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DETECTION OF ENTEROVIRUSES AND HEPATITIS A VIRUS RNA IN COW MILK BY RT-PCR

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The aim of the study is to evaluate the precence of Hepatitis A virus (HAV) and enteroviruses (EV) in milk samples. Raw milk and whey samples (n=50), collected from eight different open air markets in Samsun (Turkey) were analysed for HAV and enteroviruses by RT-PCR. Viral RNA was extracted using the rapid acid guanidinium thiocyanate-phenol-chloroform method. HAV primers were selected from sequences of the VP2 and VP4 capsid region. Enterovirus primers were selected from the 5' non-translated region, which is the most conserved region in an enterovirus genome. Four samples (8%) raw milk were positive for EV RNA. However, HAV RNA was not determined the milk samples. This is the first report on the detection of HAV and EV RNA in cow milk samples in Turkey. This study highlights the interest for contamination by HAV and EV in milk samples from different areas.

Key words: enteroviruses, hepatitis A virus, cow milk

INTRODUCTION

Foodborne viruses usually cause viral gastroenteritis, and inflammation of the stomach, and small and large intestines. The major sources of enteric viruses in contaminated foods include: (i) shellfish harvested from faecally contaminated estuaries; (ii) fruits or vegetables irrigated or washed with faecally contaminated water; and (iii) foods that become contaminated during preparation through contact with faecally contaminated surfaces or the hands of infected food handlers (Jaykus, 2000; Sair *et al*., 2002). Noroviruses (NoV), hepatitis A virus (HAV), rotavirus, astrovirus and enteric adenoviruses are known foodborne viruses, of which NoV is by far the major causative agent (Baert *et al*., 2009).

Enteroviruses are members of the *Picornaviridae* family and are classified as (+)ssRNA viruses. Enteroviruses are subgrouped into polioviruses, coxsackievirus (groups A and B), echovirus and enterovirus 68 to 71. Human enteroviruses infect millions of people worldwide and cause clinical manifestations such as aseptic meningitis, myocarditis, acute haemorrhagic conjunctivitis and other acute and chronic illnesses (Melnick, 1996).

Among the enteric viral diseases, hepatitis A virus (HAV) represents the most important public health problem. HAV belongs to the *Hepatovirus* genus of the *Picornaviridae* family (Gust *et al*., 1983). It typically has high transmission rates among young children in developing countries, in areas where crowding is common and sanitation is poor, and in households and child-care settings (Mast and Alter, 1993).

The Center for Disease Control and Prevention (CDC) reported that 21 183 cases in 1 097 foodborne outbreaks in 2007 and 6 120 cases in 199 outbreaks had viral causes (CDC, 2007). According to data from the surveillance systems of the Foodborne Viruses in Europe network the *Norovirus* was responsible for >85% (n = 3 714) of all nonbacterial outbreaks of gastroenteritis reported from 1995 to 2000 in Europe (Lopman *et al*., 2003). HAV is an increasing problem in the Western European countries because of the decrease in immunity of populations in countries with a high standard of hygiene (Vasickova *et al*., 2005). Most foodborne viruses are more resistant to heat, disinfection, and pH changes than are most vegetative bacteria (Koopmans and Duizer, 2004). According to the Health Minister of Turkey, HAV was seen in 30 662 people in 1990 of which 45 died; but in 2006, 7 137 people were affected and 5 of them died (Anonymous, 2006).

Since the isolation of human enteric viruses, and in particular HAV, is difficult, expensive and time-consuming, there is a critical need for better indicators of human-specific faecal pollution (Croci *et al.,* 2000). Although cell culture is a reliable diagnostic method, molecular methods such as RT-PCR offer more sensitive, highly specific and rapid test results in support of enterovirus (EV) diagnosis (Anonymous, 2009). The low levels of virus contamination and the presence of PCR inhibitors are the main obstacles for the application of RT-PCR for the detection of viruses in food samples (Deng *et al*., 1994; Hafliger *et al.,* 1997; Shieh *et al.,* 1999). A variety of methods of RNA extraction have been described (Atmar *et al.,* 1993; Cromeans *et al.,* 1997; Sunen and Sobsey, 1999). Total RNA isolation by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture has been described. The method provides a pure preparation of undegraded RNA at high yield and can be completed within 4 h. It is particularly useful for processing large numbers of samples and for isolation of RNA from minute quantities of cells or tissue samples (Chomcyznski and Sacchi, 1987).

