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Presence of Hepatitis E Virus in a RED Deer (Cervus elaphus) Population in Central Italy

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5 **Presence of hepatitis E virus (HEV) in a red deer (*Cervus elaphus*) population in central Italy**

6

7 Di Bartolo I.[1*§], Ponterio E.[1*], Angeloni G.[1], Morandi F.[3], Ostanello F.[2], Nicoloso S.[4], Ruggeri
8 F.M.[1]

9

10 [1] Istituto Superiore di Sanità, Dep. of Veterinary Public Health and Food Safety, Rome, Italy

11 [2] University of Bologna, Dep. of Veterinary Medical Sciences, Ozzano dell'Emilia (BO), Italy

12 [3] Monti Sibillini National Park, Visso (MC), Italy

13 [4] DREAm Italia, Arezzo, Italy

14 *Equally contributed to the paper

15

16

17 [§] Corresponding author:

18 Ilaria Di Bartolo
19 Istituto Superiore di Sanità
20 Department of Veterinary Public Health and Food Safety
21 Viale Regina Elena 299
22 00161 Rome
23 Tel.: +39 06 4990 2787; fax: +39 06 4938 7101.
24 E-mail address: ilaria.dibartolo@iss.it

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26 **Running Title: Hepatitis E virus in deer, in Italy.**

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Summary

Hepatitis E is an acute human disease caused by the Hepatitis E virus ~~RNA virus~~ (HEV). In addition to humans, HEV has been detected in several animal species and is recognized as a zoonotic pathogen. Pigs, wild boar and deer can be reservoir. In this study, we evaluated HEV prevalence in a free-living red deer (*Cervus elaphus*) population in central Italy by detecting virus-specific antibodies and RNA in sera. A total of thirty-five out of 251 red deer sera were positive for anti-HEV IgG. HEV RNA was detected in 10 of 91 sera selected randomly. Two genomic fragments targeted by diagnostic PCRs in the capsid region were sequenced, both matching with HEV genotype 3 virus. Overall results also confirmed the occurrence of HEV infection ~~also~~ in deer in Italy.

Key words: Hepatitis E, zoonoses, red deer, anti-HEV IgG, genotype 3.

Introduction

The hepatitis E virus (HEV) is a small RNA virus belonging to the family *Hepeviridae* which includes viruses causing hepatitis in man, and provisional classified in the genus *Orthohepevirus* species *Orthohepevirus* A (Smith et al., 2014). In the general human population, the disease is usually self-limiting with a mortality rate below 1%, but chronic infections have been observed particularly in transplanted patients (Scobie et al., 2013). The disease was firstly considered endemic only in developing countries, but an increasing number of sporadic cases and small outbreaks have been reported in industrialized areas, including Europe, where the disease is now recognized as a significant emerging threat to public health (Kamar et al., 2012, Ruggeri et al., 2013).

HEV is a human or zoonotic pathogen that may involve domestic and wild pigs (and other species such as deer and rabbits) as reservoir (Pavio et al., 2010, Forgach et al., 2010, Izopet et al., 2012). Mammalian HEV strains are classified in four genotypes, all of which infect humans. Genotypes 3 and 4 are zoonotic, and closely related swine and human HEV sequences have been described worldwide (Meng, 2013). Genotype 3 HEV strains routinely infect swine and occasionally other animal species, (such as deer and wild boar. Cases of human hepatitis E caused by zoonotic genotypes 3 and 4 have been attributed to consumption of raw or undercooked meat and liver from pork, wild boar and deer (Pavio et al., 2010, Colson et al., 2010). In Italy, the presence of genotype 3 HEV was described in pigs and wild boar (Di Bartolo et al., 2008, Martelli et al., 2008, Caruso et al., 2014), in food products derived from swine (Di Bartolo et al., 2012, Di Bartolo, 2014), and in sporadic human cases (La Rosa et al., 2011, Romano et al., 2011). More recently a small outbreak in humans was associated with genotype 4 HEV infection (Garbuglia et al., 2013), and this genotype was also detected in pigs farmed in Northern Italy (Monne et al., 2014).

