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Quantification and genetic diversity of Hepatitis E virus in wild boar (*Sus scrofa*) hunted for domestic consumption in Central Italy

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ABSTRACT

Hepatitis E is an emerging disease in industrialized countries. The food-borne transmission of hepatitis E virus (HEV) is associated principally with products derived from the domestic pig, the wild boar, and deer; however, few quantitative data are available on HEV loads in animals used in food production. This study assessed HEV occurrence, viral load and genetic variability in wild boar hunted for domestic consumption in the district of Viterbo (Central Italy) where high anti-HEV IgG seroprevalence values are reported in humans. A total of 332 liver and 69 intestine samples were obtained from wild boar hunted between 2011 and 2014. The liver tissue in 54 of the animals (16.3%) was HEV-positive. Viral loads in quantifiable liver samples ($n = 29$) ranged between 3.2×10^2 and 3.8×10^5 genome copies (g.c.)/g with a mean value of 1.85×10^4 g.c./g. A statistically significant positive correlation was found between viral concentration in liver and intestinal tissue, though mean viral load in the intestines was lower (3.13×10^3 g.c./g). Twenty-six samples were characterized molecularly as genotype 3 (G3) and four subtypes (a, c, f and l) were detected. Finally, twelve samples with near identical sequences were identified as G3 but could not be assigned to any of the known subtypes, and could therefore represent a potentially new subtype.

1. Introduction

Hepatitis E virus (HEV) is a non-enveloped, RNA-virus of the family *Hepeviridae* and is known to be associated with a self-limiting form of acute hepatitis in humans that usually resolves itself within 2–6 weeks. The case fatality rate is relatively low, ranging between 1% and 5% (Pavio et al., 2010) but, in pregnant women in developing countries where hepatitis E is endemic, can rise to 20% following the onset of fulminant hepatitis (Pérez-Gracia et al., 2017). Furthermore, the infection can become chronic in immunocompromised individuals (Aggarwal and Jameel, 2011; Purcell and Emerson, 2008) or evolve into acute-on-chronic liver failure in patients with chronic liver disease (Kumar and Saraswat, 2013). In developing countries, genotypes 1 and 2 (G1 and G2) predominate (Kamar et al., 2012) and HEV is responsible for large epidemics, mainly involving waterborne transmission (Aggarwal, 2011). In industrialized countries, infection is associated with travel to endemic areas or, as increasingly reported (Adlhoch et al., 2016; Aspinall et al., 2017), is autochthonous, involving genotypes 3 and 4 (G3 and G4) (Adlhoch et al., 2016; Dalton et al., 2008; Okamoto, 2007). Whereas G1 and G2 are restricted to humans, G3 and G4 are zoonotic, infecting several animal species, including those used in food production, such as the domestic pig, the wild boar and deer (Doceul et al., 2016; Johne et al., 2014). Additional genotypes of HEV (Smith et al., 2014) have been described in rabbits (G3ra), wild boar (G5 and G6), and camelids (G7) (Izopet et al., 2012; Sato et al., 2011; Takahashi et al., 2011; Woo et al., 2014).

The food-borne transmission of HEV associated with the consumption of liver, meat, sausages and offal products derived from domestic pig, wild boar, and deer has been reported in several studies conducted in various countries (Colson et al., 2010, 2012; Renou et al., 2014; Riveiro-Barciela et al., 2015; Tei et al., 2004; Yazaki et al., 2003). While other foodstuffs such as vegetables and shellfish may provide

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alternative transmission pathways for this virus (King et al., 2018; La Rosa et al., 2018; Maunula et al., 2013; Mesquita et al., 2016a; O'Hara et al., 2018; Terio et al., 2017), the risk to human health associated with the consumption of raw and undercooked pork, wild boar and deer products has been clearly established (EFSA BIOHAZ Panel, 2017), several case studies having demonstrated the association between wild boar meat consumption and sporadic HEV events (Kim et al., 2011; Li et al., 2005; Masuda et al., 2005; Saint-Jacques et al., 2016) or small outbreaks (Matsuda et al., 2003; Rivero-Juarez et al., 2017; Tamada et al., 2004).

The presence of antibodies against HEV and of viral RNA in wild boar tissue (liver, meat, blood, etc.) has been reported upon extensively in Europe (Pavio et al., 2017; Porea et al., 2018; Rivero-Juarez et al., 2018; Spancerniene et al., 2018; Weigand et al., 2018), however only a few studies have investigated viral load in the organs and tissues of wild boar (Anheyer-Behmenburg et al., 2017; Kubankova et al., 2015; Lhomme et al., 2015).

