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Hepatitis E Virus in Wild Boar from Poland

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Abstract: Hepatitis E virus (HEV) is an emerging zoonotic pathogen of growing public health concern. HEV genotype 3 is a cause of sporadic human infections in Europe, and is carried by wild boars (*Sus scrofa*). This study investigated the prevalence of HEV in free-ranging wild boars from different regions of Poland and evaluated the potential risk factors associated with infection. Serum samples (n=367) were tested serologically for HEV antibodies, and spleen samples (n=100) for viral RNA using Real-Time quantitative RT-PCR. Detection rates were analysed in relation to geographic origin, sex, and age. HEV-IgG antibodies were detected in 154/367 (41.96%) samples. Viral RNA was identified in 10/100 animals, all of which also tested seropositive. Nested reverse-transcription PCRs amplifying short genomic fragments within

ORF1 and ORF2 confirmed five viral sequences: one strain was classified as HEV-3c, related to a human strain from the Netherlands, while the other four belonged to an unclassified subtype similar to sequences reported in wild boar in Europe. Only wild boar density appeared to significantly influence HEV seropositivity. Positive animals were distributed across multiple voivodeships, suggesting widespread environmental circulation.

Keywords: hepatitis E virus; One Health; Poland; surveillance, wild boar, zoonosis

1. Introduction

Hepatitis E virus (HEV) is an emerging zoonotic pathogen which has received increasing global attention for its public health implications (Castagna et al., 2024). HEV is a quasi-enveloped, single-stranded RNA virus with positive polarity, belonging to the species *Paslahepevirus balayani*. This species is divided into eight primary genotypes (HEV-1 to HEV-8). Genotypes HEV-1 and HEV-2 are restricted to human hosts and are predominantly found in developing regions, where transmission typically occurs through the faecal–oral route, particularly via consumption of contaminated water or food (Pavio et al., 2017; Denner, 2019; Smith et al., 2014; Purdy et. al., 2022). In contrast, genotypes HEV-3 and HEV-4 can infect a broad range of animal species (Sridhar et al., 2017; Oechslin et al., 2020). They are frequently implicated in zoonotic transmission, particularly in industrialized countries, where domestic pigs and wild boar (*Sus scrofa*) represent the main reservoirs (EFSA, 2022). Wild boars are recognized as important reservoirs of HEV-3, which is responsible for most autochthonous human infections in Europe (Sridhar et al., 2017).

Over the past two decades, HEV has been detected in various wild boar populations throughout many European countries, with reported seroprevalence rates ranging from 5% to over 50%, depending on the region, sampling strategy, and detection method (Fanelli et al., 2021). The widespread presence of HEV highlights the potential risk of its transmission, not only to hunters and people handling wild game meat, but also through environmental contamination. There is, therefore, a need for systematic surveillance of HEV prevalence to understand the role of wildlife in its maintenance and transmission. Furthermore, as wild boar populations often overlap with domestic pig farming areas and human settlements, it is

important to acquire data on HEV circulation in free-ranging animals to successfully implement effective One Health surveillance strategies (Schlosser et al., 2014; Thiry et al., 2017). Therefore, the aim of this study was to determine the seroprevalence and viral presence of HEV in wild boar sampled from multiple voivodeships in Poland, to characterize its genotype, and to assess the environmental and demographic risk factors that may potentially be associated with HEV exposure.

2. Materials and methods

2.1 Materials

Between February and July 2024, blood samples were collected from 367 wild boars (162 females, 205 males) from 13 Polish Voivodeships: dolnośląskie, lubuskie, łódzkie, małopolskie, mazowieckie, opolskie, podkarpackie, pomorskie, śląskie, świętokrzyskie, warmińsko-mazurskie, wielkopolskie, and zachodniopomorskie (Fig. 1). The animals ranged from six months to four years of age (mean age: 1.67 years). Most samples were collected from hunted animals, and some from animals found dead due to fatal vehicle collisions. Hunters with appropriate wild boar hunting licenses provided samples from hunted animals. Approval from the ethics committee was not required since the study did not use live animals, nor were any killed. Wild boar are not a protected species in Poland. The material was collected by convenience sampling (non-probabilistic sampling method). Blood samples were collected post-mortem from the heart in tubes with a clot activator and transported to the laboratory at 4ºC. After centrifugation, the serum was separated and stored at −20 ◦C until further analysis. From about one-third of the animals (n=100), a fragment of spleen was collected and frozen at -20 ◦C for Real-Time quantitative PCR (RT-qPCR) analysis. All methods were performed in accordance with the relevant guidelines and regulations. The molecular analysis was carried out at the Experimental Zooprophylactic Institute of Southern Italy.

