi bir aşamasında rotavirus ile kontamine olabilmektedir. Bu çalışmada deniz ürünleri (midye, balık vb.), kırmızı et (sosis, köfte vb.) ve kanatlı et ürünlerinden (jambon, kanat vb.) 105 örnek toplanmıştır. Örnekler, RT-PCR ile rotavirus varlığı açısından test edilmiştir. Pozitif örneklerden virus izolasyonu için hücre kültürüne ekim gerçekleştirilmiş ve örneklerin genotiplendirilmesi için DNA dizilimi ve filogenetik analiz yapılmıştır. RT-PCR ile 2 midye örnek grubunda rotavirus tespit edilmiştir. Bir örnek için rotavirusun hem VP4 hem de VP7 gen bölgelerinden DNA dizi analizi yapılmış ve tespit edilen virusun grup A insan rotavirus G1P[8] olduğu belirlenmiştir. Örneklerin hücre kültürüne inokulasyonundan sonra rotavirus izolasyonu gerçekleşmemiştir. Bu çalışma, çiğ midye örneklerinde rotavirus varlığını ortaya koymaktadır.

Anahtar kelimeler: Gıda kaynaklı hastalıklar, Genotip, MA-104, Rotavirus, RT-PCR

Introduction

Acute gastroenteritis is an illness that affects people worldwide and rotavirus is one of the most common agent that causes this condition. Rotavirus infections affect people of all ages with mild symptoms in adults, but young children are affected severely. According to estimates, rotavirus infections caused diarrhea that led to the deaths of 215.000 children under the age of 5 worldwide in 2013 and 37% of diarrhea-related deaths in children under the age of 5 in 2008 (Tate et al., 2012). Children aged 4-23 months are in the risk group for severe rotavirus infections, which may include hospitalization or death. Repeated infections often occur due to insufficient immunity at this age. Immunity against rotavirus gradually increases with each subsequent infection, and therefore symptoms get milder in reinfections (Velazquez et al., 1996; Gladstone et al., 2011). There is no significant difference reported in incidence of rotavirus infections between developed and developing countries. However, mortality rates in developing countries are much higher than those in developed countries. It is reported that more than 80% of fatal rotavirus infections occur in developing countries with poor hygiene, sanitation and malnutrition (Parashar et al., 2003; 2006).

Person-to-person transmission is a common way of rotavirus infections via the fecal-oral route. Additionally, foodborne transmission plays an important role in outbreaks. Foods can be contaminated with rotaviruses by the use of sewage polluted water for irrigation or washing, results in contamination of vegetables in the field. Harvest of seafood from contaminated areas is also one of major risk. Meat can be contaminated with rotavirus in slaughterhouse if precautions are not taken. In addition,

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food can be contaminated with rotavirus in restaurants, canteens, and food courts by foodhandlers; it has been reported that food handlers are the most important source of such contamination (Koopmans and Duizer, 2004).

Foods act as a transport vehicle until they deliver the viruses to the target host (Jaykus, 2000). Putrefaction, deterioration or color changes are not observed in foods contaminated with viruses, as in bacterial contaminations. Therefore, it is not possible to perform a sensorial pre-examination of virus-contaminated food before consumption (Richards, 2001; Hasoksuz, 2008). Thus, when even foods with a high viral load consumed, no particular difference can be observed from safe food.

The aim of this study was to investigate the presence of rotavirus in ready-to-eat or raw food samples of animal origin by RT-PCR. Positive samples were subjected to cell culture-based virus isolation study and sequenced to determine the genotype of the circulating virus.

Materials and Methods

Samples and positive control

A total of 105 raw and ready-to-eat food samples were collected (Table 1) in Istanbul, exceeding the minimum required sample size for the study parameters of a 95% confidence level, 10% margin of error. The samples were brought to the laboratory with their original packages or in a sterile plastic bag in cold chain.

Bovine rotavirus B223 strain was used as positive control in molecular and cell culture based virus isolation studies.

Table 1: Raw and ready-to-eat food samples tested in the study.

