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Evidence of hepatitis E virus in milk and feces from rural cattle farms in Mazandaran province, Iran

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Abstract

Hepatitis E virus (HEV) is a zoonotic pathogen that can cause acute viral hepatitis in both humans and animals. Some animals, such as swine, rabbits, cattle, and sheep are recognized as primary reservoirs of HEV. Recently, HEV has emerged as a significant public health concern in both industrialized and developing countries. Up to now, there is no information about zoonotic sources and prevalence of HEV in wild and domestic animals in Iran. This cross-sectional study aims to determine the prevalence of HEV. Multi-stage cluster sampling approach was used to collect 400 fecal swabs and 400 milk samples between September and February 2022. These samples were collected from rural cattle dairy farms in northern Iran. The viral genome was extracted, and semi-nested reverse transcription polymerase chain reaction was used to detect the HEV RNA genome. The overall estimated herd-level prevalence of HEV was found to be 6.25% (5/80, 95.00% CI: 2.06 - 13.99%). Among the 400 fecal and 400 milk samples obtained from 80 farms, the prevalence of HEV was 3.75% (3/80, 95.00% CI: 0.78 -10.57%), and 2.50% (2/80, 95.00% CI: 0.80 - 30.74%), respectively. Positive fecal samples were detected in Abbas-abad and Amol cities, while positive milk samples were found in Tonekabon City. Based on our findings, Hepatitis E is present in the cattle population of Mazandaran province, Iran. Further, more in-depth investigations are recommended due to the zoonotic nature of this disease. It is crucial to consider control and preventive measures in order to mitigate the spread of HEV.

Introduction

Hepatitis E is a zoonotic disease caused by the hepatitis E virus (HEV), classified in the *Paslahepevirus* genus of the Hepeviridae family.[1-3] The HEV is a small, nonenveloped virus with a positive-sense single-stranded RNA genome of relatively 7.20 kb in length.[4] Hepatitis E disease causes 20 million yearly infections with 44,000 deaths in humans worldwide.[5] It primary affects young adults and has a high morbidity rate of up to 25.00% in infected pregnant women. Also, the mortality rate due to acute HEV infection varies, ranging from 0.10 - 4.00% for the general population and up to 25.00% for infected pregnant women.[6]

The HEV has a single serotype, but eight genotypes have been recognized, which can cause infections in both human and animal species.[7] Asymptomatic and self-limiting infections are common features of HEV infection, but acute liver failure can occur, leading to chronic hepatitis in immunocompromised individuals.[8] In developing countries with poor sanitation conditions, HEV-1 and HEV-2 genotypes also known as restricted human genotypes, are responsible for the extensive epidemic of acute hepatitis outbreaks in humans in disease-hyperendemic regions.[9] Genotypes five and six were isolated from HEV-infected wild boar in Japan, while HEV-7 was reported in camels in the Middle East.[10]

The main route of HEV transmission is the fecal-oral route, predominantly via contaminated water supplies and food, including raw or undercooked animal products or internal organs.[11] Genotypes HEV-1 and HEV-2 are mainly transmitted via contaminated water consumption.[12] For HEV-3 and HEV-4 genotypes, zoonotic transmission through contaminated swine meat and meat products, or contact with infected animals, particularly pigs, is proposed.[13,14] Furthermore, blood transfusions and vertical transmission (from mother to new-born child) are recognized as less common routes of transmission.[12]

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Hepatitis E is a global public health concern, particularly in Iran, where the seroprevalence of immunoglobulin G against HEV in humans was the second highest in the Middle East and North Africa.[15] In developed countries, a limited number of cases have been identified, with a significant prevalence of anti-HEV antibodies detected in a considerable proportion of blood donors or individuals in good health. Depending on the population group that was screened or the geographical region of sampling, the seroprevalence of HEV in Europe, according to systematic review analysis , has ranged from 0.60 to 52.50%.[6] The cause of this relatively elevated seroprevalence remains unclear. A potential reason for this finding could be subclinical HEV infections.

