Detection of Hepatitis E Virus in Livers and Muscle Tissues of Wild Boars in Italy

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Abstract

In industrialized countries, hepatitis E is now recognized as an emerging zoonosis. Autochthonous cases have been increased over recent years in Europe and are mainly associated with HEV-3 infections. Pigs and wild boars are considered the main reservoirs of the zoonotic HEV-3 and HEV-4 genotypes. Over the past decade, the number of wild boars has drastically increased in Europe. Due to habitats closer to humans and domestic animals, the role of wild boar as a reservoir of the zoonotic HEV is considered to be an emerging issue. ~~In Europe, HEV-3 detection has been described widely in wild boar in feces, tissues, blood and frequently in liver, which is the main organ of replication.~~ In this study, we investigated the presence of HEV RNA by a real-time RT-PCR assay in paired liver and muscle samples collected from 196 wild boars (*Sus scrofa*) hunted in the two areas of Central and Southern Italy. ~~In these areas, the presence of HEV in the wild boar populations had been already investigated two years before.~~ Twenty animals (10.2%) were HEV RNA positive in livers, 11 of which were also positive in muscles. The ORF2 and ORF1 partial viral sequences were obtained for nine paired livers and muscles, and when aligned were identical to each other. Phylogenetic analyses confirmed detection of different HEV-3 subtypes: 3c, 3f, 3i and some that were not assigned to any subtypes that have so far been identified. Results need further investigation because they are based on analyses of sequences of short genome regions. Nevertheless, we observed that the same strains were circulating in the wild boar populations from the two investigated areas, confirming persistence of the same HEV strains in the wild boar population over time.

Keywords: HEV; hepatitis E; wild boar; muscles; liver; subtype

Introduction

Hepatitis E is now considered an emerging disease in industrialized countries. Over the last decade, an increasing number of human cases have been reported in Europe probably linked to a higher awareness of clinicians but also to a wider circulation of the etiological agent the hepatitis E virus (HEV) (Ricci et al., 2017). In Europe and other industrialized countries, autochthonous cases have been linked to HEV strains belonging to the zoonotic genotype HEV-3 and HEV-4 (Ricci et al., 2017). After the first detection of HEV-3 in pigs in the US, studies conducted revealed a widespread diffusion of HEV-3 in domestic and feral pigs worldwide (Salines et al., 2017). The other zoonotic genotype HEV-4 was firstly considered endemic in Asia but over the last years, several studies reported its detection in pigs in Europe although less frequently than HEV-3 (Monne et al, 2015; Salines et al., 2017). The main reservoirs of HEV-3 and HEV-4 are pigs and wild boars. In domestic pigs, seroprevalence between 5% and 100% have been described (Pavio et al., 2017) and farm-prevalence between 12.5% to 100% (Salines et al., 2017). In wild boars, studies conducted in several European countries confirmed the circulation of HEV-3, with prevalence varying between 3.7% and 68.2% (Pavio et al., 2017) and seroprevalences ranging between 4.9% (Caruso et al., 2015) and 57.4% (Kukielka et al., 2016), but in general lower than in pigs.

Zoonotic genotypes have been also reported in other animal reservoir such as deer and mongoose, infected by HEV-3 (Doceul et al., 2016), or rabbits infected by a distinct subtype of HEV-3 which was also detected in few human patients (Abravanel et al., 2017). The occurrence of sporadic and clustered human cases of hepatitis E was linked to the consumption of undercooked or raw meat (wild boar and deer) and pork products (mostly pig liver sausages) ~~infected~~ containing HEV-3 or HEV-4. The genomic sequences of the viruses identified in leftovers and in human cases were closely related to each other (Matsuda et al., 2003, Tei et al., 2003, Yazaki et al., 2003, Li et al., 2005, Colson et al., 2010), supporting the zoonotic transmission of HEV-3 and HEV-4. The first evidence of foodborne transmission of HEV was described in Japan and linked to the consumption of raw deer (sashimi) (Tei et al., 2003). However, deer is not considered a main reservoir of the zoonotic genotype but most probably a spillover host of wild boar (Anheyer-Behmenburg et al., 2017). Clusters of human cases linked to consumption of pig liver sausages containing HEV-3 and HEV-4 have been described in France and in other European countries (Colson et al., 2010; Renou et al., 2014, Harrison & DiCaprio, 2018). Liver is the main organ of HEV replication and the virus accumulates in the bile (de Deus et al., 2007).

