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Molecular detection of hepatitis E virus in wild boar population in eastern Romania

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Summary

In industrialized countries, Hepatitis E is a recognized zoonosis, with wild boar and swine representing the main reservoirs for zoonotic genotype HEV-3 in Europe. Data related to HEV infection in wild boar population in Romania are restricted to serological surveys. Therefore, our main goal was to determine the HEV prevalence in wild boar population and to characterize HEV strains circulating in Romania. Using TaqMan real-time RT-PCR assay, we analyzed the presence of RNA HEV in 45 liver samples and five spleen samples collected from 50 wild boars. Samples were collected during the 2013–2015 hunting seasons. Nine samples of 50 were tested positive for HEV RNA, resulting an overall prevalence of 18%. Phylogenetic analysis revealed that the isolates clustered in different HEV-3 monophyletic groups, depending on the sampling county. This is the first study signalling, based on molecular analysis, the presence of HEV in wild boar population from Romania. Also, in this study, we report the detection of HEV in splenic tissue from wild boar.

KEYWORDS

hepatitis E virus, phylogenetic analysis, wild boar, zoonosis

1 | INTRODUCTION

Hepatitis E virus (HEV) is the causal agent of an acute self-limiting hepatitis in humans (Aggarwal, 2011) and of a subclinical infection in animals (Pavio, Meng, & Renou, 2010) HEV is a non-enveloped virus with an icosahedral capsid with an outer diameter of approximately 27–34 nm. HEV has a positive single-stranded RNA genome encoding three open reading frames (ORFs), which include the non-structural protein (ORF1), the capsid protein (ORF2) and a phosphoprotein that is associated with the cytoskeleton (ORF3) (Zafrullah, Ozdener, Panda, & Jameel, 1997).

The HEV genomes have been identified from several animals such as domestic and feral pigs, deer, rat, rabbits, ferrets, cutthroat trout, bats, mongoose and camels. Domestic pigs and wild boars have been recognized as the main reservoirs of zoonotic HEV. Hepatitis E virus belongs to the *Hepeviridae* family. The HEV strains isolated from humans, pigs and wild boars have been classified into the *Orthohepevirus* genus *Orthohepevirus A* species (Smith et al., 2014). Out of the genotypes of HEV belonging to this species, four main are capable of infecting humans (HEV-1 to HEV-4) (Doceul, Bagdassarian, Demange, & Pavio, 2016).

Genotypes HEV-1 and HEV-2 infect exclusively humans and are transmitted primarily via the faecal-oral route through contaminated water, while genotypes HEV-3 and HEV-4 are zoonotic agents and infect humans, pigs and other mammals and are transmitted by contact with animal reservoirs or consumption of raw or undercooked meat from infected animals (Pavio et al., 2010). Genotype HEV-3 strains isolated from humans and animals, except rabbit strains, are classified into two main clades: one major clade 3abchij (subtypes 3a, 3b, 3c, 3h, 3i and 3j) and a second clade 3efg (subtypes 3e, 3f and 3g) (Smith et al., 2016). Vina-Rodriguez et al., 2015 propose HEV-3 clustering into three monophyletic groups: "3abj", "3chi", "3feg."

The HEV strains circulating in domestic and feral pigs are genetically related to the strains identified in autochthonous human cases (Doceul et al., 2016; Pavio, Meng, & Doceul, 2015). Wild boar has been identified as a risk factor for autochthonous HEV infections in

industrialized countries such as Japan (Sonoda et al., 2004), Germany (Wichmann et al., 2008), Sweden (Widen et al., 2011) and Italy (Mazzei et al., 2015).