The HEV can also infect an increasing range of animal hosts including cattle, deer, goats, sheep, rabbits, and rats.[16,17] Investigations on HEV in ruminant populations have increased over time, with HEV infection being identified in many ruminant species.[7]

The detection of HEV in livestock and its potential to cause illness in non-human primates underscores the concern of zoonotic transfer of HEV through the consumption of meat, liver, and milk from infected animals.[18] While there is a global lack of data on the circulation of HEV in ruminants, an increasing body of data has depicted the presence of HEV in farmed ruminants highlighting them as a potentially significant source of HEV. This, necessitates further in-depth investigations on the topic.[7]

In Iran, it is customary to consume raw milk and non-pasteurized dairy products, making it important to measure the prevalence of this disease in domestic animals.[19,20] The HEV has been detected in dromedary camels, but so far, no study has investigated the existence of HEV strains among cattle and bovine milk in Iran.[21] Thus, this study aimed to determine the prevalence of HEV in milk and feces samples from rural cattle farms in Mazandaran province.

Materials and Methods

Study design and data collection. The target population of this cross-sectional study consisted of rural cattle farms located in Mazandaran province with 10 or more cows. Samples were collected from September to February 2022 from cows aged over one year old. Mazandaran province covers an area of 23,842 km^2, located in the north of Iran (approximately 57 90′ - 57 20′ E longitude; 43 30′ - 43 90′ N latitude). The natural conditions in this region, are characterized by a humid and temperate climate influenced by the sea, mountains, and forests of Alborz, making it a significant province in Iran for cattle, sheep, and goat farming. Summer temperatures average around 25.00 ˚C, while winter temperatures drop to approximately 9.00 ˚C. A multi-stage random cluster sampling design was used. The sample size was calculated using the formula for sample size calculation to detect positive sample, considering a 95.00% confidence level, 5.00% maximum allowable error, and an expected prevalence of 1.00% from the study by Go *et al.*[22] The sample size was calculated using the following equations:[23]

$$n = \frac{\ln(1-C)}{\ln(1-P)}$$

where, n = required sample size; C = confidence level, and P = prevalence.

In this equation, 300 milk and 300 feces samples were calculated. Due to the multi-stage random cluster sampling method, there was variance between the clusters and an increase in the standard error of the obtained results, the sample size was corrected through the following formulas:

$$g = \frac{Z^2\, T_s\, V_c}{d^2\, T_s - Z2\, P_{exp}\,(1 - P_{exp})}$$

where, g = number of clusters to be sampled, P_{exp} = expected prevalence, d = desired absolute precision, T_s = total number of animals to be sampled, V_c = between-cluster variance, and Z = confidence level. According to this equation, 80 clusters (herds) were calculated.

Additionally, following formula was utilized to calculate the corrected sample size for the cluster sampling method.

$$T_s = \frac{Z^2\, g\, P_{exp}\,(1 - P_{exp})}{g d^2 - Z^2\, V_c}$$

where, T_s = total number of animals to be sampled when the number of clusters is fixed, g = number of clusters, V_c = between-cluster variance, Z = confidence level, P_{exp} = expected prevalence, and d = desired absolute precision.[24]

Finally, 400 milk and 400 feces samples were considered for the study.

The research area was divided into three distinct categories: Eastern, Western, and Central regions. Then, from the list of villages in each region, five villages are randomly selected using the Excel RAND function as the primary sampling units (version 2019; Microsoft Corporation, Redmond, USA).

From the selected villages, a selection was made based on the frequency of rural cattle farms within each village, employing a sampling technique known as probability proportional to size. Subsequently, four to six cattle farms were identified as secondary sampling units. Then, five milk and five fecal samples were obtained from distinct healthy cows, originating from each of the mentioned farms. Individual samples within each farm were pooled. Each pooled sample consisted of five milk or five fecal samples. In total 80 pooled milk samples and 80 pooled fecal samples were prepared. We obtained approval from the villagers to collect these samples while maintaining the privacy of the animal owners.