In Italy, HEV seroprevalence and viral RNA presence in wild boar has been evaluated in some regions of Northern (Caruso et al., 2017; Di Profio et al., 2016; Martelli et al., 2008; Martinelli et al., 2015; Serracca et al., 2015), Central (Mazzei et al., 2015; Montagnaro et al., 2015) and Southern Italy (Aprea et al., 2018; Di Profio et al., 2016). In these studies, it emerged that HEV circulation in wild boar differs significantly from one region to the next, seroprevalence rates ranging between 4.2% and 56.2%, with HEV RNA in liver samples varying between 0% and 33.5%. However, in these studies, no quantitative information was provided on the levels of HEV in the tissues analyzed. Thus, while random sampling has confirmed the widespread occurrence of HEV in wild boar across Europe, few data exist on viral loads in animals hunted for human consumption (either directly or through meat preparations). Ultimately, this lack of detailed information hinders the development of a quantitative risk assessment for HEV transmission through the consumption of raw or undercooked wild boar cuts.

The aim of this study was to assess HEV occurrence, viral load and genetic variability in wild boar hunted for domestic consumption in the district of Viterbo (Latium region, Central Italy) where the anti-HEV IgG seroprevalence rate in the human population, in greatly exceeding the national average (Spada et al., 2018), signals an elevated level of exposure to the virus.

2. Materials and methods

2.1. Sample and data collection

Animal tissue sampling was performed as a part of the annual demographic control program of the wild boar (*Sus scrofa*) population of the Latium region, Central Italy. Through the auspices of the various hunting associations of the district of Viterbo (Northern Latium) tissue samples were obtained from wild boar hunted over three hunting seasons (2011–12, 2012–13 and 2013–14). In total, 3692 hunters were involved, the hunting taking place within latitudes 42°10′19″ and 42°50′20″ N and longitudes 11°27′14″ and 12°18′25″ E, and covering an area of 441.1 km^2 (Fig. 1). All hunted animals were *post mortem*-inspected by the local veterinary services following Regulations (EC) 2004/882, 2004/853 and Regulation (EU) 2015/1375, the latter laying down specific rules on official controls for *Trichinella* in meat. After the carcasses had been inspected, and the laboratory results received, the hunters were allowed to retain wild boar cuts for further processing, including domestic consumption or small local trade. For each slaughtered animal (n = 672), the following information was recorded: date and place of shooting, sex, age (estimated on the extent of tooth eruption), weight, race (Maremma, Hungarian or hybrid) and site of wound. Tissue samples (primarily liver and, where possible, also intestine) were obtained using a random selection criterion from approximately 50% of the animals hunted and, at final count, the sampling yielded 332 livers and 69 intestines. These were refrigerated during transport to the laboratory, where they were stored in 1 g aliquots at below −15 °C.

Fig. 1. Geographic area included in the study. The area included in the study is highlighted in dark grey. In the map are reported the districts (light grey) characterized by a high human seroprevalence of anti-HEV IgG (≥25.0%): AP: Ascoli Piceno; AQ: L'Aquila; NU: Nuoro; OG: Ogliastra; RI: Rieti; VT: Viterbo.

2.2. Sample preparation

Virus extraction was performed following the method described in Szabo et al. (2015) with slight modifications. Briefly, 1 g of coarsely chopped tissue was added to a 50 ml tube containing approximately 30 sterile glass beads (3–4 mm diameter). After adding 10 µl of process control virus (Mengovirus, strain MC_0, 1.6×10^5 $TDCI_{50}$/ml) and of 3.5 ml of TRIZOL Reagent (Life Technologies, Carlsbad, Canada), samples were homogenized for 2 min via high-speed vortexing. Following mechanical disruption of the animal tissues, the samples were incubated for 15 min at room temperature and subsequently centrifuged at 8000 × g for 20 min at 4 °C. After centrifugation, 0.7 ml of chloroform (0.2 v/v) was added to the recovered supernatant, the suspension vortexed for 15 s, and then incubated at room temperature for 15 min. Thereafter, the samples were again centrifuged at 8000 × g for 15 min at 4 °C and the aqueous phase retained; its volume (on average 2.3 ± 0.1 ml) was measured and the suspensions were stored at below −75 °C prior to nucleic acid extraction.

2.3. Nucleic acids extraction and real time RT-(q)PCR

Nucleic acids were extracted from 1 ml of sample using the NucliSens MiniMag extraction system (bioMérieux, France) following the manufacturer's instructions. RNA was eluted in 100 µl and stored at below −75 °C prior to real time PCR analysis. A negative extraction control (molecular grade water) was added to each sample extraction batch.