First indications of HEV infections in wild boars were made in 1999, when 17% of free-living pigs investigated in Australia were found positive for anti-HEV antibodies (Chandler, Riddell, Li, Love, & Anderson, 1999) but the first detection of HEV in wild boar was reported in 2004, in Japan (Sonoda et al., 2004). Across Europe, genotype HEV-3 has been previously detected in wild boars from France (Jori et al., 2016; Kaba, Davoust, Marie, & Colson, 2010; Lhomme et al., 2015), Germany (Chandler et al., 1999; Kaci, Nockler, & Johne, 2008; Schielke et al., 2009), Hungary (Forgach et al., 2010; Reuter, Fodor, Forgach, Katai, & Szucs, 2009), Italy (Caruso et al., 2015; Martelli et al., 2008; Martinelli et al., 2015; Mazzei et al., 2015), Netherlands (Rutjes et al., 2009, 2010), Belgium (Thiry et al., 2015) Sweden (Widen et al., 2011), Portugal (Mesquita, Oliveira, Coelho, Vieira-Pinto, & Nascimento, 2016), Estonia (Ivanova et al., 2015) and Spain (de Deus et al., 2008) with prevalence rates ranging from 2.5% to 25% (Martelli et al., 2008). Adlhoch et al. (2009) reported the highest HEV infection rate of 68.2%, in wild boars from Germany.

In Romania, there are limited data available regarding infection with HEV, both in humans and animals (pigs and wild boar), most studies being restricted to serological surveys. Studies that focused on the identification of HEV in domestic and wild boars from Romania were conducted particularly in livestock from eastern counties of the country. In 2014, Aniță et al., reported circulation of HEV in pig farms in eastern Romania. The phylogenetic analysis of the ORF2 sequence indicated that swine HEV isolates belonged to genotype 3. First studies on wild boar were conducted in 2009, serological surveys detecting IgG antibodies anti-HEV in 4.44% of tested animals. Since then, the serological investigations showed increased rates of anti-HEV IgG antibodies, ranging from 6.25% to 11.1% in wild boar population from the counties located in eastern Romania (Aniță, Aniță, Tănase, Ludu, & Savuța, 2009; Anita et al., 2014; Botezatu, Tănase, Aniţa, Rîmbu, & Carp-Cărare, 2014; Porea, Anita, Paslaru, & Savuta, 2015, 2016).

Therefore, our goals were to determine HEV prevalence in wild boar population from the eastern Romania, to characterize the identified strains to determine their relationship to strains previously detected from swine in Romania and with other available HEV strains isolated worldwide.

2 | MATERIALS AND METHODS

2.1 | Sample collection

Between 2013 and 2015, 45 liver samples and five spleen samples were collected from 50 wild boars during the hunting season, from five counties located in eastern Romania: Suceava, Iași, Vrancea, Galați and Botoșani (Figure 1). The wild boars from which the samples were collected were included in the Classical Swine Fever (CSF) and African Swine Fever (ASF) Surveillance and Control Programs. Following collection, samples were stored at −80°C until testing.

The age was estimated by the hunters and recorded for 37 animals. The animals were divided into three age groups: less than 1 year (n = 4), between 1 and 2 years (n = 11), older than 2 years (n = 22). The age of 13 animals could not be determined (Table 1).

2.2 | Sample preparation and RNA extraction

Tissue samples were homogenized in lysis buffer (RNeasy Mini Handbook QIAGEN kit) with β-mercaptoethanol using FastPrep24 Version 3.0. (MP Biomedicals, ref: 6003-500). The homogenized samples were centrifuged, and then RNA was extracted from the supernatants using RNeasy Mini Handbook QIAGEN kit (ref: 52906) according to the manufacturer's protocol. RNA was eluted twice with 40 μl of sterile water after 1-min incubation at room temperature. RNA was aliquoted and stored at −80°C until use.

2.3 | RT and PCR controls

Precautions were taken to prevent false-positive and false-negative results in RT-PCR amplification. In addition to spatial separation of workspaces at crucial experimental points (e.g., RNA extraction, PCR mix preparation), each experiment included several control samples: positives and negative samples for RNA extraction; negative and positives controls of RT, first PCR and nested PCR in each run.

2.4 | TaqMan real-time RT-PCR assay

HEV RNA detection was adapted from the method described by Jothikumar, Cromeans, Robertson, Meng, and Hill (2006) and performed as described by Barnaud, Rogee, Garry, Rose, and Pavio (2012). This assay targets a fragment of ORF3 which is a highly conserved region in different HEV genotypes. Real-time RT-PCR data were collected after the reaction, and the threshold cycle (Ct) was calculated. For generation of standard quantification curve, the Ct values were plotted proportionally to the logarithm of the input copy numbers of standard RNA. Standard RNA was obtained after in vitro transcription of a plasmid pCDNA 3.1 ORF2-3 HEV, as described by Barnaud et al. (2012). The results are expressed in genomic copy number per gram of tissues (GE/g).

