Hepatitis E virus detection in hunted wild boar (*Sus scrofa*) livers in Central Italy

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Abstract

Hepatitis E virus (HEV) is a zoonotic pathogen, responsible for numerous cases of infection in humans. Transmission occurs through the orofecal route, and ingestion of contaminated foods represents an important risk factor for final consumer's health. Wild animal species, in particular wild boar (*Sus scrofa*), are the main virus reservoirs; liver is the target organ, from which, through the hematic diffusion, HEV reaches different tissues and organs, as muscular one. The hygienic-sanitary critical issues connected with game meat food chain in general, and particularly wild boar, with special regards to any geographical area where this animal species can be directly in contact with humans, domestic ones (*i.e.*, domestic pig), and other wild reservoirs (*i.e.,* wild ruminants), finds favorable environmental conditions, have induced us to conduce the present scientific investigation.

During the hunting season 2019/2020, a total of 156 wild boar livers were collected from provided plucks at slaughterhouse in Ascoli Piceno. Nested RT-PCR was used for the viral RNA detection. Results demonstrated a positivity of 5.12% (8/156), and the circulation in the screened area of genotype 3 subtype c, which is frequently identified in Central Italy. HEV sanitary relevance and the emerging role of any food chains in its transmission impose further detailed studies. The molecular screening of hunted wild boars' livers can provide important information about virus's circulation in wild animal populations in a specific area.

Introduction

Every year, viral foodborne pathogens cause numerous infections with repercussions on humans, animals, and environmental health. Generally, norovirus, hepatitis A virus, rotavirus, and hepatitis E virus (HEV) are the main actors responsible for many food-related outbreaks (EFSA, 2017).

HEV is an interesting zoonotic foodborne pathogen characterized by a global incidence of 20 million (Melgaço *et al.*, 2018) cases of infection, with important repercussions on public health and economic growth in developing (60 % of the global incidence) and developed countries (Melgaço *et al.*, 2018; Shirazi *et al.*, 2018).

HEV infection mainly occurs through the ingestion of contaminated uncooked or undercooked food matrices of animal origin (*i.e.*, swine liver and meat products, wild boar meat, venison, shellfish, etc.), and contaminated wastewaters (EFSA, 2017; Denner, 2019).

HEV can be also directly transmitted between receptive hosts [*i.e.,* swine, wild boar, wild ruminants, humans (veterinarians, slaughterhouse workers, hunters, etc.)] through a direct contact with infected animals, as demonstrated in previous studies (EFSA, 2017; Pavio *et al.*, 2017; Bonardi *et al.*, 2020; Ferri and Vergara, 2021). This last route also justifies the viral circulation and its persistence in any geographical areas (EFSA, 2017).

Taxonomic classification collocates HEV in the *Hepeviridae* Family, Genus *Orthohepevirus A*, and it is a non-enveloped single-stranded-RNA virus (Smith *et al.*, 2014). Viral genome is characterized by three overlapping open reading frames (ORF). From these three ORF (1-3), ORF2 encodes for a strategical capsid protein (also named pORF2) involved in the cyto-interaction between HEV and host's specific receptors: the heparan sulfate proteoglycans (HSP), expressed by certain cytotypes such as enterocytes, hepatocytes, and myocytes (Oechslin *et al.,* 2020). This mechanism represents the evolutionary strategy adopted by the viral particles for cellular binding and infection in receptive hosts.

In immunocompetent human subjects, HEV causes sporadic self-limiting infections presenting a low mortality rate (about 2%). Conversely, in the immunocompromised patients, virus can induce several and dangerous clinical symptoms (Spada *et al.*, 2018).

HEV1 and 2 are widely diffused in developing countries causing severe gastroenteric symptoms in humans and animal species (due to contaminated water and food ingestion) (EFSA, 2017).