HEV detection was carried out using real-time RT-(q)PCR assay. For each reaction, a 5 µl aliquot of sample RNA was analyzed using the RNA

UltraSense™ One-Step qRT-PCR System (Life Technologies), 1.25 µl of RNA UltraSense enzyme mix and the following concentrations for primers and probe: 500 nM for forward primer JVHEVF (5′-GGTGGTTTC TGGGGTGAC-3′), 900 nM for reverse primer JVHEVR (5′-AGGGGTTG GTTGGATGAA-3′), and 250 nM for probe JVHEVP-MGB (5′-FAM-TGA TTCTCAGCCCTTCGC-MGB-3′) (Garson et al., 2012; Jothikumar et al., 2006). The amplification conditions were as follows: reverse transcription for 60 min at 50 °C, inactivation for 5 min at 95 °C and 45 cycles of 15 s at 95 °C, 1 min at 60 °C, and 1 min at 65 °C.

Analyses were performed in duplicate on an ABI Prism 7700 SDS system (Applied Biosystems, Foster City, CA, US) and the average concentration of the two replicate reactions used for quantification. All samples that tested close to or below the LOD_{95} of the real-time RT-(q) PCR assay (1.12 genome copies/µl ≡ 2.6×10^2 g.c./g) were re-tested in quadruplicate reactions; only samples in which at least two wells showed amplification were considered positive.

Two negative PCR controls were included in each run. PCR inhibition was ruled out using an external amplification control (*in vitro* synthesized RNA) and amplifications were considered acceptable if inhibition was ≤50%. Virus recovery from samples was assessed using the process control virus, according to Costafreda et al. (2006) with an extraction acceptability criterion of ≥1%; samples that did not achieve the extraction efficiency criterion underwent a second extraction process. A linearized plasmid containing the target sequence was used to generate the standard curve (dynamic range 1×10^0–1×10^4 copies/µl); curves with a slope lying between −3.1 and −3.6 and a $R^2 \geq 0.98$ were used for quantification. To ensure comparability of results, a conversion factor between the standard plasmid used in the study and the WHO International Standard for Hepatitis E Virus (PEI code 6329/10, 250.000 IU/ml) was calculated. For this, the WHO reference material (100 µl) was extracted using the NucliSens MiniMag system (bioMerieux), and the resulting RNA and its tenfold dilutions analyzed by real-time RT-(q)PCR in parallel with the standard plasmid. Two separate runs were performed and the average of the two results used to establish correspondence of genome copies to IU.

2.4. Sequencing and phylogenetic analysis

An RT-nested-PCR targeting the ORF2 region of the HEV genome was used to genotype and subtype the HEV-positive samples. Primers HE044 (5′-CAAGGHTGGCGYTCKGTTGAGAC-3′) and HE040 (5′-CCCT-TRTCCTGCTGAGCRTTCTC-3′) were used for the first cycle (amplicon size 506 bps) (Okamoto et al., 2001), and primers HE110-2_modified (5′-GYTCKGTTGAGACCWCBGGBGT-3′) and HE041 (5′-TTMACWGTC-RGCTCGCCATTGGC-3′) for the nested reaction, obtaining an amplicon of 467 bps (La Rosa et al., 2011; Shrestha et al., 2003). PCR was done using 5 µL of RNA and 1 µL (10 pmol) of each primer in a 50 µL reaction using the MyTaq One-Step RT-PCR Kit (Bioline, London, UK). The PCR cycling conditions were as follows: reverse transcription at 42 °C for 45 min, inactivation for 4 min at 94 °C, followed by 35 cycles of 94 °C for 30 s, 51 °C for 30 s, 72 °C for 30 s, and a final step of 72 °C for 10 min. Two microliters of the product obtained from the first PCR were used as a template in the nested PCR assay, using the MyTaq Red Mix kit (Bioline) and the same thermal cycling conditions. As a PCR positive control, RNA extracted from the WHO International Standard for HEV (PEI code 6329/10) was used. Amplifications were carried out in a T100™ Thermal Cycler (Biorad; Hercules, CA, US) and standard precautions followed to prevent PCR contamination. All positive results were confirmed by replicating the analysis in a second, independent, PCR amplification. The specific nested PCR products were confirmed by gel electrophoresis (1.5% agarose gel) visualized using GelRed Nucleic Acid Gel Stain (Biotium, CA, USA) as a staining reagent.

All PCR products were purified using a Montage PCRm96 Microwell Filter Plate (Millipore, MA, USA) and were subjected to direct automated sequencing on both strands (Bio-Fab Research, Italy). The raw forward and reverse ABI files were aligned and assembled into a consensus sequence using MEGA 7 software (Kumar et al., 2016). The consensus sequences were submitted to BLAST analysis and to the HEV Typing Tool (http://www.rivm.nl/mpf/typingtool/hev/) for genotyping.

