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Initial insights into the genomic detection of hepatitis E virus in goat milk and feces in Iran: The need for surveillance and control measures

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Abstract

Hepatitis E virus (HEV) is recognized as a novel zoonotic agent. Due to lack of data on the presence of HEV in small ruminants in Iran, the study aimed to detect the HEV RNA in goat feces and raw milk samples. To address this gap, 155 pooled samples, including 83 feces and 72 milk samples, were collected from 83 goat farms during December 2022 to December 2023 in Mazandaran province. All samples were screened for HEV RNA by conventional PCR and nested RT-PCR. A total four farms were found positive for HEV RNA, exclusively in feces samples, while all milk samples were negative. These finding propose that fecal-oral transmission still remains as the prevailing route of HEV transmission. Although, all milk samples were negative, we cannot eliminate the potential risk of HEV transmission through dairy products. This data has provided insight into the circulation of HEV in the targeted population and emphasizes the need for comprehensive surveillance and routine diagnostic programs. Furthermore, epidemiological investigations are necessary to evaluate viral dissemination among livestock and to identify potential risk factors to humans.

Introduction

In the last few years, there has been more knowledge about zoonotic infections that can be transmitted through food products has been raised (1). This is primarily due to much research on outbreaks caused by viral and

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bacterial foodborne pathogens, causing health issues and impeding the economic situation of developed and underdeveloped countries (2).

Recently, the Hepatitis E virus (HEV) has become a growing global concern. It is an emerging infectious agent that causes hepatitis E disease, resulting in acute liver inflammation. According to the World Health Organization, more than 20 million new cases of hepatitis E virus infection occur annually, and it is estimated that approximately one-third of the world population has been affected by HEV (3). HEV, formally known as Paslahepevirus balayani by International Committee on Taxonomy of Viruses, is a single-stranded RNA virus classifying into the genus *Paslahepevirus* in the family *Hepeviridae*. *Orthohepevirus* (4). Its RNA genome has a 7.2 kbp in length, which is surrounded by an icosahedron capsid. In all known HEV strains, the genome composes of three open reading frames (ORFs), including ORF1, ORF2, and ORF3. Exceptionally, in genotype 1 strains an additional ORF, ORF4, has been recognized (5). HEV involves eight distinct genotypes and can infect humans and various animal species. Genotypes 1 and 2 lead to infection only in humans and are associated with the Asia, Africa, and Central America regions (6). Genotypes 3, 4, and 7 are important regarding zoonotic concerns. Genotypes 4 and 3 have significant virulence and often result in severe disease (7). These genotypes have been frequently detected from pigs, goats, cattle, deer, wild boar, and rabbits, while genotype 7 has also been isolated in camels. Genotypes 5 and 6 have been recognized in nature but have only been described in pigs. Additionally, genotypes 7 and 8 have been identified in camels (7-9).

HEV is primarily transmitted through the fecal-oral route, which usually occurs through the consumption of water contaminated with infected host feces. Contaminated water supplies with human fecal waste have been identified as the responsible for large outbreaks of HEV infection in countries located in tropical and subtropical regions (10). The virus is shed in high quantities in feces, posing a risk to livestock farmers exposed to asymptomatic infected animals. Sporadic infections through the consumption of raw milk and infected liver, uncooked and undercooked meat, particularly pork and its derived products, have been described (11).

HEV develops a systemic disease that primarily affects the liver. It generally causes asymptomatic, self-limiting, and acute infection and rarely fatal disease (12). In humans, HEV develops flu-like clinical symptoms followed by fever, abdominal pain, arthralgia, vomiting, anorexia, skin rash, jaundice, liver failure, and dark urine (5). HEV shows a higher rate of mortality in pregnant women and young infants. Additionally, immunocompromised patients can develop chronic hepatitis (13). There are few reports concerning the abundance of HEV in the milk and feces of livestock ruminant species in developing countries (14), and the potential risk of zoonotic infections through virus transmission from small ruminants to humans has not been clear. In Iran, previous studies have documented the presence of HEV RNA in various hosts, including human, dromedary camel, and gazelles which consequently emphasized the concern over the virus transmission from animals to humans (15-17). However, no data are available regarding HEV detection in small ruminants, resulting a notable gap in understating HEV epidemiology in these animals.