HEV3 and HEV4 are usually responsible for sporadic and pauci-symptomatic cases of illnesses in the industrialized countries. These two genotypes have been identified in humans, in *Sus scrofa domesticus*, and in *Sus scrofa* (EFSA, 2017). HEV3 and 4 genotypes, isolated from animal (wild boars, wild ungulates, humans) species, present high similarities with human ones, supporting that these two genotypes are zoonotic too (Matsuda *et al.*, 2003; Colson *et al.,* 2010; Serracca *et al.*, 2015).

HEV5 and HEV6 were identified from wild boars, HEV 7 and HEV8, also named camelid-variants (Sridhar *et al*., 2017).

Among wildlife and domestic animal species, *Sus scrofa domesticus* (usually as asymptomatic hosts) has a clear role as HEV reservoir, which is closely and epidemiologically connected with other important wild species: *Sus scrofa* and wild ruminants as further source of environmental

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viral spreading (Matsuda et al., 2003; Pineda *et al.*, 2014; Pavio *et al.*, 2017; Denner, 2019; Fredriksson-Ahomaa, 2019).

In the European continent, genotype 3 is mostly identified in the cases of infection (in humans and animals) (EFSA, 2017). More specifically, HEV3 has been widely discovered from wild boar livers in different Italian regions (mainly characterized by high wild boar numbers / km^2, areas with important historical hunting traditions with high related artisanal product consumptions, etc.) (Caruso *et al.,* 2015; Aprea *et al.*, 2018; Montone *et al.*, 2019; Bonardi *et al.*, 2020).

For this reason, in the present study, wild boar liver samples collected in Ascoli Piceno province during the hunting season 2019/2020 were screened through nested RT-PCR assay, then resulting in the identification of few samples positive for HEV3 subtype c.

Materials and Methods

In the present study, a total of 156 wild boar livers (*Sus scrofa*) were collected during the hunting season 2019/2020 in the province of Ascoli Piceno, Marche region. This province is characterized by a rooted hunting tradition with special regard to the wild boar species. The hunting areas are characterized by low pig farm density, small rural communities (with low domestic swine numbers in extensive farming systems for familial consumption), and low anthropization.

Each sample was identified reporting specific information, as illustrated in Table 1. In accordance with the European Regulations (EU Reg. 853/2004; 625/2017; 624/2019) and Regional Marche Law (3/2012), all animals received *ante* and *post mortem* evaluation by the law-designed figures (*i.e.*, Veterinary Sanitary Authorities), and *Trichinella* spp. detection screening (EU Reg. 1375/2015).

All liver samples (aliquots: 25 grams) were transported under refrigerated conditions and stored at – 80°C until their processing, at the laboratories of Food Inspection Department, University of Teramo, where they were molecularly screened for HEV RNA detection.

Laboratory activities included three steps: sample homogenization, RNA nucleic acid extraction (through the TRIzol LS method, Invitrogen, Ltd, Paisley, UK), and nested RT-PCR assay. For reverse and nested PCR reactions were used primers targeting specific portions of ORF1 and ORF2 genes (Wang *et al.*, 1999), as reported in Table 2. RT-PCR and nested PCR were performed in a total volume of 25 µl, by using Qiagen® OneStep RT-PCR Kit and Green Master Mix Promega®, respectively. In the first reaction, 2.5 µl of extracted templates (RNA) was added to the mix, and successively 1 µl of the obtained products (cDNA) was used to perform nested PCR. Nested PCR products were loaded on the agarose gel for the electrophoresis assay. The obtained nitid amplicons positions were compared to a specific DNA ladder (Genetics, FastGene 50 bp or 100 bp DNA Marker) (Figure 1).

Successively, amplicons were purified by using Qiagen QIAquick® PCR Purification Kit and sequenced by BioFab Research (Rome). Nucleotide similarity was performed by using BLAST system (http://www.ncbi.nlm.nih.gov/genbank/index.html).

The datasets of this study were correlated through the chi-square statistic value (with Yates correction) applied where appropriated, using XLSTAT 2014 software (Renmond, Washington, USA). Correlations between two parameters were determined by Pearson correlation (r) coefficient analysis. Findings of P< 0.05 were considered significant.

