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First Identification of Hepatitis a Virus RNA in Mussels (*Mytilus* sp.) in Turkey

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Abstract: In this study, a total of 600 mussels collected in six different areas of the coastal Middle Black Sea region of Turkey were analysed for the presence of Hepatitis A Virus (HAV). HAV was evaluated by cell culture infection method and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). In conclusion, HAV RNA was found in 2 of 60 cell culture supernatants of the mussel samples (3.3%). This is the first study reporting the isolation of HAV in mussels in Turkey. This study highlights the interest to contamination of Hepatitis A in mussel samples from different areas.

Key words: Hepatitis A, mussel, CPE, RT-PCR, contamination, Turkey

INTRODUCTION

Hepatitis A Virus (HAV) is classified in the *Hepatovirus* genus of the *Picornaviridae* family which causes infectious hepatitis and gastroenteritis by raw or undercooked seafood consumption (Gust *et al*., 1983; Desenclos *et al*., 1991). The first documented outbreak of shellfish-borne hepatitis occurred in Sweden in 1956 when 629 cases associated with raw oyster consumption were reported (Roos, 1956). The most disturbing recent incident occurred in China in 1988 (Anonymous, 1988), Hepatitis A outbreak associated with clams involved >250,000 cases (nine deaths) in Shanghai. HAV has been successfully isolated from oysters, mussels and clams (De Medici *et al*., 2001; Chironna *et al*., 2002; Casas and Sunen, 2008).

Health Ministry of Turkey reported that in 1990 Hepatitis A was seen in 30.662 people, 45 resulted with death and in 2006, 7.137 people were affected and 5 of them died. Hepatitis A events and mortality rates were decreased in Turkey during the last decade. There isn't eligible network or database about foodborne diseases and mortality rates in Turkey. The source of Hepatitis A contamination isn't clearly known yet. Poor hygienic conditions of food and water sources have been considered but there has been no study about Hepatitis A in food. Cell culture infectivity assay, immunological and molecular techniques such as Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) nested and semi-nested RT-PCR have been used in the detection of HAV and other enteric viruses in shellfish samples (Jaykus, 2000). Cell culture methods are expensive and time consuming in fact HAV replication occurs in several days (Cliver *et al*., 1983; Croci *et al*., 1992). The Polymerase Chain Reaction (PCR) is a rapid, economical, sensitive and a specific assay for the detection of enteric viruses in clinical and environmental samples.

The low levels of virus contamination and the presence of PCR inhibitors in shellfish are the main obstacles for the application of RT-PCR for the detection of viruses in shellfish samples (Deng *et al*., 1994; Shieh *et al*., 1999).

This study investigated Hepatitis A virus as a foodborne infection in mussels in the Middle Black Sea Region using cell culture and RT-PCR techniques. Samples were collected from six different regions of Samsun city. This is the first study about Hepatitis A isolation from food in Turkey. Thus, the study will offer guidance to relevant further studies.

MATERIALS AND METHODS

Mussel processing: A total of 600 mussels were collected equally in the six different areas of the coastal Middle Black Sea region of Turkey (Samsun Province) by monthly between August 2005 and January 2006 (Fig. 1). Each sample group contained 10 mussels, therefore a total of 60 mussel samples were examined. About 25 g of mussel

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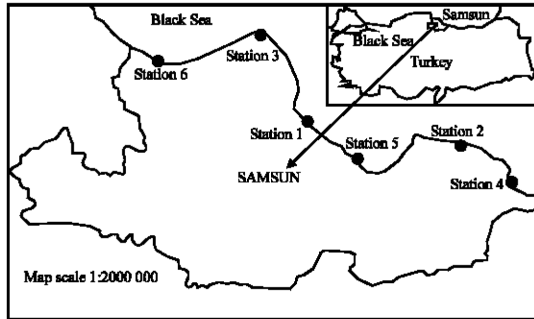

Fig. 1: Sampling areas in the Samsun Province

were homogenized in 175 mL of glycine buffer (0.1 M glycine, 0.3 M NaCl at pH 9.5) using a homogenisator (Interscience-Bag Mixer 400, France) at high setting for 3 min (Kingsley, 2007). Homogenates were centrifuged in eppendorf tubes at 12000 g for 3 min to remove the suspended solids. The supernatants were stored at -80°C until further analysis.