The phylogenetic tree was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The analysis involved 97 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding and a total of 412 positions were included in the final dataset. Evolutionary analyses were conducted in MEGA 7. Nucleotide sequences of the partial ORF2 region of the HEV strains obtained in this study were deposited in GenBank under accession numbers MH836524 to MH836549.

2.5. Statistical analysis

For the demographic characteristics of the wild boar population (age, weight), the minimum, maximum and arithmetic mean were calculated. For the quantitative results of HEV analysis on liver and intestinal tissues, the geometric mean of the quantifiable values (i.e. results above the LOQ of the method: 1.40 genome copies/µl ≡ 3.2×10^2 g.c./g) and the Pearson correlation coefficient r were calculated. Fisher's exact test, the chi-square test, and the t Student test were executed to compare significance of differences among prevalence values and means, respectively. A multivariate regression analysis was conducted to determine the factors associated with HEV detection, using sex, race and age as variables. All statistical calculations were done using MedCalc Statistical Software v18 (MedCalc Software bvba, Ostend, Belgium). Animal weight was excluded from the analysis due to collinearity with age (correlation matrix = 0.738; XlStat v 2019.1.1, Addinsoft, Boston, US).

3. Results

The population structure of the hunted wild boar (n = 672) and of the 332 wild boar tested for the presence of HEV is summarized in Table 1. The majority of the wild boar (n = 238, 71.7%) were hunted during the 2012–2013 season, while the 2011–2012 and 2013–2014 seasons accounted for 16.3% and 12.0% of the samples, respectively. Males represented 44.0% and females 48.5% of the wild boars tested; no data are available for the remaining 7.5%. The hybrid race was predominant (62.0%), followed by Maremma (28.9%) and Hungarian (0.6%). The average age of the sampled animals was 22.8 ± 14.1 months (range 2.5–102 months) and their average weight was 56.7 ± 24.1 kg (range 10–150 kg). None of the characteristics of the sampled group differed significantly from those of the population of origin.

Overall, the liver tissue from 54 animals (16.3% of the total, 95% C.I.: 12.7%–20.6%) was found positive for HEV. Positive animals were distributed evenly across the three hunting seasons (2011–2012: 14.8%; 2012–2013: 17.2%; 2013–2014: 12.5%). No statistically significant difference was detected in the prevalence of HEV in wild boars based on sex (male 17.1% *vs* female 18.0%; p = 0.8814) or, for the predominant types, based on race (hybrid 18.9% *vs* Maremma type 15.6%; p = 0.5231). On the other hand, HEV prevalence differed significantly amongst the three age groups (0–12 months *vs* 13–24 months *vs* > 24 months; $p < 0.0001$) and average age of HEV-positive animals was significantly lower than average age of the population (15.9 months as opposed to 22.8 months; p = 0.0006). Furthermore, HEV was never detected in animals older than 48 months. Differences in average weight, correlated to age, were also statistically significant (40.9 *vs* 56.7 kg; p = 0.0001). Multivariate regression analysis confirmed age to be the most relevant risk factor for HEV infection (p = 0.0001; area under the ROC curve = 0.708, 95% C.I.: 0.653–0.758).

With regard to the quantitative levels of HEV in the positive liver samples (Table 2), approximately half of the samples (n = 25) tested positive at levels close to the LOD_{95} of the method and, therefore, were

Table 1
Characteristics of the wild boar population included in the study and detection rate of Hepatitis E virus in the tested animals.

	Wild boar population	Sampled wild boars	Positive samples	% of positive samples
Hunting season				
2011–2012	113	54	8	14.8%
2012–2013	450	238	41	17.2%
2013–2014	109	40	5	12.5%
Sex				
Male	280	146	25	17.1%
Female	334	161	29	18.0%
unknown	58	25	0	0%
Race				
Hybrid	399	206	39	18.9%
Maremma type	186	96	15	15.6%
Hugarian	14	2	0	0%
unknown	73	28	0	0%
Age (months)				
0–12	199	93	30	33.3%
13–24	204	111	16	14.4%
25–48	194	95	8	7.8%
> 48	13	8	0	0%
unknown	62	25	0	0%
Total	**672**	**332**	**54**	**16.3%**
Age (months)				
Minimun	2.5	2.5	5	–
Maximum	110	102	48	–
Average	22.6	22.8	15.9	–
SD	14.1	14.1	9.7	–
Weight (kg)				
Minimun	10	10	12	–
Maximum	150	150	80	–
Average	53.6	56.7	40.9	–
SD	24.2	24.1	19.9	–

