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Investigation of Hepatitis A and E Viruses in Mussels Collected from the Bosphorus, in Istanbul, Turkey – Short Communication

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Abstract

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Hepatitis A and E viruses (HAV and HEV) are recognised as food- and water-borne infections and shellfish are a well-documented source of these viruses. The presence of HAV and HEV in mussels has not previously been investigated in Bosphorus, Istanbul, Turkey, and therefore the aim of this study was to investigate the frequency of HAV and HEV in mussels collected from this location. A total of 92 pooled samples representing 736 mussels originating along the Bosphorus coast were collected from fish distributors. RNA was extracted using the RNeasy Plant Mini Kit and a TaqMan real-time RT-PCR was performed using primers specific to HAV and HEV. Amongst the 92 pooled samples tested, three (3.3%) were found to be positive for HAV by the TaqMan real-time RT-PCR. No HEV was detected in any of the mussels tested. A 174 bp product was observed on gel electrophoresis with HAV positive samples detected by SYBR Green real-time PCR after reverse transcription. This product was sequenced and confirmed to contain HAV sequences by alignment using the data in GenBank. This is the first report describing the presence of HAV in mussels harvested in the Bosphorus, Istanbul, Turkey, indicating the potential contamination of aquatic environment and risk to public health.

Keywords: hepatitis; mussels; Turkey; virus; real-time RT-PCR

Hepatitis A and E are enterically transmitted diseases which are endemic in Asia, Africa and in the Middle East. Hepatitis A virus is recognised as a major cause of food- and water-borne viral hepatitis worldwide and is frequently associated with the consumption of contaminated shellfish, sandwiches, fresh

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vegetables, fruits and water (KOOPMANS & DUIZER 2004; COOK & RZEUZKA 2006; KOKKINOS *et al.* 2012; RODRIGUEZ-LAZARO *et al.* 2012; MAUNULA *et al.* 2013). Food-borne transmission of HEV has been associated with the consumption of undercooked pork products (COLSON *et al.* 2010; SAID *et al.* 2014), with the possibility of several thousand people being infected each year (HEWITT *et al.* 2014). HEV has also been detected in mussels, shellfish and other bivalves (DONIA *et al.* 2012).

Replication of HAV and HEV in cell cultures is difficult. Therefore, molecular techniques like reverse transcription PCR (RT-PCR), SYBR-Green real-time RT-PCR and quantitative real-time RT-PCR are preferred in most laboratories for detection of HAV and HEV in shellfish and other food matrices (SCHULTZ *et al.* 2007; PINTO *et al.* 2009; NAMSAI *et al.* 2011; BIGORAJ *et al.* 2014; POLO *et al.* 2015). HAV and HEV occurrence in shellfish has been investigated in many countries (HANSMAN *et al.* 2008; NAMSAI *et al.* 2011; CROSSAN *et al.* 2012; BIGORAJ *et al.* 2014; SUFFREDINI *et al.* 2014; POLO *et al.* 2015), but there have been no similar studies conducted in Turkey. Therefore, this study was performed to detect and investigate the frequency of HAV and HEV in mussels collected from the Bosphorus, Istanbul, Turkey.

MATERIAL AND METHODS

Mussel collection and processing of samples. Raw shelled mussels were collected each month from September 2014 to August 2015 as well as from January to August 2016 from the fish distributors who collected them along the Bosphorus coast (Kumkapı, Rumelikavağı, Sariyer, Donkey Island and Ambarlı) for consumer markets in Istanbul. Sampling was the modification of the method described by others (BOXMAN *et al.* 2006; YILMAZ *et al.* 2010). Briefly, in each month 8–10 mussels were randomly selected from a bucket/pack containing about 500 mussels. On the day of visit, 2–4 randomly selected buckets/packs were sampled depending on the number of buckets/packs the seller holds. Mussels were transported to the laboratory at 4–8°C (using a Dewar container) and processed within 24 hours. Digestive tissues were removed from each mussel, and these materials taken from 8 mussels were pooled. By this method, 92 mussel pools were formed representing 736 mussels. Approximately 2 g of pooled digestive tissue were used for RNA extraction. The tissue was

homogenised using 1-mm glass beads (0.5 g) and a cell disrupter for 40 s at 4 m/s (Ribolyser-Cell Disrupter; Hybaid, Turkey). After homogenisation, 350 ml of the supernatant was used for RNA extraction.