Goats are the one the most important livestock animals in Iran. They are widely raised for the milk and meat production (18). They breed under traditional and semi-intensive management systems in Iran. In regarding to the close contact of these animals with human and consumption of their raw milk and dairy products in rural areas may increase the zoonotic transfer of HEV from infected animals (19). Thu, this study aimed to establish the first baseline information about the presence of HEV in goat in Iran by detecting HEV genome in a range of feces and raw milk samples.

Materials and methods

Sampling

Due to evaluate the presence of HEV in goat population in the north of Iran, goat farms located in ten cities of Mazandaran province, including Chalus, Nowshahr, Nur, Amol, Babol, Babolsar, Savadkooh, Qaemshahr, Sari, and Juybar were selected (Figure 1).

Samples were taken from 83 farms during December 2022 to December 2023. Goats are not as the primary livestock species in north of Iran. For this limitation, only farms which have at least with 20 goats were included in this study. Before sampling, the farm owner's consent was obtained. A structured questionnaire to record the herd population and History of diarrhea of each farm was completed by the farmers. In addition, the hygienic condition of animal housing areas of each farm based of several criteria including the floor and walls, the provision of appropriate ventilation, the cleanliness of the water trough, the establishment of disease prevention and control measures, such as the isolation of sick or newly arrived animals, and the consistent examination of the animals was evaluated (20). Husbandry management system was categorized in two groups, including intensive (with up to 20 animals) and semi-intensive (with more than 21 animals). Samples of goat feces and milk were collected from each farm. Four animals were selected randomly based on ear tag list of each farm for sampling. In the case of male animals, only feces samples were taken. Feces samples were taken from the rectum using a swab and placed in PBS solution (pH=7.2) supplemented with antibiotics (penicillin/streptomycin) and amphotericin B. The milk samples were taken directly from the goats' udders in sterile containers. Subsequently, Individual samples within each farm were pooled. Each pooled sample consisted of four milk or four feces samples. In total, 72 pooled milk and 83 pooled feces samples were prepared. All samples were transferred to the laboratory at a temperature of 4°C and subsequently stored in a freezer at -70 °C for further analysis.

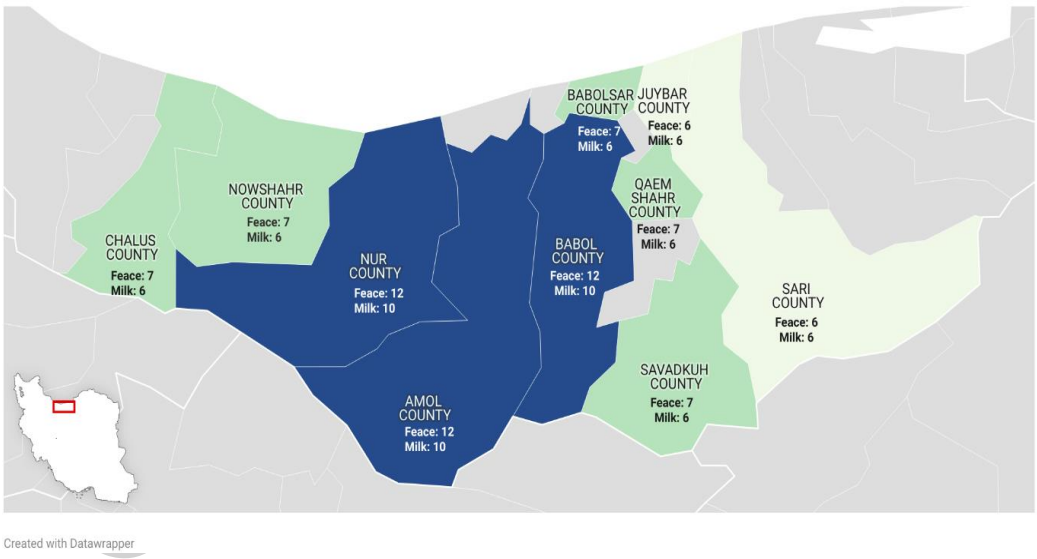

Fig. 1. Distribution of feces and milk samples in 10 studied cities of Mazandaran province, Iran. The image was generated by Datawrapper (https://app.datawrapper.de).

