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Determination of Hepatitis A Virus, *Enterobacteriaceae*, Coliform and *Escherichia coli* Contamination of Frozen Raspberries*

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Summary: Hepatitis A is transferred via fecal-oral route, direct contact from person to person or with ingestion of contaminated food. Foods like, raspberries, strawberries and blueberries are important source of hepatitis A virus (HAV). Frozen retail packaged raspberries are analyzed in this study. Frozen raspberry samples have were obtained from local supermarkets monthly between January and December 2015 in Ankara province, Turkey. The samples were evaluated for HAV contamination using ISO/TS 15216-1:2013 in duplicate and Mengovirus was used for process control. Samples that found to be contaminated with HAV were further analyzed for *Enterobacteriaceae* Coliform and *Escherichia coli.* Totally 240 samples were analyzed and 20 (8.33%) were found to be contaminated with HAV. The highest HAV prevalence was on 6th month (25%). On the contrary no HAV was found on 3rd, 4th and 10th months of analyzes. The highest *Enterobacteriaceae,* Coliform and *E. coli* counts were found on 2nd month 4.90, 4.84 and, 3.48 $\log_{10}$ cfu/g, respectively. The results indicate that even the food relies with the microbiological criteria, there is no guarantee for viral contamination. Additionally, high cost of virus analyzes is another barrier for routine control which has a direct impact on public health. Additional measures like HACCP, GHP and GMP should be applied strictly from farm to fork to avoid contamination with HAV.
Key words: Coliform, *Enterobacteriaceae, Escherichia coli,* hepatitis A, raspberries

Dondurulmuş Ahududularda Hepatit A Virüs, *Enterobacteriaceae*, Koliform ve *Escherichia coli* Kontaminasyonunun Belirlenmesi

Özet: Hepatit A virüsü (HAV), genellikle fekal-oral yolla, kişiden kişiye direkt temas veya kontamine gıda ve suyun alınmasıyla bulaşmaktadır. Dondurulmuş ahududu, çilek ve yabanmersini gibi üzümsü meyveler kategorisinde yer alan ürünler, HAV kontaminasyonu açısından önemli kaynaklar arasında yer almaktadır. Bu çalışmada piyasada paketli olarak satılan dondurulmuş ahududu örnekleri kullanılmıştır. Dondurulmuş ahududu örnekleri, 2015 yılında Ankara'da bulunan marketlerden Ocak-Aralık ayları arasında her ay 20 tane olacak şekilde toplanmıştır. Çalışma konusunu oluşturan ahududu örneklerinde HAV analizi ISO/TS 15216-1:2013 uygun olacak şekilde iki paralel olarak yapılmıştır ve proses kontrolü için Mengovirüs kullanılmıştır. HAV pozitif bulunan örneklerde *Enterobacteriaceae,* Koliform bakteri ve *E. coli* sayımları gerçekleştirilmiştir. Toplam 240 adet ahududu örneğinin 20 adetinin (%8,33) HAV pozitif olduğu tespit edilmiştir. En yüksek prevalansın 6. ayda (%25) olduğu tespit edilirken, 3, 4 ve 10. aylarda alınan örneklerde ise HAV bulunmamıştır. En yüksek *Enterobacteriaceae,* koliform ve *E. coli* sayılarının 2. ayda olduğu ve bu ayda sırasıyla 4,90, 4,84 ve 3,48 $\log_{10}$ kob/g bakteri olduğu tespit edilmiştir. Bu sonuçlar gıdaların mikrobiyolojik kriterlere göre bakteriyel ajanlar bakımından yeterli koşulları sağlıyor olduğunu gösteriyor olmasına rağmen, viral etkenler bakımından herhangi bir garanti sağlamadığını göstermektedir. Ayrıca yüksek maliyetli virüs analizleri, halk sağlığı üzerinde doğrudan etkisi olan rutin kontrollerin yapılması için bir engel oluşturmaktadır. HACCP, GHP ve GMP gibi ilave önlemler, HAV ile bulaşmayı önlemek için kesinlikle çiftlikten çatala kadar uygulanmalıdır.
Anahtar kelimeler: Ahududu, *Enterobacteriaceae, Escherichia coli,* hepatit A, koliform

Introduction

Viruses need live cells to replicate, and essentially intracellular living beings. Therefore, the amount of viruses neither increases during processing, transport and storage, nor causes any sensory changes in foods (21). All food-borne viruses are human pathogens (25). Food might be contaminated with feces, which may carry virus, bacteria or other health hazard organisms. Due to this idea food borne virus contamination usually thought to indicate fecal contamination of foods

Food-borne viruses are divided into three groups; viral gastroenteritis (Norovirus, Rotavirus and, Astrovirus), viral hepatitis (Hepatitis A and E) and other food-borne viruses (Enteroviruses) (24). Hepatitis A

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virus (HAV) belongs to the genus *Hepatovirus* of the *Picornaviridae* family. HAV is a positive sense virus that has a single helix RNA and it has a diameter of 27-32 nm and no membrane (4, 22, 24). HAV disinfectants are quite resilient to low pH, temperature and environmental conditions (22, 23).