HEV-genome detection was rarely reported in pig muscles at the slaughterhouse and it was probably linked to cross-contamination during evisceration (Di Bartolo et al., 2012). In 1134 paired livers and ham muscles from pigs at slaughterhouse, HEV was detected in 2.8% of the tested livers but in none

of the muscles (Feurer et al., 2018). Conversely, a study conducted in Germany described the detection of HEV in the muscle of 89% of wild boars whose livers were positive for HEV (Anheyer-Behmenburg et al., 2017).

In Italy, several studies reported detection of HEV-3 in wild boars, infected with different and widely heterogeneous group of subtypes. The most common subtypes detected are 3c, 3e and 3f subtypes (Martelli et al., 2008, Caruso et al., 2015, Martinelli et al., 2015, Mazzei et al., 2015, Montagnaro et al., 2015, Serracca et al., 2015, Di Profio et al., 2016, Aprea et al., 2018, Di Pasquale et al., 2019, Zecchin et al., 2019) but recently novel subtypes have been also detected, confirming the high heterogeneity of HEV-3 in wild boars (De Sabato et al., 2018c).

In this study, we investigated the presence of HEV-3 in both livers and tissue muscles of two wild boar populations from the two area of Central (Lazio) and Southern Italy (Campania). In addition, to evaluating the persistence of the virus in the same wild boar population over time, the study was conducted on wild boar populations which had already been investigated for the presence of HEV one and two years before, and tested positive for HEV-3 (Aprea et al., 2018, De Sabato et al., 2018b). ~~The aim of this study was to investigate the presence of HEV in paired muscles and livers and to evaluate the persistence of the virus in the same wild boar population over time.~~

Materials and methods

Sampling

One hundred and ninety-six paired liver and muscle samples were collected from wild boars during the hunting season from October 2017 to January 2018. 116 and 80 wild boars were hunted in Viterbo province (Lazio region, Central Italy) and Salerno province (Campania region, Southern Italy), respectively. The sampling size allowed for the detection of HEV in a large (infinite) wild boar population when the expected prevalence was at least 15% with an acceptable error of 5% (95%CI). During post-mortem inspection by the local health authority, liver and diaphragm (20-30 cm^3) were collected and transported under refrigerated conditions to the laboratory and stored at -80°C until use.

Nucleic acid extraction

Fifty mg of liver and muscle samples were cut using disposable scalpels from the inner part of the samples and used for RNA extraction. The 116 wild boars from Lazio region were tested in Istituto Superiore di Sanità in Rome as previously described (De Sabato et al., 2018b). The eighty paired liver and muscle samples from wild boars hunted in Campania region were processed at Istituto Zooprofilattico del Mezzogiorno (IZS Portici) using QIAsymphony kit with the automated extractor QIAsymphony SP/AS instruments (Qiagen, Milan, Italy), according to the manufacturer's

instructions. Both methods of nucleic acid extractions are based on the use of silica columns manually or using the automated extractor. Before homogenization, all samples were artificially contaminated with 10 µl of murine norovirus used as nucleic acid extraction control.

Recovery rate

The murine norovirus RNA was detected by Real-Time RT-PCR as previously described (Baert et al., 2008). The recovery rate was estimated by comparative cycle threshold (Ct) method (Schmittgen & Livak, 2008). ~~Nucleic acid extractions gave a recovery rate >10% for all samples. Both RNA extraction methods, manual and automated, gave similar results for muscle samples, with a mean recovery rate of 47.7%±0.45 and 40.0%±0.22, respectively. The mean recovery rate for RNA extraction from liver was 22.8%±0.47 and 45.0%±0.18 using manual and the automated system, respectively.~~

RT-qPCR for HEV

The HEV genome was detected and quantified by quantitative Real-Time RT-PCR (RT-qPCR) as previously described (Jothikumar et al., 2006, De Sabato et al., 2018b).

Nested RT-PCR for HEV sequencing

The RNA of HEV positive liver and muscle samples was analysed by nested RT-PCR amplifying a 348 bp fragment in the ORF2 (Meng et al., 1997, De Sabato et al., 2018b). The sample negative to the first protocol were analysed by a second nested RT-PCR amplifying 493 bp within the ORF2 region and overlapping with the region amplified with the first protocol (Boxman et al., 2017). A 800 bp fragment within the Methyltransferase region (ORF1) was also amplified from paired liver and muscle samples by nested RT-PCR (ORF1) (Erker et al., 1999, Di Bartolo et al., 2016). The DNA amplicons were sequenced by Eurofins Genomics (Germany).

