Molecular survey of HEV infection in wild boar population in Italy

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

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Summary

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

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

Introduction

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

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

Materials and Methods

Sampling

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

Sample preparation and nucleic acid extraction

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

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

RT-qPCR for HEV

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

Limit detection of HEV RNA

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

Nested-RT-PCR for HEV sequencing

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

Phylogenetic analyses

Nucleotide sequence similarity was analyzed with the BLAST server (http://www.ncbi.nlm.nih.gov/genbank/index.html). A 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 1000 bootstrap replications. The sequences were

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

Statistical analysis

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

Results

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

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

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

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

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

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

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

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

Discussion

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

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

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

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

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

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

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No conflict of interest to declare

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

Municipality	area (km^2)	Altitude* min	max	mean	HEV RNA-positive/examined	Prevalence (%)	95% CI	P
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.