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ORIGINAL ARTICLE

Detection of Hepatitis A Virus and Enteroviruses in Raw Milk and Some Dairy Products From Dakahlia Governorate, Egypt Using Real-time PCR

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Abstract

BACKGROUND: The present study aimed to evaluate the presence of hepatitis A virus (HAV) and enterovirus (EV) in raw milk and dairy products and highlight the potential significance of real-time polymerase chain reaction (PCR) in the detection of foodborne viruses.
METHODS: A total of 165 samples of dairy products (including milk cream and Kareesh cheese, 15 samples each) and 135 raw milk samples were randomly collected from different households with either hand milking or bulk tanks in Dakahlia Governorate, Egypt, over 28 months from July 2020 to November 2022, using real-time PCR as a diagnostic method.
RESULTS: Only two milk samples were positive for HAV (1.48%, 2/135), while no milk samples tested positive for EV. Additionally, two cream samples (13.33%, 2/15) tested positive for HAV in dairy products; however, there were no positive samples in the Kareesh cheese. Dairy products and milk were completely free of EV.
CONCLUSION: HAV was recovered from raw milk and milk products, while all the tested samples were completely free from EV, indicating that HAV is more prevalent in the environment than EV. This study highlights the urgent need for more precise and sensitive viral recovery techniques to identify food-borne viruses that pose a risk to human health.

Keywords: Hepatitis A virus and enteroviruses, Milk and dairy products, Real-time RT-PCR

1. Introduction

Outbreaks and illnesses caused by food-borne pathogens have a significant impact on health, not only through illness but also through the costs associated with actions taken to mitigate their effects on communities [1]. According to a recent analysis by specialists in risk assessment, food-borne viruses are among the top priorities for food safety [2].

Enteric viruses such as hepatitis A virus (HAV) and enteroviruses are global public health concerns that have led to significant food-borne outbreaks and financial losses. HAV is one of the most prevalent viruses associated with foodborne outbreaks [3]. The virus has a single antigenic type and is considered a member of the genus Hepatovirus in the family Picornaviridae [4]. The virus is a non-enveloped, positive-sense, single-stranded RNA virus, and its infectious dose is roughly estimated to be 10−100 virions with a long incubation period (2−7 weeks) [5].

Infected individuals can shed large numbers of viruses (10^6−10^8 particles/g) which starts two weeks after infection and continues for five months, resulting in infectious hepatitis and jaundice [5].

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Children are more susceptible than adults, and developing nations, particularly those in certain regions of Africa, Asia, and the Middle East, have higher infection rates [6].

Numerous foodborne HAV outbreaks have been reported worldwide. A recent study indicated that between 1988 and 2018, global HAV outbreaks were primarily associated with contaminated food [3]. An important outbreak related to frozen berries included 1589 cases in 13 countries of the European Economic Area, with Italy having the highest number of cases (1438) [7]. A total of 148 outbreaks linked to the consumption of raw milk products were reported in the United States between 1998 and 2011, resulting in 2348 illnesses, and two fatalities [8]. Also, the frequency of outbreaks linked to raw dairy products is approximately 150 times higher than that of pasteurized dairy products per unit of product consumed [9]. The symptoms of the disease range from moderate jaundice and inflammation to sudden liver failure [10].

Enterovirus (EV) is a positive-sense single-stranded RNA virus that belongs to the family Picornaviridae, ranges from 20 to 30 nm in diameter, and has been associated with several human and animal diseases [11]. Infection by EV has been linked to a variety of clinical syndromes, such as encephalitis, meningitis, gastroenteritis, respiratory illnesses, and paralytic syndromes, in addition to asymptomatic infection [12]. A food-borne outbreak occurred in 1914 due to consumption of raw milk contaminated with EV, which led to a poliomyelitis outbreak [13].

HAV and EV (among other human enteric pathogens) can spread during the process of milking or through contaminated surfaces, including milk utensils and milk tanks, as well as the hands of infected handlers [14]. Because of the low infectious dosage of these viruses, the population may be seriously at risk from even minute amounts of enteric viruses found in milk [15,16]. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) is still the most frequently used method for detecting viruses in food because of its speed, ease of use, sensitivity, and specificity [3]. There is little information regarding the contamination of milk and dairy products with foodborne viruses. Therefore, the present study aimed to detect the potential occurrence of HAV and EV in raw milk and dairy products in Dakahlia Governorate using real-time PCR.

2. Materials and methods

2.1. Samples collection

A total of 165 samples were obtained, including 135 raw milk samples and 30 dairy products (milk cream and Kareesh cheese, 15 each). Samples were randomly collected from various dairy sources in Dakahlia Governorate, Egypt, from July 2020 to November 2022. Raw milk and dairy products were collected from either small household farms representing hand milking (71 samples), or from local markets representing bulk tanks (94 samples). According to the season, summer, autumn, and winter were represented by (60, 31, and 74 samples), respectively. All samples were collected aseptically in sterile plastic bags and bottles, kept in coolers, and immediately transferred to the laboratory for further processing.