Phylogenetic analyses

Nucleotide sequence similarities were analysed by the BLAST server (http://www.ncbi.nlm.nih.gov/genbank/index.html) and by the Genotyping Tool of HEVnet (https://www.rivm.nl/mpf/typingtool/hev) (Mulder et al., 2019). The maximum likelihood (ML) phylogenetic tree was constructed with the Tamura–Nei parameter model as suggested by the MEGA 7 software model test (http://www.megasoftware.net) based on 1,000 bootstrap replications. The dataset used for the phylogenetic analysis includes HEV-3 subtype reference strains (HEV-3a to -3c and HEV-3e to -3j) (Smith et al., 2016), novel subtypes recently described (HEV-3k, HEV-3l) (Miura

et al., 2017, De Sabato et al., 2018a), and HEV strains detected in Europe available on NCBI database and HEVnet typing tool.

The sequences were submitted to NCBI GenBank under accession numbers: WB147VT2017, MK889015, MK888997; WB150VT2017, MK888998; WB160VT2017, MK888999; WB164VT2017, MK889000; WB165VT2017, MK889001, MK889014; WB142VT2017, MK889002, MK889013; WB119VT2017, MK889003; WB161VT2017, MK889004, MK889017; WB125VT2017, MK889005, MK889016; WB110VT2017, MK889006, MK889018; WB142VT2017M MK889009, MK889019; WB165VT2017M, MK889008, MK889020; WB147VT2017M, MK889007, MK889021; WB125VT2017M, MK889011, MK889022; WB161VT2017M, MK889010, MK889023; WB110VT2017M, MK889012, MK889024; WB/HEV/NA01ITA18, MK978789, MK978795; WB/HEV/NA02ITA18, MK978788, MK978794; WB/HEV/NA03ITA18, MK978790, MK978796; WB/HEV/NA01ITA18M, MK978797, MK978791; WB/HEV/NA02ITA18M, MK978798, MK978792; WB/HEV/NA03ITA18M MK978799, MK978793.

Statistical analysis

The McNemar's test was used to evaluate any differences between HEV positivity in the liver and/or muscles from the same subjects (paired samples). Confidence intervals (95%CI) were calculated by binomial (Clopper-Pearson) "exact" method based on the β distribution.

To assess a possible correlation between amount of genome in paired liver and muscle samples, the Pearson correlation coefficient *r* was calculated.

Analyses were conducted using SPSS software, version 25 (IBM SPSS, Armonk, NY, USA).

Results

Molecular detection and quantification of HEV-RNA

During the hunting season 2017-2018, 196 paired muscles and livers were sampled from wild boar hunted in two Italian provinces in Central (Lazio region) and Southern Italy (Campania region).~~from where HEV positive wild boars had already been detected one and two years before. However, in the first study only livers were sampled and analysed.~~

Nucleic acid extractions gave a recovery rate >10% for all samples. Both RNA extraction methods, manual and automated, gave similar results for muscle samples, with a mean recovery rate of 47.7%±0.45 and 40.0%±0.22, respectively. The mean recovery rate for RNA extraction from liver was 22.8%±0.47 and 45.0%±0.18 using manual and the automated system, respectively.

Samples were analysed for HEV by RT-qPCR. Overall, 10.2% (20/196; 95%CI 6.3-15.3) of livers tested positive for HEV-RNA, with 12.1% (14/116; 95%CI 6.8-19.4) and 7.5% (6/80; 95%CI 2.8-15.6) prevalence detected in wild boars hunted in Lazio and Campania regions, respectively.

Eleven animals (55.0%, 11/20), 5 from Lazio region and 6 from Campania region, were positive for HEV in both muscle and liver. None of the animals were positive in muscle only, while 4.6% (9/196) were positive in liver only. A significantly higher HEV prevalence ($p<0.05$) was observed in liver than in muscle (McNemar's test =7.11; p=0.008), (Table 1).

The median value of viral load was 1.4×10^7 GE/g, ranging from 4.6×10^2 to 1.2×10^9 GE/g in liver, 2 samples were non quantifiable because under the LOQ of the method. In the muscle, the median value of viral load was 1.4×10^5 GE/g ranging between 1.5×10^4 and 1.4×10^6 GE/g. Results of viral load in the two sample types were compared using the Pearson coefficient, a significant positive correlation was observed (*r*: 0.93; $p<0.01$).

