

Alma Mater Studiorum Università di Bologna  
Archivio istituzionale della ricerca

Molecular survey of HEV infection in wild boar population in Italy

This is the final peer-reviewed author's accepted manuscript (postprint) of the following publication:

*Published Version:*

De Sabato, L., Ostanello, F., De Grossi, L., Marcario, A., Franzetti, B., Monini, M., et al. (2018). Molecular survey of HEV infection in wild boar population in Italy. *TRANSBOUNDARY AND EMERGING DISEASES*, 65(6), 1749-1756 [10.1111/tbed.12948].

*Availability:*

This version is available at: <https://hdl.handle.net/11585/644598> since: 2018-11-28

*Published:*

DOI: <http://doi.org/10.1111/tbed.12948>

*Terms of use:*

Some rights reserved. The terms and conditions for the reuse of this version of the manuscript are specified in the publishing policy. For all terms of use and more information see the publisher's website.

This item was downloaded from IRIS Università di Bologna (<https://cris.unibo.it/>).  
When citing, please refer to the published version.

(Article begins on next page)

This is the peer reviewed version of the following article:

Molecular survey of HEV infection in wild boar population in Italy

which has been published in final form at <https://doi.org/10.1111/tbed.12948>.

This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.

1   **Molecular survey of HEV infection in wild boar population in Italy**

2

3   Running title: Hepatitis E virus detection in wild boar in central Italy

4

5   De Sabato Luca<sup>a,b</sup>, Ostanello Fabio<sup>c</sup>, De Grossi Luigi<sup>d</sup>, Marcario Anita<sup>d</sup>, Franzetti Barbara<sup>e</sup>, Di  
6   Bartolo Ilaria<sup>a§</sup>

7

8   <sup>a</sup> Department of Food Safety, Nutrition and Veterinary Public Health, Istituto Superiore di Sanità,  
9   Viale Regina Elena, 299, 00161, Rome, Italy

10   <sup>b</sup> Department of Sciences, University Roma Tre, Viale Guglielmo Marconi, 446, 00146, Rome, Italy

11   <sup>c</sup> Department of Veterinary Medical Sciences, University of Bologna, Via Tolara di Sopra, 50,  
12   40064, Ozzano dell'Emilia, BO, Italy

13   <sup>d</sup> Zooprophyllaxis and Research Institute of Latium and Tuscany "M. Aleandri", Strada Terme 4a,  
14   01100 Viterbo, Italy

15   <sup>e</sup> Italian National Institute for Environmental Protection and Research, via Ca' Fornacetta, 9, 40064  
16   Ozzano dell'Emilia (BO), Italy

17

18

19   § Corresponding author at:

20   Ilaria Di Bartolo  
21   Istituto Superiore di Sanità  
22   Department of Veterinary Public Health and Food Safety  
23   Viale Regina Elena 299  
24   00161 Rome  
25   Tel.: +39 06 4990 2787;  
26   E-mail address: [ilaria.dibartolo@iss.it](mailto:ilaria.dibartolo@iss.it)

27

28

## 1    **Summary**

2    Hepatitis E virus (HEV) is an RNA virus causing an acute generally self-limited disease in humans.  
3    An increasing number of autochthonous cases linked to zoonotic transmission of HEV genotype 3  
4    have been reported over the last 10 years in Europe. Pigs and wild boars are considered the main  
5    reservoirs. The principal route of transmission in Europe is foodborne, linked by direct or indirect  
6    evidence to the consumption of raw or undercooked pork products and wild boar meat. In this study,  
7    we sampled 92 wild boar (*Sus scrofa*) livers during active surveillance in five municipalities in  
8    Central Italy throughout the hunting season 2016-2017. HEV RNA was detected in 52.2% of liver  
9    sampled with prevalence ranging from 0.0% to 65.7%. HEV positive wild boars were detected in all  
10   but one area of hunting. Phylogenetic analysis showed that strains clustered within the two subtypes  
11   HEV-3c and HEV-3f and displayed a wide range of phylogenetic diversity. Several strains were  
12   circulating in the areas investigated; animals possibly belonging to the same family group hunted by  
13   the same team were infected with a unique strain (100% nucleotide identity). Since wild animals are  
14   a proven source of HEV transmission to humans and pigs, the high prevalence observed (mean  
15   52.2%) poses a question on the risk of consuming wild boar meat and thus this subject deserves  
16   further investigations.

17

18   **Keywords:** Hepatitis E virus, wild boar, zoonosis, genotype 3, subtype, Italy