Biological sample collection. Fecal samples were obtained by rectal swabs and transferred in sterile containers containing 10.00% (w/v) phosphate buffered saline (pH = 7.20) supplemented with Penicillin-Streptomycin and Amphotericin B (Biowest, Nuaillé, France) to prevent bacterial and fungal growth. The samples were put next to ice and transported to the laboratory, where they were kept at – 80.00 ˚C. Milk samples were collected directly from the cow's teats, kept in sterile containers, and transported to the laboratory with the same method as the fecal samples.

Sample preparation and viral genome extraction. Fecal samples were centrifuged at 4,000 rpm for 15 min at 4.00 ˚C and the clarified supernatant was stored at – 80.00 ˚C for virus genome extraction. Milk samples were centrifuged at 8,000 rpm for 15 min. Three phases were generated in the milk samples: top layer (containing fat), intermediate layer (without fat), and bottom layer (semi-solid layer). The intermediate layer was retrieved and kept at – 80.00 ˚C for further analysis. To validate the efficiency of our viral genome extraction from samples and to rule out a possible inhibitory effect of cow's feces and milk on RNA detection, milk, and fecal samples were inoculated with 150 µL of 10^8 embryo infective dose 50% Newcastle disease virus (NDV) as a control sample.[25] Subsequently, total RNA extraction was done using a Viral RNA/DNA extraction kit (iNtRON Biotechnology Inc., Seongnam-si, South Korea) according to the manufacturer's instructions. Briefly, a 300 µL volume of each sample was used. After chemical and enzymatic lysis and two washing steps, the RNA was yielded in a 60.00 µL elution buffer. A and B primers were synthesized to extract a 363 base pair segment of the viral genome using the same HEV method.

Reverse transcriptase polymerase chain reaction (RT‑PCR) for HEV and NDV RNA detection. The solution was incubated at 25.00 ˚C for 10 min, then at 47.00 ˚C for 60 min, and finally at 85.00 ˚C for 5 min in the PCR device. To detect the HEV genome, two-step RT-PCR amplifications were conducted. In the first step, cDNA synthesis reactions were done by using the Easy cDNA Synthesis Kit (Parstous, Mashhad, Iran) in a 20.00 µL reaction mixture containing 5.00 µL of RNA, 10.00 µL of 2.00 X RT Buffer Mix, 2.00 µL Enzyme Mix (H- minus MMLV and RNase inhibitor) and 3.00 µL diethyl pyrocarbonate water. The thermal program was performed according to the manufacturer's instructions. In the next step, semi-nested and conventional PCR assays were done. For semi-nested PCR assay, the primer pair HEV-CS and HEV-CAS is an internal primer, and the primer pair HEV-CSN and HEV-CASN is an external primer.[26] In conventional PCR assay, primer pair HEV-F and R were kindly provided by used by Dr. Mokhtari (Table 1).[27] For NDV detection, a primer pair that has been already designed by Kant *et al.* was used (Table 2).[28] All PCR reactions were carried in the volume of 25.00 µL. The thermal profile for amplification included 35 cycles of 95.00 ˚C for 50 sec (denaturation step), followed by annealing at 56.00, 52.00, or 55.00 ˚C for 60 sec (depending on the primer pairs), and extension at 72.00 ˚C for 50 sec, with a final incubation at 72.00 ˚C for 8 min. The PCR products were electrophoresed on 1.50% Agarose gel and the amplified fragments were stained with a safe stain.

Statistical analysis. The prevalence of HEV was determined by calculating the proportion of samples that tested positive out of the total number of examined samples, along with 95.00% confidence intervals. Data analysis was carried out using SPSS software (version 26.0; SPSS Inc., Chicago, USA).