The ORF2 and ORF1 partial viral sequences were obtained for nine paired livers and muscles (Accession Numbers: MK888997-MK889024 and MK978788- MK978799). The sequenced strains obtained from paired livers and muscles belonging to the same animal were identical (100% nt.id.). No differences were observed between ORF1 and ORF2 phylogenetic trees (data not shown) and, to determine the subtypes, analyses were conducted using ORF2 regions. No evidence of recombination was observed in any of the strains sequences in both ORF1 and ORF2 genomic fragments. Six additional sequences in the ORF2 were obtained from animals in which virus was present only in the livers.

~~The obtained HEV sequences were subjected to phylogenetic analysis, including HEV-3 subtype reference strains, novel subtypes and HEV strains detected in Europe available online~~.

Phylogenetic analyses and subtyping assignment of HEV-3 strains

The Italian HEV strains detected in this study, based on the phylogenetic analysis (Figure 1), were classified into three subtypes: HEV-3f, -3c, -3i, while some strains, due to the high divergence with other HEV-3 strains were not classified into any HEV subtype.

The WB110VT2017, WB125VT2017 and WB161VT2017 ORF2 sequences from liver and muscle clustered within the HEV-3f subtype clade. The three Italian strains shared ~~among each other~~ a 99.5% nucleotide identity (nt.id.); 86.0% nt.id. with the HEV-3f reference strain (E116-YKH98C, AB369687) and an even higher percentage (up to 92.0% nt.id.) with the human and swine HEV-3f strains detected in Europe. The WB110VT2017, WB125VT2017 and WB161VT2017 ORF2 sequences shared only 84.0% nt.id. with two strains detected in the same hunting area one year before (WB02VT2016 and WB03VT2016).

One strain, namely WB119VT2017 clustered within the HEV-3c subtype, showing 95.0-97.0% nt.id. with other viral sequences from wild boar, swine and human strains detected in Italy. All the aforementioned strains belong to the HEV-3c subtype, allowing the provisional assignment of WB119VT2017 to the HEV-3c, because only a short sequence was available. A strict nucleotide correlation, 92.0-93.5% nt.id., was observed between WB119VT2017 and 5 strains detected one year before (WB47VT2016, WB52VT2016, WB17VT2016, WB27VT2016, WB21VT2016) in the same area.

Six virus strains, namely WB147VT2017, WB150VT2017, WB164VT2017, WB165VT2017, WB160VT2017 and WB142VT2017, displayed nucleotide sequence identities ranging between 98.8% and 100%. They formed a monophyletic cluster ~~The six Italian viral strains formed a monophyletic cluster~~ with 11 HEV strains detected in wild boar hunted in the same area one year before. Sequenced ORF2 fragments of the virus strains collected in 2016 and 2017 diverged from 1.0% to 5.7%. The Italian strains clustered in the same subtype and displayed a high nucleotide identity (91.7-92.5%) with the WB/HEV/NA17ITA15 strain detected in Southern Italy (Campania region) (MF959764) in 2015 and classified by whole genome analyses in the HEV-3i subtype. However, strains detected in this study displayed a low nucleotide identity (83.0-86.0%) with the HEV-3i reference strain BB02 (FJ998008). These divergent results could be due to the short ORF2 fragments sequenced which did not allow for a definitive assignment of the virus subtype (Figure 1, cluster indicated by 3*).

~~The divergent results obtained by comparison with two HEV-3i strains, the reference BB02 (FJ998008) and the WB/HEV/NA17ITA15 (MF959764), could be due to the short ORF2 fragments sequenced in this study and do not allow for a definitive assignment of the detected strains (Figure 1, cluster indicated by 3*).~~

Similarly, it was not possible to definitively assign the subtype of three strains (WB/HEV/NA01ITA18, WB/HEV/NA02ITA18 WB/HEV/NA03ITA18) detected in wild boars hunted in Campania region, due to the similar nucleotide distance from the 3i subtype reference strain (BB02, FJ998008; *p*-distance 0.13, 86.5%-87.0% nt.id.) and the other HEV-3 subtype reference strains (*p*-distance: 0.11-0.18). Strains, WB/HEV/NA01ITA18, WB/HEV/NA02ITA18 and WB/HEV/NA03ITA18, shared a nt.id. ranging between 97.2-99.0%, and displayed 96.0-97.7% nucleotide identities with the 3i strain WB/HEV/NA17ITA15 (MF959764). For these 3 strains, the nucleotide sequences were obtained from both liver and muscle, revealing in all cases a 100% nt.id. from paired samples analysed. Furthermore, the three HEV strains clustered together with the Italian strain WB/HEV/NA17ITA15 (MF959764) detected in wild boars in the same geographical area 2 years before.