The aim of this study was to investigate the presence of HAV and EV in cow milk samples collected from eight different open-air markets in the Samsun region in the Middle Black Sea, Turkey. Viral RNA extraction from milk and whey samples were also compared by the phenol:chloroform extraction method and RT-PCR techniques. To our knowledge is the first study on hepatitis A and enteroviruses in milk samples using RT-PCR in Turkey, and should thus provide guidance for further studies.

MATERIAL AND METHODS

Milk samples

In this study, 50 raw cow milk samples were collected from eight different open-air markets (bazaars) in Samsun, on the coast of the Middle Black Sea, in the Turkey, between January and March 2008. Milk samples were collected in 500 mL sterile glass jars and then sent to the laboratory under refrigerated conditions. HAV and EV RNA were extracted from milk and milk whey samples.

Extraction of milk serum

Milk samples were placed in 20 mL plastic vials. A total of 1 mL 0.3% rennet was added to the samples and clotting was allowed to take place at 37 ^{o}C for 20 min. Milk serum was filtered through filter paper (Whatman No. 40). The filtrate was centrifugated at 3000 rpm for 5 min. The upper layer of milk cream was removed. The lower layer of clear milk was added to cryo tubes and stored at –80 ^{o}C (Alais, 1974).

Viral RNA extraction

Viral RNA was extracted from the milk and milk whey samples using an acid guanidium-phenol-chloroform-isoamyl alcohol method as described by Chomcyznski and Sacchi (1987). Briefly, 400 μL milk was mixed in an equal volume of denaturing solution of 4 mol/L guanidinium isothiocyanate, 0.5% laurosylsarcosine (Sarcosyl), and 0.1 mol/L β-mercaptoethanol in 25 mmol/L sodium citrate (pH 7.0). After phenol:chloroform extraction, 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^{o}C. The concentration and purity of RNA were measured by absorbance at 260 and 280 nm wavelengths using a UV spectrophotometer (Helios Gamma*,* Thermo Spectronic*,* Mercers Row*,* Cambridge, UK).

Reverse transcriptase-polymerase chain reaction

HAV-specific primers were selected from published sequences of the VP2 and VP4 capsid region on the basis of 100% sequence homology among the HAV strains FG (Venuti *et al.,* 1985).Then the primers were checked for cross-reactivity with other enteric viruses by computer analysis (Advanced Blast Programme). Enterovirus primers were selected from the 5' non-translated region, which is the most conserved region of the genome of enteroviruses (Kopecka *et al.,* 1988). The primer sequences are reported in Table 1.

Complementary DNA synthesis was initiated by incubation of tubes at 70^{o}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 Tris-HCl, 25 mmol/L KCl, 4 mmol/L $MgCl_2$, 10 mmol/L DTT, 50 ng random hexamer primers, 200 U Moloney murine leukaemia virus reverse transcriptase (MMLV-RT) (MBI, Fermentas, Lithuania), and 10 U of RNase inhibitor. The reaction mixture was incubated first at 25^{o}C for 10 min, followed by a second incubation at 37^{o}C for 1 hour. Moloney murine

leukaemia virus reverse transcriptase was then inactivated by holding at 70°C for 10 min.

The polymerase chain reaction amplification was performed as described 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 Tris-HCl (pH 8.8), 20 mmol/L $NH_4(SO_4)_2$, 1.5 mmol/L $MgCl_2$, 10 pmol of each primer, 0.2 mmol/L 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 s at 95°C, 10 s at 37°C, and 1 min at 70°C, repeated for 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 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 a 370 bp genome fragment for HAV and a 420 bp fragment for EV.

Table 1. Primer sequences

Primer	Sequence	Localization	References
Hepatitis A	5' CAG ACT GTT GGG AGT GG 3'	762–778	Beneduce *et al.,* 1995
Hepatitis A	5' TTT ATC TGA ACT TGA AT 3'	1131–1147	
Enteroviruses	5' CAA GCA CTT CTG TTT CCC CGG 3'	160–180	Izuka *et al.,* 1987*
Enteroviruses	5' ATT GTC ACC ATA AGC AGC CA 3'	580–599	

*Position in reference to the Coxsackievirus B1 sequence

RESULTS

Milk and milk whey samples were investigated for the presence of HAV and EV by RT-PCR. Four samples (8%) raw milk were positive for EV RNA with an amplicon fragment size of 420 bp in RT-PCR (Figure 1). But, none of the milk whey samples analysed contained EV RNA. In addition to this, HAV RNA was not determined in the milk and milk whey samples (Table 2).