not quantifiable. Twenty-nine samples (8.7% of the tested samples) showed HEV levels ≥ LOQ; concentrations ranging from 10^4 to 10^5 genome copies/g (g.c./g) were those most frequently detected (3.9% of samples). Overall, HEV concentration in positive, quantifiable liver tissues ranged between 3.2×10^2 and 3.8×10^5 HEV g.c./g (data not shown) with a geometric mean value of 1.85×10^4 HEV g.c./g. Given the conversion factor obtained through comparison of the plasmid standard with the WHO International Standard PEI code 6329/10 (1 g.c./μl = 1.11 IU/μl), these concentrations were equivalent to a range of 3.6×10^2–4.2×10^5 HEV IU/g and a geometric mean value of 2.05×10^4 HEV IU/g. No significant differences ($p > 0.05$) were detected for mean quantitative values based on hunting season, sex or race of the animal. With regard to the age of the animals, no significant difference in HEV loads were detected according to age group (Table 2), but the proportion of wild boars showing HEV levels ≥ LOQ declined progressively with age, decreasing from 18.3% (age group 0–12 months) to 9.9% (age group 13–24 months) and, finally, 1.4% (25–36 months).

With reference to the intestinal samples analyzed, HEV RNA was detected in 26 samples, all obtained from animals in which the virus was detected also in the liver tissue. HEV loads ranged from not quantifiable (6 samples) up to 1.67×10^5 g.c./g, with an average contamination – expressed as the geometric mean of the quantifiable samples – of 3.13×10^3 g.c./g. This viral load is significantly lower than that calculated for liver tissue ($p = 0.0049$). The Pearson's r, based on the log-transformed data of 19 wild boars in which quantifiable data were available for both liver and intestinal tissue, revealed a statistically significant positive correlation between the two tissues ($r = 0.5248$; $p = 0.0211$).

With regard to molecular characterization, the ORF2 region of 26 of the 54 positive samples (48.1%) was successfully amplified by nested PCR. Sequencing revealed all these samples to belong to genotype 3. In total, four different subtypes were detected by comparing the partial ORF2 sequences with prototype sequences held in the RIVM database: 3a ($n = 3$), 3c ($n = 4$), 3f ($n = 5$) and 3l ($n = 2$), the latter being a provisional subtype proposed by Xia et al. (2008). Furthermore, 12 sequences were identified as G3 but could not be assigned to any of the known subtypes following comparison with the GenBank and RIVM databases. These sequences showed only 93% nt identity with the closest HEV sequence available in GenBank (KC782933) and ≤89% nt identity with the closest sequences belonging to subtype G3h.

The phylogenetic tree based on the partial ORF2 region is shown in Fig. 2. The tree contains the 26 study sequences along with 71 genomic

Table 2
Hepatitis E viral loads according to hunting season, sex, race and age of the tested animals.

	N° of tested samples	N° of positive samples	Detection at LOD level	Viral loads					Geometric mean of quantifiable samples (g.c./g)
				LOQ-10^3 g.c./g	10^3–10^4 g.c./g	10^4–10^5 g.c./g	10^5–10^6 g.c./g	Total	
Hunting season									
2011–2012	54	8	5 (9.3%)	0	0	1	2	3 (5.6%)	1.18×10^5
2012–2013	238	41	18 (7.6%)	3	6	10	4	23 (9.7%)	1.46×10^4
2013–2014	40	5	2 (5.0%)	0	1	2	0	3 (7.5%)	1.80×10^4
Sex									
Male	146	25	11 (7.5%)	1	2	7	4	14 (9.6%)	3.11×10^4
Female	161	29	14 (8.7%)	2	5	6	2	15 (9.3%)	1.14×10^4
unknown	25	0	–	–	–	–	–	–	–
Race									
Hybrid	206	39	17 (8.3%)	3	5	11	3	22 (10.7%)	1.51×10^4
Maremma type	96	15	8 (8.3%)	0	2	2	3	7 (7.3%)	3.51×10^4
Hungarian and unknown	30	0	–	–	–	–	–	–	–
Age (months)									
0–12	93	30	13 (14.0%)	3	4	6	4	17 (18.3%)	1.53×10^4
13–24	111	16	5 (4.5%)	0	3	6	2	11 (9.9%)	2.45×10^4
25–48	95	8	7 (7.4%)	0	0	1	0	1 (1.1%)	2.31×10^4
> 48	8	0	–	–	–	–	–	–	–
unknown	25	0	–	–	–	–	–	–	–
Total (%)	**332**	**54 (16.3%)**	**25 (7.5%)**	**3 (0.9%)**	**7 (2.1%)**	**13 (3.9%)**	**6 (1.8%)**	**29 (8.7%)**	**1.85×10^4**

g.c./g = genome copies per gram.
LOD = limit of detection (2.6×10^2 c.g./g); LOQ = limit of quantification (3.2×10^2 c.g./g).