2.5 | Nucleotide sequencing and sequence analysis

Positive samples by TaqMan real-time RT-PCR assay were analyzed using the RT-nested PCR, amplifying a 348 nt fragment of ORF2, using two sets of degenerate primers (Cooper et al., 2005). All oligonucleotides were purchased from MWG Biotech AG (Eurofins MWG, Ebersberg, Germany). The assay was adapted from the method described by Cooper et al. (2005) and performed as described by Bouquet et al. (2011).

The nested PCR products were analyzed after migration on an agarose (1%) gel and ethidium bromide staining. The expected final product of the nested RT-PCR was 348 bp.

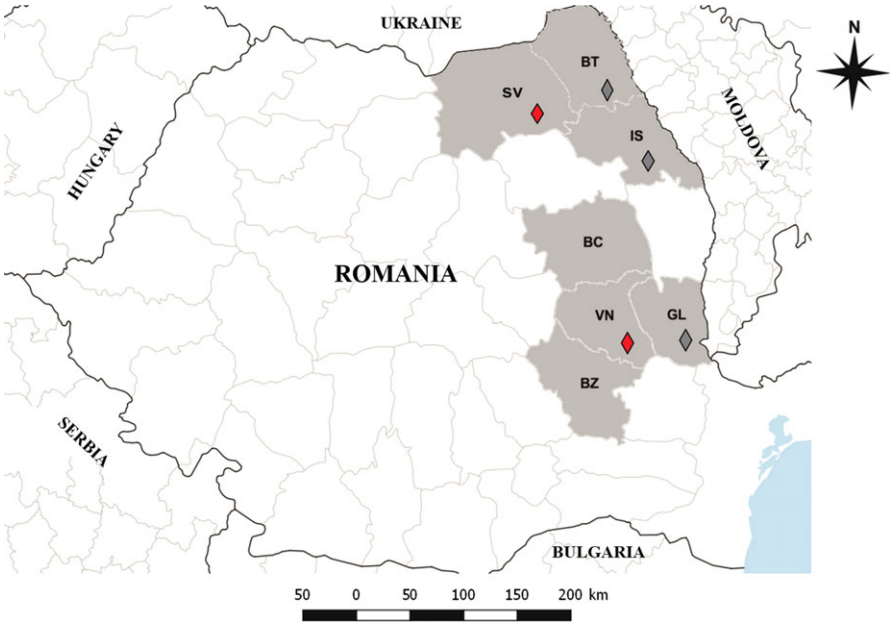

FIGURE 1 Counties searched in previous and the current study for HEV infection in wild boar population in Romania. (▭) Counties where studies for HEV infection have been undertaken. (♦) Counties where wild boar HEV has been detected in this study. (♦) Counties where negative wild boar HEV has been identified in this study. SV, Suceava; BT, Botoșani; IS, Iași; BC, Bacău; VN, Vrancea; GL, Galați; BZ, Buzău

TABLE 1 Data on the tested samples and obtained prevalence of HEV infection

				Age							
	Tissue			<1 year		1–2 years		>2 years		Age unknown	
County	Type of tissue	No. tested	No. positive	No. tested	No. positive	No. tested	No. positive	No. tested	No. positive	No. tested	No. positive
Vrancea	Spleen	5	2	–	–	–	–	5	2	–	–
	Liver	3	0	2	0	–	–	1	0	–	–
Suceava	Liver	30	7	1	0	11	2	15	5	3	1
Iasi		10	0	1	0	–	–	–	–	9	0
Botoșani		1	0	–	–	–	–	1	0	–	–
Galați		1	0	–	–	–	–	–	–	1	0
Total		50	9 (18%)	4	0 (0%)	11	2 (18.18%)	22	7 (31.81%)	13	1

The positive amplicons were sequenced with nested PCR inner primer (3158N) by MWG Biotech AG (Eurofins MWG). Nucleotide sequences were analyzed and edited individually using Bioedit software. Phylogenetic tree was generated with MEGA 7 using the neighbour-joining method and the p-distance model. The number of bootstrap replicates was 1,000. The generated sequences were aligned with the best hits to our sequences in GenBank and with the reference strains of each HEV-3 subtype (Smith et al., 2016). An human HEV-1 strain (EF530670.1/) was used as outgroup.