Table 2. The RT-PCR positivity of enteroviruses and hepatitis A virus in milk and milk whey

	Milk (n=50)	%	Milk-Whey (n=50)	%
Enteroviruses	4	8	0	0
Hepatitis A	0	0	0	0

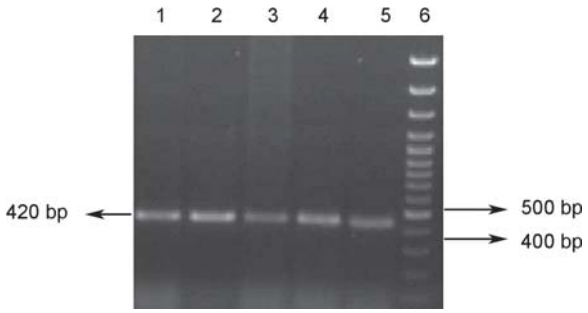

Figure 1. The PCR products amplified from Enterovirus RNA extracted in milk samples. Lane 1: Positive control; Lanes 2–5: Positive result for Enteroviruses in milk samples; Lane 6: 100 bp DNA ladder

DISCUSSION

Milk serves as an excellent protective culture medium for certain microorganisms. Pathogenic organisms in the milk are derived from the dairy animal itself, the human handler or the environment. These organisms can be excreted through the udder directly into the milk, or orginate from the skin and mucous membranes of the animal or milker and contaminate the milk (Kaplan *et al*., 1952).

No research on enteroviruses and hepatitis A virus in milk samples in Turkey has been performed to date using the RT-PCR method. Therefore, this study is important as it provides the first viral data for milk in Turkey. This study was done using raw milk sold at bazaars, which is consumed by many people as it is cheaper than pasteurized milk. Raw milk represents a serious public health risk due to bacterial, viral, and parasitic diseases. Brucellosis is an important zoonotic infection that is a public health problem in our country and was previously detected in raw cattle milk sold in bazaars in Samsun (Terzi, 2006).

Infectious hepatitis is considered to be one of the most serious viral diseases for which milk may be an important vehicle of transmission (Ballance 1954). The consumption of faecally contaminated foods is recognized as the predominant mode for the transmission of human enteric viruses, which are increasingly recognized as a significant public health concern (Sair *et al*., 2002).

The application of nucleic acid amplification for the detection of viral contamination is hindered by three factors: (i) large sample volume, (ii) low level contamination and (iii) the presence of inhibitors of enzymatic nucleic acid amplifcation. RT-PCR is a powerful technique for detecting the nucleic acid sequence of an organism and for differentiating between types of enteric viruses, such as enteroviruses, astroviruses rotaviruses, and adenoviruses (Casas and Sunen, 2001).

Recently, many RT-PCR assays have been developed for the molecular typing of enteroviruses. Phylogenetic studies also suggest that the 5' non-translated region (NTR) of all enteroviruses contains highly conserved RNA

secondary structures (Chapron *et al*., 2000). In this study, EV were detected in four milk samples (8%) using primers in the 5'-NTR region.

Similarly, the isolation of EV from dairy cattle has been reported by several researchers Dingman (1916) and Knapp *et al.* (1921) reported a milk-borne epidemic of poliomyelitis. Aycock (1927) described a larger epidemic in England. Of a total of 75 cases, 69 were associated with milk from one dealer. All the 43 paralytic cases used this milk. The milk was apparently pasteurized by the flash method only, in muggy weather, and was not bottled (Gordon and Brown, 1949).

In this study HAV was not detected in cow milk by RT-PCR. HAV infections, commonly known as infectious hepatitis, can cause liver damage leading to death (Hollinger and Tricehurst, 1996). HAV is an increasing problem in the Western countries of Europe because of the decrease in immunity of populations in countries with high standards of hygiene. The virus is most commonly transmitted via the faecal–oral route, either by direct contact with an HAV-infected person or by ingestion of HAV-contaminated food or water (Vasickova *et al*., 2005). Murphy *et al.* (1945) reported a hepatitis outbreak of 10 cases in Georgia, transmitted by milk. Some foodborne HAV outbreaks in Sweden have also been reported. Several hundred people became infected by HAV after consumption of HAV-contaminated oysters (Lund and Lindqvist, 2004). In 1976 a dish washer in a restaurant in Stockholm was infected by HAV. Twenty-eight consumers of the salad became HAV infected and two of the consumers died (Lund and Lindqvist, 2004). Terzi *et al*. (unpublished data) reported that HAV was detected in mussels obtained from the Black Sea region of Turkey.

In conclusion, to prevent milkborne outbreaks, the milk must be pasteurized and kept cool, the pasteurizing equipment must function adequately, and precautions must be taken to prevent post-pasteurization contamination. In addition, molecular epidemiological techniques can be successfully applied to identify and characterize an outbreak of HAV and EV in contaminated milk samples.