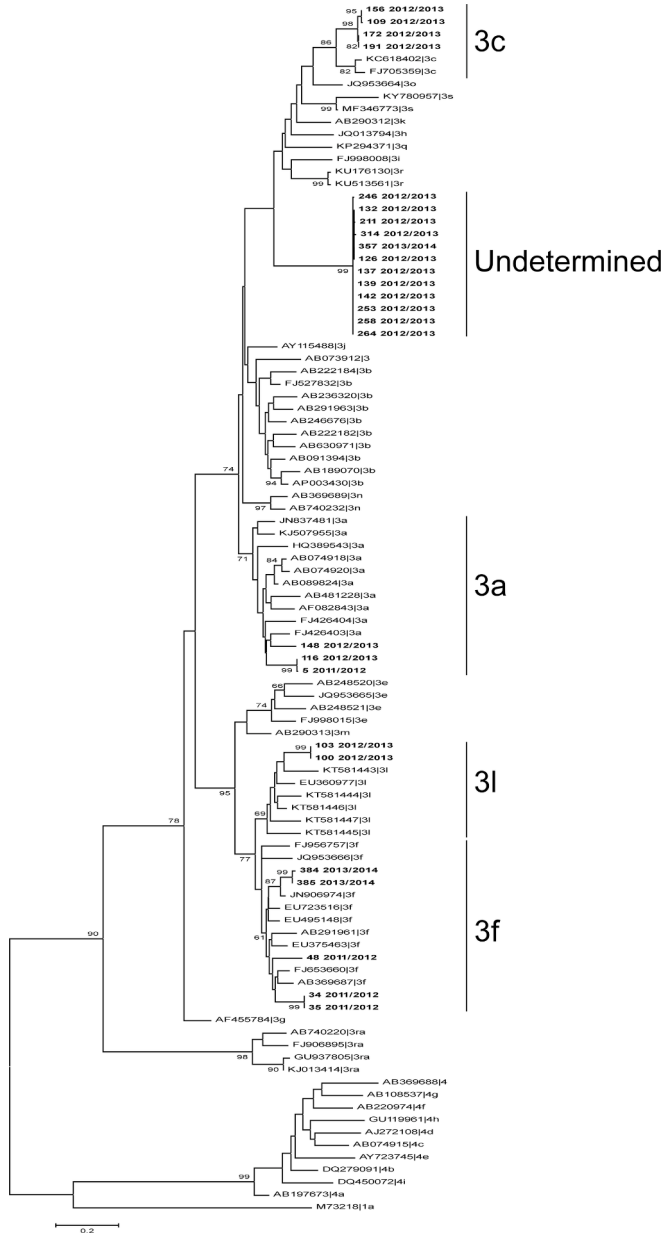

Fig. 2. Phylogenetic tree of the partial ORF2 sequence of HEV positive samples. The tree was inferred by using the Maximum Likelihood method based on the General Time Reversible model. The analysis involvd 97 nucleotide sequences. Codon positions included were 1st + 2nd + 3rd + noncoding and a total of 412 positions were included in the final dataset. Evolutionary analyses were conducted in MEGA 7. Subtypes were defined according to the HEV typing tool (http://www.rivm.nl/mpf/typingtool/hev/).

sequences retrieved from GenBank representing subtypes G3a to G3j, G3ra, G4a to G4i (Smith et al., 2016), and the provisional subtypes G3k to G3s according to the HEV Genotyping Tool. The 14 identified study sequences grouped into four supported clusters along with the corresponding subtypes 3a, 3f, 3c and 3l, in agreement with the BLAST analysis. The remaining 12 "undetermined" sequences, instead, formed a distinct and well-supported clade (100% bootstrap) without reference sequences, confirming that these samples cannot be classified down to subtype.

The detected subtypes displayed strong geographic clustering despite originating, in several cases, from animals hunted in different seasons. Subtypes 3a, 3f and 3l were mostly detected in the northern part of the sampling area, while the subtype 3c and the strains of the

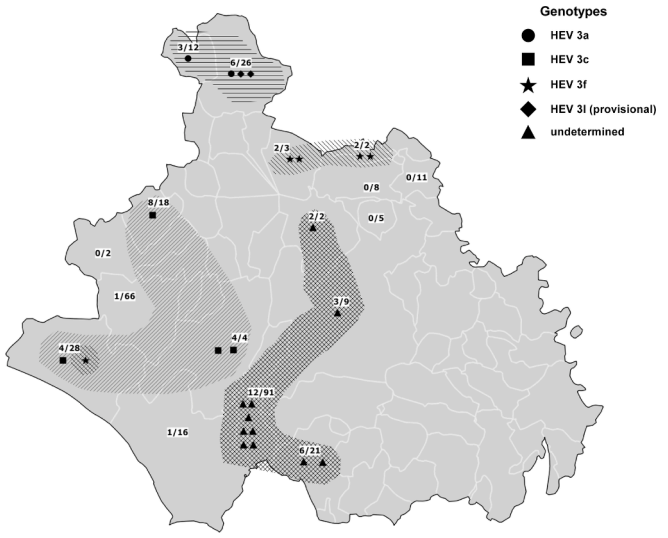

Fig. 3. Spatial distribution of the Hepatitis E positive wild boar and of the detected genetic subtypes.
The symbols in the map describe the spatial distribution of the genotypes detected in the study. For each hunting zone is reported the number of HEV positive samples on the total number of collected samples.