3 | RESULTS

3.1 | Detection of HEV RNA in wild boar liver and spleen samples

A total of 50 tissues samples (45 liver samples and five spleen samples) from wild boar originating from five counties located in the eastern part of Romania were analyzed by TaqMan real-time RT-PCR for the detection of the HEV RNA. Nine samples were tested positive with an overall prevalence of 18%. The HEV viral load was between 7.8×10^3 and 3.14×10^9 GE/g tissues. HEV RNA was detected in seven of 45 liver samples tested and in two of five spleen samples tested. The obtained data show that in the two positive spleen samples with Ct values 33.91 and 30.15, the copy number of viral genome was 1.71×10^5GE/g and 2.19×10^6GE/g tissue, respectively. Positive wild boars for HEV originated from two counties: Vrancea (two positive spleen samples) and Suceava (seven positive liver samples), which are located on opposite sides of the analyzed area (Figure 1). HEV RNA was detected in wild boars aged between 1 and 2 years (2/11-18.18%) and in animals older than 2 years (7/22-31.81%) (Table 1).

3.2 | HEV Sequence analysis

The positive samples by TaqMan real-time RT-PCR were analyzed by RT-Nested PCR, amplifying a 348 bp fragment of ORF-2. Following this reaction, five nested RT-PCR products were obtained: one of the two positive spleen samples and four of the seven positive liver