2.2. Samples preparation

The collected milk (2.5 mL) or dairy products (2.5 g) were homogenized in 7.5 mL of beef glycine buffer adjusted to pH 9.5 and stirred for 30 min with a magnet stirrer. Thereafter, it was centrifuged at 5000 rpm for 20 min and filtered with a Whattman membrane of 142 mm diameter and 0.45 μm pore size, followed by organic flocculation as a final step. The same procedures were performed for the Kareesh cheese samples, except for the organic flocculation step.

2.3. Extraction of viral RNA

Viral RNA was extracted from the collected samples using the WizPrep™ Viral DNA/RNA Mini Kit (Wizbiosolutions, Republic of Korea), according to the manufacturer's instructions.

2.4. Real-time PCR for quantification of HAV and enterovirus

RT-PCR using the TaqMan probe was performed to quantify HAV and EV genome copies in a one-step RT-qPCR amplification assay. The primers and probes that were used are listed in Table 1.

For HAV, RT-PCR was performed using HAV primers and probes (Thermo fisher) according to the method described by Costafreda *et al.* [4] in a total volume of 25 μl containing 2.5 μl buffer (10×), 1 μl dNTPs (10 mM), 0.7 μl forward primer, 0.7 μl reverse primer (both of them stock 100 μM), 0.3 μl probe, 14.8 μl nuclease-free water, and 5 μl extracted RNA. Amplification was performed using an RT-PCR thermal cycler (Rotor-Gene Q; Qiagen). The thermocycling profile was 50 °C for 1 h for the reverse transcription reaction, at 95 °C for 10 min as a hot start, and 45 cycles of PCR as follows: 95 °C for 15 s for denaturation, 60 °C for 1 min for annealing, and 70 °C for 1 min for extension. Fluorescence was

Table 1. Primers and probes used for quantification of hepatitis A virus and enterovirus.

Virus	Primers (′5-3′)	Probe (′5-3′)	Reference
Hepatitis A virus (HAV)	Forward (HAV 68) ′5-TCACCGCCGTTTGCCCTAG-3′ Reverse (HAV 240) ′5-GGAGAGCCCTGGAAGAAAG-3′	′5-CCTGAACCTGC AGGAATTAA-3′	**Costafreda *et al.* (2006)** [4]
Enterovirus (EV)	Forward (EV-UTR) ′5-ACATGGTGCGAAGAGTCTATTGAGCT-3′ Reverse (EV-UTR) ′5-CGACTACTTTGGGTGTCCGTGTTTC-3′	′5-VICTCCGGCCCCTGAA TGCGGCTAAT-MGB-NFQ-3′	**Cabrerizo *et al.* (2014)** [17]

evaluated at the end of each cycle and the results were interpreted according to the standard curve.

For EV, RT-PCR was performed using primers and probes (Thermo fisher) according to the method described by Cabrerizo *et al.* [17] in a total volume of 45 μl containing 4.5 μl buffer (10×), 2 μl dNTPs (10 mM), 1.1 μl forward primer, 1.1 μl reverse primer (both stock 100 μM), 0.7 μl probe, 30.6 nuclease-free water, and 5 μl of extracted RNA. Amplification was performed in a real-time PCR thermal cycler (Rotor-Gene Q; Qiagen). The thermocycling profile was 50 °C for 2 min for the reverse transcription reaction, at 95 °C for 10 min as a hot start, and 45 cycles of PCR as follows: 95 °C for 15 s for denaturation, and 60 °C for 1 min for annealing-extension. Fluorescence was evaluated at the end of each cycle and the results were interpreted according to the standard curve.

3. Results

3.1. Detection of HAV and enterovirus

Two samples each of raw milk (1.48%, 2/135) and two samples of cream (13.33%, 2/15) were positive for HAV, whereas none of the tested samples were positive for enteroviruses. The positive milk samples contained 4.32 × 10 and 5.12 × 10 genome copies/ml with Ct values of 39.36 and 39.08, respectively, while the positive samples of milk cream contained 7.27 × 10 and 9.13 × 10 genome copies/g with Ct values of 38.56 and 37.83, respectively (Table 2). Based on the seasonal distribution, the positive samples were determined in winter (4.05%, 3/74), and autumn (3.22%, 1/31) (Table 3). Three positive samples originated from small household farms (4.22%, 3/71), and one sample from local markets (1.06%, 1/94) (Table 4).

Table 3. Prevalence of hepatitis A virus and enterovirus in the examined samples according to season.

Identified Virus	Spring ($n = 0$)	Summer ($n = 60$)	Autumn ($n = 31$)	Winter ($n = 74$)
HAV	0	0	1 (3.22%)	3 (4.05%)
EV	0	0	0	0

4. Discussion

Given that the available information about the occurrence of enteric viruses in raw milk and dairy products in the Dakahlia governorate is scarce, the present study was conducted to detect HAV and EV in raw milk and dairy products.