The first evidence of possible zoonotic transmission of HEV from wildlife animals derives from cases of human HEV infection related to consumption of sika deer (*Cervus nippon*) and wild boar meat (Tei et al., 2003, Sonoda et al., 2004). In addition to domestic animals, wild species (wild

boar, deer, wild rat, mink, moose) living within or at margin of the same areas might also act as a source or reservoir of novel HEV strains eventually infecting man (Johne et al., 2014, Ruggeri et al., 2013). Information on wild animal strains is still poor and the possible contribution of other mammals as HEV reservoir is unknown.

In Italy, data on HEV spread are available on farmed pigs and wild boar (Di Bartolo et al., 2008, Martinelli et al., 2013), but no survey of HEV infection in deer had been conducted previously.

In this study, we collected serum samples from wild deer and investigated the presence of anti-HEV IgG antibodies by an in-house Enzyme Linked Immunosorbement Assay (ELISA) using the recombinant capsid protein of a swine genotype 3 strain as antigen (Ponterio et al., 2014). We also investigated HEV RNA in serum samples from wild deer, by Real-Time Reverse Transcription (RT) PCR.

Materials and methods

Sampling

During three hunting seasons (2007–2008, 51 sera; 2008–2009, 103 sera and 2009–2010, 97 sera) in the province of Pistoia (44°00' N, 11°00'E; 80–1,200 m altitude) two hundred fifty one deer sera were collected between October and January.

The red deer (*Cervus elaphus*) individuals sampled belonged to a wild population living in the Apennins mountain ridge (44°06' N, 11°00' E) between Tuscany and Emilia-Romagna regions (Bologna, Pistoia, Prato and Florence provinces constitute the deer areal of the Central Apennins Tosco-Emiliano-Romagnolo). The deer population in this area is estimated to be 3,800 individuals (Nicoloso, 2010) and is managed by an Interregional Technical Commission. In this area, deer herds are thinned every year by hunting, as planned by the local authority. A set of rules defines if, where and at what times hunting can take place.

ELISA based on ORF2 capsid protein for IgG detection in red deer

To evaluate the presence of IgG antibodies against HEV in sera, an ELISA test described previously was used (Ponterio et al., 2014), with few modifications. This test is based on a recombinant capsid protein from an Italian swine genotype 3 HEV, expressed using the baculovirus recombinant system. Briefly, the partial purified capsid protein was used to coat 96-well microplates (Nunc MaxiSorp® plate, Thermo Scientific, Illkirch Cedex, France). After incubation overnight, the unbound antigen was removed and the red deer sera were added at a dilution of 1:200, in triplicate. The same test was conducted against a heterologous antigen (bovine norovirus VLPs (Zakhour et al., 2010), data not shown), as a negative control test. The ELISA microplate wells were incubated with a peroxidase conjugated rabbit anti-deer IgG antibody (1:500) (KPL, Guildford, UK). The optical density (OD) was measured at 450nm. The cut off was determined as the OD mean value + 1 standard deviation (SD), calculated on 251 examined red deer sera. All assays included a positive deer serum (kindly provided by Rutjes SA, Laboratory for Zoonoses and Environmental Microbiology, RIVM, Bilthoven, NL).

HEV-RNA detection by Real Time RT-PCR

Total RNA was extracted from 160 µl of sera (~~no.~~ N = 91) using the Qiamp-Viral RNA extraction mini kit (Qiagen, Hilden, Germany), following the manufacturer's instruction, and was eluted in 60 µl of diethylpyrocarbonate (DEPC) water. Detection of HEV was performed by duplex one-step Real-Time ~~reverse transcription (~~RT-PCR targeting both HEV and an internal amplification control (IAC), as previously described (Di Bartolo et al., 2012). All experiments included negative and positive controls, with and without IACs. For interpretation of results, if the observed IAC Ct value was as expected and the cycle threshold (Ct) value for HEV was not detectable or was ≥40, the sample was considered to be negative (Di Bartolo, 2014). The Real-Time RT-PCRs were run in Statagene MX3005P QPCR system (Agilent, Santa Clara, USA).