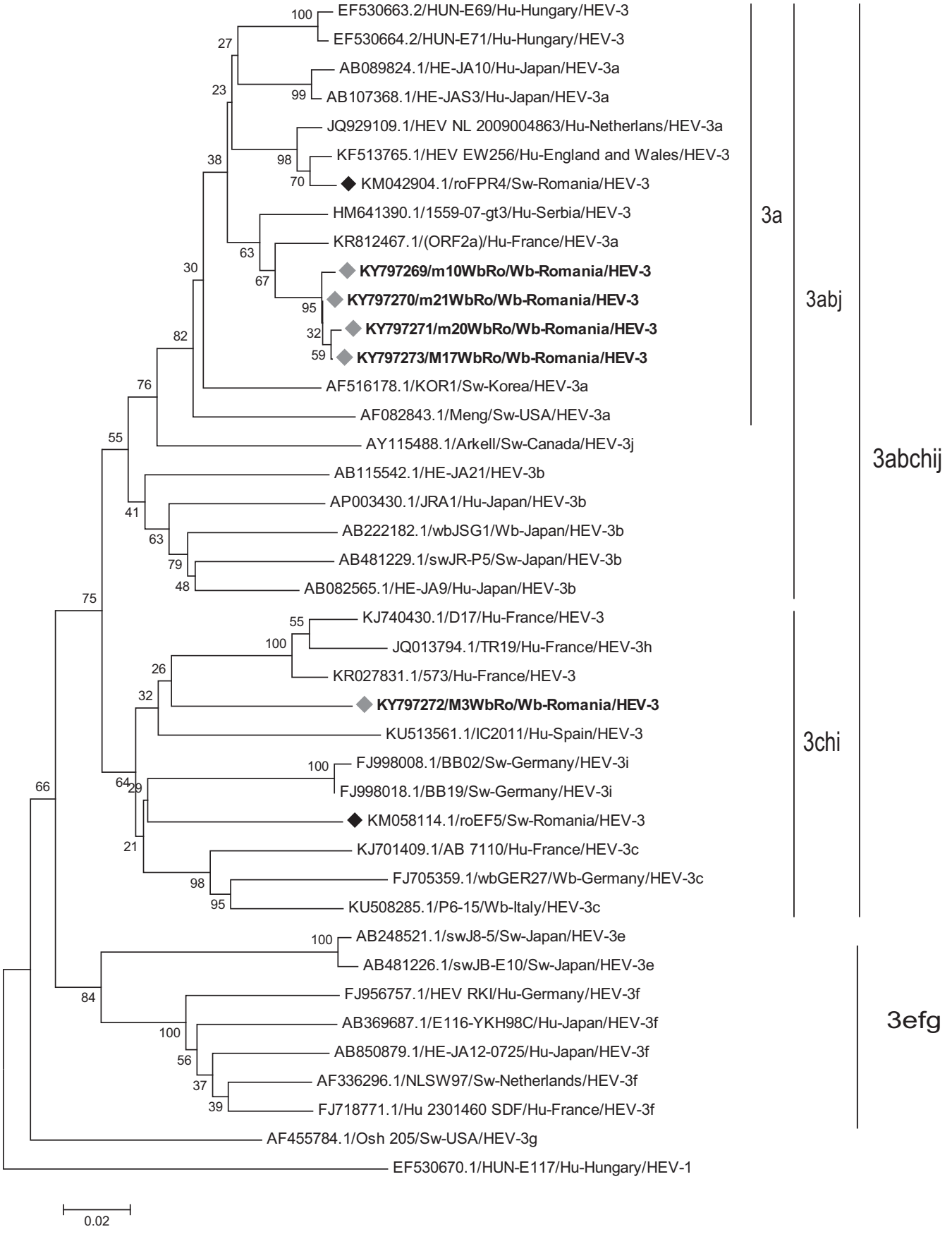
EF530663.2/HUN-E69/Hu-Hungary/HEV-3
EF530664.2/HUN-E71/Hu-Hungary/HEV-3
AB089824.1/HE-JA10/Hu-Japan/HEV-3a
AB107368.1/HE-JAS3/Hu-Japan/HEV-3a
JQ929109.1/HEV NL 2009004863/Hu-Netherlans/HEV-3a
KF513765.1/HEV EW256/Hu-England and Wales/HEV-3
KM042904.1/roFPR4/Sw-Romania/HEV-3
HM641390.1/1559-07-gt3/Hu-Serbia/HEV-3
KR812467.1/(ORF2a)/Hu-France/HEV-3a
KY797269/m10WbRo/Wb-Romania/HEV-3
KY797270/m21WbRo/Wb-Romania/HEV-3
KY797271/m20WbRo/Wb-Romania/HEV-3
KY797273/M17WbRo/Wb-Romania/HEV-3
AF516178.1/KOR1/Sw-Korea/HEV-3a
AF082843.1/Meng/Sw-USA/HEV-3a
AY115488.1/Arkell/Sw-Canada/HEV-3j
AB115542.1/HE-JA21/HEV-3b
AP003430.1/JRA1/Hu-Japan/HEV-3b
AB222182.1/wbJSG1/Wb-Japan/HEV-3b
AB481229.1/swJR-P5/Sw-Japan/HEV-3b
AB082565.1/HE-JA9/Hu-Japan/HEV-3b
KJ740430.1/D17/Hu-France/HEV-3
JQ013794.1/TR19/Hu-France/HEV-3h
KR027831.1/573/Hu-France/HEV-3
KY797272/M3WbRo/Wb-Romania/HEV-3
KU513561.1/IC2011/Hu-Spain/HEV-3
FJ998008.1/BB02/Sw-Germany/HEV-3i
FJ998018.1/BB19/Sw-Germany/HEV-3i
KM058114.1/roEF5/Sw-Romania/HEV-3
KJ701409.1/AB 7110/Hu-France/HEV-3c
FJ705359.1/wbGER27/Wb-Germany/HEV-3c
KU508285.1/P6-15/Wb-Italy/HEV-3c
AB248521.1/swJ8-5/Sw-Japan/HEV-3e
AB481226.1/swJB-E10/Sw-Japan/HEV-3e
FJ956757.1/HEV RKI/Hu-Germany/HEV-3f
AB369687.1/E116-YKH98C/Hu-Japan/HEV-3f
AB850879.1/HE-JA12-0725/Hu-Japan/HEV-3f
AF336296.1/NLSW97/Sw-Netherlands/HEV-3f
FJ718771.1/Hu 2301460 SDF/Hu-France/HEV-3f
AF455784.1/Osh 205/Sw-USA/HEV-3g
EF530670.1/HUN-E117/Hu-Hungary/HEV-1
0.02
3a
3abj
3abchij
3chi
3efg

FIGURE 2 Phylogenetic tree based on the alignment of the generated sequences with the best hits to our sequences in GenBank and with the reference strains of each HEV3 subtype[6]. Outgroup: human HEV-1 strain (EF530670.1/). The wild boar HEV strains obtained in our study are highlighted in boldface and through grey diamonds. The black diamonds indicated the swine HEV strains previously characterized in eastern Romania. The tree was generated with MEGA 7 using the neighbour-joining method and the p-distance model. The number of bootstrap replicates was 1,000

samples. To characterize the HEV strains, the positive products were sequenced and analyzed. The characterization of viral strains circulating in wild boar population analyzed in this studies based on a fragment of ORF2, indicated that all isolates belonged to genotype 3.