It has been documented that food could be act as a vehicle for transmission of human enteric viruses [15,18,19]. Large outbreaks of hepatitis and gastroenteritis of suspected food-borne origin have been reported in the literature [20–24]. According to El-Senousy *et al.* [25] enteric viruses, especially HAV and enteroviruses, are of great importance in the food industry because they are related to many food-borne outbreaks, either due to consumption of unpasteurized milk or using contaminated water in cleaning milking utensils or during food preparation throughout production, packaging, and distribution processes. Extremely high numbers of viruses shed in stools of infected individuals (10^5 to 10^{13}/g feces), and human enteric viruses are transmitted mainly through the oral–fecal route, which can occur directly between individuals or via consumption of contaminated food or water.

Table 2. Prevalence of hepatitis A virus and enterovirus in the examined samples.

Identified virus	Raw milk ($n = 135$)	Milk cream ($n = 15$)	Kareesh cheese ($n = 15$)
HAV	2 (1.48%)	2 (13.3%)	0
EV	0	0	0

Table 4. Prevalence of hepatitis A virus and enterovirus in the examined samples according to scale.

Identified Virus	Small household farms ($n = 71$)	Local markets ($n = 94$)
HAV	3 (4.22%)	1 (1.06%)
EV	0	0

In the present study, the majority of tested samples were negative for both HAV and EV. Only two samples were positive for HAV. These results are consistent with those reported by Mortazavi *et al.* [26]. Zaher *et al.* [27] found HAV in 4.75% (19/400) of milk samples, and Pakbin *et al.* [28] identified HAV in 25.81% (71/492) of the tested raw milk samples. Terzi *et al.* [29] detected EV in 8% (4/50) of tested raw milk samples, whereas HAV was not found in any of the tested samples. Shalaby *et al.* [30] identified EV in 7.33% (11/150) of the raw milk samples. Regarding the presence of HAV in dairy products, virus was detected in 13.3% (2/15) of tested cream samples. These results were higher than those reported by Zaher *et al.* [27] who found HAV in 5.5% (22/400) of ice cream samples and 4.5% (18/400) of cottage cheese samples. Neither HAV nor EV were detected in any of the examined Kareesh cheeses. Variations in viral recovery might be due to different contamination levels in the examined samples or the suitability of the viral extraction method to some types of milk products more than the other types.

The data in Table 3 illustrate that three positive samples were recorded in winter (4.05%, 3/74) and one in autumn (3.22%, 1/31), while no positive samples were detected during summer, indicating that HAV infection is more common in winter and autumn than in summer and spring, which may be attributed to the survival of viruses at lower temperatures because HAV and EV are heat-labile viruses that are sensitive to high temperatures [31−34].

The results in Table 4 show that the positive samples from household farms were higher than those found in samples collected from markets at 4.22% (3/71) and 1.06% (1/94), respectively. These results were compatible with those reported by Shalaby *et al.* [30] in which EV was detected in 10% (5/50) of farmers' house milk samples and 4% (2/50) of market samples. High levels of contamination found in samples collected from households could be related to a poor sanitized environment and fecal contamination of milk samples or water used to clean milking utensils. In contrast, bulk tanks exhibited lower levels of contamination. This might be due to the mixing of milk from different sources, free and contaminated, which dilutes the virus titer in the milk.

Sensitive and reliable techniques for the accurate detection of enteric viruses in milk and milk products are required to ensure their safety. Determination of HAV markers in milk samples is critical. Based on the data in this study, it is clear that the inhibitory substances found in milk have an impact on HAV detection tests. Although it has been previously documented that diluting the milk matrix enhances the performance of HAV molecular analysis, diluting the milk samples is not the optimal strategy, particularly if the contaminated milk sample has a low HEV load. Hennechart-Collette *et al.* [35] reported that whey proteins, besides casein, negatively affect viral genome recovery, resulting in reduced sensitivity and false-negative results.

5. Conclusion

Preventive strategies, such as hygienic measures and manufacturing practices, must be included in production chains of milk and dairy products to minimize contamination and transmission of food-borne viruses to humans and to prevent the occurrence of food-borne outbreaks.

Declarations

Ethics information

The current research work was conducted according to standards of Research Ethics committee, Faculty of Veterinary Medicine, Mansoura University.

Availability of data and materials

Data in the present study is available.

Conflicts of interest

The authors declare that there is no any conflict of interest in the current research work.

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Authors' contribution

Ahmed Elhady performed experiments and wrote the first draft of the manuscript. Waled Morsy El-Senousy and Seham F. Hassan performed the molecular experiments at the Environmental Virology Laboratory, National Research Center, Egypt. Mayada Gwida and Maha Al-Ashmawy supervised the entire research work and revised the manuscript. All the authors have read and approved the final version of the manuscript for publication.

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