Red Meat	Qty.	Seafood	Qty.	Poultry Meat	Qty.
Ground meat from butcers	5	Raw fish (internal organs)	5	Spicy raw chicken wings	5
Packaged ground meat from supermarkets	5	Raw mussels	10	Raw chicken	5
Packaged meatballs from supermarkets	5	Internal organs of frozen fish from supermarkets	5	Raw chicken wings	5
Sudjuk	5	Packaged mussel from supermarkets	5	Schnitzel	5
Salami	5	Raw squid from restaurants	5	Turkey Ham	5
Sausage	5			Sausage	5
Raw kebab from restaurant	5			Salami	5
				Turkey sausage	5
Total	**35**		**30**		**40**

Homogenization

The samples were dissected with sterile disposable scalpels, diluted with PBS in a sterile plastic bag and homogenized with stomacher (Seaward Stomacher 400C) at 260 RPM for 20 min. The supernatant was transferred to 50 ml tubes and centrifuged with 4000 rpm at 4°C for 35 minutes, then supernatant transferred to 15 ml tubes in duplicate, one was stored at -20°C for molecular studies and the other at -80°C for cell culture-based isolation studies.

Molecular diagnose

RNA isolation was performed by using Roche High Pure Viral Nucleic Acid Kit (Cat. no:11858874001) according to the manufacturer's instructions. RT-PCR used to detect the VP4 and VP7 gene regions of rotavirus by using 'Con3/Con2' (Gentsch et al., 1992) and 'sBeg9/End9' (Gouvea et al., 1994) primers (Table 2). For each sample, 5µl of the isolated RNA product including 0.8 µl DMSO, 0.6 µl forward and reverse primers was incubated in a dry block heater at 94°C for 5 minutes, and then kept on ice for 2 minutes. After that cDNA synthesis carried out by using Promega Reverse Transcription System (Cat. No: A3500) according to manufacturer instructions. Promega, GoTaq® G2 Flexi DNA Polymerase (catalog no: M7805) kit used for PCR applications. The PCR conditions are shown in Table 3 and 4 for amplification of VP4 and VP7 genes. Agarose gel electrophoresis carried out to visualise the DNA fragments.

Table 2. Primers used for molecular diagnosis.

Gene Region	Primer	Sequence	Lenght (bp)
VP4	Con3	TGG CTT CGC CAT TTT ATA GAC A	877
	Con2	ATT TCG GAC CAT TTA TAA CC	
VP7	sBeg9	GGC TTT AAA AGA GAG AAT TTC	1062
	End-9	GGT CAC ATC ATA CAA TTC TAA TCT AAG	

Sequencing

PCR amplicons were visualized by agarose gel electrophoresis and purified by Roche High Pure PCR Product Purification Kit (Catalog No: 11732668001) according to the manufacturer's instructions. Sanger sequencing method performed in ABI 3130XL Genetic Analyzer device using the same primers as for RT-PCR amplification. Mega X software used for nucleotides alignment with CLUSTRAL W algorithm. For the construction of the phylogenetic tree, sequences obtained from GenBank, and the full names of the sequences were shortened in G1 and P[8] groups. Phylogenetic tree was evaluated by Neighbor-Joining method using 1000 replicates bootstrap analysis.

Table 3. PCR conditions used for detection of VP4.

Master Mix (per sample)		PCR programme			
5X Green GoTaq® Flexi Buffer	5µl	Initial denaturation	94°C	3 min	1 cycle
MgCl2 (25mM)	3µl	Denaturation	95°C	1 min	35 cycles
dNTP (10mM each)	1µl				
Primer (F) (20 pmol/ µl)	1µl	Annealing	52°C	2 min	
Primer (R) (20 pmol/ µl)	1µl				
GoTaq® G2 Flexi DNA Polymerase (5u/µl)	0.5µl	Extension	72°C	1 min	
Template DNA	5µl				
Water	34.5µl	Final extension	72°C	7 min	1 cycle
Total	50 µl				

Cell culture based virus isolation:

For the RT-PCR positive samples, homogenates which were prepared before and kept at -80°C in 15ml tubes, were thawed at room temperature, centrifuged at 4000 rpm at 4°C for 30 minutes. After the supernatant was passed through membrane filters with a pore diameter of 0.2 µm, inoculated onto MA-104 cell monolayers (ATCC, CRL-2378.1) according to previous protocol with minor modifications (Arnold et al., 2009). Briefly the homogenates incubated at 37°C for 1 hour with the concentration of 10µg/ml trypsin (Sigma Aldrich, T4799). Following the incubation, the homogenate diluted with EMEM to reach a final concentration of 2 µg/ml trypsin and inoculated on the MA-104 cell monolayer. Five subsequent blind passages were performed**.**

Table 4. PCR conditions used for detection of VP7.