The isolates obtained from wild boar in Suceava County (m10wbRO, m20wbRO, m21wbRO and M17wbRO, GenBank accession numbers: **KY797269**, **KY797270**, **KY797271**, and respectively **KY797273**) had 98%–99% nucleotide sequences homology with each other and clustered within the subtype 3a based on alignment with the best hits of our sequences in GenBank (Figure 2). Blast analysis showed also that these sequences had a maximum of 97% nucleotide similarities with a French human isolate (GenBank accession number KR812467.1), 95% nucleotide similarities with a Dutch human isolate (GenBank accession number JQ929109.1), 94% nucleotide similarities with two human strains isolated in Japan (GenBank accession numbers AB089824 and AB107368), and 93% nucleotide similarities with a swine strain isolated in Korea, clustered in subtype 3a. Also, these isolates presented 94% nucleotide similarities with a human isolate from Serbia (GenBank accession number HM641390.1), and with two human strains isolated in Hungary (GenBank accession numbers EF530663 an EF530664).

The isolate obtained from the wild boar's spleen in Vrancea County (M3wbRO, GenBank accession number **KY797272**) based on the alignment with sequences of reference strains of each HEV-3 subtype (Smith et al., 2016) clustered within the "3chi" group and seems to belong to subtype 3h (Figure 2). Therefore, for framing this HEV strain in subtype 3h, full genomic sequence should be performed. This isolate presented a maximum 90% nucleotide similarities with a French human isolate (GenBank accession number KR027831.1) and 89% nucleotide similarities with two human strains, one from France (GenBank accession number KJ740430.1) and one from Spain (GenBank accession number KU513561.1).

4 | DISCUSSION

Hepatitis E is an emerging zoonosis, and the wild boar reservoir has been identified in different countries as a risk factor for autochthonous HEV infections. In Romania, the previous studies conducted in wild boar population in eastern counties indicated the presence of HEV based on the identification of the anti-HEV IgG antibodies (Aniță et al., 2009; Porea et al., 2015, 2016).

In this study, 50 samples collected from wild boars from eastern Romania were analyzed for the presence of HEV RNA, resulting in an overall prevalence of 18%. We previously conducted serological studies in wild boar population from the same area, during the same hunting seasons. The results indicated the presence of HEV with different seroprevalence rates: 9.61% (5/52) in 2015 and 10.29% (7/68) in 2016 (Porea et al., 2015, 2016). After analyzing wild boar samples from five counties, we detected HEV-positive animals only in Vrancea and Suceava counties. This might be due to the uneven distribution of the samples in the study area. However, previous serological studies conducted on wild boar sera revealed anti-HEV IgG antibodies seroprevalence of 11.1% in Iasi county (Porea et al., 2015) and 10.52% in Galaţi county (Porea et al., 2016). Therefore, we cannot exclude the presence of HEV in wild boar populations in the three negative counties identified in this study.

The HEV RNA was detected in wild boars aged between 1 and 2 years (2/11-18.18%) and in animals older than 2 years (7/22-31.81%) (Table 1). The results of this study are similar with those previously described in Europe. These aspects have suggested that the HEV in wild boar can be detected for a long time during the life of animals as a consequence of an incomplete immune response with subsequent reinfections or of the chronicity of HEV infection in wild boar (Adlhoch et al., 2009; Rutjes et al., 2010).