Conventional RT-PCR for HEV genome amplification and sequence analyses

Samples positive by Real Time RT-PCR were further analyzed by conventional two-step RT-PCR. Reverse transcription (RT) was performed using random hexamer primers at 42°C for 1 h with

119 GoScript reverse transcriptase (Promega Italia, Milan, Italy), and was followed by PCR and nested
120 PCR with primers 3156–3157 (first run) and 3158–3159 (nested-PCR) (Huang et al., 2002), which
121 amplified y a 320 bp region of the capsid protein gene (ORF2). A second nested RT-PCR using
122 primers HEVORF1con-s1/HEVORF1con-a1 (first PCR) and HEVORF1con-s2/HEVORF1con-a2
123 (nested-PCR), which anneal in the ORF1 and amplify a fragment of 287 bp in the
124 Methyltransferase (Erker et al., 1999), was also performed to further confirm positive samples. All
125 steps from RNA extraction to nested-PCR included negative controls.
126 The HEV positive samples were sequenced by the BioFab Research srl (Rome, Italy). Sequences
127 were aligned with known HEV sequences retrieved from GenBank and analyzed using the
128 Bionumerics software packages v6.0 (Applied Maths, Kortrijk, Belgium), for strain genotype
129 identification and for comparison with known sequences. A phylogenetic tree was drawn using the
130 Neighbor-Joining method with the Kimura 2 correction factor, using the Molecular Evolutionary
131 Genetic Analysis (MEGA 6) software (Tamura et al., 2013). Confidence values at the nodes were
132 obtained by performing 500 bootstrap analyses.
133 Sequences were submitted to GenBank under the accession nos. KF706392-93.
134 **Statistical analysis**
135 Two-hundred fifty one sera were collected from red deer (*Cervus elaphus*), 101 of which were
136 males and 150 females, aged from 7 months to 14 years. The ages of the red deer were assessed
137 from tooth sections, and were then grouped into age classes of biological meaning (according to
138 reproductive and social status) as follows: class 0 (females and males up to 11 month old; no. N =
139 79), class 1 (females and males up to 23 month old; no. N = 27), class 2 (females over 2 years and
140 males up to 4 years old; no. N= 130), class 3 (males over 4 years; no. N = 15). The first three
141 classes were considered as family groups while class 3 included only the solitary males that came
142 close to family groups only during the breeding season. Prevalence for HEV seropositive and for
143 HEV RNA positive was statistically analyzed by sex, age-classes, and social groups by chi square
144 test or Fisher's exact test.

145

Results

146 A total of 35 out of 251 sera tested (13.9%; 95% CI: 9.9-18.9) were positive for anti-HEV IgG
147
148 antibodies by ELISA (Table 1). The OD values of anti-HEV IgG in deer sera ranged from 0.001 to
149 0.808 (mean: 0.137; SD: 0.143).
150 No significant sex-related differences in seroprevalence were detected (males: 10.9%; females:
151 16.0%), but differences were observed between the four age classes (described in materials and
152 methods), showing a significantly ($P<0.05$) higher seroprevalence in young animals (Table 1).
153 Seropositive animals were identified only in family groups (14.8%).
154 To identify recent HEV infections, the presence of HEV RNA was investigated by Real-Time RT-
155 PCR, in 91 sera selected randomly stratified by sex, age and hunting season. HEV RNA was
156 detected in 10 out of 91 sera (Table 2), showing a prevalence of 11% (95% CI: 5.4-19.3). Not
157 significant differences in prevalence were detected in relation to sex (males 14.6% *vs.* females
158 8.0%), whereas a statistically significant difference was observed in the prevalence of viremia
159 among age groups, which was 8.8%, 0.0%, 7.9% and 50.0% in class 0, 1, 2 and 3 respectively;
160 which represent increasing animals age (as described in materials and methods). Differences were
161 also observed between the two social groups investigated, showing a significantly ($P<0.01$) higher
162 prevalence in solitary males (50.0%) than in family groups (7.2%).
163 The 10 samples positive by Real-Time RT-PCR were also tested by two conventional HEV RT-
164 PCR assays in order to determine the genotype of HEV involved. Only two out of ten positive
165 samples resulted positive by RT-PCR, and only one of them was positive by both ORF1 and ORF2
166 tests. The samples that were positive also by conventional RT-PCR gave the lowest Ct values (36-
167 37) in the Real Time RT-PCR, that is considered an assay with higher sensitivity than conventional
168 RT-PCR (Gyarmati et al., 2007). Sequence analysis was possible only for the two ORF2 PCR
169 products, which showed a 96.7% nucleotide identity to each other. The phylogenetic tree (Fig. 1)
170 drawn using the two sequences (250 bp; Acc. no. KF7006392-93) confirmed a close correlation

