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

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

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

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

68 boar, deer, wild rat, mink, moose) living within or at margin of the same areas might also act as a
69 source or reservoir of novel HEV strains eventually infecting man (Johne et al., 2014, Ruggeri et
70 al., 2013). Information on wild animal strains is still poor and the possible contribution of other
71 mammals as HEV reservoir is unknown.
72 In Italy, data on HEV spread are available on farmed pigs and wild boar (Di Bartolo et al., 2008,
73 Martinelli et al., 2013), but no survey of HEV infection in deer had been conducted previously.
74 In this study, we collected serum samples from wild deer and investigated the presence of anti-HEV
75 IgG antibodies by an in-house Enzyme Linked Immunosorbement Assay (ELISA) using the
76 recombinant capsid protein of a swine genotype 3 strain as antigen (Ponterio et al., 2014). We also
77 investigated HEV RNA in serum samples from wild deer, by Real-Time Reverse Transcription
78 (RT) PCR.
79
80 **Materials and methods**
81 **Sampling**
82 During three hunting seasons (2007–2008, 51 sera; 2008–2009, 103 sera and 2009–2010, 97 sera)
83 in the province of Pistoia (44°00’ N, 11°00’E; 80–1,200 m altitude) two hundred fifty one deer sera
84 were collected between October and January.
85 The red deer (*Cervus elaphus*) individuals sampled belonged to a wild population living in the
86 Apennins mountain ridge (44°06’ N, 11°00’ E) between Tuscany and Emilia-Romagna regions
87 (Bologna, Pistoia, Prato and Florence provinces constitute the deer areal of the Central Apennins
88 Tosco-Emiliano-Romagnolo). The deer population in this area is estimated to be 3,800 individuals
89 (Nicoloso, 2010) and is managed by an Interregional Technical Commission. In this area, deer
90 herds are thinned every year by hunting, as planned by the local authority. A set of rules defines if,
91 where and at what times hunting can take place.
92 **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

GoScript reverse transcriptase (Promega Italia, Milan, Italy), and was followed by PCR and nested PCR with primers 3156–3157 (first run) and 3158–3159 (nested-PCR) (Huang et al., 2002), which amplified ~~y~~ a 320 bp region of the capsid protein gene (ORF2). A second nested RT-PCR using primers HEVORF1con-s1/HEVORF1con-a1 (first PCR) and HEVORF1con-s2/HEVORF1con-a2 (nested-PCR), which anneal in the ORF1 and amplify a fragment of 287 bp –in the Methyltransferase (Erker et al., 1999), was also performed to further confirm positive samples. All steps from RNA extraction to nested-PCR included negative controls.

The HEV positive samples were sequenced by the BioFab Research srl (Rome, Italy). Sequences were aligned with known HEV sequences retrieved from GenBank and analyzed using the Bionumerics software packages v6.0 (Applied Maths, Kortrijk, Belgium), for strain genotype identification and for comparison with known sequences. A phylogenetic tree was drawn using the Neighbor-Joining method with the Kimura 2 correction factor, using the Molecular Evolutionary Genetic Analysis (MEGA 6) software (Tamura et al., 2013). Confidence values at the nodes were obtained by performing 500 bootstrap analyses.

Sequences were submitted to GenBank under the accession nos. KF706392-93.

Statistical analysis

Two-hundred fifty one sera were collected from red deer (*Cervus elaphus*), 101 of which were males and 150 females, aged from 7 months to 14 years. The ages of the red deer were assessed from tooth sections, and were then grouped into age classes of biological meaning (according to reproductive and social status) as follows: class 0 (females and males up to 11 month old; ~~no.~~ N = 79), class 1 (females and males up to 23 month old; ~~no.~~ N = 27), class 2 (females over 2 years and males up to 4 years old; ~~no.~~ N= 130), class 3 (males over 4 years; ~~no.~~ N = 15). The first three classes were considered as family groups while class 3 included only the solitary males that came close to family groups only during the breeding season. Prevalence for HEV seropositive and for HEV RNA positive was statistically analyzed by sex, age-classes, and social groups by chi square test or Fisher's exact test.