Spiking of the norovirus and RNA extraction. For extraction control, human stool positive for Norovirus GII was spiked into 2 grams of digestive tissue as described previously (YILMAZ *et al.* 2010). The tissue was homogenised as above. After homogenisation, the supernatant was collected and used for RNA extraction. Viral RNA was extracted from test samples and Norovirus GII spiked samples using a RNeasy® Plant Mini Kit (Qiagen, Germany) as described by the manufacturer. The amount of RNA in the extracted material of all samples was measured using a Nano-Drop spectrophotometer (Nano-Drop 1000c; Thermo Scientific, USA). Final concentration was adjusted prior to RT and the conditions of the real-time PCR for NoV Genogroup II were the same as described previously (YILMAZ *et al.* 2010).

Reverse transcription and detection of HAV and HEV. Reverse transcription was performed in two steps as described previously (YILMAZ *et al.* 2011). For the first step, 9 ml of RNA template (about 950 ng) was used. For the second step, a total volume of 20 ml reaction mixture was prepared consisting of 10 ml RNA/primer mixture from the first step. Thirty milliliters of nuclease-free water was added to each cDNA sample and kept at –70°C until required.

For the TaqMan real-time RT-PCR to detect HAV and HEV RNA, the total volume of 25 ml reaction mixture was prepared. The location and target region of the primers used for the TaqMan real-time RT-PCR are summarized in Table 1. All primers and probe were synthesised by Qiagen (Turkey). The method used for the TaqMan real-time RT-PCR for HAV (COSTAFREDA *et al.* 2006) and HEV (JOTHIKUMAR *et al.* 2006) were similar as described previously. In case of positivity found in a TaqMan real-time RT-PCR assay, a real-time RT-PCR was performed by using SYBR-Green and the product was visualised by agarose gel (1.5%) electrophoresis and sequenced by a commercial company (Refgen, Turkey).

Controls included during extraction and TaqMan real-time RT-PCR. For the positive controls, mussel sample spiked with human stool containing norovirus GII, and cDNA from HAV-positive sera (obtained previously in Turkey) and HEV-RNA (kindly supplied by Professor Wim van der Poel, Wageningen University) were used. For the negative controls, RNA extracted from uncontaminated mussels (giving

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Table 1. Primers and probe used in this study

Primers	Sequences (5'–3')	Product size (bp)	Target gene	Target sequences	References
HAV68	TCACCGCCGTTTGCCTAG			68–85 ^a	
HAV150	FAM-CCTGAACCTGCAGGAATTAA–MGB	174	5'UTR	150–169	COSTAFREDA <i>et al.</i> (2006)
HAV240	GGAGAGCCCTGGAAGAAAG			241–223	
JVHEVF	GGTGGTTTCTGGGGTGAC			5.237–5.254 ^b	JOTHIKUMAR <i>et al.</i> (2006)
JVHEVP	FAM-TGATTCTCAGCCCTTCGC–BHQ	70	capsid	5.260–5.277	
JVHEVR	AGGGGTTGGTTGGATGAA			5.306–5.289	
HAV BR5F	TTGTCTGTACAGAACAATCA	360	VP1/VP2A junction	2.952–2.973 ^c	REUTER <i>et al.</i> (2006)
HAV newRT	AGC AGT CAC TCC TCT CCA G			3.294–3.311	

^aNC_001489; ^bNC_001434; ^cM14707

consistent negative real-time RT-PCR signals) and reaction mixture with nuclease-free water in place of template were included.