Hepatitis A is generally transmitted through fecal or oral means, direct contact between people, or the intake of contaminated food and water (16, 22, 23). Food can be contaminated with HAV both during growing and later phases (27). Nearly 1.5 million hepatitis A cases occur worldwide each year (16). Marine crustaceans, fresh vegetables and fruits, salads, milk and, convenience food products that cause outbreaks most (1, 5, 23, 27, 28). Due to low infectious dose and long incubation period of the disease in foodborne hepatitis A outbreaks, the cause cannot be determined in 50% of the cases (3, 27). Contamination of foods usually is heterogynous, and the contaminating particle numbers are lower than the numbers in feces (2).

Such fruits as raspberries and strawberries are considered to be an important source of HAV contamination (10, 29). These fruits can be contaminated due to the usage of waste water during agricultural irrigation, fertilization or later processing stages conducted by infected personnel (8, 29, 30, 31). Hepatitis A outbreaks occur when these products are put on sale unpacked and touched with bare hands in markets. Additionally, they are not put through multi-phase washing processes so that their shelf-lives or sensory qualities are not affected (14). The risks posed by fruits are regarded as serious by European Union Prompt Alarm System. The records of the year 2013 alone reveal 13 cases, all of which report HAV contamination of grape-like plants (11). The studies conducted in Turkey are mostly seroprevalence studies, and studies regarding the presence of HAV in food products are very limited. This study can be accepted as the first report for HAV contamination and prevalence of frozen raspberries and it is first to represent possible link and correlation between fecal contamination and HAV contamination.

Material and Methods

In this study, samples of packaged frozen raspberry [Rubusideaus, (Ln.)], have been analyzed. 20 samples were obtained from local supermarkets monthly between January and December 2015 in Ankara province, Turkey. After the samples were obtained, they were brought to the laboratory and analyzed within 2 hours.

The HAV analysis of the raspberry samples in this study was performed according to ISO/TS 15216-1:2013 (20). *Mengovirus* was used for process control. All samples were analysed in two parallels. Enterobacteriaceae counting was performed according to ISO 21528-2 method (18), coliform bacteria counting ISO 4832 (19) and *Esherichia coli* counting ISO 16649-2 (17) methods. HAV analyzes were held after elution and concentration of virus particles and real time PCR was used to demonstrate the contamination. For this purpose the raspberry samples were mixed for 20 min with an equal volume of buffer containing 0.25 mol l^{-1} Tris base and 1.92 mol l^{-1} glycine (Sigma, St Louis, MO, USA), and the liquid was transferred to a centrifuge tube. The tubes were centrifuged at 9.000 *g* for 30 min at 4°C. The supernatants were transferred to another tube, and 0.05 volumes of 10 mg ml^{-1} bovine serum albumin (Promega, Madison, WI, USA) and 0.1 volume of 5 mol l^{-1} sodium chloride were added to each sample. The samples were mixed well by inversion. An equal volume of 17% polyethylene glycol (Sigma) was added to each sample, and the samples mixed by inversion and incubated overnight at 4°C. Following overnight incubation, the samples were centrifuged at 9.000 *g* for 30 min at 4°C, and the pellets were stored for further extraction.

After HAV recovery from samples, the viral pellet was dissolved in 1 ml of acid guanidinium thiocyanate–phenol–chloroform mixture (Biotecx Laboratories Inc., Houston, TX, USA). Total RNA was precipitated from the aqueous phase by the addition of an equal volume of 100% isopropanol, washed with 75% ethanol and solubilized in RNase-free water. During RNA extraction, special care was taken to avoid cross-contamination by separating pre- and post-PCR work areas and using aerosol-resistant tips. Following conventional PCR, a NRT-PCR was performed using a set of internal primers, forward, reverse, and a TaqMan probe (FAM). The primer sequences are shown in table 1. The TaqMan probe, containing a 5′ reporter dye and a downstream 3′ quencher, hybridized to a specific sequence of the target HAV sequence amplified by the NRT-PCR. The amplification reactions were carried out in a total volume of 25 µl containing 1× PCR Master-mix (Applied Biosystems), 12.5 pmol of each primer, 15 pmol fluorescence-labelled probe, nuclease-free water and 2.5 µl of the conventional PCR product. The NRT-PCR assay was performed using SmartCycler II (Cepheid, Sunnyvale, CA, USA). The following amplification protocol was used: 1 cycle at 95°C for 2 min, 50 cycles at 95°C for 15 s and 60° C for 40 s. Data analysis was performed using avai-

Table 1. Primer sequences used for detection of hepatitis A virus

Primers	5′ to 3′
Forward	AGG CTA CGG GTG AAA CCT CTT AG
Reverse	CGC CGC TGT TAC CCT ATC C
TagMan Probe	AAT ACT TCT ATG AAG AGA TGC C