19

## 1    **Introduction**

2    Hepatitis E is an acute viral disease caused by Hepatitis E virus (HEV) and characterized by the fecal-  
3    oral route transmission. (Kamar *et al.*, 2017). HEV is a non-enveloped single strand RNA virus  
4    classified in the family *Hepeviridae* and the genus *Orthohepevirus* (Purdy *et al.*, 2017). The genus  
5    includes the *Orthohepevirus A* species divided into 7 genotypes. The genotypes HEV-1 and HEV-2,  
6    restricted to humans, circulate in endemic area (Asia and Africa) causing several outbreaks linked to  
7    the ingestion of contaminated water. In non-endemic area (industrialized countries), ~~most of the~~  
8    infections by HEV-1 and HEV-2 are related to travel in endemic area. Furthermore, in the last 10  
9    years, , ~~HEV is considered an under recognised pathogen, in the last 10 years,~~ an increasing number  
10    of autochthonous infections have been described linked to the zoonotic transmission of the genotype  
11    3 and 4 and is now increasingly recognized as endemic also in some developed regions. HEV-3 and  
12    -4 in industrialized countries are zoonotic and linked by direct or indirect evidence to the consumption  
13    of raw pork products (mainly liver sausages) and undercooked wild boar meat (Kamar *et al.*, 2017).  
14    The latter genotypes infect humans and several animal species among which pigs and wild boars are  
15    the main reservoirs (Ricci *et al.*, 2017). More recently, novel hosts of HEV-3 and HEV4 have been  
16    described in rabbit and yak, respectively, and novel genotypes in wild boar (HEV-5, -6) and camel  
17    (HEV-7) (Woo *et al.*, 2016, Smith *et al.*, 2014, Lee *et al.*, 2016, Takahashi *et al.*, 2011). In Europe,  
18    HEV-3 is the most frequent in humans, pigs and wild boar. HEV-4 which is mainly found in Asia, it  
19    was only recently detected in Italy in pigs and one human case (Monne *et al.*, 2015, Garbuglia *et al.*,  
20    2013). The genotypes HEV-5 and HEV-6, have only been detected in Japanese boar (*Sus scrofa*  
21    *leucomystax*) so far (Takahashi *et al.*, 2011). The presence of HEV-3, the most common genotype in  
22    Europe, has been extensively described in pig populations, with high seroprevalence which increases  
23    with age (up to 100%) (Pavio *et al.*, 2017). The peak of infections in pigs is after the loss of maternal  
24    immunity: between 3 and 8 weeks of age, the virus is secreted on feces and/or is detected in liver with  
25    prevalence ranging between 8–30% in weaners, 20–44% in growers and 8–73% fatteners/finishers  
26    (Pavio *et al.*, 2017). Wild boar is also susceptible to HEV infection, displaying seroprevalences

1 ranging between 4.9% (Caruso *et al.*, 2015) and 57.4% (Kukielka *et al.*, 2016). Among European  
2 countries, different percentages of HEV-RNA detection in liver samples were reported ranging  
3 between 3.7% (Caruso *et al.*, 2015) and 68.2% (Adlhoch *et al.*, 2009). Wild boar HEV positive  
4 animals were detected in each age classes, including juveniles of 4 months of age, and animals older  
5 than 24 months (Martelli *et al.*, 2008, Sonoda *et al.*, 2004). Interestingly, a recent study described  
6 detection of HEV RNA in 89% of muscle sampled from wild boar HEV positive in liver (Anheyer-  
7 Behmenburg *et al.*, 2017). Several phylogenetic studies on HEV wild boar strain sequences showed  
8 relatedness to human and swine strains suggesting an important role of the wild boar as reservoirs of  
9 the virus and as a possible source of infections for breeding pigs and humans (Spahr *et al.*, 2018). In  
10 Italy, the area of the wild boar distribution is nearly 77% of the country (232,000 km<sup>2</sup>) while the  
11 population size is estimated at over 1.000.000 units (ISPRA, 2017). According to the national and  
12 regional regulations, hunting on grounds is allowed from October to January. The meat and entrails  
13 of wild boars are used for direct human consumption or to produce sausages and salami. In Italy, the  
14 prevalence of HEV in wild boar ranges between 1.5% from feces and 1.9% up to 33.7% from liver  
15 or bile tested. This could be a regional difference or could also be partially linked to different  
16 specimens that have been tested (feces, bile or liver). The Italian wild boar HEV strains were  
17 sequenced in short genome regions and classified into -3c, -3e, -3f subtypes and some strains for  
18 which the subtype could not be determined, confirming the high heterogeneity of HEV in wild boars  
19 (Martelli *et al.*, 2008, Caruso *et al.*, 2015, Martinelli *et al.*, 2015, Mazzei *et al.*, 2015, Montagnaro *et*  
20 *al.*, 2015, Serracca *et al.*, 2015, Di Profio *et al.*, 2016, Aprea *et al.*, 2017). In this study, we  
21 investigated the occurrence of HEV in wild boars hunted in Lazio Region (Central Italy), where the  
22 wild boar population is distributed over 60% of the territory and the number of animals hunted per  
23 year is probably lower than the annual growth rate (ARSIAL *et al.*, 2014). In order to determine virus  
24 circulation and characterize strains detected, liver samples were tested for HEV by Real-Time  
25 reverse-transcription PCR (RT-qPCR) and genotyped by sequencing and phylogenetic analyses.

## 1    **Materials and Methods**

### 2    **Sampling**

3    During the hunting season from October 2016 to January 2017, 92 individual liver samples were  
4    collected from wild boars (*Sus scrofa*) hunted in 5 municipalities (A, B, C, D, E) located in Viterbo  
5    Province (3.612 km<sup>2</sup>, Lazio Region, Central Italy). It is reasonable to assume that contacts among  
6    animals living in the investigated area named A, B and C and those living in D and E area are likely  
7    rare because a highway separates the two subareas. Some geographical features of the 5  
8    municipalities, about 20-50 km apart, are reported in Table 1 (ISTAT, 2018). In each municipality,  
9    hunting areas with dimensions of 25-400 hectares are assigned to specific hunting teams. In the  
10    hunting districts where the 5 municipalities are located, 3,374 wild boars were killed during the  
11    hunting season 2016-17 (Sex Ratio 1:0.96; killing average density of 17.3 wild board/km<sup>2</sup> of hunting  
12    ground) (ISPRA, 2017).