RT-PCR for sequencing. Samples found to be positive for HAV by TaqMan real-time RT-PCR were subjected to sequencing by using the methods described previously (COSTAFREDA *et al.* 2006; REUTER *et al.* 2006). The primers used in RT-PCR for sequencing are given in Table 1.

RESULTS AND DISCUSSION

Amongst the 92 pooled mussel samples (representing 736 mussels), 3 (3.3 %) were found to be positive for HAV. HEV was not detected in any mussel sample. A HAV positive signal was obtained using the TaqMan real-time RT-PCR in 3 mussel pools collected in December and November 2014 and July

2015; CT values were 34, 34.3 (Figure 1), and 38, respectively. Positive mussel samples showed a real-time RT-PCR signal in both undiluted and 10-fold diluted cDNA. 174 bp products were observed on gel electrophoresis with HAV positive samples detected by SYBR Green real-time RT-PCR. This product was sequenced and confirmed to contain HAV sequences by alignment using the data in GenBank. Despite several attempts, we were unsuccessful in amplifying a 360 bp product from HAV positive mussels (CT values 34, 34.3, and 38) for sequencing by RT-PCR. Using NoV Genogroup II positive controls (human stool diluted 1/10) the real-time PCR for NoV Genogroup II virus showed the threshold cycle (CT) value between 25 and 28 and a 100-bp amplicon by agarose gel. For HAV and HEV positive controls, CT of 29.7 (Figure 1) and 28 (data not shown) were obtained, respectively. No CT value was obtained with negative controls.

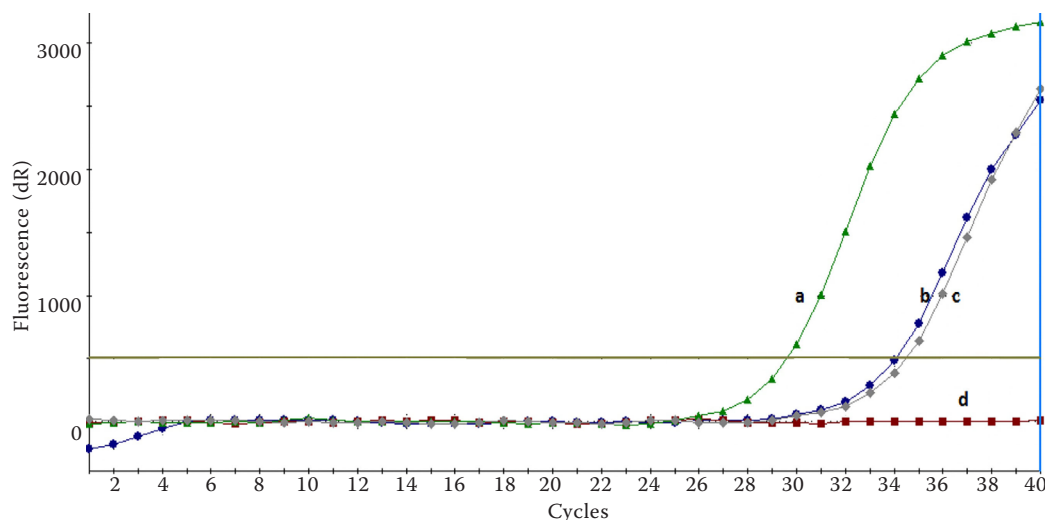


Figure 1. Real-time PCR results of 2 of the 3 positive mussel pools: a – HAV positive control; b – positive mussel pool; c – another positive mussel pool; d – negative control

HAV and HEV are important food- and water-borne viruses causing hepatitis and the majority of the patients require hospitalisation. The viruses are usually transmitted by vegetables, fruits, sandwiches, undercooked sea products and water (KOOPMANS & DUIZER 2004; KOKKINOS *et al.* 2012; MAUNULA *et al.* 2013). Mussels have been described as the bivalves which are most contaminated with human enteric viruses (GABRIELI *et al.* 2007). In Turkey, mussels are frequently consumed and they are either fried or steam cooked. Neither of these cooking methods may result in a temperature in the mussels digestive organs high enough to inactivate viruses (KIRKLAND *et al.* 1996; McDONNELL *et al.* 1997; CROCI *et al.* 2005). Therefore, such mussels may pose a risk to human health in Turkey, however this has not been investigated until now. Therefore, this study was performed to investigate the presence of HAV and HEV in mussels collected from the coast of Bosphorus, Turkey.

