Geographical restriction of Hepatitis E virus circulation in wild boars (*Sus scrofa*) in Emilia-Romagna region, Northern Italy

Silvia Bonardi,[1] Virginia Filipello,[2] Enrico Pavoni,[2] Valentina Carta,[2] Luca Bolzoni,[3] Margherita Corradi,[4] Stefano Gilioli,[4] Marina Nadia Losio[2]

[1]Department of Veterinary Science, Unit of Food Inspection, University of Parma; [2]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER), Section of Brescia; [3]Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna (IZSLER), Risk Analysis and Genomic Epidemiology Unit, Parma; [4]Management Body for Parks and Biodiversity "Emilia Occidentale", Sala Baganza, Italy

Abstract

Hepatitis E virus (HEV) is a single-strand RNA virus that causes an acute viral hepatitis in humans. Among its eight recognized genotypes, HEV-3 and HEV-4 are zoonotic, infecting humans, pigs and wild boars. Recently, HEV-3 has been also detected in red deer, which represents another reservoir of HEV. Consumption of raw pork products (mainly liver sausages), undercooked wild boar meat, raw wild boar liver and deer meat has been responsible for foodborne HEV human worldwide. From November 2018 to March 2019, liver samples collected from 97 wild boars hunted in Emilia-Romagna region (Northern Italy) were tested for HEV RNA. The hunting area included two territories for an extension of 33 km^2, named A (about 13 km^2, natural park, deciduous wood) and B (about 20 km^2, cultivated fields in proximity of a river) areas. Distance between the two areas ranged between 8 to 10 km. A total of 73 wild boars were hunted in area A, and 24 in area B. HEV RNA was detected by Real-time RT–PCR in 23/73 liver samples of wild boars living in area A only (31.5% - 95% CI: 22.0-42.8%). The HEV sequences (n=13) clustered within genotype 3. The majority of positives belonged to animals < 12 months (12/25; 48%), followed by subadults (13-24 months) (7/16; 43.8%) and adults (4/32; 12.5%). This difference was found to be statistically significant (p=0.0024). In absence of pig farms, the restriction of HEV-positive animals to a well-defined territory of 13 km^2 (Boschi di Carrega Regional Park) could hypothetically be related to the presence of red deer (*Cervus elaphus*), which lived in area A at the beginning of the hunting season. Further studies are needed to confirm or deny our hypothesis.

Introduction

Hepatitis E virus (HEV) is a single-strand RNA virus that in humans causes an acute viral hepatitis after an incubation period of 4–5 weeks, but the infection is often asymptomatic. However, even if the mortality rate is generally low (0.5%), it can reach 25% in pregnant women (Farshadpour *et al*., 2018). HEV is divided in into eight recognized genotypes. HEV-1 and HEV-2 are restricted to humans and circulate in developing countries, where they are endemic, causing outbreaks linked to contaminated water (Doceul *et al*., 2016). HEV-3 and HEV-4 are zoonotic, infecting humans and animal species among which pigs and wild boars are the main reservoirs. Over the last 10 years, HEV-3 and HEV-4 human infections have been observed increasingly in industrialized countries linked commonly to the consumption of raw pork products (mainly liver sausages) but also undercooked wild boar meat (Pavio *et al*., 2017). Concerning the role of wildlife animals in the zoonotic transmission of HEV, the first evidence was derived from cases of human HEV infection due to consumption of sika deer (*Cervus nippon*) and wild boar meat (Tei *et al*., 2003; Sonoda *et al*., 2004).The more recent genotypes are HEV-5 and HEV-6 detected in Japanese wild boars, and HEV-7 and HEV-8 detected in camels (Sridhar *et al*., 2017). The survival of HEV in the environment has been reported in several studies, in particular Johne *et al*. (2016) demonstrated that HEV particles remain infective after one month at room temperature and after more than 2 months at 4°C. In pigs, the virus is mainly excreted in feces, leading to an accumulation of HEV in the environment that is pivotal for the spread of infection (Andraud *et al*., 2013). In this perspective, HEV contamination of water or the environment in the vicinity of pig farms, especially around slurry storage facilities, may persist for a long time and may represent a transmission route for the wild fauna (Kasorndorkbua *et al*., 2005).