Discussion

Results obtained in this study confirmed the circulation of HEV-3 in wild boar populations from two provinces of Lazio and Campania region (Central and Southern Italy, respectively). In this study, an overall prevalence of 10.2% has been reported, in line with previous European studies (Di Pasquale et al., 2019, Wang et al., 2019, Mesquita et al., 2016, Spancerniene et al., 2019, Porea et al., 2018) but lower compared to results obtained in the previous studies conducted in animals hunted in the same areas of Lazio (12.1% *vs* 30.0-64.0%) and Campania (7.5% *vs* 12.3%) regions one and two years before (De Sabato et al., 2018b, Aprea et al., 2018). Results may depend on geographical differences in the investigated areas.

In fact, in the area of Lazio region, Viterbo province (Central Italy), two previous studies showed a moderate prevalence variability depending on the year of sampling (2013 *vs* 2014; 12.4% to 17.2%) in an area of 400 Km^2 (Di Pasquale et al., 2019) and a significant variable prevalence depending on the area of hunting (areas of 29.0-113.8 Km^2) ranging from 0.0% to 64.0% (De Sabato et al., 2018b). The median GE value detected in liver was 1.4×10^7 GE/g a comparable value to those reported in other European studies (Anheyer-Behmenburg et al., 2017, Di Pasquale et al., 2019) ~~and as previously described, (Anheyer-Behmenburg et al., 2017)~~ with a higher titre in the liver than in the muscle (1.4×10^5 GE/g). The presence of HEV-3 in muscles has been observed in 55.0% of animals tested positive in liver, 11 animals (5.6%) were positive in both muscle and liver and three were only positive in the liver. None of the animals were HEV positive in muscle only. Comparing the viral load in livers and muscles, a positive correlation was observed indicating a higher level of HEV in liver. This is the main organ of viral replication where the virus should accumulate ~~and a higher level and frequency of HEV detection is expected~~. The presence of the virus in muscles from animals found HEV-positive in the liver was also observed in Germany, with 89.0% of animals HEV-positive in the liver also HEV-positive in the muscle (Anheyer-Behmenburg et al., 2017). Results obtained in this study and in our study, ~~This result~~ showed the ~~high~~ probability of detecting HEV in both liver and muscle ~~but~~ and also confirmed that liver is more frequently positive. In our study, the HEV-3 RNA detected in the muscle is probably due to viremia that we could not confirm because blood was not available to evaluate the presence of HEV RNA. ~~The possible link to the viremia, live open questions concerning the precautions measures to be taken during slaughtering which could help the bleeding of the animals reducing the risk of cross-contamination.~~ Conversely, as recently hypothesized for pigs co-infected with Porcine reproductive and respiratory syndrome virus (PRSSV), the virus could replicate even in muscles, leading to a higher risk of contaminated meat since the HEV infection is

asymptomatic (Salines et al., 2019). The origin of the virus in the muscle remains unknown and deserves further investigation.

~~In Italy and in many other European countries, wild boar is frequently consumed and both livers and muscles are used to produce commercial or traditional foods such as sausages, which are usually eaten raw, after preservative treatments (e.g. salting, short seasoning) whose effects on HEV survival are unknown, with evident risks of infection for consumers.~~

The sequence analyses performed in this study, confirmed a wide heterogeneity of HEV-3 strains, revealing different subtypes circulating in the Lazio region (Viterbo province, Central Italy) and in the Campania region (Salerno province, Southern Italy). It is important to underline that since only partial short genomic sequences of ORF2 and/or ORF1 have been performed and used for the phylogenetic analysis, the sequence variability observed could be even higher.

These results suggest that strains strictly related or identical (up to 100%) to each other constantly circulate in wild boar; such as WB/HEV/NA01ITA18 and WB/HEV/NA02ITA18 which display 97.7% nt.id. with WB/HEV/NA17ITA15 detected in the same hunting area two years before. The constant circulation of some strains in the same hunting area was confirmed by the detection in this study of six identical viral strains, WB142VT2017, WB147VT2017, WB150VT2017, WB164VT2017, WB165VT2017 and WB160VT2017 that displayed 98.8% nt.id. with strains detected one year before in the same area.