Interesting is that in our study, two of nine RNA HEV-positive samples are splenic tissue. The research carried out by different authors has shown that HEV can be identified in spleen tissue samples collected from domestic pigs, both in experimental (Lee, Ha, Ahn, & Chae, 2009; Williams et al., 2001) as well as in natural infections (Choi & Chae, 2003). Anheyer-Behmenburg et al., 2017; detected RNA HEV in the spleen and also kidney tissues of wild boars and deer from Germany. Our results might reflect the suggestions from the literature indicating that HEV replicates and accumulates in tissues other than the liver (de Deus et al., 2007; Williams et al., 2001).

Different prevalence rates of HEV in wild boars from Europe were observed. Various factors were suggested to be involved in prevalence differences of HEV such as: the type of tissue analyzed, animals stage of infection at the moment of sampling, the different diagnostic techniques, the ecological differences of the wild boar populations and the phenotypic characters (Adlhoch et al., 2009; Caruso et al., 2015; Jori et al., 2016). The prevalence of HEV obtained in our study (18%) is lower than the prevalence reported on bile tissue samples in Germany (68.2%) (Adlhoch et al., 2009) and Italy (25%) (Martelli et al., 2008) but it is similar to the prevalence rates obtained from other tissue samples in different studies performed in Spain (19.6%) (de Deus et al., 2008) and Estonia (16%) (Ivanova et al., 2015). The HEV prevalence from our study is higher compared to studies performed in Hungary (12.2%, 11%) (Forgach et al., 2010; Reuter et al., 2009), France (2.5%, 2.3%) (Jori et al., 2016; Kaba et al., 2010) and Sweden (8.2%) (Widen et al., 2011), although a generally higher number of samples have been analyzed in these countries. The prevalence detected emphasizes a high circulation of HEV infection in wild boar population from eastern Romania.

The HEV strains isolated from wild boar during this study were characterized. To our knowledge, this is the first study that focused on the identification of hepatitis E virus in wild boar samples in Romania. Results showed that different HEV-3 subtypes are circulating in two counties which were found to have HEV-positive wild boar. Studies carried out in Germany reported the isolated HEV strains from wild boar to be clustered according to their geographical origin (Adlhoch et al., 2009; Schielke et al., 2009). In 2014, Anita et al. (2014), conducted a study in swine and humans in eastern Romania revealing HEV circulation in pig farms and the exposure of human population. Based on phylogenetic analysis of the ORF2 sequence, swine HEV isolates belonged to genotype 3. The fact that HEV strains isolated from wild boars in Suceava county are framed in one subtype and, moreover, are related to a swine HEV strain previously characterized in eastern Romania (GenBank accession number KM042904) (Anita et al., 2014), shows that wild boars and pigs could represent an endemic source for human HEV infections. However, the fact that in this study the different subtypes of HEV 3 were identified in wild boars from two geographically distant counties in the analyzed area shows that wild boar can represent a source of HEV infection with the different subtype for other animals and from humans.

The phylogenetic analysis also revealed that the identified HEV strains in wild boars in Romania were genetically related to human HEV strains circulating in Europe. Some studies highlighted that HEV genotypes 3a, 3c, 3h and 3i detected in wild boars showed a high sequence identity to human HEV strains from autochthonous hepatitis E cases (Forgach et al., 2010; Mazzei et al., 2015; Rutjes et al., 2010; Schielke et al., 2009). Subtype 3a has been described in humans in England and Portugal. In Germany, HEV subtype 3a strains with high sequence homology were isolated from patients with acute HEV infection and swine from the same geographic region (Wenzel et al., 2015). Recently, HEV-3a was detected in Southeastern France in autochthonous human infection (Saint-Jacques, Tissot-Dupont, & Colson, 2016). In a study conducted in Sweden, HEV strains isolated from humans showed relatedness with strains from swine and wild boars in the same county (Widen et al., 2011). The serological studies regarding the infection with hepatitis E virus in humans in Romania reveal increased rates of IgG anti-HEV antibodies from human population in northeast of the country (Anita et al., 2014; Vâță et al., 2015). These results together with those obtained in our study suggest a possible correlation with the presence of HEV in swine and wild boars from this area. To confirm this assumption, further research should be undertaken to identify and to characterize the hepatitis E virus from human cases.

Globally, studies have showed that the strains isolated from animals, humans, food and from environment (water sewage) are related, highlighting that the HEV is a public health issue that must be analyzed based on the "One Health" concept.

CONFLICT OF INTEREST

The authors have no conflict of interests to disclose.

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