TRADITION AND MODERNITY IN VETERINARY MEDICINE, 2025, vol. 10, No 1(18): 3–10

ABSENCE OF HEPATITIS E VIRUS CIRCULATION AMONG EUROPEAN BROWN HARE DURING 2015 IN TWO BULGARIAN DISTRICT

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(Submitted: 6 November 2024; Accepted: 10 January 2025; Published: 25 June 2025)

ABSTRACT

The aim of this study was to assess the presence of zoonotic hepatitis E virus genotype 3 (HEV-3) in 43 liver samples from European Brown Hares in two administrative regions of Bulgaria (Plovdiv and Burgas) in 2015. Nested and Real time RT-PCR were used for HEV nucleic acid detection. In addition, liver transudate was tested for the presence of anti-HEV class IgG antibodies by ELISA (ID Screen® Hepatitis E Indirect Multi-species, IDVet, Grabels, France) following the manufacturer's protocol. No positive samples for the presence of nucleic acid were proven in the current research by nested and Real time RT-PCR. Anti-HEV antibodies were also not detected. Further studies need to be conducted to assess the presence of the virus in the European Brown Hare population in Bulgaria in order to establish the role of this host in the epidemiology of the disease in humans as well.

Key words: Hepatitis E virus, wildlife, PCR, ELISA, Bulgaria.

Introduction

Hepatitis E virus (HEV) strains which can cause infections in mammals and birds are classified in the subfamily *Orthohepevirinae* of the family *Hepeviridae* (https://ictv.global/taxonomy/taxondetails?taxnode_id=20132053&taxon_name=Hepatitis%20E%20virus). Among the strains of the virus, 4 main genotypes (HEV 1 to HEV 4) are recognized in humans. Genotypes HEV 1 and HEV 2 infect only humans and circulate mainly in nondeveloped countries. Genotypes 3 and 4 infect humans but also other mammals and are responsible for zoonotic transmission of the infection causing hepatitis associated with the consumption of raw or undercooked meat, such as pork liver sausages and wild boar meat (Dalton *et al.*, 2008).

Genotype 3 has been reported in humans and various animal species such as: pigs, goats, sheep and rabbits (Doceul *et al.*, 2016; Johne *et al.*, 2014; Long *et al.*, 2017; Mesquita *et al.*, 2018 d.; Wu *et al.*, 2015). HEV 3 and HEV 4 are mainly distributed among pigs, with wild boar being the main reservoir of infection and, to a lesser extent, deer (Pavio *et al.*, 2017). Several more genotypes of the virus have been described in recent years in wild pigs in Japan (HEV 5 and HEV 6) and in camels in the Middle East (HEV 7) and China (HEV 8) (Smith *et al.* , 2020; Lee *et al.*, 2016). Of the newly discovered genotypes, only HEV 7 is zoonotic, as it was also detected in a human who consumed poorly heat-treated camel meat and milk (Woo *et al.*, 2016). Rabbit strains of HEV are genetically very close to HEV 3, but in phylogenetic analysis of the isolates form a separate monophyletic clade from other HEV 3 (Smith *et al.*, 2020; Schemmerer et.al., 2022). The

nucleotide sequence of rabbit HEV is 76-79% identical to that of other HEV 3 strains (Corman *et al.*, 2019).

The zoonotic potential of rabbit HEV has been confirmed following its detection in acutely and chronically infected patients in Switzerland (Sahli *et al.*, 2019), Spain (Rivero-Juarez *et al.*, 2020), France (Abravanel *et al.*, 2017), and in blood donors in Ireland (Baylis *et al.*, 2022). The distribution of HEV is widespread nowadays, however the full range of animal species that can act as reservoirs for HEV has not been studied in detail. The first reported case of rabbit HEV infection was in domestic European rabbits (Oryctolagus cuniculus) raised on a farm in China (Zhao *et al.*, 2009). Infection in rabbits is asymptomatic, although subclinical hepatitis has been observed with experimental infection (Li *et al.*, 2020). After experimental infection, the virus was detected in the feces of infected rabbits 2 days after infection, and the duration of viral shedding was 5 weeks (Wang *et al.*, 2017). Multiple studies have reported the presence of rabbit HEV in domestic, wild and laboratory rabbits in different countries around the world, such as China in domestic rabbits-11.4% (Li *et al.*, 2021), United States of America-16.4% (Cossaboom *et al.*, 2011) and Australia-3.5 % (Jenckel *et al.*, 2021).