Fig. 1

2.2 Serological analysis

Before testing, serum samples were allowed to thaw at room temperature. The serological analysis was performed using the NovaTec Vetline Hepatitis E (HEV) commercial ELISA kit (Gold Standard Diagnostics, Davis, California, US), detecting IgG; according to the manufacturer's instructions. Briefly, the plates were covered with specific antigens able to bind corresponding antibodies in the sample. Ten µL of the samples, diluted with 1 mL of dilution buffer, was added to the wells and incubated for one hour at 37°C. After washing the wells, a horseradish peroxidase (HRP) labeled conjugate was added and incubated for 30 minutes at room temperature. Following that, a second wash was performed to remove unbound conjugate. Next, tetramethylbenzidine (TMB) substrate was added to visualize the complex. Sulphuric acid was added to stop the

reaction, which produced a yellow colour; the intensity of the colour was proportional to the amount of specific antibodies detected. The optical density of each sample was read using an EPOCH spectrophotometer (BioTek Instruments Inc., US) at a wavelength of 450 nm and calculated following the manufacturer's instructions.

2.3 Viral nucleic acid extraction

Nucleic acid extraction was performed using the King Fisher Flex System (Thermo Fisher Scientific, Waltham, Massachusetts, USA) with the Mag Max Viral Pathogen Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's protocol. Briefly, spleen samples (25 mg) were individually homogenized by a Tissue Lyser (Qiagen GmbH, Hilden, Germany) at 30 Hz for 5 min in a 2 mL tube containing 1 mL of phosphate-buffered saline (PBS) and a 4.8 mm stainless steel bead. The homogenates were centrifuged and 200 µL of supernatant underwent nucleic acid extraction. The nucleic acids were eluted in 80 µL of elution buffer and immediately analysed through RT-qPCR. A negative process control (NPC), containing 200 µL PBS instead of sample, was added at each extraction run. Furthermore, prior to extraction, each sample (including NPC) was spiked with 5µL murine norovirus (10^7 PFU mL-1; plaque-forming units) as an external amplification control (EPC) to evaluate the possible presence of PCR inhibitors and to assess the recovery rate (Di Bartolo et al. 2012; Iaconelli et al. 2015; Amoroso et al., 2021).

The murine norovirus RNA was detected by RT-PCR as previously described (Baert et al., 2008). The recovery rate was calculated by the comparative cycle threshold (Ct) method (Schmittgen & Livak, 2008).

2.4 HEV RT-qPCR screening and quantification

To screening for HEV, the four main genotypes known to infect humans and animals were searched with a broad-range RT-PCR using the following primers and probe (Jothikumar et al., 2006):

- Forward primer HEV-F: 5’-GGTGGTTTCTGGGGTGAC-3’
- Reverse primer HEV-R: 5’-AGGGGTTGGTTGGATGAA-3’
- Probe HEV-P: FAM-5’-TGATTCTCAGCCCTTCGC-TAMRA-3’

The reactions were run on a QuantStudio 5 Real-Time PCR thermal cycler (Thermo Fisher Scientific, Waltham, Massachusetts, USA) using the following thermal profile: reverse transcription at 50°C for 15 min, initial denaturation at 95°C for 15 min, followed by 45 cycles of 95°C for 15 s and 60°C for 60 s. Each reaction was carried out in a final volume of 25 µL containing 5 µL nucleic acid extract, 12.5 µL AgPath-ID™ One-step RT-PCR kit (Applied Biosystem by Thermo Fisher Scientific), 0.625 µL each primer (10 µM), 1.25 µl probe (2 µM), 1 µl RT-PCR enzyme mix and 4 µL nuclease-free water. A reference strain of HEV-3e (Acc. No. OP558159; Ianiro et al., 2023) was used as a PCR-positive control (PC). Samples with Ct < 37 were considered positive.

The results were analyzed as described previously (Di Bartolo et al. 2012; Iaconelli et al. 2015; Aprea et al. 2016): if the Ct of the EPC in the sample was comparable to that of the EPC in the NPC, the sample was analyzed as undiluted. However, if the difference between the two Cts was at least 3, or a multiple of 3, the sample was analysed diluted by at least 1:10, with one decimal dilution every three threshold cycles of difference. Quantification was carried out by the mean of a standard curve constructed by amplifying serial dilutions of the quantified standard positive control (from 2.5x 10^5 to 2.5 genome equivalents / µL). The log genome equivalent (GE) value was plotted against the *Ct* number, and the results were expressed as the number of GE per g of spleen tissue (GE/g).