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REFERENCES

1. *Alais C*, 1974, Science du lait. In: Sepaic (ed). Principes des Techniques Laitieres, Paris, 636-65.
2. Anonymous, 2006, General Management of Basic Health Service Annual of 2006. http://www.saglik.gov.tr/TR/istatistik/2006/menu.doc, Accessed 5 January 2009.
3. Anonymous, 2009, Rapid Detection of Enterovirus Using Real-time PCR. https://www.labcorp.com. Accessed 20 July 2009.
4. *Atmar RL, Metcalf TG, Neill FH, Estes MK*, 1993, Detection of enteric viruses in oysters by using the polymerase chain reaction, *Appl Environ Microbiol,* 59, 631-5.
5. *Aycock WL*, 1927, A milk-borne epidemic of poliomyelitis, *Am J Epidemiol,* 7, 791-803.

6. *Baert L, Debevere J, Uyttendaele M*, 2009, The efficacy of preservation methods to inactivate foodborne viruses, *Int J Food Microbiol,* 131, 83-94.
7. *Ballance GA*, 1954, Epidemic of infective Hepatitis in an Oxford College, *Br Med J,* 1, 1071-14.
8. *Beneduce F, Pisani G, Divizia M, Panaž A, Morace G*, 1995, Complete nucleotide sequence of cytopatic hepatitis A virus strain isolated in Italy, *Virus Res,* 18, 299-309.
9. CDC, 2007, Annual Listing of Foodborne Disease Outbreaks, United States. Center for Disease Control and Prevention. http://www.cdc.gov/foodborneoutbreaks/outbreak_data.htm Accessed July 20, 2009.
10. *Casas N, Sunen E*, 2001, Detection of enterovirus and hepatitis A virus RNA in mussels (*Mytilus* spp.) by reverse transcriptase-polymerase chain reaction, *J Appl Microbiol,* 90, 89-95.
11. *Chapron CD, Ballester NA, Fontaine JH, Frades CN, Margolin AB*, 2000, Detection of astroviruses, enteroviruses, and adenovirus types 40 and 41 in surface waters collected and evaluated by the information collection rule and an integrated cell culture-nested PCR procedure, *Appl Environ Microbiol,* 66, 6, 2520-5.
12. *Chomcyznski P, Sacchi N*, 1987, Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction, *Anal Biochem,* 162, 156-9.
13. *Croci L, Medici DD, Scalfaro C, Fiore A, Divizia M, Donia D et al*., 2000, Determination of enteroviruses, hepatitis A virus, bacteriophages and *Escherichia coli* in Adriatic Sea mussels, *J Appl Microbiol,* 88, 293-8.
14. *Cromeans TL, Nainan OV, Margolis HS*, 1997, Detection of hepatitis A virus RNA in oyster meat, *Appl Environ Microbiol,* 63, 2460-3.
15. *Deng MY, Day SP, Cliver DO*, 1994, Detection of hepatitis A virus in environmental samples by antigen-capture PCR, *Appl Environ Microbiol,* 60, 1927-33.
16. *Dingman JC*, 1916, Report of a possible milkborne epidemic of infantile paralysis. New York State, *J Med,* 16, 589-90.
17. *Gordon C, Brown D*, 1949, The possible significance of milk and water in the spread of virus ýnfections, *Am J Public Health,* 39, 764-71.
18. *Gust I, Coulepsis A, Feinstone S, Locarnini S, Moritsugu Y, Najera R et al.*, 1983, Taxonomic classification of HAV, *Intervirology*, 20, 1-7.
19. *Hafliger D, Gilgen M, Luthy J, Hubner P,* 1997, Semi-nested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood, *Int J Food Microbiol,* 37, 27-36.
20. *Hollinger FB, Tricehurst JR*, 1996, Hepatitis A virus. In: Fields BN, Knipe DM, Howley PM (eds.), Fields Virology, Vol. 1. 3rd edition. Lippincott-Raven Publishers, Philadelphia, PA.
21. *Izuka N, Kuge S, Nomoto A*, 1987, Complete nucleotide sequence of genome of coxsackievirus B1, *Virology,* 156, 64-73.
22. *Jaykus LA*, 2000, Enteric viruses as žemerging' agents of foodborne disease, *Irish J Agric Food Res,* 39, 245-55.
23. *Kaplan MM, Abdussalam M, Bijlenga G*, 1952, Diseases transmitted through milk. Veterinary Public Health Unit, Division of Communicable Diseases, World Health Organization, Geneva, Switzerland.
24. *Koopmans M, Duizer E*, 2004, Foodborne viruses: an emerging problem, *Int J Food Microbiol,* 90, 23-41.
25. *Kopecka H, Prevot J, Girard M, Fuchs F, Aymard M*, 1988, Inte're ^ t des sondes ARNc (ribosondes) synthe'tise'e *in vitro* dans la detection des ente'rovirus par hybridation mole'culaire, *Annals Ins Pasteur – Virol,* 139, 217-25.
26. *Knapp AC, Godfrey ES, Aycock WL*, 1926, An outbreak of poliomyelitis apparently milkborne, *JAMA,* 87, 635-9.
27. *Lopman BA, Reacher MH, Van Duijnhoven Y, Hanon FX, Brown D, Koopmas M,* 2003, Viral gastroenteritis outbreaks in Europe, 1995–2000, *Emerg Infect Dis,* 9, 90-6.
28. *Lund F, Lindqvist R*, 2004, Virus in food and drinking water in Sweden. Norovirus and Hepatitis A virus. National Food Administration, Sweden, Report 22.
29. *Mast E, Alter MJ,* 1993, Epidemiology of viral hepatitis, *Semin Virol,* 4, 273-83.