Among consumers, hunters are particularly exposed to foodborne hepatitis E, if they are used to eat undercooked wild boar meat (Rivero-Juarez *et al*., 2017). In order to minimize the risk of HEV infection, especially vulnerable groups of consumers (e.g. persons with a weakened immune system or with pre-existing liver injury) should thoroughly cook meat, liver and meat products derived from wild boars, pigs and red deer, ensuring a minimum internal temperature of 71°C for 20 min (EFSA, 2017).

In Italy, HEV-3 and HEV-4 have been described mainly in pigs, with one human case linked to HEV-4 (Garbuglia *et al*., 2013). HEV prevalence in wild boar is variable, partly due to regional differences but also due to the variety of specimens tested and methods applied (Caruso *et al*., 2017; De Sabato *et al*., 2018a) that hamper comparison between studies. The role of wild fauna and the implications for diffusion dynamics remain therefore unclear. The aim of this study was to investigate the occurrence of HEV in livers collected from wild boars hunted in Italy and destined to human consumption. The animals were hunted under depopulation programs in force in Emilia-Romagna region, Northern Italy.

Correspondence: Silvia Bonardi, Department of Veterinary Science, University of Parma, Strada del Taglio 10, 43126, Parma, Italy.
Tel.: +39.0521.032744.
E-mail: silvia.bonardi@unipr.it

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Materials and Methods

Sample collection

From November 2018 to March 2019, a total of 97 wild boars were hunted in Emilia-Romagna region (Northern Italy) where wild boars are thinned every year by hunting, according to specific depopulation plans set by the local authorities. The hunting territory included one municipality in Parma province for an extension of approximately 33 km^2. Wild boars were hunted in two different areas, named A and B (Figure 1). Area A has an extension of about 13 km^2; it is a deciduous wood alternated with bushy areas (Boschi di Carrega Regional Park, 44°42' N; 10°12' E; 120-270 m altitude). Area B has an extension of 20 km^2 and is characterized by both cultivated and bushy areas in the proximity of the river Taro (76-112 m altitude). Wild boar density in the two areas is quite different, with about 20 animals/ 100 ha in area A *vs.* 5-7/100 ha in area B. The desired density should not be higher than 3-4/wild boars /100 ha. Distance between area A and B is between 7 to 10 km. The two areas are separated by inhabited areas and a high-speed road; in addition, some traits of area A are surrounded by fences. Neither pig farms, nor backyard swine are present in the study areas. A portion of the liver (50 g approximately) was collected from each animal, stored in sterile containers and frozen at -20°C until use. Only animals dead less than 5 hours were included in the study. Gender, pregnancy status and age of the animals were recorded. The age was determined based on tooth eruption (Saez-Royuela *et al*., 1989); the animals were considered "young" (class 0) when ≤12-month-old, "sub-adults" (class 1) when 13–24 month-old and "adults" (class 2) when >24 month-old.

RNA extraction and purification

For each liver sample 450 mg were homogenized with QIAzol Lysis Reagent (Qiagen, Hilden, Germany) according to producer instructions and spiked with 10 µL of a titrated suspension of Mengovirus process control (1.6 × 10^5 TCID50 per ml; strain MC0). RNA has then been purified with the NucliSENS® MiniMag Kit (bioMérieux, Marcy-l'Étoile, Francia). The eluted RNA was conserved at -80 °C until use.

One-Step Real-time RT–PCR detection of HEV

HEV RNA was detected using a One-Step real-time RT–PCR based on the primers and probe described by Jothikumar *et al*. (2006). All PCRs were executed on a Bio-Rad CFX96 system (Bio-Rad, Hercules, CA, USA) with the thermal profile and reaction mix described by Di Pasquale *et al*. (2019). Mengovirus amplification was carried out as described in ISO 15216-1:2017 (ISO, 2017).

Genotyping

To determine HEV genotype a fragment of the ORF1 region of the viral genome was sequenced using the primers described by Wenzel *et al*. (2011). The obtained sequences were aligned with reference strains reported in literature and retrieved on the NCBI Nucleotide database (https://www.ncbi.nlm.nih.gov/nucleotide/) (Sridhar *et al*., 2017).