In Europe HEV was reported in France: 7.0% in farmed rabbits and 22.9% in wild rabbits (Izopet *et al.*, 2012); Netherlands: 22.8% in farmed, 0.0% in wild, 60.0% in pet rabbits (Burt *et al.*, 2016); Germany: 17.1% in wild rabbits, 25.0% in feral European rabbits (Hammerschmidt *et al.*, 2017; Ryll *et al.*, 2018) and in Poland: 14.9% in slaughtered farmed rabbits (Bigoraj *et al.*, 2020). In the Iberian Peninsula 372 wild rabbits and 78 Iberian hares (Lepus granatensis) were tested negative for HEV (Caballero-Gomez *et al.*, 2020), while in Portugal, a seroprevalence of 4.1% was reported (Lopes *et al.*, 2020). The increased number of rabbits kept as pets and their close contact with their owners is another potential route for transmission of infection to humans, as supported by the detection of HEV-3 in a pet rabbit (Caruso *et al.*, 2015) and presence of anti-HEV antibodies in other domestic rabbits (Di Bartolo *et al.*, 2016). All of these findings largely confirm the need to investigate the role of rabbits as hosts and reservoirs of HEV in the human infection cycle. Our study was prompted by data on the circulation of HEV-3 among the wild boar population in Bulgaria (Tsachev *et al.*, 2021) and lack of information about virus circulation in wild and domestic rabbits. It is for this reason that we decided to examine 43 samples of rabbits shot in 2015, in 2 different areas of southern Bulgaria.

Materials and Methods

Samples

The sampling of Rabbits included in this study was performed during the hunting period which for this species is allowed from 1st of October to 31st of December. The samples were collected from 43 adult European brown hares in the southern Bulgaria, Europe (Fig.1). The studied regions were selected based on the available data for higher hare population density (5 to 8 animals/100 ha) (Zhelev, 2015). Animals were caught in December 2015. All hares were without gross lesions in any organs observed during the pathological examination. During the necropsy livers were collected from all hares.

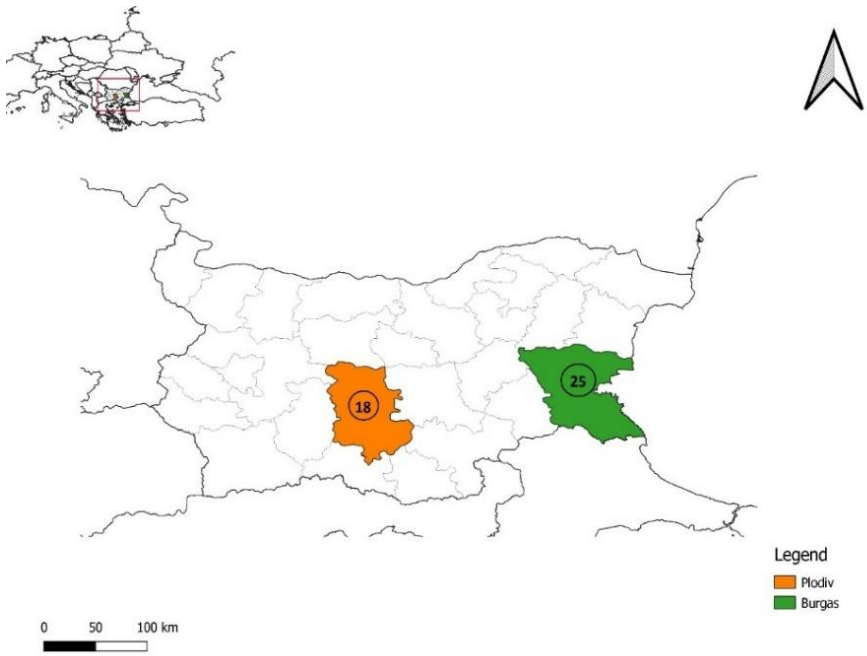

Figure 1: Map of Bulgaria showing the sampling area and the numbers of livers collected.

Nucleic acid extraction

The liver samples were homogenized with antibiotic media containing Eagle's Minimum Essential Medium (EMEM), 10 U/ml penicillin, 0.1 mg/ml streptomycin, 0.25 mg/ml gentamicin and 2.5 µg/ml amphotericin B. After 24 h incubation at +4°C, the suspensions were centrifuged at 2000 g for 15 min and the supernatants were used for extraction. Total/viral RNA was extracted with IndiSpin® Pathogen Kit (Indical Bioscience, Leipzig, Germany) following the manufacture's protocol.