RNA extraction, RT-PCR assays: Viral RNA was extracted from the homogenized mussel tissues and cell culture supernatants of mussel samples using an acid guanidium-phenol-chloroform-isoamyl alcohol mixture as described by Chomcyzynski and Sacchi (1987). Briefly, 400 μL supernatant from obtained mussels and cell supernatants were mixed in a denaturing solution of 4 mol L^{-1} guanidinium isothiocyanate, 0.5% laurosylsarcosine (Sarcosyl) and 0.1 mol L^{-1} β-mercaptoethanol in 25 mmol L^{-1} sodium citrate (pH 7.0). After phenol/chloroform extraction, the RNA was precipitated twice with isopropanol and washed with 70% ethanol. The RNA pellet was air-dried, re-suspended in 20 μL of DEPC-treated water and stored at -80°C.

All samples were tested by RT-PCR using primers. VP2 and VP4 primers were described previously (Beneduce *et al*., 1995). These primers allowed the amplification of capsid region of HAV strain FG. Complementary DNA synthesis was initiated by incubation of tubes at 70°C for 5 min to denature probable secondary structures in the RNA. The synthesis of cDNA was carried out in a mixture of 25 mmol L^{-1} Tris-HCl, 25 mmol L^{-1} Kcl, 4 mmol L^{-1} $MgCl_2$, 10 mmol L^{-1} DTT, 50 ng random hexamer primers, 200 U Moloney murine leukemia virus reverse transcriptase (MMLV-RT) (MBI, Fermentas, Lithuania) and 10 U of RNase inhibitor. The reaction mixture was incubated first at 25°C for 10 min followed by a second incubation at 37°C for 1 h. Moloney murine leukemia virus reverse transcriptase was then inactivated by holding at 70°C for 10 min. Polymerase chain reaction amplification was performed as described

Table 1: Hepatitis A virus primer sequences

Primer (5'→3')	Localization	Product size (bp)
Primer 1 5′ CAGACTGTTGGGAGTGG 3′	762-778	385
Primer 2 5′ TTTATCTGAACTTGAAT 3′	1131-1147	-

elsewhere (Croci *et al*., 2000) with minor modifications. Briefly, amplification was carried out by adding 3 μL of cDNA to the master mix containing 75 mmol L^{-1} Tris-HCl (pH 8.8), 20 mmol L^{-1} NH_4 $(SO_4)_2$, 1.5 mmol L^{-1} $MgCl_2$, 10 pmol of each primer, 0.2 mmol L^{-1} dNTP and 0.5 U of Taq DNA polymerase (MBI, Fermentas, Lithuania). The amplification was completed in 30 μL of total reaction mixture in a thermal cycler (Thermo, USA). The steps of amplification on the thermal cycler were set up as follows: 25 sec at 95°C, 10 sec at 37°C and 1 min at 70°C, repeated 30 cycles. Amplification was terminated by final extension at 70°C for 5 min. The resulting DNA products (amplicon) were analysed on agarose gel (2%) in the presence of 0.5 μg mL^{-1} ethidium bromide after electrophoresis at 80 V for 30 min. The DNA bands were observed under ultraviolet light. A positive RT-PCR result was indicated by amplification of the 385 bp genome fragment for Hepatitis A (Table 1).

Cell culture inoculation: Hep-2 cell line was grown in Eagles Minimal Essential Medium (EMEM, Biological Industries, Israel) supplemented with 10% fetal bovine serum (Biological Industries, Israel), HEPES 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) (Sigma Aldrich, St. Louis, USA) and antibiotics in standart concentration (Biological Industries, Israel). Virus extraction was performed according to the method of Croci *et al*. (1999) with some modifications. Briefly, supernatants were resuspended in approximately 4 volumes of cell culture medium (EMEM suplemented with 10% foetal bovine serum, tris buffer and antibiotics) and centrifuged at 3000 g (Eppendorf 5702 R, Germany) for 15 min at 4°C. Supernatants were stored at -80°C until examined. Final dilutions were inoculated in 0.2 mL amount onto Hep-2 cells grown to confluency in 24-well microplates. The cultures incubated at 37°C in 5% CO_2 atmosphere controlled daily for appearence of cytopathic effect (Olympus CKX41, Japan). After 7 days, the culture medium was harvested by freze-thawing and then were centrifuged at 3000 g for 15 min. The supernatants of all samples were examined for nucleic acid of HAV using PCR.