Conversely, within the same subtype different strains can be found, confirming the high heterogeneity of HEV in wild boar. Within the subtype 3f, the strains sequenced diverged from 1.0% to 8.0%. Despite the divergence at the nucleotide level of HEV strains, no substitutions were found at the amino acid level in strains sampled in this and in the previous studies conducted 1 and 2 years before in the same areas (Aprea et al., 2018, De Sabato et al., 2018b). The high degree of conservation of the amino acid sequences of strains indicates the adaptation of HEV in wild boar populations. ~~ensuring .~~ These facts may explain the maintenance of the same HEV strains in wild boar population over time ~~allowing for the circulation of the HEV over time~~ (Kukielka et al., 2016).

The present study has some limits, since neither epidemiological data such as age, sex nor sera for antibodies or viremia detection were available. In the absence of this information, it remains unknown if age, sex or viremia are correlated to the prevalence observed or to the probability of detection of HEV-RNA in muscles. However, the study aimed at confirming if ~~the risk of detection of~~ HEV-3 was present in livers and muscles ~~exists~~ in wild boar; in matrices that are intended for human consumption. Results obtained in this study showed the urgent need to implement surveillance of HEV in wild boar and in game. ~~The constant presence of HEV in wild boar populations observed over two years poses a risk, as wild boar is destined for human consumption.~~ The presence of HEV in muscles, although

not necessarily linked to replication of the virus, but to incomplete bleeding, is frequent and its presence deserves further investigation since muscles are consumed directly generally well-cooked and are also used to produce sausages. In the absence of specific control measures for HEV, hunters should be informed about implementing procedures during slaughtering.

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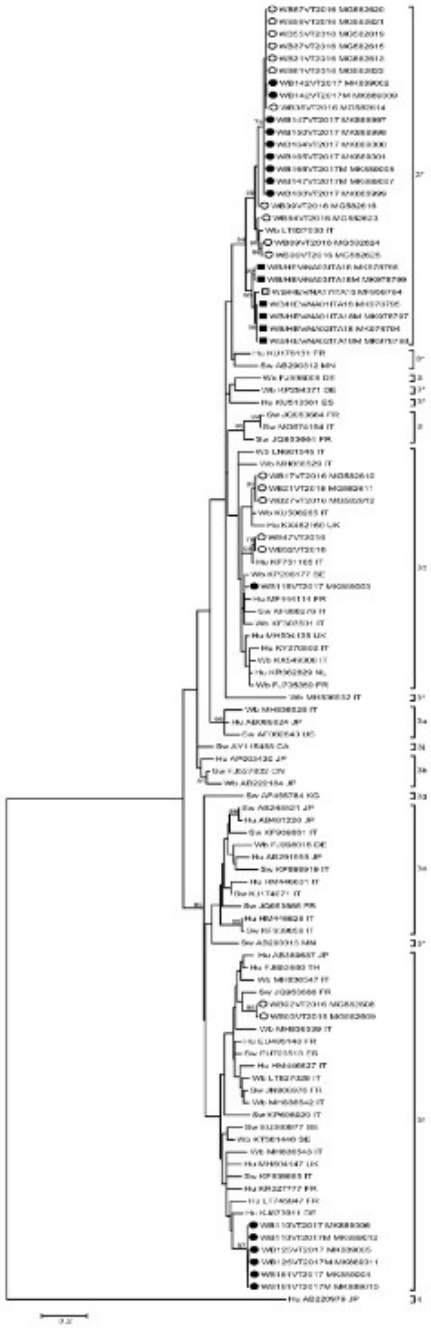
No conflict of interest to declare

Table 1. Results of HEV-RNA detection in paired liver and muscle samples from wild boars

		HEV RNA in liver (%)			***p* value**
		Positive	Negative	Total	
HEV RNA in muscle (%)	Positive	11 (5.6)	0 (0.0)	11 (5.6)	0.008
	Negative	9 (4.6)	176 (89.8)	185 (94.4)	
	Total	20 (10.2)	176 (89.8)	196 (100.0)	

Fig. 1 Maximum likelihood phylogenetic tree based on 300 nt fragment within the HEV ORF2 region of 105 HEV-3 strains from human, swine and wild boar including HEV-3 reference strains. The HEV-4 strain was used as outgroup. Bootstraps values > 70 are indicated. Representative swine, human, wild boar strains are included. Each entry includes host (Hu: human, Sw: swine, Wb: wild boar), accession number and country origin of the strain. The sequences obtained in this study from Central and Southern Italy are indicated with circle and square, respectively, followed by the name of the strain and the accession number. The letter M indicates sequenced strain obtained from muscle. Sequences collected in 2016 and 2015 are indicated with solid and empty symbols, respectively



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