undetermined subtype were predominantly detected in the western and southern parts of the sampling area (Fig. 3).

4. Discussion

Hepatitis E is considered an emerging disease in industrialized countries, due to an increase in autochthonous cases (Adlhoch et al., 2016; Aspinall et al., 2017). In the last few years, occurrence of hepatitis E in subjects without a travel history to endemic areas has been repeatedly reported also in Italy (Biliotti et al., 2018; Bouamra et al., 2014; Festa et al., 2014; La Rosa et al., 2011; Stroffolini et al., 2015; Tarantino et al., 2016). Data provided by the Integrated Epidemiological Surveillance System for Acute Hepatitis (SEIEVA) shows that approximately 80% of the Hepatitis E cases reported for Italy in 2017 were autochthonous (SEIEVA, 2017). In addition, a recent nation-wide retrospective study on blood donors revealed the average national anti-HEV IgG seroprevalence rate to be 8.7% and, also, highlighted the great variation in this rate according to geographic region and district. Six areas of insular and peninsular Central Italy (Ascoli Piceno, L'Aquila, Nuoro, Ogliastra, Rieti and Viterbo) had seroprevalence values exceeding 25% and these values could find cause in local eating customs, including the traditional consumption of dried liver sausages, and/or in intensive environmental HEV contamination (Spada et al., 2018).

This study focuses on assessing HEV occurrence and viral load in wild boar hunted for domestic consumption in the Viterbo district (Central Italy), where high human seroprevalence rates occur. As described, population management of wild boar is conducted annually in accordance with regional and local guidelines and includes hunting teams being assigned to designated areas. After sanitary inspection and clearance, the wild boar carcasses are shared out amongst the hunters for local trade and domestic consumption; this encompasses the families, relatives and acquaintances of the approximately 3700 registered hunters. In our study, HEV RNA was detected in 16.3% of the 332 liver samples obtained, viral prevalence showing no significant variation over the three-year (2011–2014) surveillance period. These results agree with those obtained previously in the adjoining region of Abruzzo, where 13.7% of the livers tested were HEV positive (Aprea et al., 2018). HEV prevalence rates in wild boar from Viterbo and Abruzzo are however significantly higher than those reported for Northern Italy, where the rates range between zero (Martinelli et al.,

2015) and 3.7% (Caruso et al., 2015). Interestingly, these regional differences in wild boar HEV occurrence mirror seroprevalence rates found in man (Spada et al., 2018). More specifically, the high anti-HEV IgG detection rates reported from the districts of L'Aquila and Viterbo (31.6% and 25.0%, respectively) correspond to the high HEV RNA prevalences found in wild boar, namely 13.7% (Aprea et al., 2018) and 16.3% (this study), whereas in Northern Italy, the low human seroprevalence rates in blood donors correlates with the absence of HEV in wild boar (Di Profio et al., 2016; Martinelli et al., 2015).

In the current study, HEV occurrence did not differ inter-annually, nor according to sex or animal race, indicating wild boar HEV infection dynamics to be relatively stable over the longer term. However, HEV infection rates differed significantly with regard to average wild boar age (15.9 months in HEV-positive animals *vs.* 22.8 months in all animals). In addition, the HEV detection rate differed amongst age groups, peaking at 33.3% in juveniles (0–12 months), subsequently declining to 14.4% in subadults (13–24 months), 7.8% in adults (24–48 months) and 0% in old animals (> 48 months). These results reflect those reported earlier by Kubankova et al. (2015) and Risalde et al. (2017) and confirm that, while in the domesticated pig HEV RNA usually is detected in young animals (< 6 months of age) (Di Bartolo et al., 2011; Leblanc et al., 2007; Seminati et al., 2008), in the wild boar it may be detected in all age groups, including adults (Martelli et al., 2008; Mesquita et al., 2016b; Michitaka et al., 2007; Montagnaro et al., 2015; Schielke et al., 2015). In wild boar, increasing age correlates with a systematic decline in HEV RNA detection rate and viral load, revealing that the Viterbo district population of *Sus scrofa* comes into contact with the virus early on in life. Age stratification of HEV infection rates under specific management conditions, was also reported upon by de Deus et al. (2008). Therefore, if the culling and consumption of wild boar was to be restricted to older animals, in particular in areas where HEV prevalence rates are high, this should, overall, reduce the risk to human health.