2.5 HEV RNA Detection by Conventional Reverse Transcription PCR

Any identified RT-qPCR positive samples were subjected to two conventional reverse transcription PCR procedures (QIAGEN OneStep RT-PCR Kit; Qiagen, Hilden, Germany) followed by nested PCRs (Go Taq, Promega, Madison, WI, USA). A pan-*Hepeviridae* test was applied; this test is able to amplify all genotypes of *Paslahepevirus balayani* by

annealing within a 300 bp in the RNA-dependent RNA polymerase region (RdRp) of the ORF1 (Drexler et al., 2012).

A second nested PCR, amplifying a short genomic region within ORF2 of 412bp was also used to detect the four HEV (HEV-1 to HEV-4) genotype strains (Mizuo et al., 2002).

The PCR products were stored at −20 °C until use, or were immediately run using agarose gel electrophoresis (1.5%). The amplicons obtained by conventional PCR were subjected to sequencing (Eurofins, Germany) and the obtained sequences were submitted to the NCBI database as PX406261-65 and PX435863-PX435865.

2.6 Phylogenetic analysis

The identified sequences were compared with the HEV sequences on the NCBI database using BLASTn (Basic Local Alignment Search Tool-nucleotide) search. For each sequence, the five most similar sequences were selected. The obtained ORF1 sequences were used to build dataset A (n = 5), together with 18 HEV-3 reference sequences (subtype a-m), the 25 most similar sequences, three Polish sequences from humans downloaded from NCBI, and one HEV-4 sequence as outgroup (Accession LC022745.1). The dataset B was built using the obtained ORF2 sequences (n = 3), as well as 18 HEV-3 reference sequences (subtype a-m), the 12 most similar sequences, 23 Polish sequences from swine and wild boar, and one HEV-4 sequence as outgroup (Accession LC022745.1). The datasets were used to build the Maximum Likelihood (ML) phylogenetic tree; construction was performed using IQ-TREE2 (v.1.6.10) with the best fit model indicated by the Model Finder and involving 1000 bootstrap.

2.7 Data elaboration and statistical analysis

Due to the low number of samples investigated by RT-qPCR, only the results of the specific HEV antibodies were subjected to statistical analysis. For this purpose, all doubtful serological results were regarded as negative. For wild boars from which samples were taken, the hunting location was given as county level. To explain the risk of HEV in wild boars for each county, the following data were collected: the number of pigs,

the number of free-ranging wild boars, the number of free-ranging deer, the area of water, and wetlands. Data on the number of pigs were obtained from the Polish Veterinary Inspection, and on the number of wild boars and deer from the State Forests database (https://www.bdl.lasy.gov.pl/portal/).

The area covered by water and wetland was calculated based on Corine Land Cover (CLC) (https://clc.gios.gov.pl), from which vector layers of land cover were obtained for the last map update, i.e. for 2018. In addition, vector layers of county boundaries were obtained from the GIS support portal (https://gis-support.pl/). Then, CLC categories representing the appropriate cover types were selected (4.1.1 inland marshes, 4.1.2 peat bogs, 5.1.1 water courses, and 5.1.2 water bodies). The area of the cover types together was calculated for each county in QGIS (version 3.22). At the same time, the area of each county was calculated in QGIS. To standardize the values obtained for the counties, all the data obtained, *viz.* the number of pigs, wild boars and deer, were converted to km^2. The area of water and wetlands was converted to the percentage of the area they occupy in each county.

The probability of HEV antibody occurrence was assessed using a generalized linear mixed model. For this purpose, serological results were assumed as a dependent binary variable. The following explanatory variables were included in the model: pig density (PIGS), wild boar density (BOAR), deer density (DEER), percentage of the area occupied by water and wetlands (WATER), as well as the sex of the examined wild boar (SEX) and their age (AGE). Region ID was included as a random factor to account for variations in mammal populations (PIGS, BOARS and DEERS) and cover types (WATER) across different regions. Model selection was based on the corrected Akaike information criterion (AICc). The best-fit models were identified by the lowest AICc score. Backward elimination was performed to find the best-fit model.