Results

In this study, 8 out of 156 (5.12%, 95% CI: 1.6-8.4%) wild boar livers resulted positive for HEV RNA detection through the usage of specific primers targeting regions belonging to ORF1 and ORF2 genes.

In Figure 1, it is possible to observe nitid amplicons related to the ORF2 nested PCR products (145 bp).

Basing positive rates data on the age of infected subjects, 5 out of 8 (62.5% - 95% CI: 29.0-96.0%) positive livers were adult animals and 3 out of 8 (37.5% - 95% CI: 4.0-71.0%) pre-puberal/puberal subjects (Table 1), and none of positive female animals resulted pregnant (positive animal gender is reported in Table 3). There was also a significant relationship among age groups represented by a chi-square statistic value (with Yates correction) 21.8832. The p-value was < 0.00001. Significant at p<0.05.

Positive animals were hunted in close geographical areas (range of 20-40 km) in the province of Ascoli Piceno, as reported in Table 3.

All sequenced positive samples were analyzed through the BLAST system (http://www.ncbi.nlm.nih.gov/genbank/index.html), confirming that all identified HEV strains belonged to genotype 3. They showed high nucleotide similarity (96% nt identity) with HEV3 subtype c identified in wastewaters, in Abruzzo region (GenBank accession number: MN062008.1 and MN062007.1) by Di Profio *et al.,* (2019).

Table 1. Hunted wild boars' livers screened for HEV RNA detection.

Sex	Estimated age*
94 F	24 P 70 A
62 M	22 P 40 A

F: Female; M: Male; HEV: hepatitis E virus; RNA: Ribonucleic acid. Age estimation was based on extent of tooth eruption and weight. From this estimation animals were classified as: P = puberal: weight between 15-40 Kg and estimated age between 13-24 months. A = adult: weight > 40 Kg and estimated age between 24-48 months.

Table 2. Primers used for HEV RNA detection through nested RT-PCR assay.

Gene	Primer	Oligonucleotide sequence (5'↘3')	Annealing (T°C)	Amplicon's size	Reference
ORF1	ConsORF1-s1	CTGGCATYACTACTGCYATTGAGC	48°C	418 bp	Wang *et al*., 1999
	ConsORF1-a1	CCATCRARRCAGTAAGTGCGGTC			
	ConsORF1-s2	CTGCCYTKGCGAATGCTGTGG	50°C	287 bp	
	ConsORF1-a2	GGCAGWRTACCARCGCTGAACATC			
ORF2	ConsORF2-s1	GACAGAATTRATTTCGTCGGCTGG	48°C	197 bp	
	ConsORF2-a1	CTTGTTCRTGYTGGTTRTCATAATC			
	ConsORF2-s2	GTYGTCTCRGCCAATGGCGAGC	50°C	145 bp	
	ConsORF2-a2	GTTCRTGYTGGTTRTCATAATCCTG			

ORF, Overlapping Open Reading Frame; HEV, hepatitis E virus; RNA, ribonucleic acid. Nucleotides = Y: T or C; R: A or G; K: G or T; W: A or T.

Discussion

In specific Italian regions (Abruzzo, Campania, Lazio, Marche, Toscana, etc.), wild boars have become a relevant food source (liver and meat products), and the consumption of wild boar products increases every year (Aprea *et al.*, 2018).

Ingestion of contaminated (raw or undercooked) liver or meat products is the basis from which HEV infects humans, and especially traditional home-made foodstuffs permit HEV persistence in specific geographical areas (Spada *et al*., 2018).

Depending on geographical and epidemiological aspects, previous studies have identified the same genotype (HEV3), but different subtypes from wild boar liver samples in specific Italian regions: 3a in Lazio (Di Pasquale *et al.*, 2019), 3c in Abruzzo (Aprea *et al.*, 2018), 3e and f in Toscana (Caruso *et al.*, 2015), 3a in Emilia-Romagna (Parma) and 3f in Lombardia (Sondrio) (Arnaboldi *et al.*, 2021).