The presence of HAV and HEV in shellfish has been investigated in many countries by using RT-PCR or RT-qPCR (FORMIGA-CRUZ *et al.* 2002; PINTO *et al.* 2009; NAMSAI *et al.* 2011; SUFFREDINI *et al.* 2014; POLO *et al.* 2015). Detection of HAV was performed in shellfish collected from five European countries – Greece, Spain and United Kingdom – and HAV was detected in 4, 3 and 1 sample, respectively, but not in Sweden (FORMIGA-CRUZ *et al.* 2002). In Spain, between 10.1–75% of the shellfish samples were found to be positive for HAV (BOSCH *et al.* 2001; PINTO *et al.* 2009; MANSO *et al.* 2010; POLO *et al.* 2015). However, in Italy 0.9 and 14.1% of the tested shellfish samples were positive for HAV (MACALUSO *et al.* 2006; SUFFREDINI *et al.* 2014). In Poland, HAV was detected in 9 (7.5%) of the shellfish (BIGORAJ *et al.* 2014). In the USA, HAV was present in 4.4% of the shellfish analysed (DEPAOLA *et al.* 2010). In Thailand, it was found that 3.8% of the shellfish and 2.9% of the cockles and 6.5% of mussels were contaminated with HAV, respectively (NAMSAI *et al.* 2011). In Japan, HAV was detected only in one of the 57 shellfish packages (HANSMAN *et al.* 2008). In China, 4.3% of tested shellfish was found to be positive for HAV (MING *et al.* 2014). In the present study, 3 (3.3%) of 92 mussel pools representing 736 mussels were found to be positive for HAV. Results of this study are similar to what was found in the USA, China (DEPAOLA *et al.* 2010; MING *et al.* 2014) but different from Spain, Poland and Thailand (BOSCH *et al.* 2001; PINTO *et al.* 2009; MANSO *et al.* 2010;

NAMSAI *et al.* 2011; BIGORAJ *et al.* 2014; POLO *et al.* 2015). The difference in detection rate may be because of the viral extraction methods, the test and primers used, the season the samples were taken and the place of samples taken (high or low contaminated areas) (COSTAFREDA *et al.* 2006; RUTJES *et al.* 2006; DE RODA HUSMAN *et al.* 2007; SCHULTZ *et al.* 2007; CROCI *et al.* 2008; UHRBRAND *et al.* 2010; POLO *et al.* 2015).

In comparison with HAV, the detection rate reported for HEV is either absent or low in many countries. In Italy no HEV was detected in eleven samples analysed (LA ROSA *et al.* 2012). In the United Kingdom, HEV was detected in 1 shellfish sample out of the 48 samples analysed (CROSSAN *et al.* 2012). In China, the overall HEV-positive detection rate was approximately 17.5% per kilogram of shellfish (GAO *et al.* 2015). In Thailand, no HEV was detected in any of the tested samples (NAMSAI *et al.* 2011). All shellfish were found to be negative in a study conducted in Denmark (KROG *et al.* 2014). Similar results were obtained in France (GRODZKI *et al.* 2014). Similarly, no HEV was detected in any of the samples analysed in the present study. Results of the above studies and this study indicate that mussels do not seem to be playing a major role in transmission of HEV to people. In contrast, HEV has been associated with the consumption of undercooked pork products (COLSON *et al.* 2010; SAID *et al.* 2014).

CONCLUSIONS

HAV was found in 3.3% of the pooled mussel samples collected from Bosphorus in Istanbul and may pose a risk to public health and contamination of aquatic environment. HEV was not detected in any of the mussel samples tested, indicating that mussels may not be a significant vehicle for this virus in this region of Turkey.

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