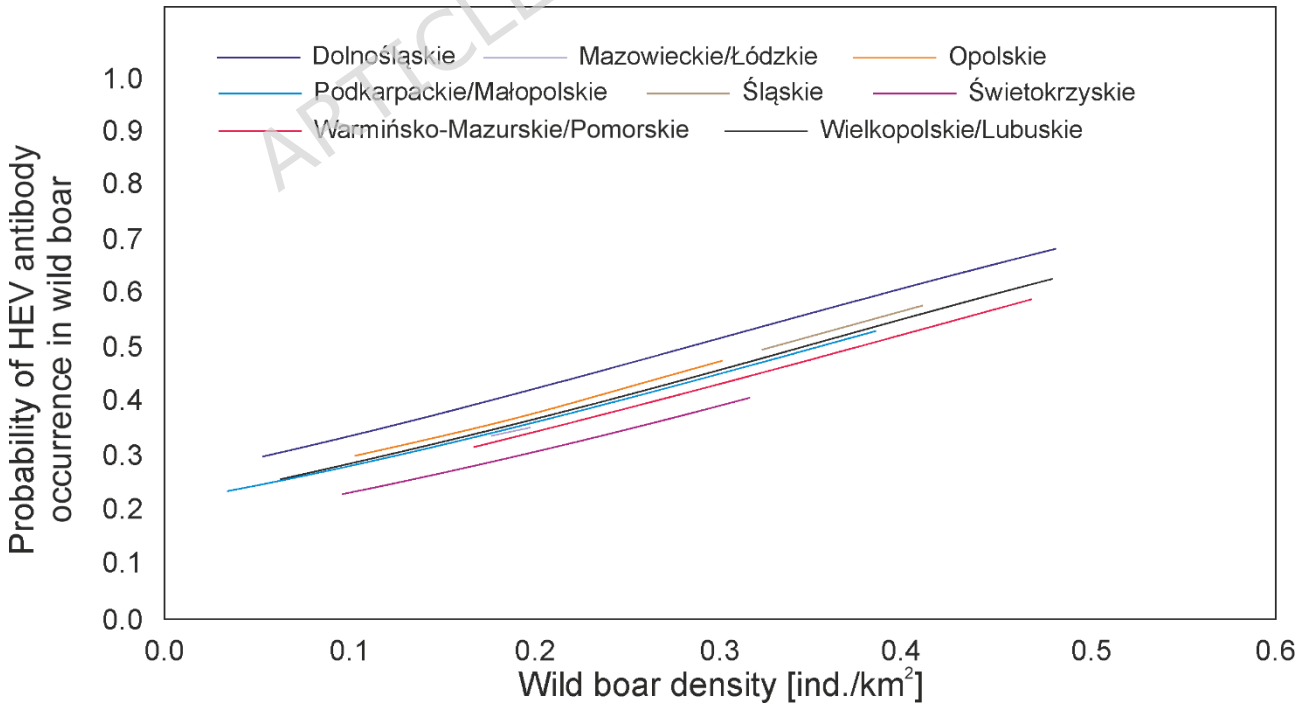
3. **Results**

3.1 HEV seroprevalence

Specific HEV antibodies were detected in 154/367 (41.96%) wild boars. The results of 12/367 (3.27%) samples were doubtful and hence considered negative. The results for each voivodeship were as follows: dolnośląskie (63/122, 51.64%), lubuskie (2/15, 13.33%), łódzkie (7/21, 33.33%), małopolskie (2/5, 40%), mazowieckie (7/16, 43.75%), opolskie (13/26, 50%), podkarpackie (5/17, 29.41%), pomorskie (5/6, 83.33%), śląskie (14/23, 60.87%), świętokrzyskie (20/74, 27.03%), warmińsko-mazurskie (4/17, 23.53%), wielkopolskie (8/14, 57.14%), and zachodniopomorskie (4/11, 36.36%).

Only the density of wild boars (BOAR) showed a significant effect on the probability of finding HEV antibodies (Table 1). Other variables (PIGS, DEER, WATER, SEX, AGE) were excluded in the model selection process. The probability of HEV antibodies in wild boar increased with the density of wild boars (Fig. 2). It should be noted, however, that the estimated probability of HEV antibodies is not high and exceeds 0.5 at a wild boar density of about 0.4 ind. /km^2.

Fig. 2



3.2 The probability of HEV antibody occurrence in wild boar according to wild boar density based on a generalized linear mixed model. Each line represents the probability calculated for a given region.

Of the wild boars analysed for the presence of antibodies, 100 (46 seropositive and 54 seronegative) underwent molecular detection of HEV RNA by RT-qPCR. The presence of viral RNA in the spleen (analysed in duplicate), was confirmed in 10/100 (10%) samples, with a mean Ct value of 32 (see Table 2).