Results

Four hundred milk and 400 feces samples were collected from 80 rural cattle farms in Ramsar, Tonekabon, Abbasabad, Nur, Babol, and Amol cities. The average number of cattle on these farms was 30, with an average age of 5.41 ± 1.34 years. The cattle belonged to indigenous, Holstein, Simmental, and Montbéliarde breeds. Using molecular methods, the herd-level prevalence of the HEV was determined to be 6.25% (5/80, 95.00% CI: 2.06 - 13.99%). The prevalence of the HEV in fecal and milk

Table 1. Primers used in polymerase chain reaction (PCR) reaction for hepatitis E virus (HEV) detection in fecal and milk samples collected from cattle in Mazandaran, Iran (2022).

PCR types	Primers	Sequence 5′ to 3′	Product length (bp)	Annealing temperature (˚C)	References
Conventional PCR	HEV-F	TTCCACCACCCAGCAGTATT	145	56.00	27
	HEV-R	GGCATTCTCAACGAGCAGTT			
RT-PCR	HEV-CS	TCGCGCATCACMTTYTTCCARAA	470	52.00	26
	HEV-CAS	GCCATGTTCCAGACDGTRTTCCA			
	HEV-CSN	TGTGCTCTGTTTGGCCCNTGGTTYCG	325	55.00	26
	HEV-CASN	CCAGGCTCACCRGARTGYTTCTTCCA			

RT: Reverse transcription.

Table 2. Primers used for reverse transcription polymerase chain reaction for Newcastle virus CI1

Primers	Sequence 5′ to 3′	Product length (bp)	Annealing temperature (˚C)	Reference
NDV-A^5	TTGATGGCAGGCCTCTTGC	363	52.00	28
NDV-B^1	GGAGGATGTTGGCAGCATT			

NDV: Newcastle disease virus

samples was 3.75% (3/80, 95.00% CI: 0.78 - 10.57%) and 2.50% (2/80, 95.00% CI: 0.80 - 30.74%), respectively. The positive milk samples were detected in Tonekabon city while positive fecal samples belonged to Abbasabad and Amol cities (Figs 1 and 2). All positive samples came from indigenous breeds.

Fig. 1. Geographical locations of cities that contained positive hepatitis E virus samples.

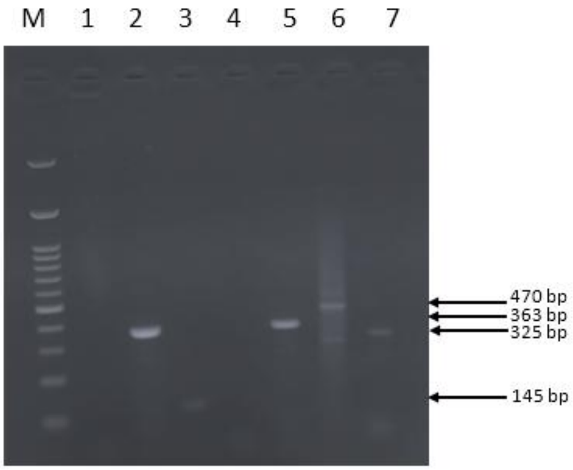

Fig. 2. Amplification of hepatitis E virus (HEV) and Newcastle disease virus by conventional and semi-nested reverse transcription polymerase chain reaction. M = Marker (100 bp), Lanes 1 and 4: Negative control, Lanes 2 and 5: Extraction control for milk and fecal samples, respectively, Lane 3: Positive sample by HEV-F and R primer pair (convention polymerase chain reaction), Lane 6: Positive sample by HEV-CS and HEV-CAS primer pair (external primer) and Lane 7: Fragment amplified by HEV-CSN and HEV-CASN primer pair (internal primer).

Discussion

Hepatitis E virus poses a significant public health concern on a global scale, given its status as a notable foodborne pathogen transmissible from animals to humans.[13] This cross-sectional survey aimed to determine the prevalence of HEV in fecal and milk samples from cattle in Mazandaran province, Iran.