171 between the Italian deer HEV strains with other reported human, swine and deer strains belonging
172 to genotype 3 subtype ~~e.~~ ~~including~~ Moreover, the Italian deer strains showed a nucleotide identity of
173 90% and 91.5% ~~including~~ with human (Romano et al., 2011) (Acc. no. HM446628) and swine
174 (Acc. no. KJ174071) strains identified previously in Italy, which showed a nucleotide identity of
175 90% and 91.5%, respectively.
176

177 **Discussion**

178 This study represents the first report on the presence of HEV in wild red deer in Italy, and confirms
179 that genotype 3 HEV has circulated in this animal population at least since the 2007-2008 hunting
180 season, when the first HEV-positive animal in this study was sampled. Our findings of 13.9% HEV
181 seropositive and 11% viremic animals are similar to those obtained in a previous study conducted in
182 Spain in 2000-2009, showing 13.6% and 10.4% of HEV seropositive and viremic animals,
183 respectively (Boadella et al., 2010), and slightly higher than the 5% seroprevalence obtained in The
184 Netherlands in 2006-2008 (Rutjes et al., 2010). Previous studies conducted in areas adjacent to
185 those examined in this study showed ~~that~~ a 25% prevalence of HEV RNA in bile samples collected
186 from wild boars ~~in central Italy~~ (Martelli et al., 2008), sharing the same habitat with deer enrolled in
187 this study. Another study conducted in Italy, reported a 10.2% antibody seroprevalence among wild
188 boars from an area farther in the North of the country (Martinelli et al., 2013), in line with results
189 obtained in other European countries (Carpentier et al., 2012, Rutjes et al., 2010).
190 The anti-HEV seroprevalence in both wild species (13.9% for deer in this study and 10.2% for wild
191 boar (Martinelli et al., 2013)) is significantly lower than in farmed pigs in northern Italy, where up
192 to 92.6% of 9–10 month old animals were shown to have anti-HEV antibodies (Di Bartolo et al.,
193 2011). The lower mean antibody seroprevalence observed in deer in Italy might be due to the lower
194 density of wildlife deer populations compared to farmed swine, since infection is transmitted
195 through the oral fecal route and the probability of infection is related to both density and closeness
196 of individuals (Di Bartolo et al., 2008). Although deer are social animals organized in herds, the

197 sexes stay mainly divided. Bucks travel alone or band together in bachelor's clubs for most of the
198 year. Until the breeding season, a mature buck almost never stays with a “doe unit”, or a group of
199 does and fawns. This social behavior might contribute to explain the remarkably higher prevalence
200 of HEV viremia observed among solitary males in this study (50%). In fact, males are normally
201 abated a few weeks after the end of the breeding period, during which they may have come in
202 contact with an HEV shedding family group or contaminated environment and might contract the
203 infection. It is well known that, in other species, viremia beginning 2 weeks post infection and can
204 last 2-4 weeks (Ruggeri et al., 2013).
205 Differently than obtained with swine sera (Ponterio et al., 2014), the ELISA test generated
206 relatively low OD values and a high background when applied to deer sera, as also reported
207 previously by others (Matsuura et al., 2007). Our ELISA values could be due to low anti-HEV
208 antibody levels in deer or to lower avidity of deer antibodies for the swine HEV antigen used.
209 However, the swine HEV ELISA was shown to detect anti-HEV antibodies in both human and
210 swine sera with comparable sensitivity (Ponterio et al., 2014). Beside technical problems, other
211 possible explanations could be a lower antibody titer due to a fading immunity in deer, which could
212 only be an occasional host of HEV genotype 3 strains, or the lack of frequent boosters due to a
213 reduced circulation of HEV in deer populations compared to swine.
214 Also the presence of HEV RNA in animals older than 12 months, suggesting that mature red deer
215 can be susceptible and immunologically naïf, might also be due to a reduced HEV circulation in the
216 habitat of this species. An alternative possible explanation is that older individuals could be re-
217 infected as a consequence of an incompletely protective immunity or short-lasting immunity.
218 The results of this study confirm that also red deer can be infected with HEV in nature, and may
219 represent an additional reservoir for HEV. In fact, the HEV genome sequences from deer were
220 similar to HEV sequences detected previously in both swine and humans. Since deer and wild boar
221 often share common pasture areas, they might transmit viral strains from each other and act as