Master Mix (per sample)		PCR programme			
5X Green GoTaq® Flexi Buffer	10µl	Initial denaturation	94°C	3 min	1 cycle
MgCl2 (25mM)	5µl	Denaturation	95°C	1 min	35 cycles
dNTP (10mM each)	1µl				
Primer (F) (20 pmol/ µl)	1µl	Annealing	55°C	2 min	
Primer (R) (20 pmol/ µl)	1µl				
GoTaq® G2 Flexi DNA Polymerase (5u/µl)	0.5µl	Extension	72°C	1 min	
Template DNA	5µl				
Water	26.5µl	Final extension	72°C	10 min	1 cycle
Total	50 µl				

Results

Two raw mussel sample groups were positive for rotavirus. Sequence data were obtained from one of the positive sample for both VP4 and VP7 regions using the same primers for detection. The sequence data submitted to GenBank (Accession number OP598880 and OP598881) and compared with the sequences available on sequence database to determine the G/P genotype. The phylogenetic analysis showed that VP7 sequence was belong to G1 and VP4 was P[8] (Figure 1, 2). Both G1 and P[8] sequences presented high nucleotide identity (>99%) with Human Rotavirus A Wa (G1P[8]) backbone strain.

Virus isolation studies carried out for the two positive samples on MA-104 cell monolayers but no CPE were observed after 5 blind passages.

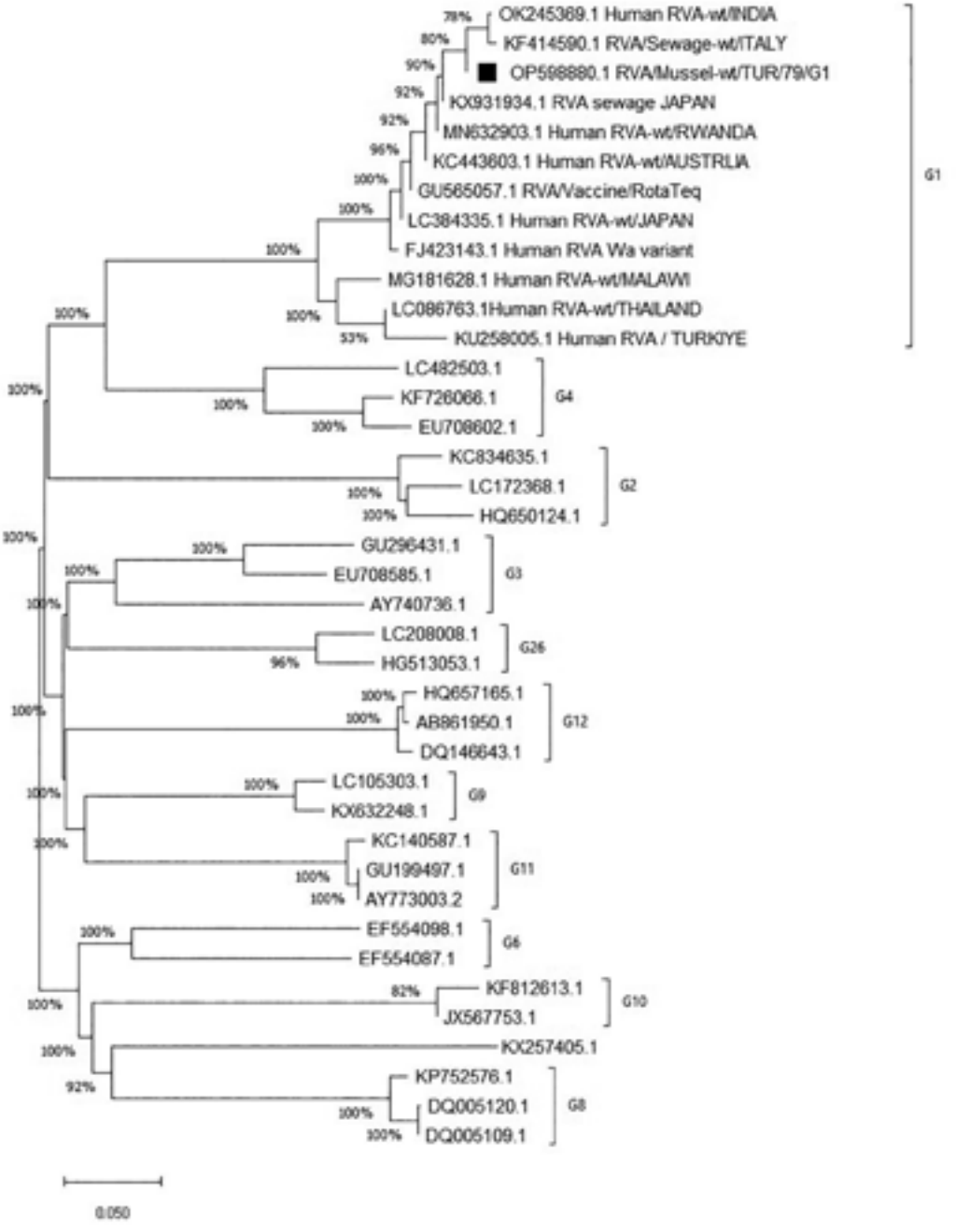

Figure 1. Phylogenetic tree of the nucleotide sequences of positive sample based on VP7.