Preparation of samples

All samples were thawed spontaneously at a temperature of 4°C. For the stool samples, the swab was initially squeezed into the body of the tubes containing the sample. Subsequently, the suspended particles were precipitated by centrifuging at 12000 RPM for ten minutes. Finally, 300 ml of sediment-free supernatant was used for genome extraction. For the milk samples, in order to separate the fat part, the samples were centrifuged at 8000 RPM for 15 minutes. Then, 300 ml of skim milk was used for genome extraction. To show the efficiency

and reliability of the extraction method applied in this study, 150 µl of control virus (Newcastle virus, genotype VII ~10^8 EID50/ml) was added to milk and feces samples. For negative control Phosphate buffered saline (PBS) was used.

Genome extraction

The extraction of the virus's genetic material was conducted by using the Viral DNA/RNA extraction kit (Intron, South Korea) according to the manufacturer's instructions. Briefly, the samples were subjected to one step of chemical and enzymatic lysis by lysis buffer (provided by the kit) and proteinase K, respectively. Subsequently, the viral genome was extracted using a two-step washing procedure and eluted in a volume of 45 µL and stored at -80 °C until next step.

cDNA synthesis

Single-stranded cDNA was created using Easy™ cDNA Synthesis Kit (Parstous®, Iran) according to the manufacturer's instructions. The 20 µl of reaction mix contained 5 µl RNA extract, 10 µl of 2x buffer-soultion (including RT buffer, 1mM dNTP mixture, 8 mM MgCl2, Oligo d (t)16 and random hexamer primers and stabilizer), 2 µl Enzyme Mix, and 3 µl of DEPC-treated water. Subsequently, after short vortex of mixture, it was incubated for 10 min at 25°C and 60 min at 47°C. Finally, the reaction was finished by heating at 85 °C. Then products were stored at -80 °C until next step.

PCR

To detect the viral genome, nested PCR and conventional PCR were applied using distinct primer pairs. The first-round nested PCR was carried out with primer pairs HEV-CAS/HEV-CS and HEV-CASN/HEV-CSN serving as external and internal primer pairs, respectively. Furthermore, the second-round nested PCR was done with primer pairs HEV-S11/HEV-A12 and HEV-S12/HEV-A11 which served as external and internal primer pairs, respectively. The conventional PCR method also utilized primers HEV-F and HEV-R which targets ORF2 of HEV genome and amplify 145 bp fragment. For control virus, the 362 bp fragment related to Newcastle virus fusion protein was synthesized by primers NDV-A (F) and NDV-B(R). The information about all primers is listed in Table 1. The PCR amplifications were prepared in a final volume of 25 µl. The reaction mix composing 4 µl of cDNA, 12.5 µl of 2x PCR Master mix (Ampliqon®, Denmark), 0.4 pmol of each forward and reverse primers, and 17 µl of nuclease-free water was prepared. The PCR thermal scheme consisted of 35 cycles of denaturation at 95 °C for one minute, followed by annealing at a designated temperature specific to each pair of primers for one minute (table 1), and extension at 72 °C for 1 min.

Table 1. Sequence of primers used for detection of HEV RNA in this study.

PCR type	Target	Primer	Sequence (5´to 3́)	Amplicon (bp)	Annealing	Ref.
Conventional RT-PCR (HEV)	ORF2	HEV-F	TTCCACCACCCAGCAGTATT	145	56	(16)
		HEV-R	GGCATTCTCAACGAGCAGTT			
		HEV-CS	TCGCGCATCACMTTYTTCCARAA	470	52	(21)
	ORF3	HEV- CAS	GCCATGTTCCAGACDGTRTTCCA			
Nested RT-PCR (HEV)		HEV-CSN	TGTGCTCTGTTTGGCCCNTGGTTYCG	325	55	(21)
		HEV-CASN	CCAGGCTCACCRGARTGYTTCTTCCA			
		HEV-S11	AGGCTCCTGGCRTYACTACTG	1283	61	(22)
	ORF-2	HEV- A12	GCGGCACTGGGCRTAAAACT			
		HEV-S12	GCCYTGGCGAATGCTGTG	1198	62	(22)
		HEV-A11	GGCCRGGAATGTAATCACG			
Conventional RT-PCR (NDV)	F protein	NDV-A(F)	TTGATGGCAGGCCTCTTGC	362	52	(23)
		NDV-B(R)	GGAGGATGTTGGCAGCATT			