222 vessels for the evolution of novel HEV strains, which may eventually be transmitted to man via
223 zoonotic and/or foodborne routes.

224 In Italy, deer is used to produce meat products such as fresh or dried sausages, and in several areas
225 controlled hunting is allowed to maintain the proper population size. During the three hunting
226 seasons regarded in this study, a mean of approximately 510 red deer per year were harvested in the
227 four provinces, and 777 hunters handled nearly 50 tons/year of raw deer meat (Nicoloso, 2010).
228 This could determine possible risks of infection through the consumption of undercooked meat,
229 contact with carcasses of infected animals or manipulation of live animals.

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Fig. 1. Phylogenetic tree based on the 290 bp sequences of the ORF2 fragment. Representative porcine, human~~s~~ and deer strains are included. Each entry includes host (Hu: human, Sw: swine, W~~wb~~: wild boar), accession number, and countries origin of strains. Strains detected in this study are in bold. Bootstrap values >70 are indicated.

350

351 Table 1

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Table 1. HEV seroprevalence according to deer age, sex and social group (251 animals)

	Group	HEV-seropositive /total*	Prevalence %	95% CI for prevalence	*p*
Age class	0 (females and males ≤ 11 month)	19/79	24.1	15.1-35.0	0.012
	1 (females and males ≤ 23 month)	3/27	11.1	3.9-28.1	
	2 (females > 2 yr and males ≤ 4 yr)	13/130	10.0	5.4-16.5	
	3 (males > 5 yr)	0/15	0.0	0.0-21.8	
	total	35/251	13.9	9.9-18.9	
Sex	Males	11/101	10.9	5.6-18.7	0.272
	Females	24/150	16.0	10.5-22.9	
	total	35/251	13.9	9.9-18.9	
Social group	Family (age classes 0-2)	35/236	14.8	10.6–20.0	0.140
	Solitary (males > 5 yr)	0/15	0.0	10.5-22.9	
	total	35/251	13.9	9.9-18.9	

* Number of deer sera tested positive by ELISA with the rORF2 antigen/total sera for each group

353

354 Table 2

Table 2. HEV RNA positivity according to deer age, sex and social group

	Group	HEV-RNA positive deer/total*	Prevalence %	95% CI for prevalence	*p*
Age class	0 (females and males ≤ 11 month)	3/34	8.8	1.9-23.7	0.002
	1 (females and males ≤ 23 month)	0/11	0.0	0.0-28.5	
	2 (females > 2 yr and males ≤ 4 yr)	3/38	7.9	1.7-21.4	
	3 (males > 5 yr)	4/8	50.0	15.7-84.3	
	total	10/91	11.0	5.4-19.3	
Sex	Males	6/41	14.6	5.6-29.2	0.337
	Females	4/50	8.0	2.2-19.2	
	total	10/91	11.0	5.4-19.3	
Social group	Family (age classes 0-2)	6/83	7.2	2.7-15.1	0.004
	Solitary (males > 2 yr)	4/8	50.0	15.7-84.3	
	total	10/91	11.0	5.4-19.3	

355 *Number of deer sera tested positive by Real-Time RT-PCR/ total sera tested for each group

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