All the animals found positive for viral RNA were also seropositive to HEV antibodies, with a relative prevalence of 21.7 % (10/46 samples). None of the seronegative samples were positive for RNA. Quantification, carried out by a standard curve which equation was: y= -3,368x+40,296 (R^2=0,9971), showed viral amounts ranging from $7.59x10^4$ to $3.13x10^5$ GE/g of spleen tissue, with a mean quantity of $2.03x10^5$ GE/g (see Table 2).

3.3 Sequence and phylogenetic analysis

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Five RT-q PCR-positive liver samples were sequenced in the 3'-ORF1 region, of which three were also sequenced in the ORF2 fragment region. The BLASTn search confirmed the presence of HEV-3 genotype sequences. The phylogenetic tree constructed using ORF1 sequences (Fig. 3) found the Polish sequences to form two clusters. The first cluster included Pol435_2024, Pol1120_2024, Pol1820_2024, Pol515_2024 with an unclassified reference sequence (MF959764, WB/HEV/NA17ITA15, named here as HEV-3*), sharing respectively 93.2, 94.2, 94.2 and 90% nucleotide identity. The WB/HEV/NA17ITA15 reference sequence was identified in a wild boar in Italy and recently recognized as a novel subtype, although unnamed (Purdy et al., 2022). The second cluster included Pol1755_2024 as a HEV-3c subtype sequence, sharing 96% nucleotide identity with the HEV-3c reference sequence (FJ705359, wbGER27). The phylogenetic tree built with ORF2 sequences (Fig. 4) indicated the presence of HEV-3c and unclassified sequences. The first cluster included Pol435_2024, Pol1120_2024 clustering with WB/HEV/NA17ITA15 and sharing with it 91% nucleotide identity.

Sequence Pol1755_2024 clustered and shared 95.9% nucleotide identity with the HEV-3c reference wbGER27.

Fig. 3.