HEV nested RT-PCR and Real time RT-PCR

First detection of HEV RNA was performed according to the method of Boxman *et al.* (2017). Briefly, 5 µL of the extracted RNA were reverse transcript with a gene specific HEV primer using smART First Strand cDNA Synthesis Kit (EURx Ltd, Gdansk, Poland) in accordance with the manufacturer's instructions. Subsequently, in a first PCR, 10 µL of the synthesized cDNA were amplified with Perpetual Taq DNA Polymerase (EURx Ltd, Gdansk, Poland). For the first PCR step, the forward primer was from positions 5909±5934 of the HEV genome sequence (Accession Number M73218), and the reverse primer was complementary to positions 6512±6534. The nested PCR was carried out with 1 µL of the First PCR template and set of primer sequences complimentary respectively to positions 5948±5985 (forward primer) and 6479±6513 (reverse primer). The amplification products were analyzed by 1.5% agarose gel electrophoresis. Additionally, 25 µL of extracted RNA were analyzed by RealStar HEV RT-PCR kit 2.0 (Altona diagnostics, Germany) in accordance with the manufacturer's instructions. The assay includes a heterologous amplification system (Internal Control) and four quantification standards calibrated against the 1st World Health Organization International Standard for Hepatitis E Virus RNA Nucleic Acid Amplification Techniques (NAT)-Based Assays (PEI code 6329/10). The test runs were considered valid at the generated standard

curve control parameter (R^2) of ≥0.98. The determined manufacturer linear range of the RT-PCR was from 1E+01 to 1E+07 IU/µl.

Serology

Forty-three livers transudate was obtained from all hares. Liver samples were frozen at -80 C and thawed at room temperature. The resulting liquid was collected after a few hours (Navarro *et al.*, 2020) and used to tested for presence of Hepatitis E antibodies with commercially available ELISA (ID Screen® Hepatitis E Indirect Multi-species, IDVet, Grabels, France) following manufacturer's protocol.

Results

A total of 43 samples from adult European brown hares were analyzed with nested RT-PCR and Real time RT-PCR to detect HEV RNA. The viral RNA was not detected in all of the tested samples either with nested RT-PCR (Fig. 2), or Real time RT-PCR (Fig. 3).

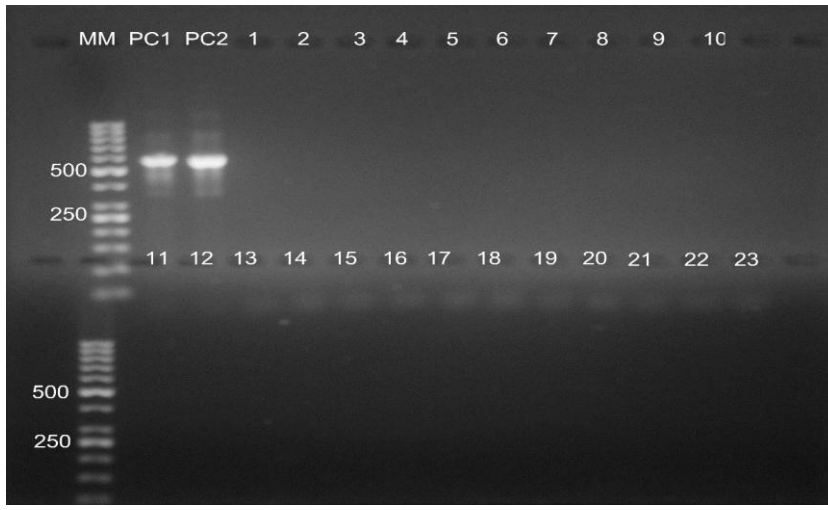

Figure 2: Electrophoresis of HEV nested RT-PCR. MM = molecular marker; PC1 = Positive Control swine HEV isolate; PC2 = Positive Control human HEV isolate;

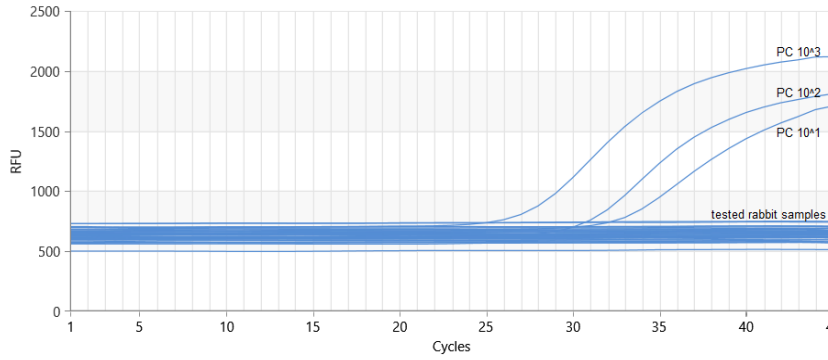

Figure 3: Real time RT-PCR visualizing the amplification curves of tested samples and the Positive controls (PC).