RESULTS AND DISCUSSION

Sixty mussel samples were collected from six different sampling sites in this study. The Cytopathic Effect (CPE)

Table 2: Determination of Hepatitis A virus in mussel samples collected from six areas of Samsun Province

District No.	Sample (n)	Cell culture (CPE) Positive	Cell culture (CPE) Negative	Cell culture (PCR) Positive	Cell culture (PCR) Negative	Mussel tissue (PCR) Positive	Mussel tissue (PCR) Negative
1	10	-	10	-	10	-	10
2	10	-	10	-	9	-	10
3	10	-	10	-	10	-	10
4	10	2	8	1	9	-	10
5	10	7	3	1	9	-	10
6	10	-	10	-	10	-	10
Total	60	9	51	2	58	-	60

of the samples was evaluated in Hep 2 cells and nine samples were found to be CPE positive. RT-PCR was performed on supernatants of the inoculated cell cultures which were observed CPE positive and negative. End of the assay, only two of them (3.3%) were found to be positive. On the other hand, RT-PCR was performed on also all mussel tissues but none of them were positive for Hepatitis A RNA (Table 2).

As a result of consuming various shellfish products from contaminated water sources foodborne infections caused by pathogenic bacteria and viruses arise. Over 100 virus species cause diseases in human through contaminated water products (Bosch, 1998). Hunting for fishery products from the waste contaminated water is one the reasons underlies the enteric virus infections of human. Consumption of mussels and other sea products from contaminated water leads to viral infections such as Enteroviruses, Norwalk virus and Hepatitis A virus (Munian-Mujika *et al*., 2003). One of the important factors in the occurrence of HAV infections is consumption of shellfish. Turkey is surrounded by sea on three sides and boasts a large capacity for producing and consuming sea products. The port cities of Turkey and coastal regions in these cities (including the coast of the Samsun region) are being contaminated by ship, industrial and sewer wastes. However, no research on viral contamination has been performed so far on water products hunted or collected from these regions. Therefore, this study is important as being the first viral research on shellfish in Turkey. In this study, the presence of HAV was examined in mussel samples collected from six coastal areas of Samsun using cell culture and RT-PCR methods. *In vitro* studies were supported with RT-PCR as HAV grows hardly in cell cultures. RT-PCR was performed on cell culture supernatants and also directly from mussel tissues to determine viral RNA. HEp-2 cells were used for inoculation from mussel samples.

Nine out of 60 (15%) samples were CPE positive. Thereafter, RT-PCR was applied to all samples (both CPE positive and CPE negative) and only two of them revealed a 385 bp fragment corresponding to HAV-RNA. The RT-PCR test was also directly applied to the mussel tissue samples; however there wasn't any proof of positive HAV-RNA probably due to the fact that HAV contains a RNA genome and direct extractions from mussel tissues could lead to viral RNA degradation depending up the physical and chemical conditions. Nine samples were found to be positive in cell culture inoculations and two of them were positive for HAV-RNA by means of the RT-PCR method.

These two samples came from the Samsun zone 4 and zone 5 indicating that these regions may be contaminated by ship and sewer wastes. In the study performed by Croci *et al*. (2000), 13 mussel samples out of 36 (36.1%) collected from the Adriatic sea were reported to be contaminated with HAV and such high incidence was associated with high HAV resistance in the sea. Macaluso *et al*. (2006) found a HAV genome presence of 14.1% in 170 bivalve molluscs by means of PCR. Coelho *et al*. (2003) found a positive rate of 22% in shellfish. The positive HAV presence found in shellfish including mussels, constitutes a risk for consumer health. These studies explicitly reveal that sea products offered for public consumption pose an HAV risk. In addition, it is very likely that samples yielding CPE in cell cultures yet being not clear of HAV-RNA in the study may contain other enteroviral pathogens such as enteroviruses, polioviruses, coxsackievirus echoviruses or adenoviruses.

CONCLUSION

The results will benefit both public and health offices concerned about Hepatitis A infections caused by consumption of HAV-contaminated shellfish and producers who need reliable methods for quality control of commercial production.

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