30. *Melnick JL*, 1996, Enteroviruses: polioviruses, coxsackieviruses, echoviruses, and newer enteroviruses. In: Straus SE (ed.). Fields Virology, 3rd edn. Lippincott-Raven, Philadelphia, 655-712.
31. *Moll W, Davis AD*, 1959, Isolation and characterization of cytopathogenic enteroviruses from cattle with respiratory disease, *Am J Vet Research,* 20, 27.
32. *Moscovici C, Laplaca M, Maisel J, Kempe CH*, 1961, Studies of bovine enteroviruses, *Am J Vet Research,* 22, 852.
33. *Murphy WJ, Petrie LM, Work SD*, 1946, Outbreak of infectious hepatitis, apparently milk-borne. *Am J Public Health* 36, 169-73.
34. *Sair AI, D'Souza DH, Moe CL, Jaykus LA*, 2002, Improved detection of human enteric viruses in foods by RT-PCR, *J Virol Methods,* 100, 57-69.
35. *Shieh YC, Calci KR, Baric RS*, 1999, A method to detect low levels of enteric viruses in contaminated oysters, *Appl Environ Microbiol,* 65, 4709-14.
36. *Sunen E, Sobsey MD*, 1999, Recovery and detection of enterovirus, hepatitis A and Norwalk virus in hardshell clams (*Merceneria mercenaria*) by RT-PCR methods, *J Virol Meth,* 77, 179-87.
37. *Terzi G*, 2006, The investigation of *Brucella* antibody with milk ring test and agglutination test in milk collected from Samsun region, *TAF Prev Med Bull,* 5, 3, 196-203.
38. *Vasickova P, Dvorska L, Lorencova A, Pavlik I*, 2005, Viruses as a cause of foodborne diseases: A review of the literature, *Vet Med Czech,* 50, 3, 89-104.
39. *Venuti A, Di Russo C, Del Grosso N, Patti AM, Ruggeri F, De Stasio PR et al*., 1985, Isolation and molecular cloning of a fast-growing strain of human hepatitis A virus from its double-stranded replicative form, *J Virol,* 56, 2, 579-98.
40. *Zell R, Martin S, Sidigi K, Henke A, Stelzner A*, 1999, Functional features of the bovine enterovirus 5'-non-translated region, *J Gen Virol*, 80, 2299-309.

DETEKCIJA ENTEROVIRUSNE I HEPATITIS A VIRUSNE RNK U MLEKU KRAVA METODOM RT-PCR

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SADRŽAJ

Cilj ovih ispitivanja je bio da se odredi prisustvo enterovirusa (EV) i hepatitis A virusa (HAV) u mleku krava. Ukupno je analizirano 50 uzoraka sirovog mleka uzetih na pijaci u mestu Samsun u Turskoj i analizirano metodom RT-PCR. Virusna RNK je ekstrahovana iz mleka i mlečne surutke, brzim kiselim gvanidin-tiocijanat-fenol-hloroform metodom. Prajmeri za HAV su izabrani iz VP2 i VP4 sekvenci kapsidnog regiona. Prajmeri za enterovirus si izabrani iz 5' "ne-translatornog" regiona koji predstavlja izrazito konzervisani region enterovirusnog genoma. Od ukupno 50 uzoraka, četiri (8%) je bilo pozitivno na EV RNK dok HAV RNK nije dokazana ni u jednom. Ovo je u Turskoj prvi izveštaj o pokušaju detekcije EV i HAV RNK u kravljem mleku.