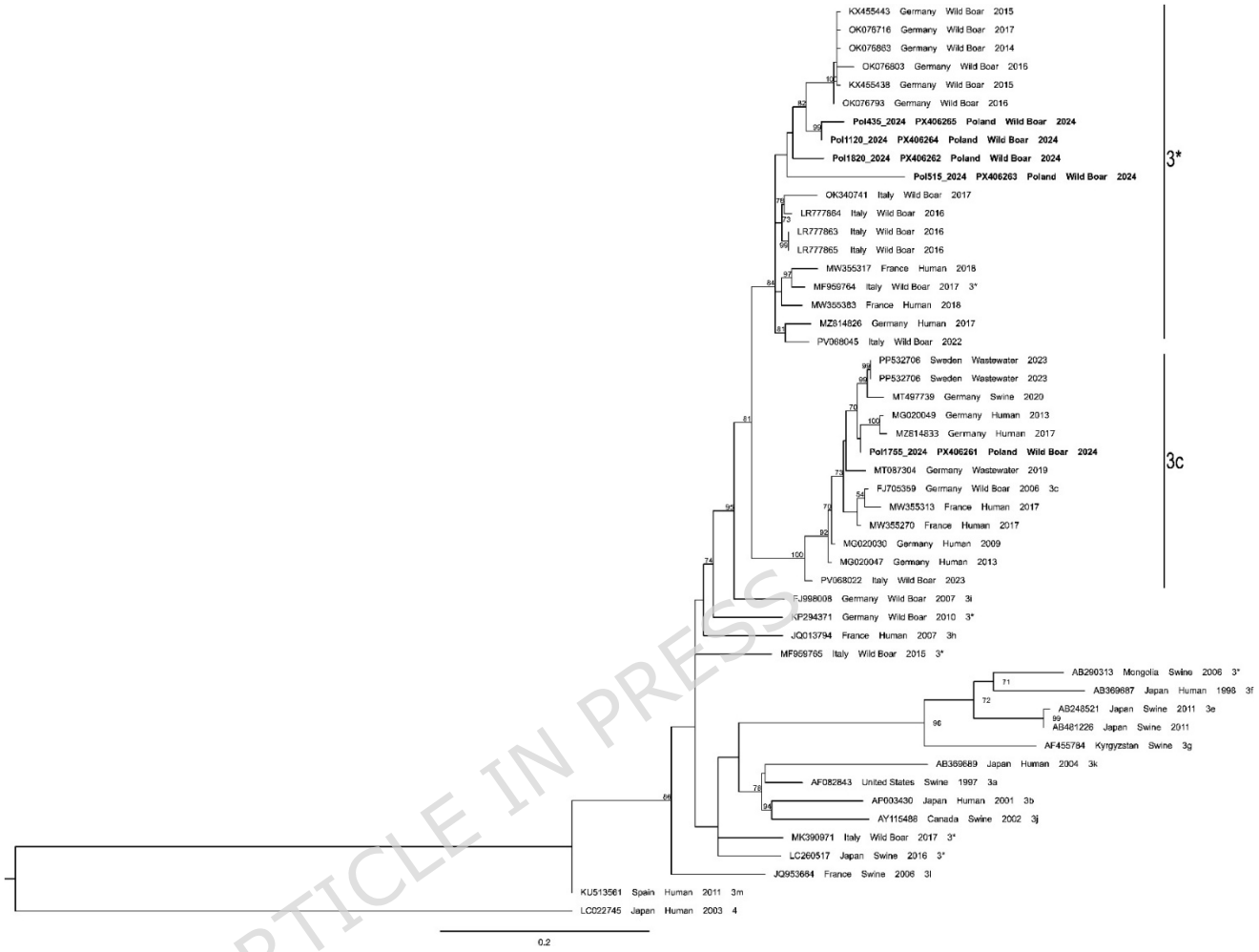
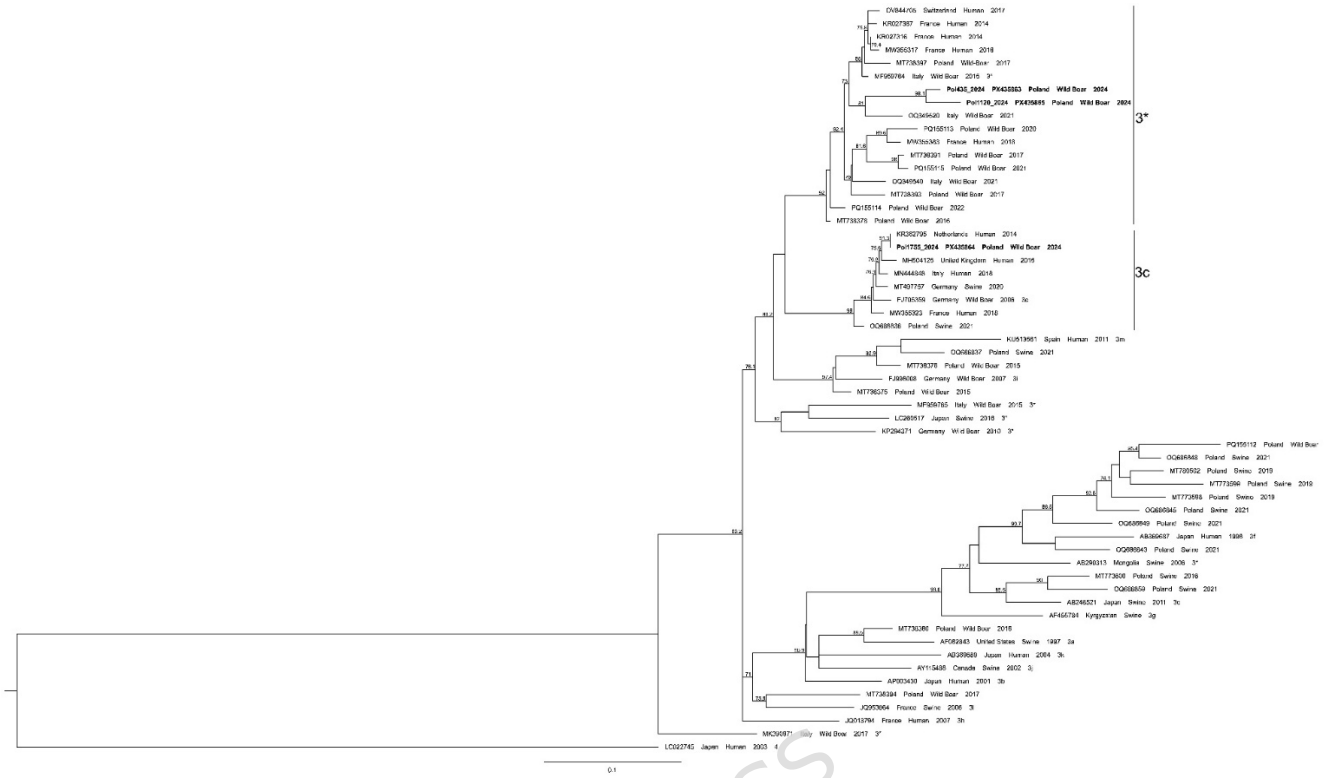


Fig. 4.