13

### 14    **Sample preparation and nucleic acid extraction**

15    One-hundred mg of liver sample was cut by scalpel in the inner part of the organ. Samples were  
16    homogenized in 650 µl of lysis buffer (RLT) with zirconia beads, using a mechanical disruptor  
17    (Tissue Lyser, Qiagen, Milan, Italy) for three runs of 2 min at 46 oscillations s<sup>-1</sup>. After centrifugation  
18    at 5000 x g per 20 minutes, the total RNA was extracted by the RNeasy Mini kit (Qiagen, Milan,  
19    Italy), according to the manufacturer's instructions and eluted in 100 µl of nuclease free water. Liver  
20    samples were artificially contaminated with 5 µl of a suspension of murine norovirus (MuNoV, strain:  
21    MNV-IT1 Acc. no. KR349276), which was used as sample process control. The RNA of MuNoV  
22    from spiked samples was detected by Real-Time RT-PCR as previously described (Di Bartolo *et al.*,  
23    2015). The recovery rate was estimated by comparative cycle threshold (Ct) method (Schmittgen &  
24    Livak, 2008).

25    All spiked samples were positive for MuNoV, mean ± SD recovery rate of 12% ± 8.2.

26

## 1 **RT-qPCR for HEV**

2 The HEV genome was detected by quantitative Real-Time RT-PCR (RT-qPCR) as described by  
3 (Jothikumar *et al.*, 2006) using the QuantiFast Pathogen +IC Kits (Qiagen, Milan, Italy) including  
4 the internal control (Internal Control Assay, ICA). For interpretation of results, if the observed ICA  
5 cycle threshold (Ct) value was as expected (comparable to the Ct value obtained in negative control,  
6 where only water was added as template) and the Ct value for HEV was not detectable or was  $\geq 39$ ,  
7 the sample was considered to be negative. Quantification of HEV Genome Equivalent (GE) was  
8 performed using a synthetic RNA reference standard (Di Bartolo *et al.*, 2015). The limit of detection  
9 (LOD) was 14 GE/ $\mu$ l calculated using ten-fold dilution series of known amount of HEV-specific  
10 RNA molecules and defined as the lowest dilution detectable in all 10 replicates.

## 11 **Limit detection of HEV RNA**

12 One positive homogenated liver sample (prepared in PBS) was ten-fold diluted and extracted as  
13 described above in M&M. The limit of detection, calculated as the lowest dilution with at least one  
14 positive out of three triplicates, was 43.000 GE/g.

## 15 **Nested-RT-PCR for HEV sequencing**

16 The RNA (21 liver samples) was analyzed by nested RT-PCR using the OneStep RT-PCR Kit  
17 (Qiagen, Milan Italy) for retro-transcription and PCR amplification and the Phusion High-Fidelity  
18 PCR Master Mix (Thermo Fisher Scientific, Rodano, Italy) for the nested-PCR, as previously  
19 described (Monini *et al.*, 2015). The nested RT-PCR amplified a 348 bp region within Open Reading  
20 Frame 2 (ORF2) of HEV genome (Huang *et al.*, 2002). The DNA amplicons were sequenced by  
21 Eurofins Genomics (Germany).

## 22 **Phylogenetic analyses**

23 Nucleotide sequence similarity was analyzed with the BLAST server  
24 (<http://www.ncbi.nlm.nih.gov/genbank/index.html>). A Maximum Likelihood (ML) phylogenetic tree  
25 was constructed with the Tamura-Nei parameter model as suggested by the MEGA 7 software model  
26 test (<http://www.megasoftware.net>) based on 1000 bootstrap replications. The sequences were



1 submitted to NCBI GenBank under accession numbers: WB02VT2016 (MG582608), WB03VT2016  
2 (MG582609), WB17VT2016 (MG582610), WB21VT2016 (MG582611), WB27VT2016  
3 (MG582612), WB31VT2016 (MG582613), WB35VT2016 (MG582614), WB37VT2016  
4 (MG582615), WB39VT2016 (MG582616), WB47VT2016 (MG582617), WB52VT2016  
5 (MG582618), WB55VT2016 (MG582619), WB57VT2016 (MG582620), WB59VT2016  
6 (MG582621), WB61VT2016 (MG582622), WB84VT2016 (MG582623), WB89VT2016  
7 (MG582624), WB90VT2016 (MG582625).

## 8 **Statistical analysis**

9 Statistical analysis was performed using SPSS software (SPSS Statistics ver. 23; IBM Corp., Chicago,  
10 IL). Comparison of prevalence observed for HEV RNA-positive animals by municipality, was  
11 conducted using Pearson chi-square test. The significance limit was set at  $P < 0.05$ . Confidence  
12 intervals were calculated by binomial (Clopper-Pearson) “exact” method based on the  $\beta$  distribution.

13

## 14 **Results**

15 ~~During the hunting season from October 2016 to January 2017, 92 individual liver samples were~~  
16 ~~collected from wild boar hunted in 5 municipalities (A, B, C, D, E) distant 20-50 km and located in~~  
17 ~~the Viterbo Province (Lazio Region, Central Italy, Fig. 1).~~

18 HEV RNA was detected in liver by RT-qPCR in 52.2% (48/92; 95% C.I. 41.5-62.7) of wild boars  
19 sampled, ranging between 0.0% (area C) and 65.7% (area B; Table 1, Fig. 1). A significant difference  
20 ( $P < 0.05$ ) was observed in HEV-RNA prevalence in each of the five areas (A-E) (Table 1). The  
21 median viral load was  $10^7$  GE/g ranging between  $8.05 \times 10^5$  and  $2.4 \times 10^{10}$  GE/g. Twenty-one positive  
22 liver samples selected to represent at least one sample for municipalities were further analyzed by  
23 conventional nested RT-PCR amplifying a 348 bp genome fragment within the ORF2 (capsid  
24 protein). Eighteen out of the 21 samples were positive. However, 3 positive samples by RT-qPCR,  
25 all belonging to animals hunted in the area D, were not further confirmed by nested RT-PCR.  
26 Amplicons obtained were sequenced and subjected to phylogenetic analysis, including in the analyses

1 representative reference of HEV-3 subtype strains (Smith *et al.*, 2016), and human, swine and wild  
2 boar strains detected in both Europe and Italy available on NCBI database  
3 (<https://www.ncbi.nlm.nih.gov>) (Fig. 2).