Statistical analysis

We assessed the probability of freedom from HEV in the HEV-negative area assuming as design prevalence that one estimated in the HEV-positive area. Since the wild boar population size is unknown, the calculation was based on the binomial distribution function.

In addition, we assessed through logistic regressions whether a significant difference in the detection of HEV was observed in wild boar sampled in the HEV-positive area as a function of sex and age. Specifically, to test the effect of age, we introduced the ordinal explanatory variable *age group*, where the sampled animals were subdivided in three age classes: young, sub-adults, and adults. The ordinal variable *age group* was included in the model using orthogonal polynomials (Chihara, 1978). Orthogonal polynomials represent a useful tool to take in to account in regression models for the linear and higher degree effects of ordinal explanatory variables on the response variable. For variables with *k* ordered levels, orthogonal polynomials with degrees from 1 to *k*-1 can be used. Since *age group* is characterized by three ordinal levels, orthogonal polynomials with degrees 1 (representing the linear effect of the *age group*) and 2 (representing the quadratic effect of *age group*) were tested. Moreover, for female individuals only, we tested through logistic regression whether the detection of HEV was significantly affected by the *pregnancy status* in addition to the *age group*. The models providing the best prediction were assessed through log-likelihood ratio tests (Venables and Ripley 2002). We used the odds ratio (OR) as effect-size statistics in logistic regressions.

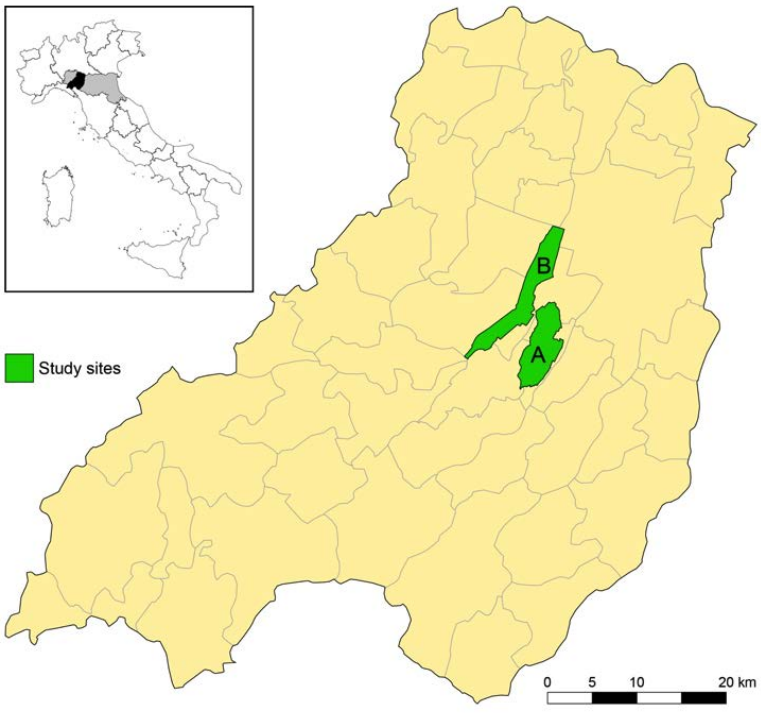

Figure 1. The hunting areas in Parma province, Emilia-Romagna region, Northern Italy. Area A has an extension of about 13 km² (deciduous wood; Boschi di Carrega Regional Park). Area B has an extension of about 20 km² (both cultivated and bushy areas in the proximity of the river Taro).

Results

Age, gender and pregnancy status of sampled animals in the two areas are reported in Table 1. HEV was detected in 23/73 wild boar livers sampled in area A (apparent prevalence 31.5% - 95% CI: 22.0-42.8%) (Table 2) and was not detected in the 24 wild boar livers sampled in area B. Genotype was determined for 13 strains that shared 99.99% identity and corresponded to HEV-3. The probability of freedom from HEV in area B, estimated through binomial distribution function, was highly significant (p_{free} = 0.99989).