In our study, the overall herd-level prevalence of the HEV was 6.25%. The prevalence of HEV contamination in milk samples was 2.50%, which was lower than the estimated prevalence in Turkey, where Demirci *et al.* found that using the qRt-PCR method, the rate of HEV RNA contamination in the raw cow milk samples was 29.16%.[29] However, our estimate was greater than that of Egypt, according to Sayed *et al.* found that all 480 samples taken from Assiut City's dairy shops, grocery stores, street vendors, and 12 rural dairy farms tested negative for HEV RNA using qPCR and nested PCR. On the other hand, 1 out of 40 milk samples (or 2.50 percent) from one rural farm contained HEV-RNA contamination.[30]

The differences in the prevalence of HEV contamination in cow milk could be due to geographical differences in HEV circulation or due to the utilization of distinct methods of extraction and detection of HEV that could result in dissimilar sensitivities and specificity of the measuring tests utilized in the studies.[31]

Although data on the risk of HEV transmission by milk consumption is still scarce,[31] a study carried out in China has shown that the consumption of raw bovine milk and the inoculation with pasteurized HEV-contaminated cow milk can induce active infection in rhesus macaques,[32] the evidence of HEV circulation in cattle even in low prevalence has been suggested.[33] Moreover, the consumption of raw dairy products has been associated with HEV seropositivity in a meta-analysis.[34]

In Iran, the consumption of raw milk is common, especially in the rural areas where it is a routine habit.[35] Therefore, the isolation of HEV in our study could present a significant public health issue in Mazandaran province. However, even with pasteurization, there are circumstances where not all viral loads of HEV can be inactivated, potentially leading to consumers being infected through pasteurized milk ingestion.[36]

In this study, the prevalence of HEV in the collected fecal samples was 3.75%. This percentage was higher than the study by Geng *et al*. where no positive samples were detected out of 467 fecal samples collected from dairy farms in Hebei Province, China.[25], Additionally, our findings showed a higher prevalence of HEV RNA in sera and liver samples collected from cattle in Turkey with the RT-PCR method, where 1 out of 194 sera samples (0.51%) and 0 out of 100 liver samples (0.00%) were found to be positive.[37]

There are numerous factors contributing to the occurrence of hepatitis infection within rural farms. Farm management and housing are crucial in determining the prevalence of HEV. Agricultural operations that exhibit sporadic maintenance of feeding stations and overall farm sanitation are insufficient to experience elevated incidences of HEV outbreaks. Additionally, animals move to graze, and keeping mixed species of ruminants (small and large ruminants) together in rural farms is common practice. Furthermore, rural farms often have direct contact with rodents.[18]

The HEV is the most prevalent cause of viral hepatitis and cases of HEV-hepatitis infections have been constantly increasing in Europe and the Middle East countries such as

Egypt and Iran.[15,38] Due to the public health importance of HEV, knowledge of the host range of HEV and their role in its epidemiology of HEV are crucial issues to control this emerging pathogen.[39,40] To our knowledge, this is the first investigation of the prevalence of HEV in bovine milk and fecal samples in Iran.

Based on our findings, the HEV is present in the cattle population of Mazandaran province, north of Iran. The overall herd-level prevalence of the HEV was estimated to be 6.25%. The prevalence of HEV in fecal and milk samples collected from cattle was 3.75 and 2.50%, respectively with the positive milk samples belonging to Tonekabon and positive fecal samples from Abbasabad and Amol cities. The isolation of HEV in the raw milk sample could pose a major public health issue in the region, as consumption of unpasteurized dairy products, especially raw milk from infected cows is the primary route of transmission to humans. Therefore, more thorough investigations are recommended. In addition, considering this disease is zoonotic, control and preventive measures should be implemented. Finally, future genotyping studies are suggested to understand the circulating strains of HEV and its epidemiology in ruminants and their products.

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Conflict of interest

The authors declare no conflict of interest.

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