4 The wild boar strain sequences clustered according to the hunter team, displaying a high nucleotide  
5 identity 98-100% (nt. id.) within each group of animals hunted the same day.

6 In the area A, 2 HEV strains were identified. Two identical wild boar sequences (WB02VT2016 and  
7 WB03VT2016) were assigned to HEV-3f subtype showing 89% nucleotide identity (nt. id.) with the  
8 -3f prototype strain (AB369687) and up to 93% with several -3f strains originated from swine,  
9 humans and wild boar (Fig. 2). The two Italian wild boar strains were related to two strains detected  
10 in human cases occurring in The Netherlands and in Italy (JX645331, HM446627) and to one Italian  
11 wild boar strain (LT827027) displaying a nt. id. of 93.3%, 90.5% and 89%, respectively.

12 Five strain sequences from areas B and E displayed a nt. id. each of 92.5% and 92% nt. id. with the  
13 wild boar -3c prototype strain (FJ705359), and were assigned to HEV-3c. HEV strains from the area  
14 B (WB17VT2016, WB21VT2016, WB27VT2016) were related (96.7% nt. id.) to both human HEV  
15 strain from The Netherlands (KR362779) and wild boar strain (KU508285) previously detected in  
16 Central Italy but in an area apparently not linked with the hunting area of the present study (Di Profio  
17 *et al.*, 2016). Two sequence strains of HEV-3c, WB47VT2016 and WB52VT2016, from animals  
18 hunted in the area E, showed a high nt. id. (98.4%) with a strain detected in an acute case of hepatitis  
19 E occurring in Northern Italy (KF751185).

20 Four HEV strains from the area A (WB55VT2017, WB57VT2017, WB59VT2017, WB61VT2017)  
21 and 7 from the area B (WB31VT2017, WB35VT2017, WB37VT2017, WB39VT2017,  
22 WB84VT2017, WB89VT2017, WB90VT2017), clustered together forming two subgroups with  
23 95.3% nt. id., in a separate clade than the other HEV-3c (86.62% nt. id.) and out of the cluster of  
24 HEV-3hi (86.4% nt. id.). The 11 sequence strains correspond to animals hunted in three different  
25 days from three teams in areas on the edge between A and B. Sequences from animals hunted by the  
26 same team were identical (99.3-100% nt. id.) and related to two Italian wild boar strains (KX549309,

1 LT827030) and one French human strain (KR027387) showing nt. id. 95%. Those sequences did not  
2 cluster with any reference HEV strains and were not assigned to any known subtype.

3

#### 4 **Discussion**

5 In this study, the mean HEV-RNA prevalence observed was 52.2%, significantly higher than in the  
6 previous studies conducted in Italy, where HEV-RNA was detected in 1.9% up to 33.7% of liver or  
7 bile collected from wild boars (Martelli *et al.*, 2008, Serracca *et al.*, 2015). The difference can be  
8 explained for several reasons, depending on sampling strategy, the age of the examined animals,  
9 duration of storage before analyses (Schielke *et al.*, 2009), the population density and the frequency  
10 of contact with other wild or domestic receptive species. The high density and the contact with other  
11 animal species are considered risk factors for several infections. In the studied areas, during the  
12 hunting season 2016-2017, an average of 17.3 wild boars/km<sup>2</sup> were hunted (ISPRA, 2017). This high  
13 density could partially justify the observed high HEV prevalence, although this hypothesis in the  
14 absence of the exact density value has to be proved yet. No sequence data are available on area D  
15 because the 3 samples positive by RT-qPCR were not further confirmed by nested RT-PCR. We  
16 suppose that this is due heterogeneity of sequences that we will investigate in the future.

17 In the present study, animals were hunted during four months in five small areas, 20-50 km apart,  
18 separated by geographical barriers (Table 1). Strains detected from animals hunted on the same day  
19 by the same team showed 100% nt. id. The other HEV strains detected were shown to be different  
20 both among the different hunting areas and within the same hunting area. This result is interesting  
21 because we are able to conclude that more strains were circulating but that animals belonging to the  
22 same family group shared one unique strain (100% nt. id.). Wild boars hunted on the same day by the  
23 same hunting team may belong to the same family group, since except for the old males, wild boar  
24 live in groups consisting of interrelated females and their litters (Briedermann, 1986, Kaminski,  
25 2005). However, some studies reported aggregations of unrelated adult females (with their litters) in  
26 family group, mainly due to intensive hunting activity, breaking down the structure of family groups

(Gabor, 1999, Brün, 2008, Iacolina, 2009). Indeed, we detected the same strains circulating in wild boars hunted along the border between A and B areas, where the probability of contact between animals can be considered high (i.e. WB35VT2017, WB61VT2017, WB31VT2017, WB37VT2017, WB55VT2017, WB57VT2017, WB59VT2017). However, we have also detected different HEV strains circulating in the same area (eg. WB27VT2017 vs WB31VT2017). As explained above, the intensive hunting can determine movement of animals escaping from one area to other, joining to new family group. This could increase contact among animals explaining different HEV strains detected within the same area. We may assume that animals (area A, B, C vs area E and D) belong to different metapopulations with limited contacts with other groups or subpopulations and, accordingly, different HEV strains within the same area were also observed. Indeed, wild boar shows a sedentary behavior and short dispersal distances as well as daily movements (< 10-12 km; Morelle et al., 2015) the geographical distances that separate the different sampling areas (eg about 30 km between A and E, about 20 km between D and E and about 15 km between C and D) are relative wide.