Among age groups, the majority of positives in the infected area belonged to the young age group (12/25; 48%), followed by the sub-adult (7/16; 43.8%) and adult age groups (4/32; 12.5%). The logistic regression models with degree-1 and degree-2 orthogonal polynomials showed a significantly linear decrease in HEV detection with the age group (OR = 0.37; CI95% = 0.19-0.68), while quadratic effects were not observed (OR = 0.71; CI95% = 0.24-1.95). Moreover, we did not find any significant relationships between animal gender and HEV detection both in the univariable logistic regression (OR = 0.87; CI95% = 0.25-2.77) and in the multivariable regression including age and sex (OR = 0.55; CI95% = 0.13-1.94). The results of the model selection were summarized in Table 3. Analogously, we did not find any significant relationships between the pregnancy status of female individuals and HEV detection both in the univariable logistic regression (OR = 0.37; CI95% = 0.11-1.16) and in the multivariable regression including age and pregnancy status (OR = 1.44; CI95% = 0.30-8.77).

Discussion

Since *Suidae* are considered the main animal reservoir of HEV worldwide (Pavio *et al*., 2017), in Italy as well as in other EU countries pigs and wild boars have been frequently tested to shed light on this important zoonosis. In Italy, HEV-3 has been previously described in pigs as well as in wild boars (Di Bartolo *et al*., 2008; Caruso *et al*., 2015), in pork products (Di Bartolo *et al*., 2012, 2015) and in autochthonous, not travel-related human cases of hepatitis E (Romanò *et al*., 2011). Reports from EU countries largely confirm the role of pigs and wild boars in the epidemiology of HEV infection (Berto *et al*., 2012; Kukielka *et al*., 2016; Porea *et al*.2018; Spancerniene *et al*., 2018; Wenzel *et al*., 2011).

In accordance with the infectious dynamics in pigs, whose HEV faecal shedding period corresponds mainly to 3-4 months of age and decreases after 6 months of age (Salines *et al*., 2017), the majority of HEV-positive wild boars tested in this study belonged to class 0 (≤ 12 months). The statistical analyses showed that the detection of HEV RNA significantly decrease with animal age, thus suggesting that the immunity status due to previous contacts with HEV was effective against re-infections. On the contrary, neither gender nor pregnancy status influenced HEV infection status in the wild boar population tested.

Table 1. Distribution of the wild boars in the two hunting areas by gender, pregnancy status, and age class. Age class legend: Young (<12 months), Sub-adults (13-24 months), and Adults (>24 months).

Area	Gender	Age class			Total
		Young	Sub-adults	Adults	
A	Male	9	3	4	18
	Female (No. pregnant)	16 (1)	13 (11)	28 (17)	55 (29)
B	Male	6	1	7	14
	Female (No. pregnant)	4 (1)	1 (1)	5 (4)	10 (6)
Total		35 (2)	18 (12)	44 (21)	97 (35)

Table 2. Prevalence of HEV-positive liver samples of wild boars of different age groups in area A.

Age class	No. of animals in the whole territory (M/F)	No. of animals in area A (M/F)	No. of positives in area A (M/F)	Prevalence among age classes in area A, %
Young	35 (15/20)	25 (9/16)	12 (3/9)	48.0
Sub-adults	18 (4/14)	16 (3/13)	7 (1/6)	43.8
Adults	44 (11/33)	32 (4/28)	4 (2/2)	12.5
Total	97 (30/67)	73 (16/57)	23 (6/17)	31.5

M: males; F: females.

Table 3. Model selection for HEV occurrence in wild boar livers sampled in area A obtained from logistic regressions. Models were compared using log-likelihood ratio test. The models with Df degree of freedom are shown, with the log-likelihood (loglik), and the p-value of the comparison with the "Df – 1" best model.

Model	loglik	*Df*	p
~ 1[(a)]	-45.49	1	-
~ sex	-45.46	1	0.83
~ age degree-1[(b)]	-40.18	2	0.0011
~ age degree-1 + age degree-2[(c)]	-39.95	3	0.49
~ age degree-1 + sex	-39.71	3	0.33

[a]Null model; [b]degrees-1 orthogonal polynomial; [c]degrees-2 orthogonal polynomial.