In HEV positive samples the average viral load, expressed as the geometric mean, was 1.85×10^4 g.c./g and, in some samples, HEV load exceeded 10^5 g.c./g. This mean value falls within those provided in the literature, the average viral load in wild boar livers in South-Western France being 6.31×10^2 g.c./g (Lhomme et al., 2015), in the Czech Republic 1.86×10^7 g.c./g (Kubankova et al., 2015) and in Germany 2.26×10^7 g.c./g (Anheyer-Behmenburg et al., 2017). Quantitative data are of paramount importance in risk assessment, particularly when considering those culinary recipes that are based on wild boar cuts, including cooked dishes (such as wild boar stew and *ragu* sauces) as well as several traditional raw meat preparations like dried sausages (occasionally including liver), salami, ham and tenderloin. Interestingly, in our study 25 of 54 liver samples (45%) had viral loads close to or below LOD_{95}, requiring repeated testing. Three hypotheses may explain low viral loads: (i) the animals had only recently come into contact with the virus; (ii) the animals were about to clear infection, and (iii) chronic HEV persistence in target tissues (Bouwknegt et al., 2008; Risalde et al., 2017; Schlosser et al., 2014, 2015). The quantitative analysis also revealed a positive correlation to exist between the viral loads found in the liver and the corresponding intestine but, on average, HEV load was consistently lower in the latter (3.13×10^3 g.c./g compared to 1.85×10^4 g.c./g). HEV levels in tissues of naturally infected animals used in food production have seldom been assessed. On average, viral load in wild boar muscle tissue was approximately 4 logs lower than that found in the corresponding liver (Anheyer-Behmenburg et al., 2017). These findings differ from those obtained in other studies on the domestic pig, in which detection of HEV in liver was not associated to virus presence in other muscle tissues as ham and loin (Feurer et al., 2018; Leblanc et al., 2010). Additional studies, using harmonized quantitative analytical methods, are needed to establish the diagnostic significance of different animal tissues to ensure HEV absence in the raw materials used in food production.

Molecular typing of HEV-positive samples confirmed the preponderance of genotype G3 in wild animals in Italy (Aprea et al., 2018; Caruso et al., 2015; Di Bartolo et al., 2017; Di Profio et al., 2016; Martinelli et al., 2015; Montagnaro et al., 2015; Serracca et al., 2015) and, furthermore, revealed that a variety of subtypes (3a, 3c, 3f and the provisional subtype 3l) circulate within the Viterbo area. To our knowledge, this is the first report on the occurrence of subtype 3a in an animal reservoir in Italy, this subtype being seldom encountered in wild boar globally (Miyazaki et al., 2016; Oliveira-Filho et al., 2014; Sato et al., 2011; Takahashi et al., 2014). Besides the four known G3 subtypes, a fifth group of sequences was detected during the study that could not be subtyped. This group comprised 12 almost identical sequences obtained from wild boar hunted between 2012 and 2014 within different areas of the district. Interestingly, the closest identity for this undetermined subtype (93% nt) was a sequence (GenBank KC782933) associated with a case of acute hepatitis in a patient with underlying health conditions (chronic lymphocytic leukemia), who is thought to have contracted HEV in March 2012, while on a hunting expedition to neighboring Tuscany. These results provide evidence for a more widespread circulation of a potentially new G3 subtype in Central Italy, and further studies are ongoing to provide full genome characterization of these sequences. Finally, strong geographic subtype clustering may be due to the isolation of some wild boar subpopulations amongst the numerous hunting reserves. The long term effect of this segregation on HEV subtype differentiation remains to be investigated.

5. Conclusion

In industrialized countries, hepatitis E virus is responsible for an increasing number of autochthonous cases of acute viral hepatitis. The food-borne transmission of HEV is becoming increasingly important, particularly with regard to the consumption of raw products derived from pork, wild boar and deer. This study highlights the significant occurrence (16.3%) and average viral load (1.85×10^4 g.c./g) of HEV in the livers of wild boar hunted for domestic consumption in Central Italy. Furthermore, HEV genotype 3 was found to comprise several subtypes; these variants occurred in separate geographic clusters and included one variant that could not be identified to a known subtype. The results indicate that, in order to lower the risk of HEV infection, the cuts of meat derived from animals potentially implicated in the food-borne transmission of HEV, should be tested as part of the national food safety program, especially when used for products that traditionally are consumed raw, such as dry sausages and salami containing liver.

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