The age of animals were not available, but if we consider that the peak of births is in spring we expect that during the hunting season (October-January) wild boars are older than 6 months. Wild boars of this age are those usually intended for human consumption. This result confirms previous findings that adult pigs showed a lower probability of infection (Di Bartolo *et al.*, 2008) while wild boars can be infected at different ages (Martelli *et al.*, 2008). This can be linked to the chronic infection described in wild boar (Schlosser *et al.*, 2015) or continuous re-infection due to incomplete or short-lasting immunity (Anheyer-Behmenburg *et al.*, 2017).

In this study, we observed a median viral load of  $10^7$  GE/gr. This value is comparable to previous studies (Anheyer-Behmenburg *et al.*, 2017, Kamar *et al.*, 2017). In the absence of *in vitro* cultivation, detection of HEV-RNA does not confirm the viability of the virus. However, the observed viral load in liver, that is the main site of virus replication, deserves attention since liver is also used to produce regional food specialties such as liver sausages that could be consumed raw.

1 This study confirmed a wide heterogeneity of sequenced wild boar HEV strains that belonged to at  
2 least two subtypes HEV-3c and HEV-3f. The sequence analyses revealed that HEV-3c is frequent, as  
3 observed in other studies conducted in both Italy and Europe (Aprea *et al.*, 2017, Serracca *et al.*,  
4 2015, Schielke *et al.*, 2009, Anheyer-Behmenburg *et al.*, 2017, Thiry *et al.*, 2017a, Di Profio *et al.*,  
5 2016, Vina-Rodriguez *et al.*, 2015, Dorn-In *et al.*, 2017, Rutjes *et al.*, 2009, Rutjes *et al.*, 2010). In  
6 Italy, HEV-3c is less frequent detected in pigs, where the main subtypes are HEV-3f and HEV3-e.  
7 Eleven HEV strains detected from animals hunted in the border of area A and B, shows nucleotide  
8 identity <86.5% with reference sequences HEV-3c and HEV-3-hi (Fig. 2), suggesting a possible local  
9 evolution but not allowing a definitive assignment to the subtypes known so far.

10 Furthermore, HEV strains sequenced in this study displayed higher nucleotide identity with human  
11 and wild boar strains than with HEV strains detected in pigs.

12 We observed a high nucleotide identity with a human strain detected in Italy linked to consumption  
13 of figatelli (pork liver sausages) (Garbuglia *et al.*, 2015). Human HEV infection after ingestion of  
14 uncooked liver and meat of wild boars was reported in Japan and Spain, respectively (Rivero-Juarez  
15 *et al.*, 2017, Li *et al.*, 2005). In Italy, one human case (Giordani *et al.*, 2013) was supposed to be  
16 linked to wild boar meat consumption because the patient had never travelled outside Italy and  
17 declared to have consumed wild boar meat. In the same area, wild boar HEV strains related to those  
18 detected in the human case were also reported (Mazzei *et al.*, 2015). Wild boars might represent a  
19 source of autochthonous HEV transmission to humans in those regions where consumption of wild  
20 boar meat is common or where there is frequent contact between pigs and wild boars. Indeed, the  
21 transmission of HEV between domestic and wild swine has been clearly demonstrated (Thiry *et al.*,  
22 2017b). Moreover, wild boars intended for human consumption are mainly captured by hunting and  
23 game meat follows a food chain different from pigs, where rules for food safety could be less strict.  
24 Furthermore, the exposure to wild boars carcasses could be a relevant source of risks for hunters  
25 (Schielke *et al.*, 2015). Our findings suggest that wild boar consumption and circulation of HEV in

1 sylvatic populations deserve further investigation and special attention by wild life managers,  
2 veterinarian and hunters.

## 4 Acknowledgements

5 We would like to acknowledge Edoardo Vignolo for his valuable technical support and the ATCVT1  
6 and ATCVT2 for their support in gathering data.