PCR product analysis

The 1.5% agarose gel electrophoresis in 1X TAE buffer was used for PCR amplicons electrophoresis. The gel was stained with a safe stain (CinnaGen, Iran). All samples were loaded alongside a 100 bp ladder (CinnaGen, Iran), as a size marker. Samples that displayed bands corresponding to the expected size were identified as positive.

Results

All control samples, including both milk and feces containing live NDV, were positive for the NDV genome, proving the accuracy of the extraction method. From a total of 83 tested goat farms, HEV RNA was detected in four goat farms, representing a frequency of 4.82%. HEV RNA was exclusively detected from feces samples, while all tested milk samples were negative. Among all studied cities, the HEV RNA was detected only in Nur, Amol, and Babol cities (Table 2). In addition, the majority of the HEV-positive animals were older than eight years, kept under poor to moderate hygienic conditions, and reared in semi-intensive management systems. A history of diarrhea was recorded in these herds (Table 3).

Discussion

The zoonotic nature of HEV poses an important public health concern in many developed and developing countries. Prior studies have revealed that the consumption of HEV-infected animal products including undercooked or raw meat, raw milk, and other dairy products and direct contact with livestock are the main route for virus transmission between animals and humans (24, 25). Research on HEV has increased significantly over the last decade, illustrating several risk factors associated with its transmission, including poor hygiene condition, consumption of contaminated water, and living in rural and mountain areas, where close contact with infected animals is more common (25).

Table 2. Positive rate of hepatitis E virus in goat farms in the cities of Mazandaran province.

Cities	Number tested (Goat farm)	Positive	Positive rate (%)
Chalus	7	0	0
Nowshahr	7	0	0
Nur	12	2	16.66
Amol	12	1	8.33
Babol	12	1	8.33
Babolsar	7	0	0
Savadkooh	7	0	0
Qaemshahr	7	0	0
Sari	6	0	0
Juybar	6	0	0
Total	83	4	4.82

Prior molecular and serological studies have also confirmed the circulation of HEV in wild wildlife populations (16, 26). Therefore, these findings have led to the suggestion that wild and domestic animals may also be a possible source of HEV infection in humans. The proximity of human residential districts to wild animals in rural and mountain environments may also facilitate the HEV zoonotic transmission. Iran is reported to be an endemic country for HEV. According to a study conducted by Amiri Dehnoyi et al. during 2017-2019, out of 50 samples taken from gazelles, three were positive for HEV, one from a gazelle under one-year-old, and two from animals over one-year-old (16). In another study, 53 healthy camels in southeastern Iran (Sistan and Baluchestan Province) were tested for the HEV RNA and results showed that 56.6% of the samples (30 individuals) were positive (15). In a human, a study screening 700 blood samples for the presence of HEV IgG and IgM antibodies as well as HEV RNA by standard enzyme-linked immunosorbent assay and nested polymerase chain reaction, respectively. The results showed that 50 out of 700 blood samples (7.1%) were positive for anti-HEV antibodies. HEV RNA was detected in 7/50 (12%) of the antibody-positive samples (17). Considering the broad host range of HEV and the lack of clear data associated with the rate of HEV infection in livestock animals in Iran, this study was designed to investigate the presence of HEV RNA in domestic goat. Two type of samples were tested. In the first phase, we focused on feces samples. Our result revealed a low frequency of HEV RNA among the studied samples. Prior studies have similarly showed the relatively low prevalence of the HEV in small ruminants. It was ranged from 5.3% in sheep livers in China (27) to 10.4% and 9.2% in sheep and goat feces in Italy, respectively (18, 28). The relatively high prevalence in Italy may be described by common practice of mixed livestock management, including the co-rearing of pigs with sheep and goats, which critically elevated the risk of cross-species infections (7).