According to the pathogenesis of HEV in swine, liver samples can be useful in HEV RNA molecular screenings to identify infected subjects (wild boars) avoiding their entrance in the game meat food chains.

In our investigation, genotype 3c was involved in the positive cases. 5.12% (5.12% - 95% CI: 1.6-8.4%) liver samples presented HEV RNA similarly to the value 3.7% (HEV 3e and 3f), 1.9% (HEV 3e, c, f), and 1.2% (HEV 3f) reported in Toscana (Caruso *et al.*, 2015), in Liguria (Serracca *et al.*, 2015), and Lombardy (Sondrio province) (Arnaboldi *et al.*, 2021) regions, respectively. On the other hand, our results were lower than 31.5% and 23.8% (HEV 3a) in Emilia-Romagna (Parma province) (Bonardi *et al.*, 2020; Arnaboldi *et al.*, 2021), 13.7% (HEV 3c) in Abruzzo region (Aprea *et al.*, 2018; Lo Presti *et al.*, 2020), and 16.3% (HEV 3a, c, f, and l) in Lazio region (Di Pasquale *et al.*, 2019).

These disparities, regarding circulating subtypes and positivity rates, are strongly influenced by the environmental aspects, investigated geographical areas, and wild animals' number that compose a specific population representing HEV reservoirs for other mammalian species (free range pig farms; humans, etc.) due to the sharing of the same habitat (Spancerniene *et al.*, 2018; Lo Presti *et al.,* 2020).

HEV prevalence, obtained in this qualitative preliminary study, finds explanation from any geographical characteristics of the screened area: low wild boar density (2,5 subjects / 100 ha), low swine intensive farm density, low anthropization level (with a high ecosystem conservation), and low wild ruminant density (<1 subject / 100 ha).

In this case, it is possible to suppose that HEV infection can be occurred through the direct contact with any free-range pigs or from contaminated area fertilized with contaminated pig manure (due to HEV structural characteristics which guarantee long environmental persistence [Andraud *et al.*, 2013]) commonly used in agriculture (improved also by the urbanization phenomenon). In fact, in the screened province, there are small communities that traditionally breed one or two swine for domestic consumption as extensive farms.

Following the above-mentioned considerations, our positive cases find a similar prevalence to those ones discovered by Arnaboldi *et al.* (2021) in Sondrio province:

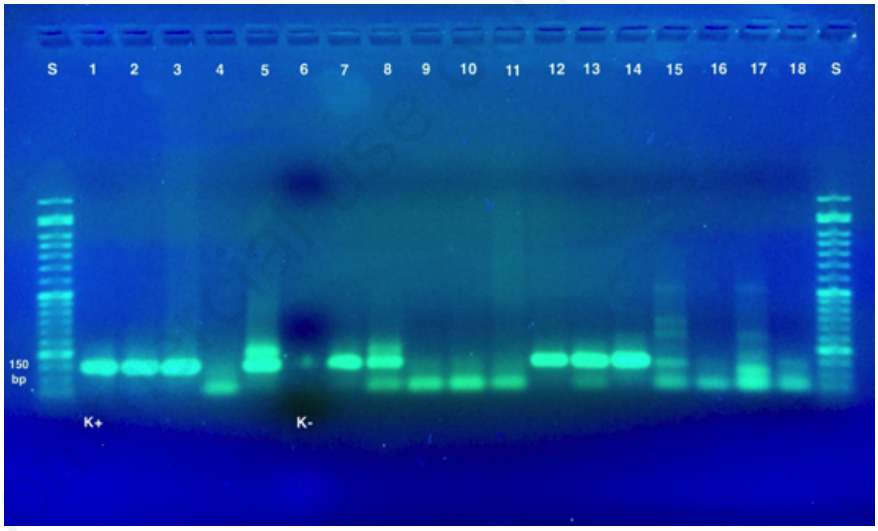

Figure 1. Electrophoresis gel (agarose 2%) in which it is possible to observe nitid positive amplicons: nested PCR products (145 bp): ORF2 gene. Wells loading: S = DNA ladder 50 bp (Genetics® FastGene 50 bp DNA Marker) loaded into the first and last wells. Each line corresponds to 50 bp. 1 = K+ / Positive control (ATCC® VR-3258SD RNA fragment). 6 = K- / Negative control. 2, 3, 5, 7, 8, 12, 13, 14 = Positive samples (samples ID and respective information are reported in Table 3). 4, 9, 10, 11, 15, 16, 17, 18 = Negative samples.