## 8 No conflict of interest to declare

## 10 References

- 11 Adlhoch, C., A. Wolf, H. Meisel, M. Kaiser, H. Ellerbrok and G. Pauli, 2009: High HEV presence in four  
12 different wild boar populations in East and West Germany. *Veterinary microbiology*, **139**, 270-278.
- 13 Anheyer-Behmenburg, H. E., K. Szabo, U. Schotte, A. Binder, G. Klein and R. Johne, 2017: Hepatitis E Virus  
14 in Wild Boars and Spillover Infection in Red and Roe Deer, Germany, 2013-2015. *Emerging infectious  
15 diseases*, **23**, 130-133.
- 16 Aprea, G., M. G. Amoroso, I. Di Bartolo, N. D'Alessio, D. Di Sabatino, A. Boni, B. Cioffi, D. D'Angelantonio,  
17 S. Scattolini, L. De Sabato, G. Cotturone, F. Pomilio, G. Migliorati, G. Galiero and G. Fusco, 2017:  
18 Molecular detection and phylogenetic analysis of hepatitis E virus strains circulating in wild boars in  
19 south-central Italy. *Transboundary and emerging diseases*.
- 20 ARSIAL, O. f. r., R. Lazio and U. d. S. d. T. DAFNE, 2014: Indagine sullo status del cinghiale (*Sus scrofa* L.)  
21 nel Lazio e sulle interazioni con le attività antropiche: base conoscitiva per l'assestamento faunistico  
22 venatorio della specie nel Lazio – Relazione preliminare – luglio 2014.
- 23 Briedermann, L., 1986: Schwarzwild (Wild boar). *VEB Deutscher, Landwirtschaftsverlag Berlin*.
- 24 Brün, J., Keuling, O., 2008: Socio-spatial behaviour and genetics of wild boar in North-Eastern Germany.  
25 *Abstracts of the 7th International Symposium on Wild Boar (Sus scrofa) and Sub-order Suiformes*.
- 26 Caruso, C., P. Modesto, S. Bertolini, S. Peletto, P. L. Acutis, A. Dondo, S. Robetto, W. Mignone, R. Orusa,  
27 G. Ru and L. Masoero, 2015: Serological and virological survey of hepatitis E virus in wild boar  
28 populations in northwestern Italy: detection of HEV subtypes 3e and 3f. *Archives of virology*, **160**,  
29 153-160.
- 30 Di Bartolo, I., G. Angeloni, E. Ponterio, F. Ostanello and F. M. Ruggeri, 2015: Detection of hepatitis E virus  
31 in pork liver sausages. *International journal of food microbiology*, **193**, 29-33.
- 32 Di Bartolo, I., F. Martelli, N. Inglese, M. Pourshaban, A. Caprioli, F. Ostanello and F. M. Ruggeri, 2008:  
33 Widespread diffusion of genotype 3 hepatitis E virus among farming swine in Northern Italy.  
34 *Veterinary microbiology*, **132**, 47-55.
- 35 Di Profio, F., I. Melegari, V. Sarchese, S. Robetto, G. Marruchella, M. C. Bona, R. Orusa, V. Martella, F.  
36 Marsilio and B. Di Martino, 2016: Detection and genetic characterization of hepatitis E virus (HEV)  
37 genotype 3 subtype c in wild boars in Italy. *Archives of virology*, **161**, 2829-2834.
- 38 Dorn-In, S., K. Schwaiger, M. Twaruzek, J. Grajewski, C. Gottschalk and M. Gareis, 2017: Hepatitis E Virus  
39 in Wild Boar in Northwest Poland: Sensitivity of Methods of Detection. *Foodborne pathogens and  
40 disease*, **14**, 103-108.
- 41 Gabor, T. M., Hellgren, E.C., Van Den Bussche, R.A., Silvy, N.J., 1999: Demography, sociospatial behaviour  
42 and genetics of feral pigs (*Sus scrofa*) in a semi-arid environment. *Journal of Zoology*, **247**, 311–322.