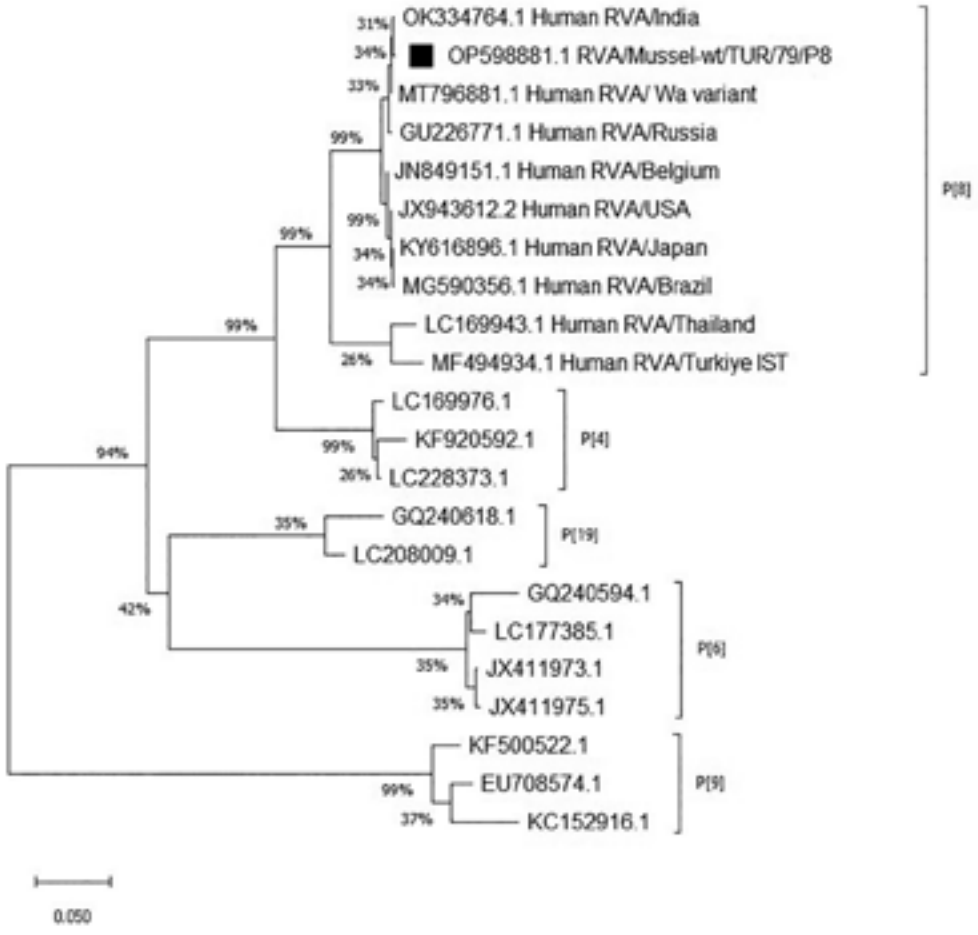

Figure 2. Phylogenetic tree of the nucleotide sequences of positive sample based on VP4.

Discussion and Conclusion

The most common cause of acute gastroenteritis in children under the age of five is rotavirus, and everyone experiences rotavirus infection at least once in their lifetime (Parashar et al., 2009). Studies conducted in Türkiye have also reported that rotavirus is one of the most common agents causing gastroenteritis (Bicer et al., 2014, Balkan et al., 2016; Tapisiz et al., 2019).