The results of the nested RT-PCR and Real Time RT-PCR were also confirmed serologically and none of the transudates were positive for antibodies (0/43).

Discussion

Like other foods from animal origin, rabbit meat is tested before it enters the food chain. When hunting wild rabbits, the carcas is examined only during the necropsy and the statement is based only of gross pathological findings. HEV infection in rabbits is mostly asymptomatic (Doceul *et al.*, 2016) with changes in serum alanine aminotransferase (ALAT) levels and with the presence of multifocal lymphohistiocytic infiltrates and hepatocellular necrosis (Krawczyński *et al.*, 2011). However, these changes are difficult to detect during routine pathological examination. Even a detailed inspection during the necropsy could not detect the infected rabbits entering the food chain.

In general, meat and meat products are one of the main routs of transmitting bacterial infectious diseases, causing so-called foodborne infections (Gonçalves-Tenório *et al.*, 2018). In this aspect, rabbit meat is considered relatively safe, as cases of foodborne infections caused by its consumption are not often reported (European commission, 2014). Human infection with HEV-3ra has been reported in various European countries (Abravanel *et al.*, 2017; Rivero-Juarez *et al.*, 2020; Baylis *et al.*, 2022). People in close contact with rabbits are at the highest risk of infection, as confirmed by the significantly higher seroprevalence found in rabbit slaughterhouse workers (Geng *et al.*, 2019). However, it is still unclear how rabbit HEV-3ra may contribute to the epidemiology of HEV infection in humans (Rivero-Juarez *et al.*, 2020). In France, the risk of infection was higher in individuals who declared that they had eaten meat from domestic and wild pigs and meat from shot wild rabbits, but there was no direct evidence of HEV-3ra transmission through food (Legrand-Abravanel *et al.*, 2010).

Sharing the same habitats with infected wild boars, wild rabbits could also be infected. In our study, we could not identify positive samples, both in serological and molecular biological testing. The main difference between our study and these one in wild boar in Bulgaria (Tsachev *et al.*, 2021), apart from the type of animals studied, is the geographical area from which they originate. In our study, the samples were from the regions of Plovdiv and Burgas, while in the other one, the samples were collected from western Bulgaria. Anyway, this is the first study on the prevalence of HEV-3ra among the wild rabbit population in Bulgaria. The absence of anti-HEV antibodies in rabbits in this study should be interpreted with caution, due to the limited number of liver transudates analyzed (n = 43). Liver transudates are a good alternative sample for the detection of antibodies against HEV in the absence of serum (Navarro *et al.*, 2020). The negative results for the presence of antibodies against HEV in the examined liver transudates in our study correspond with results from Spain (Caballero-Gomez *et al.*, 2020) and Italy (De Sabato *et al.*, 2023). The present study employed a combination of diagnostic real-time qualitative RT-PCR (Altona Diagnostics) and genotyping PCRs for the analysis of rabbit samples. The same real-time qualitative PCR test was employed for the determination of viral load not only for rabbit HEV but also for novel HEV isolates in Irish blood donors (Baylis *et al.*, 2022). The confirmed sensitivity of the Altona Diagnostics realStar® HEV RT-PCR for HEV-3ra isolates was the rationale behind the implementation of the test in the present analysis of rabbit liver samples.

The number of rabbits sampled in the study was very small and we cannot rule out the circulation of HEV among them. However, in another recent study in wild rabbits, using the same type of samples, no viral RNA and antibodies against it were detected (De Sabato *et al.*, 2023).

Conclusion

There is no evidence of the HEV circulation in wild rabbits in the studied regions in Bulgaria during 2015. However, further studies should be conducted to establish the current circulation of the virus and to monitor fluctuations in HEV epidemiology and the subsequent risk of HEV-3ra transmission to humans and other wild mammals. Information on the circulation of HEV in this type of animal is scarce, not only in Bulgaria, but also worlwide. Studies in wild and farmed rabbits will provide us with useful information about the circulation of the virus and its spread among these populations, as well as its possible transmission from them to humans.

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