- 1 Garbuglia, A. R., A. I. Alessandrini, N. Pavio, S. Tesse, S. Grignolo, C. Viscoli, D. Lapa and M. R.  
2 Capobianchi, 2015: Male patient with acute hepatitis E in Genoa, Italy: figatelli (pork liver sausage)  
3 as probable source of the infection. *Clinical microbiology and infection : the official publication of*  
4 *the European Society of Clinical Microbiology and Infectious Diseases*, **21**, e4-6.
- 5 Garbuglia, A. R., P. Scognamiglio, N. Petrosillo, C. M. Mastroianni, P. Sordillo, D. Gentile, P. La Scala, E.  
6 Girardi and M. R. Capobianchi, 2013: Hepatitis E virus genotype 4 outbreak, Italy, 2011. *Emerging*  
7 *infectious diseases*, **19**, 110-114.
- 8 Giordani, M. T., P. Fabris, E. Brunetti, S. Goblirsch and L. Romano, 2013: Hepatitis E and lymphocytic  
9 leukemia in Man, Italy. *Emerging infectious diseases*, **19**, 2054-2056.
- 10 Iacolina, I., 2009: Nonkin Associations in Wild Boar Social Units. *Journal of Mammalogy*, **90**, 666-674.
- 11 Jothikumar, N., T. L. Cromeans, B. H. Robertson, X. J. Meng and V. R. Hill, 2006: A broadly reactive one-  
12 step real-time RT-PCR assay for rapid and sensitive detection of hepatitis E virus. *Journal of*  
13 *virological methods*, **131**, 65-71.
- 14 Kamar, N., J. Izopet, N. Pavio, R. Aggarwal, A. Labrique, H. Wedemeyer and H. R. Dalton, 2017: Hepatitis  
15 E virus infection. *Nature reviews. Disease primers*, **3**, 17086.
- 16 Kaminski, G., Brandt, S., Baubet, E., Baudoin, C., 2005: Life-history patterns in female wild boars (*Sus*  
17 *scrofa*): mother–daughter postweaning associations. *Canadian Journal of Zoology*, **93**, 474-480.
- 18 Kukiela, D., V. Rodriguez-Prieto, J. Vicente and J. M. Sanchez-Vizcaino, 2016: Constant Hepatitis E Virus  
19 (HEV) Circulation in Wild Boar and Red Deer in Spain: An Increasing Concern Source of HEV  
20 Zoonotic Transmission. *Transboundary and emerging diseases*, **63**, e360-368.
- 21 Lee, G. H., B. H. Tan, E. C. Teo, S. G. Lim, Y. Y. Dan, A. Wee, P. P. Aw, Y. Zhu, M. L. Hibberd, C. K. Tan,  
22 M. A. Purdy and C. G. Teo, 2016: Chronic Infection With Camelid Hepatitis E Virus in a Liver  
23 Transplant Recipient Who Regularly Consumes Camel Meat and Milk. *Gastroenterology*, **150**, 355-  
24 357 e353.
- 25 Li, T. C., K. Chijiwa, N. Sera, T. Ishibashi, Y. Etoh, Y. Shinohara, Y. Kurata, M. Ishida, S. Sakamoto, N.  
26 Takeda and T. Miyamura, 2005: Hepatitis E virus transmission from wild boar meat. *Emerging*  
27 *infectious diseases*, **11**, 1958-1960.
- 28 Martelli, F., A. Caprioli, M. Zengarini, A. Marata, C. Fiegna, I. Di Bartolo, F. M. Ruggeri, M. Delogu and F.  
29 Ostanello, 2008: Detection of hepatitis E virus (HEV) in a demographic managed wild boar (*Sus scrofa*  
30 *scrofa*) population in Italy. *Veterinary microbiology*, **126**, 74-81.
- 31 Martinelli, N., E. Pavoni, D. Filogari, N. Ferrari, M. Chiari, E. Canelli and G. Lombardi, 2015: Hepatitis E  
32 virus in wild boar in the central northern part of Italy. *Transboundary and emerging diseases*, **62**, 217-  
33 222.
- 34 Mazzei, M., R. Nardini, R. Verin, M. Forzan, A. Poli and F. Tolari, 2015: Serologic and molecular survey for  
35 hepatitis E virus in wild boar (*Sus scrofa*) in Central Italy. *New microbes and new infections*, **7**, 41-  
36 47.
- 37 Monini, M., I. Di Bartolo, G. Ianaro, G. Angeloni, C. F. Magistrali, F. Ostanello and F. M. Ruggeri, 2015:  
38 Detection and molecular characterization of zoonotic viruses in swine fecal samples in Italian pig  
39 herds. *Archives of virology*, **160**, 2547-2556.
- 40 Monne, I., L. Ceglie, D. I. M. G. A. Natale, S. Zamproga, A. Morreale, E. Rampazzo, G. Cattoli and L.  
41 Bonfanti, 2015: Hepatitis E virus genotype 4 in a pig farm, Italy, 2013. *Epidemiology and infection*,  
42 **143**, 529-533.
- 43 Montagnaro, S., C. De Martinis, S. Sasso, R. Ciarcia, S. Damiano, L. Auletta, V. Iovane, T. Zottola and U.  
44 Pagnini, 2015: Viral and Antibody Prevalence of Hepatitis E in European Wild Boars (*Sus scrofa*) and  
45 Hunters at Zoonotic Risk in the Latium Region. *Journal of comparative pathology*, **153**, 1-8.
- 46 Pavio, N., V. Doceul, E. Bagdassarian and R. John, 2017: Recent knowledge on hepatitis E virus in Suidae  
47 reservoirs and transmission routes to human. *Veterinary research*, **48**, 78.
- 48 Purdy, M. A., T. J. Harrison, S. Jameel, X. J. Meng, H. Okamoto, W. H. M. Van der Poel, D. B. Smith and C.  
49 Ictv Report, 2017: ICTV Virus Taxonomy Profile: Hepeviridae. *The Journal of general virology*, **98**,  
50 2645-2646.
- 51 Ricci, A., A. Allende, D. Bolton, M. Chemaly, R. Davies, P. S. F. Escamez, L. Herman, K. Koutsoumanis, R.  
52 Lindqvist, B. Norrung, L. Robertson, G. Ru, M. Sanaa, M. Simmons, P. Skandamis, E. Snary, N.  
53 Speybroeck, B. Ter Kuile, J. Threlfall, H. Wahlstrom, I. Di Bartolo, R. John, N. Pavio, S. Rutjes, W.  
54 van der Poel, P. Vasickova, M. Hempen, W. Messens, V. Rizzi, F. Latronico, R. Girones and E. P. B.  
55 Hazards, 2017: Public health risks associated with hepatitis E virus (HEV) as a food-borne pathogen.  
56 *Efsa J*, **15**.

1 Rivero-Juarez, A., M. Frias, A. Martinez-Peinado, M. A. Risalde, D. Rodriguez-Cano, A. Camacho, I. Garcia-  
2 Bocanegra, F. Cuenca-Lopez, J. C. Gomez-Villamandos and A. Rivero, 2017: Familial Hepatitis E  
3 Outbreak Linked to Wild Boar Meat Consumption. *Zoonoses and public health*, **64**, 561-565.

4 Rutjes, S. A., F. Lodder-Verschoor, W. J. Lodder, J. van der Giessen, H. Reesink, M. Bouwknegt and A. M.  
5 de Roda Husman, 2010: Seroprevalence and molecular detection of hepatitis E virus in wild boar and  
6 red deer in The Netherlands. *Journal of virological methods*, **168**, 197-206.

7 Rutjes, S. A., W. J. Lodder, F. Lodder-Verschoor, H. H. van den Berg, H. Vennema, E. Duizer, M. Koopmans  
8 and A. M. de Roda Husman, 2009: Sources of hepatitis E virus genotype 3 in The Netherlands.  
9 *Emerging infectious diseases*, **15**, 381-387.

10 Schielke, A., V. Ibrahim, I. Czogiel, M. Faber, C. Schrader, P. Dremsek, R. G. Ulrich and R. Johne, 2015:  
11 Hepatitis E virus antibody prevalence in hunters from a district in Central Germany, 2013: a cross-  
12 sectional study providing evidence for the benefit of protective gloves during disembowelling of wild  
13 boars. *BMC infectious diseases*, **15**, 440.

14 Schielke, A., K. Sachs, M. Lierz, B. Appel, A. Jansen and R. Johne, 2009: Detection of hepatitis E virus in  
15 wild boars of rural and urban regions in Germany and whole genome characterization of an endemic  
16 strain. *Virology journal*, **6**, 58.

17 Schlosser, J., A. Vina-Rodriguez, C. Fast, M. H. Groschup and M. Eiden, 2015: Chronically infected wild boar  
18 can transmit genotype 3 hepatitis E virus to domestic pigs. *Veterinary microbiology*, **180**, 15-21.