The growing industrial food production sector, changing food consumption habits as a result of crowded cities worldwide, and spread of international trade in food have increased the risk of foodborne diseases. Poor hygiene conditions in the food industry can cause serious gastroenteritis cases in humans. Since foodborne viruses are highly contagious, the source of infection is not attributed to food until the first cases are detected, and can be defined as an epidemic transmitted person-to-person (Newell et al., 2010). Food can be contaminated with rotavirus in various ways, such as using waste water for irrigation on the field, or zoonotic transmission may occur through consumption of meat, contaminated by sick animals at the slaughterhouse (Machnowska et al., 2014; Jones and Muehlhauser, 2017). Food-handlers are also one of the main source of contamination. A case of acute gastroenteritis occured in schools in some parts of Japan, and studies identified it as a rotavirus-related case that may result from lunch (Hara et al., 1978). In a similar gastroenteritis case in Fukui, Japan, where 3000 people were affected in 7 primary schools, rotavirus was identified as the cause. Although the virus was not detected in any food or water samples, it was interpreted as foodborne due to the fact that lunch was distributed to all 7 schools from a single source (Matsumoto ve ark., 1989). In a rotavirus outbreak

in a mother and child sanatorium in Germany, rotavirus was detected in a sample of potato stew and sequence comparison with a stool sample showed that two viruses were identical (Mayr et al., 2009).

In this study, while rotavirus was not detected in red and poultry meat products, 2/15 (13%) raw mussel samples were positive for both the VP4 and VP7 gene regions. Although our sample size of raw mussels was limited, the results are in agreement with the studies conducted in Italy that found 9% and 12% positivity of rotavirus in raw mussels collected from production sites on the coast yards (Fusco et al.,2017; 2019). In a study conducted in Brazil, rotavirus was detected in all 11 mussels collected from the sea coast (Keller et al., 2013), and 5.4% of bivalve shellfish were positive in Thailand (Kittigul et al., 2014). Our results contrast with a study from Istanbul, in which the presence of rotavirus investigated with multiplex real-time PCR in 52 groups (1350 pieces) of mussels collected in April, and none of the samples was positive (Ghalyoun and Unver Alcay, 2018). This disagreement might be due to the sample collection period of two studies. It has been reported that the incidence of rotavirus infections increases in winter (Patel et al., 2013; Gundeslioglu et al., 2018). In this study, the samples were collected in December and January, this factor can explain the dissimilarity of results.

The sequence analyses showed that the virus detected from the mussels in this study was a human rotavirus A G1P[8] type. It has been reported that rotavirus G1P[8] is the most common genotype in human rotavirus infections globally (Banyai et al., 2012; Doro et al., 2014). In Türkiye, studies showed that G1P[8] is also the predominant genotype causing gastroenteritis in humans. (Ceyhan et al.,2009; Bozdayi et al., 2008; Altindis et al., 2016). The sequence of the sample presented high nucleotide identity with Wa backbone strain. It has been reported that G1P[8] genotypes are usually associated with the Wa-like backbone (Rasebotsa et al., 2020), which is also genetically close to Rotarix and Rotateq vaccine strains. Evidence of vaccine virus shedding has been reported previously (Yoshikawa et al., 2019; Simsek et al., 2022), but the genetic data in the current study are insufficient to make such an interpretation.

The fact that the rotavirus detected in the mussel samples was of human origin and the samples were raw, suggests that this contamination most likely did not originate from a food-handler. It is more likely that, mussels harvested from a coast of city where probably sewage-contaminated stream meets the sea. As mussels are bivalves and they are filter-feeders, environmental viruses from the contaminated water concentrate in their body during the filtering.

Isolation studies were carried out in cell culture from food samples that were positive for rotavirus and no CPE were observed. With this result, it was thought that the rotavirus detected in mussel samples was inactive. This inactivation may have occurred due to the long period of time between harvest in the coastyard and collection from the market.

Our study has some limitations regarding the method used for molecular diagnosis of food products. It is widely agreed that various chemical ingredients in food matrices can affect the results of molecular detection of targeted nucleic acid region (Piskata et al., 2019). Although, ISO/TS 15216-1:2013 standard was defined for detection of hepatitis A virus and norovirus in food, there has no standard diagnose method defined for detection of rotavirus. Still, the method in this standard could have been adapted by using an internal positive control for the efficiency of molecular diagnose which could have enhanced the accuracy of the negative results obtained in this study.

In conclusion, the results of this study demonstrate that mussels can be contaminated with rotaviruses, posing a threat to public health if consumed undercooked. Although there are designated restricted areas for harvesting mussels that are free from sewage contamination, the mussels investigated in this study were likely not collected from these safe zones. Our findings reinforce the importance of strictly adhering to harvesting mussels only from these restricted areas to ensure they are free from contamination.

Conflict of interest: The authors declared that there is no conflict of interest.

Acknowledgments: This study is produced from the first author's PhD thesis. Views and opinions expressed are however those of the author(s) only and do not necessarily reflect those of the Republic of Türkiye Ministry of Agriculture and Forestry. Neither the the Republic of Türkiye Ministry of Agriculture and Forestry can be held responsible for them.

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