Table 3. The number of HEV RAN positive feces samples based on studied variables.

Risk factors	Variables	Positive feces samples
Average Age (year)	Young(<1)	0
	Adult(1-8)	1
	Old(>8)	3
Husbandry management systems	Intensive	0
	Semi-intensive	4
Hygienic condition	Poor	2
	Moderate	2
	Good	0
History of diarrhea in the herd	Yes	3
	No	1

In Iran, mixed farming farms of pigs with other ruminants are uncommon, which may explain the lower positivity rate of HEV obtained in this study. Furthermore, epidemiological data proposed the role of pigs as potential reservoirs of HEV and also documented a widespread virus distribution in many countries, including the United Kingdom, China, Italy, Japan, and Canada (29-34). Seasonal migration of small ruminants to

highland pastures during the summer season, due to access to the fresh food, is common. This type of rearing system was observed in all positive cities in this study. Therefore, we proposed that the shared pastures containing HEV reservoir or infected animals may be the origin of the HEV detected in this study.

Due to the zoonotic nature of hepatitis E disease, milk samples were also collected. The consumption of unpasteurized and contaminated milk could potentially elevate the risk of human infection. In addition, it has been proposed that pasteurized milk may possible source of infection if the virus is not completely inactivated (35). HEV RNA was not detected from any milk samples which is consistent with previous studies (7, 36). In general, HEV RNA was found in milk in very low level (14). For instance, Demirci et al, reported HEV RNA in 12 (18.5%) out of 65 goat milk samples (37). In another study, Mokhtar et al. in 2020, detected HEV RNA in only 2 of 280 (0.7%) goat milk samples in Egypt tested (38). In Europe, data on HEV in milk and its products are limited. In one study that was conducted in the Czech Republic showed 1.4% (4/290) of tested samples was positive (14). The relatively low detection rate of HEV RNA in milk samples may be explained by lower viral loads, geographical variation in HEV prevalence, differences in approaches for viral genome extraction and detection of HEV, as well as the presence of inhibitory components, including enzymes, antibodies, which can impede the HEV molecular detection assays (39).

The source of the HEV RNA in the positive feces samples remains unclear. Based on detailed information from our positive farms, seasonal migration to highland pastures occurs regularly during the summer. Consequently, this proposed that the wild animals, especially wild boars could be served as a source of infection for goats. Kubankova et al, showed that the relatively high HEV positivity rate among wild boars in the Czech Republic (40). Therefore, it can be inferred that contaminated highland pastures could be a source of infection for small ruminants. Various studies have showed the higher level of viral load in feces samples (41). By considering the isolation of the virus genome in feces samples, our data suggests that the fecal-oral remains a predominant route of HEV transmission. However, the lack of positive milk samples dose not exclude the potential transmission risk through dairy products. Furthermore, the source of HEV RNA identified in goat feces remains unclear.

Conclusion

This data confirmed the presence of HEV RNA in domestic goats, in Iran. several limitation in this study, including sampling from only one province of the country, small sample size and low positive rate, which may have decreased the statistical power to describe the risk factor attributed to the HEV circulation. Although, pooled sampling strategy is cost-effective, may have not accurately reflected the true prevalence of HEV and potentially underestimating the true positive rate. According to the Food and Agriculture Organization of the United Nations, to confirm the reservoir role and source of pig for HEV infection. To assess the reservoir role for domestic ruminants, more robust investigations are required. Conducting large and accurate studies worldwide using the sampling of biological fluids and tissues, including serum, feces, milk, and tissue samples from liver, in combine with the application of standardized and improved diagnostic methods, would also be effective in determination the role of ruminants in the epidemiology of HEV and the risk of disease transmission to humans. In addition, serological surveillance for HEV detection among individuals with occupational exposure to ruminants, such as slaughterhouse workers, veterinarians, farmers, and hunters, would be useful to better understand the role of ruminants as a reservoir of HEV concerning hosts and the occupational risks associated with contact with them.

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Conflict of Interests

The authors declare no conflict of interest.

Ethical Approval

We declare that all ethical points attributed to animal care and ethics committees of the university were considered in the present research.

Artificial Intelligence Statement

We declare that no artificial intelligence was used in this study.

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