In our study, HEV-3-positive animals were confined to a well-defined territory of 13 km^2, *i.e* the Boschi di Carrega Regional Park (Parma province), characterized by the absence of pig farms as well as backyard swine. In Parma province, pigs are commonly reared following strict biosecurity procedures, in accordance with Parma Ham Consortium guidelines and third countries export requirements. Direct contact between domestic and feral pigs is therefore to exclude, such as between pig farm personnel/equipment and wild boars. On the contrary, indirect contact between pigs and wild boars could not be completely excluded, favoured by the use of pig manure in agriculture and the consequent dispersion of virus particles in large cultivated areas. However, the appropriate treatment of manure required for intensive pig farming procedures reduces the risk of environmental pollution and indirect contact between farmed and feral pigs. Interestingly, at the very beginning of the hunting season (October 2018), a wintry familiar group of red deer (*Cervus elaphus*) composed of about 13 animals (female adults, female sub-adults and young deer) was living in the park. HEV infection has been recently demonstrated in red deer in Italy. For example, a free-living red deer population was found to be positive for HEV RNA and HEV antibodies in 11.0% and 13.9% of 251 serum samples, respectively, and HEV subgenotype 3e could be identified in a subset of sera (Di Bartolo *et al*., 2017). The deer strains showed 90.0% and 91.5% nucleotide identity with human (Romanò *et al*., 2011) and porcine strains previously identified in the country (Di Bartolo *et al*., 2017). In other countries, recent surveys have detected HEV RNA or HEV antibodies in samples from wild boars and red deer collected in the same geographical areas, such as in the Netherlands (Rutjes *et al*., 2010), Spain (Kukielka *et al*., 2016) and Lithuania (Spancerniene *et al*., 2018), thus confirming the epidemiological role of red deer in the maintenance of HEV infection among wildlife.

In Italy, recent surveys have confirmed the role of wild boars as carriers of HEV. In different regions of the country, wild boars were found to be infected by HEV-3 subtype 3a (Di Pasquale *et al*., 2019), as well as subtypes 3e and 3f (Caruso *et al*., 2015), subtypes 3e, 3c and 3f (Serracca *et al*., 2015), subtypes 3c and 3f (De Sabato *et al*., 2018a) and the novel subtype 3i, never identified before either in wild boars or in pigs (De Sabato *et al*., 2018b). The zoonotic potential of HEV-3 strains isolated from wild boars and showing high similarity with human HEV sequences has been demonstrated in Tuscany region, Central Italy, following a case of human infection in a municipality bordering a wild boar hunting area (Mazzei *et al*., 2015).

Conclusions

In Europe, HEV is endemic but the burden of the disease for humans is still unknown because the disease is not under EU surveillance. To better monitor epidemiology and human exposure to HEV, the collation of HEV data from human and animal populations from different countries would be crucial and serve the "One Health" approach to protecting human health (Adlhoch *et al*., 2016). The present study confirms the role of wild boars as reservoir of HEV-3 in Northern Italy, in accordance with previous data (Caruso *et al*., 2015; De Sabato *et al*., 2018a). Nevertheless, a suggestive hypothesis on the epidemiology of HEV infection in wild game can be formulated. In fact, considering the characteristics of industrial pig farming in Parma province, the infection status of the HEV-positive wild boars could be related to recent contacts with red deer. Our hypothesis is supported by the following circumstances: *i)* the presence of red deer in the same restricted geographical area at the beginning of the hunting season; *ii)* the resistance of HEV in the environment (Johne *et al*., 2016), thus favouring its transmission to animals not living in close contact but sharing the same pasture areas; *iii)* the circulation of HEV-3 among red deer in Italy (Di Bartolo *et al*., 2017); *v)* the absence of free-range pig farms in the area; *iv)* the segregation of neighbouring domestic pigs in intensive farms characterised by high biosecurity measures. To confirm or deny our hypothesis, future studies involving HEV testing of red deer samples should be carried out.

In Italy, the role of pigs in the transmission of HEV to wild boars has been suggested (Caruso *et al*., 2015), but in areas where pigs are strictly segregated from wild game the epidemiological situation involving other animal populations should be investigated. In shared habitats, interspecies transmission of HEV-3 between wild boars and red deer might occur and involve humans via zoonotic/foodborne routes (Di Bartolo *et al*., 2017). Since hunters are the consumers at higher risk of HEV foodborne infection, more information should be supplied to this category by the competent authorities at EU level. In addition, more information on the effectiveness of different cooking practice in the mitigation of HEV foodborne infection should be accessible to all consumers.

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