Table 2. Prevalence of HAV in frozen raspberries with contamination levels of *Enterobacteriaceae,* Coliform and *E. coli* in HAV positive samples (log_{10} cfu/g ± Sd)

Month	HAV (%) (P/T)*	*Enterobacteriaceae*	Coliform	*E. coli*
1	10 (2/20)	3.70±0.02	3.44±0.08	2.30±0.43
2	5 (1/20)	4.90	4.84	3.58
3	0 (0/20)	-	-	-
4	0 (0/20)	-	-	-
5	5 (1/20)	4.00	2.30	$<10^2$
6	25 (5/20)	3.86±0.98	3.63±0.30	2.69±0.74
7	15 (3/20)	4.22±1.22	2.77±1.34	$<10^2$
8	5 (1/20)	4.96	3.49	$<10^2$
9	10 (2/20)	3.82±0.15	$<10^2$	$<10^2$
10	0 (0/20)	-	-	-
11	20 (4/20)	4.63±0.32	$<10^2$	$<10^2$
12	5 (1/20)	3.82	2.85	2.30

*Positive samples/Total samples

lable software (Applied Biosystems, 7500 Fast Real-Time PCR system Software). The cycle threshold (Ct) values were determined from the mean baseline signals during the early cycles of amplification. The amount of virus measured with NRT-PCR was determined using a standard curve.

Enterobacteriaceae, coliforms and *E. coli* counts were held using Violet Red Bile Dextrose Agar, Violet Red Bile Lactose Agar, and Tyrptone Bile X-glucoronide Agar (Merck, Darmstadt, Germany) respectively. For counting 25 g of raspberries were diluted using 225 ml peptone water and 0.1 mL of these dilutions were streaked on previously mentioned agars.

Results

Twenty (8.33%) of a total number of 240 raspberry samples were determined to be HAV-positive. While the highest prevalence (25%) was seen in the sixth month, HAV was not found in the samples that were taken in the third, fourth and tenth months. It was found that HAV contamination at eleventh month was 20%, 15% on seventh month, 10% on first and ninth months. Likewise on second, fifth, eighth and twelfth months it was 5%. At the same months *Enterobacteriaceae* was 4.90, 4.00, 4.96 and 3.82 log_{10} cfu/g and coliform was 4.84, 2.30, 3.49 and 2.85 log_{10} cfu/g. All HAV positive results were also positive for *Enterobacteriaceae.* However, on ninth and eleventh months no coliforms were found, together with HAV. Likewise, no *E. coli* contamination was accompanying to HAV on fifth, seventh, ninth and eleventh months. On ninth and eleventh months no *E .coli* contamination was recorded with HAV. In all HAV positive samples, highest *Enterobacteriaceae* contamination was recorded at 8th month (4.96 log_{10} cfu/g), coliform (4.84 log_{10} cfu/g) and *E. coli* (3.58 log_{10} cfu/g) was on the second month. *E. coli* numbers were higher than regulation limit on first, second, sixth and twelfth months with the contamination levels of 2.30±0.43, 3.58, 2.69±0.74 and 2.30 log_{10}cfu/g The HAV prevalence in raspberries, the numbers of *Enterabacteriaceae,* coliform and *E. coli* are shown in Table 2.

Discussion and Conclusion

While the studies on outbreaks due to hepatitis A in grape-like fruits mainly focus on determining the source of the outbreak and HAV incidence in humans (7, 12, 13, 15, 26), sufficient literature on hepatitis A prevalence in these products is lacking worldwide. Widespread consumption of the above-mentioned products without heat-treatment highlights the importance of viral contamination in these products. Also, it is known that HAV is not inactivated with freezing, a method of food preservation and the agent can survive freezing temperature for long periods (6). The fact that HAV was detected in this study shows that the agent can survive freezing conditions.

The fact that there is no control in any production stages to eliminate HAV and it is consumed directly is the reason why these products become the cause of frequent outbreaks (29). These products generally have acidic compositions (pH 2.5-3.3) and contain approximately 5% of sugar. HAV is more stable against heat-treatment in low pH and high sugar concentration (9, 10).

The data obtained in the present study show similarity with the studies (21,25) that report no bacterial indicators of fecal contamination in some of the products with HAV and no correlation between viral agents and bacterial agents. These food products pose a risk in terms of viral contamination even if they meet the required microbiological conditions (25).

In conclusion, the fact that food products meet the required microbiological conditions does not offer any guarantee in terms of viral agents, what is more, it is continuing to of importance in terms of public health as a result of the economic difficulties in applying analyses to routine. In order to eliminate the risk, it is necessary to focus on the areas where contamination

first occurs. The contaminations that may occur during and after processing need to be prevented by using such systems as HACCP, GHP and GMP efficiently. Also, care must be taken to prevent the risk posed by these products that are imported from endemic areas to the people in non-endemic areas. The imported products have to be checked with a fast and reliable method. Studies must be increased to build various chemical and physical barriers to minimize the contamination of grape-like fruits with HAV.

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