Table 3. Positive animals to the molecular screening for HEV RNA detection.

Samples ID	Sex of positive animals	Estimated age*	Geographical localization
2	F	A	Vena Piccola (AP)
3	M	P	Rotella (AP)
5	F	A	Vena Piccola (AP)
7	F	A	Roccafluvione (AP)
8	F	A	Venarotta (AP)
12	F	A	Vena Piccola (AP)
13	M	P	Venarotta (AP)
14	F	P	Roccafluvione (AP)

F: Female; M: Male. Age estimation was based on extent of tooth eruption and weight. *From this estimation animals were classified as: P = pre and puberal: weight between 15-40 Kg and estimated age between 13-24 months. A = adult: weight > 40 Kg and estimated age between 24-48 months.

especially for low wild fauna density and for any common ecological and environmental characteristics of the studied area.

Our scientific hypothesis, conducted in this preliminary study, will be further enriched with serological and biomolecular screenings of free-range pigs and wild ruminants, as possible other actors involved in the viral life cycle, which were never screened before.

There are also two other important aspects that must be considered: age and gender of positive subjects.

Conversely to our results (62.5% 5/8 positive livers belonged to adult animals and 37.5% 3/8 pre-puberal/puberal subjects), Di Pasquale *et al.* (2019) found specular positivity rates: 55.5% (30/54 positive animals) in juveniles, 29.6% (16/54) in sub adults (classified as pre/puberal subjects), and 14.9% (8/54) in adults. Similar data reported by Di Pasquale *et al.* (2019), were also observed by other research groups (Martinelli *et al.,* 2015; Bonardi *et al.,* 2020). These differences can be explained by sample collection; indeed, in the hunting season reported in our study, pre-puberal/puberal and adult subjects were mostly collected, whereas nonjuvenile animal was included in the sampling.

The results of this study confirm that HEV is an autochthon pathogen that survives in regional environments with a strong hunting tradition and related food consumption and wild boar could likely represent a strategical wild reservoir (Szabo *et al.*, 2015). In particular, among the different existing genotypes, HEV3 is likely the most represented, as supported by its frequent detection also in HEV-foodborne cases of infection in Central Italy (Spada *et al.*, 2018). Luckily, genotype 3 is generally responsible for asymptomatic conditions and only sporadically determines severe symptoms that require hospitalization. Nevertheless, it should be considered by Public Sanitary Authorities that have the important role to guarantee safe food products for the final consumers.

Veterinary official inspection activities could benefit from molecular biology analyses performed in laboratory which can provide precious information regarding the safety about game products for all consumers and can support the accomplishment of epidemiological surveys on the environmental circulation of certain HEV genotypes and subtypes.

This approach is necessary due to the increasing of wild boars' food chains in any national areas and more specifically, in Central Italy, where regional game food (uncooked or undercooked) productions and consumptions could be crucial for the infection for humans, highlighting the necessity to improve surveillance screenings.

Conclusions

HEV has become an important microbiological factor that require more attention by Sanitary Authorities. Its presence poses questions about repercussions on food safety, game meat food production chain and economic aspects.

HEV represents a crucial public health issue that has also repercussions on productive activities, therefore this condition requires an implementation of preventive measures, leading to practical applicability of preventive medicine as mean of One-Health approach.

Hepatic aliquots screenings of hunted wild boars, through molecular biology assays, can provide important information that permit to monitor the viral circulation.

An interdisciplinary perspective which includes environmental aspects directly correlated to animal and human health is auspicated.

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