4. Discussion

The current study contributes to the growing body of evidence regarding the circulation of HEV in wild boar populations across Europe. Our findings confirm the presence of HEV antibodies in serum samples and HEV-RNA in spleen from wild boars originating from multiple Polish voivodeships. These results align with earlier reports from other European countries, including Germany (Schielke et al., 2009), Italy (Aprea et al., 2018; De Sabato et al., 2020; De Massis et al., 2022), and Spain (Kukielka et al., 2016; Caballero-Gómez et al., 2019), where HEV prevalence in wild boar has been documented as a significant public health concern. The detection of HEV RNA in apparently healthy wild boars supports the hypothesis that these animals can serve as asymptomatic reservoirs of infection (Wu et al., 2011; Aprea et al., 2018). Given the popularity of wild boar meat and hunting practices in Poland, the zoonotic risk posed by direct contact with blood and tissues or consumption of undercooked meat is considerable. This is particularly relevant in light of reports of genotype 3 HEV infections in humans linked to consumption of game meat (Takahashi et al., 2012; European Food Safety Authority, 2017).

Our study revealed an HEV-seroprevalence of 41.96% and an HEV-RNA occurrence of 10%. These results are in accordance with those of previous studies, which note a prevalence of 17.2-49% (serology) and 9.4-12% (RNA) (Larska et al., 2015; Weiner, 2016; Dorn-In et al., 2017; Kozyra et al., 2020); however, in those studies, HEV-RNA detection was conducted on different matrices (faecal samples or liver tissue) (Dorn-In et al., 2017; Kozyra et al., 2020). Detection rates are known to vary between organ types, and detailed data about the detectability of HEV RNA in the spleen are scarce (Fanelli et al., 2021). Our present findings indicate a mean viral RNA concentration of $2.03x10^5$ GE per gram of spleen tissue, which is higher than the value previously reported in muscle ($1.5x10^4$ GE/g), but lower than that in liver tissue ($1.4x10^7$ Ge/g) (De Sabato et al. 2020). Another study conducted on field samples reported a lower level of detection in the spleen compared to the liver (Anheyer-Behmenburg et al., 2017); as such, it is possible that the prevalence in our study may be underestimated.

Nevertheless, our analyses indicate that wild boar density had a significant effect on the probability of detecting HEV antibodies, which is consistent with previous reports in which high wild boar density was linked to increased HEV infection rates across Europe (Mughini-Gras et al., 2017; Castanga et al., 2024) Our results found that HEV detection rates vary between voivodeships, which may reflect differences in ecological conditions, host population density, and anthropogenic factors. This geographic pattern is consistent with previous findings from Germany and the Czech Republic, where regional hotspots of HEV circulation in wildlife were observed (Adlhoch et al., 2016; Strakova et al., 2018).

Phylogenetic analysis showed that the sequences characterized belonged to HEV-3, specifically two subtypes: HEV-3c and an unclassified HEV-3 subtype. HEV-3c is a common subtype in Europe, mainly in the Netherlands (Hogema et al., 2021), Germany (Schemmerer et al., 2022), and Belgium (Suin et al., 2019), and it is found in both humans and wild boar. Indeed, the HEV-3c sequence detected closely matched a human case from the Netherlands (Table 3). The other four HEV-3 sequences clustered separately, with the nucleotide identity and clustering suggesting they represent a distinct subtype. Their closest reference strain was the Italian wild boar WB/HEV/NA17ITA15, which has not yet been formally assigned a subtype due to the lack of at least three full genomes required for official subtype assignment (Purdy et al., 2022). Sequence analyses confirmed the circulation of HEV strains across Europe and highlighted the importance of molecular typing for tracing the origin and spread of cases.

This is the second Polish study to report HEV subtypes in wild boar. Kozyra et al. (2021) identified HEV-3a, 3i, and some unclassified strains related to 3h. In Poland, human HEV-3c and 3i subtypes have also been reported in blood donors, and 3c in pigs but the 3c strains from these and earlier studies did not cluster together. Unfortunately, due to the limited number of available sequences, it is not possible to draw specific conclusions about phylogenetic relationships and transmission dynamics.