19 Schmittgen, T. D. and K. J. Livak, 2008: Analyzing real-time PCR data by the comparative C(T) method.  
20 *Nature protocols*, **3**, 1101-1108.

21 Serracca, L., R. Battistini, I. Rossini, W. Mignone, S. Peletto, C. Boin, G. Pistone, R. Ercolini and C. Ercolini,  
22 2015: Molecular Investigation on the Presence of Hepatitis E Virus (HEV) in Wild Game in North-  
23 Western Italy. *Food and environmental virology*, **7**, 206-212.

24 Smith, D. B., P. Simmonds, J. Izopet, E. F. Oliveira-Filho, R. G. Ulrich, R. Johne, M. Koenig, S. Jameel, T. J.  
25 Harrison, X. J. Meng, H. Okamoto, W. H. Van der Poel and M. A. Purdy, 2016: Proposed reference  
26 sequences for hepatitis E virus subtypes. *The Journal of general virology*, **97**, 537-542.

27 Smith, D. B., P. Simmonds, S. Jameel, S. U. Emerson, T. J. Harrison, X. J. Meng, H. Okamoto, W. H. Van der  
28 Poel and M. A. Purdy, 2014: Consensus proposals for classification of the family Hepeviridae. *The*  
29 *Journal of general virology*, **95**, 2223-2232.

30 Sonoda, H., M. Abe, T. Sugimoto, Y. Sato, M. Bando, E. Fukui, H. Mizuo, M. Takahashi, T. Nishizawa and  
31 H. Okamoto, 2004: Prevalence of hepatitis E virus (HEV) infection in wild boars and deer and genetic  
32 identification of a genotype 3 HEV from a boar in Japan. *Journal of clinical microbiology*, **42**, 5371-  
33 5374.

34 Spahr, C., T. Knauf-Witzens, T. Vahlenkamp, R. G. Ulrich and R. Johne, 2018: Hepatitis E virus and related  
35 viruses in wild, domestic and zoo animals: A review. *Zoonoses and public health*, **65**, 11-29.

36 Takahashi, M., T. Nishizawa, H. Sato, Y. Sato, Jirintai, S. Nagashima and H. Okamoto, 2011: Analysis of the  
37 full-length genome of a hepatitis E virus isolate obtained from a wild boar in Japan that is classifiable  
38 into a novel genotype. *The Journal of general virology*, **92**, 902-908.

39 Thiry, D., A. Mauroy, C. Saegerman, A. Licoppe, T. Fett, I. Thomas, B. Brochier, E. Thiry and A. Linden,  
40 2017a: Belgian Wildlife as Potential Zoonotic Reservoir of Hepatitis E Virus. *Transboundary and*  
41 *emerging diseases*, **64**, 764-773.

42 Thiry, D., N. Rose, A. Mauroy, F. Paboeuf, L. Dams, S. Roels, N. Pavio and E. Thiry, 2017b: Susceptibility  
43 of Pigs to Zoonotic Hepatitis E Virus Genotype 3 Isolated from a Wild Boar. *Transboundary and*  
44 *emerging diseases*, **64**, 1589-1597.

45 Vina-Rodriguez, A., J. Schlosser, D. Becher, V. Kaden, M. H. Groschup and M. Eiden, 2015: Hepatitis E virus  
46 genotype 3 diversity: phylogenetic analysis and presence of subtype 3b in wild boar in Europe.  
47 *Viruses*, **7**, 2704-2726.

48 Woo, P. C., S. K. Lau, J. L. Teng, K. Y. Cao, U. Wernery, T. Schountz, T. H. Chiu, A. K. Tsang, P. C. Wong,  
49 E. Y. Wong and K. Y. Yuen, 2016: New Hepatitis E Virus Genotype in Bactrian Camels, Xinjiang,  
50 China, 2013. *Emerging infectious diseases*, **22**, 2219-2221.



**Table 1.** HEV-RNA prevalence obtained from wild boar hunted in the five examined municipalities (A-E). Some geographical features are reported.

| Municipality | area<br>(km <sup>2</sup> ) | Altitude* |     |      | HEV RNA-<br>positive/examined | Prevalence (%) | 95% CI      | P     |
|--------------|----------------------------|-----------|-----|------|-------------------------------|----------------|-------------|-------|
|              |                            | min       | max | mean |                               |                |             |       |
| A            | 29.1                       | 63        | 326 | 131  | 16/25                         | 64.0           | 42.5 - 82.0 | 0.010 |
| B            | 33.0                       | 74        | 364 | 179  | 23/35                         | 65.7           | 47.8 - 80.9 |       |
| C            | 105.0                      | 220       | 600 | 400  | 0/5                           | 0.0            | 0.0 - 52.2  |       |
| D            | 113.8                      | 125       | 963 | 299  | 6/17                          | 35.3           | 14.2 - 61.7 |       |
| E            | 84.2                       | 36        | 213 | 129  | 3/10                          | 30.0           | 6.7 - 65.3  |       |
| Total        |                            |           |     |      | 48/92                         | 52.2           | 41.5 - 62.7 |       |

\*meter above sea level.

**Fig. 1.** Maps of hunting area (in grey), number of HEV positive animals/total animals investigated per area is reported.

**Fig. 2.** Phylogenetic tree based on the 302-bp sequences of the ORF2 fragment. Representative porcine, human and wild boar strains are included. Each entry includes host (Fig: figatelli; Hu: human, Sw: swine, Wb: wild boar), accession number and countries origin of strains. Strains detected in this study are in bold. Bootstrap values >70 are indicated.