Our study confirms the presence of HEV among free-ranging wild boar in Poland. The detection of both viral RNA and antibodies in several regions, including eastern and central parts of the country, indicates that HEV is circulating in the natural environment. These results contribute valuable data to the understanding of HEV epidemiology in Central Europe and emphasize the necessity of incorporating wildlife surveillance into broader public health strategies. Given the zoonotic potential of HEV and the popularity of wild boar meat, particularly among hunting communities, awareness campaigns and risk mitigation efforts are recommended.

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Author contributions

A.D. research design and coordination, collecting samples, data analysis, writing – original draft; D.K. data analysis, statistical analysis, revision of the manuscript, K.M., M.P., E.K. laboratory work, data analysis; K.A., K.F.H. study coordination, data analysis, revision of the manuscript. E.D.L. data analysis; N.D.A. revision of the manuscript; F.S. data analysis; M.L. data analysis; G.F. revision of the manuscript; I.D.B. data analysis, writing – original draft; L.D.S. data analysis; M.G.A. data analysis, writing – original draft, revision of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability statement

All detailed data are available for request in the corresponding authors. The datasets generated during the current study are available in the NCBI database repository, PX406261-65 and PX435863-PX435865.

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Additional Information

The authors declare no competing interests.

Consent for publication

All authors have read and agreed to the published version of the manuscript.

Figure legends

Figure 1. Location and number of wild boars analysed (white numbers). This map was created using open source software: vector graphic design program (Inkscape 1.4.2, inkscape.org) and the Python programming language (Python 3.14.0, python.org). GIS data (country and voivodeships shape files) were downloaded from publicly available government database (geoportal.gov.pl).

Figure 2. The probability of HEV antibody occurrence in wild boar according to wild boar density based on a generalized linear mixed model. Each line represents the probability calculated for a given region.

Figure 3. Phylogenetic analysis based on the 270 nt fragment of the partial RdRp region within ORF1, including the five sequences obtained in this study (entries highlighted in bold), 26 HEV-3 sequences obtained from the NBCI database, 18 HEV-3 references and a HEV-4 sequence used as outgroup. Sequence entries are reported as GenBank Accession Number, Country, Host species and Collection Date.

Figure 4. Phylogenetic analysis based on the 390 nt fragment of the partial capsid protein region within ORF2, including the three sequences obtained in this study (entries highlighted in bold), 35 HEV-3 sequences from the NBCI database, 18 HEV-3 references and a HEV-4 sequence used as outgroup. Sequence entries are reported as GenBank Accession Number, Country, Host species and Collection Date.

Tables

Table 1. Effect of wild boar density (BOAR) on the HEV antibody occurrence in wild boar.

Source	Estimate	Standard error	t	p	Lower CI	Upper Ci

Intercept	-1.245	1.783	-0.729	0.466	-4.807	2.206
BOAR	3.772	1.048	3.600	<0.001	1.712	5.832

Table 2. Wild boars positive for the detection of HEV RNA in spleen.

ID	Sex	Age (year)	Weight (Kg)	Voivodeship	Poviat	Site	RT-qPCR (Ct)	HEV RNA (GE/g tissue)
435	M	3	67	Małopolskie	gorlicki	Bystra, 49.658944; 21.114819	**27.11**	**3.13x10^5**
515	M	2	ND	Wielkopolskie	pleszewski	51.93319250; 17.77005070	**30.8**	**2.26x10^5**
519	F	1	ND	Wielkopolskie	ostrowski	Hocina	**29.82**	**2.49x10^5**
990	M	2	63	Małopolskie	nowosądecki	Piwniczna	**32.9**	**1.76x10^5**
1036	F	2	37	warmińsko-mazurskie	olsztyński	Kępajny, gmina Barczewo	**37.16**	**7.59x10^4**
1120	F	1.5	30	Zachodniopomorskie	policki	Dobieszczyn Karpin gm. Police	**27.67**	**3.02x10^5**
1121	F	2	30	Zachodniopomorskie	policki	Trzebieradz Karpin gm. Police	**34.6**	**1.35x10^5**
1731	F	2	48	Mazowieckie	nowodworski	Kazuń Nowy	**35.9**	**1.04x10^5**
1755	M	3	70	Małopolskie	bocheński	Bochnia	**32.1**	**1.95x10^5**
1820	F	1.5		Wielkopolskie	pilski	Motylewo	**29.5**	**2.56x10^5**

M= male; F=Female; ND= not determined; GE/g= genome equivalent per gram of tissue