145

146 **Results**

147 A total of 35 out of 251 sera tested (13.9%; 95% CI: 9.9-18.9) were positive for anti-HEV IgG
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

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

Discussion

This study represents the first report on the presence of HEV in wild red deer in Italy, and confirms that genotype 3 HEV has circulated in this animal population at least since the 2007-2008 hunting season, when the first HEV-positive animal in this study was sampled. Our findings of 13.9% HEV seropositive and 11% viremic animals are similar to those obtained in a previous study conducted in Spain in 2000-2009, showing 13.6% and 10.4% of HEV seropositive and viremic animals, respectively (Boadella et al., 2010), and slightly higher than the 5% seroprevalence obtained in The Netherlands in 2006-2008 (Rutjes et al., 2010). Previous studies conducted in areas adjacent to those examined in this study showed ~~that~~ a 25% prevalence of HEV RNA in bile samples collected from wild boars ~~in central Italy~~ (Martelli et al., 2008), sharing the same habitat with deer enrolled in this study. Another study conducted in Italy, reported a 10.2% antibody seroprevalence among wild boars from an area farther in the North of the country (Martinelli et al., 2013), in line with results obtained in other European countries (Carpentier et al., 2012, Rutjes et al., 2010).

The anti-HEV seroprevalence in both wild species (13.9% for deer in this study and 10.2% for wild boar (Martinelli et al., 2013)) is significantly lower than in farmed pigs in northern Italy, where up to 92.6% of 9–10 month old animals were shown to have anti-HEV antibodies (Di Bartolo et al., 2011). The lower mean antibody seroprevalence observed in deer in Italy might be due to the lower density of wildlife deer populations compared to farmed swine, since infection is transmitted through the oral fecal route and the probability of infection is related to both density and closeness 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

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

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

References

Boadella, M., M. Casas, M. Martin, J. Vicente, J. Segales, J. de la Fuente and C. Gortazar, 2010: Increasing contact with hepatitis E virus in red deer, Spain. *Emerging infectious diseases,* 16, 1994-1996.

Carpentier, A., H. Chaussade, E. Rigaud, J. Rodriguez, C. Berthault, F. Boue, M. Tognon, A. Touze, N. Garcia-Bonnet, P. Choutet and P. Coursaget, 2012: High hepatitis E virus seroprevalence in forestry workers and in wild boars in France. *Journal of clinical microbiology,* 50, 2888-2893.

Caruso, C., P. Modesto, S. Bertolini, S. Peletto, P. L. Acutis, A. Dondo, S. Robetto, W. Mignone, R. Orusa, G. Ru and L. Masoero, 2014: Serological and virological survey of hepatitis E virus in wild boar populations in northwestern Italy: detection of HEV subtypes 3e and 3f. *Archives of virology*.

Colson, P., P. Borentain, B. Queyriaux, M. Kaba, V. Moal, P. Gallian, L. Heyries, D. Raoult and R. Gerolami, 2010: Pig liver sausage as a source of hepatitis E virus transmission to humans. *The Journal of infectious diseases,* 202, 825-834.

Di Bartolo, I., Angeloni, G., Ponterio, E., Ostanello, F., Ruggeri, F.M., 2014: Detection of hepatitis E virus in pork liver sausages. *International Journal of Food Microbiology*.

Di Bartolo, I., M. Diez-Valcarce, P. Vasickova, P. Kralik, M. Hernandez, G. Angeloni, F. Ostanello, M. Bouwknegt, D. Rodriguez-Lazaro, I. Pavlik and F. M. Ruggeri, 2012: Hepatitis E virus in pork production chain in Czech Republic, Italy, and Spain, 2010. *Emerging infectious diseases,* 18, 1282-1289.

Di Bartolo, I., F. Martelli, N. Inglese, M. Pourshaban, A. Caprioli, F. Ostanello and F. M. Ruggeri, 2008: Widespread diffusion of genotype 3 hepatitis E virus among farming swine in Northern Italy. *Veterinary microbiology,* 132, 47-55.

Di Bartolo, I., E. Ponterio, L. Castellini, F. Ostanello and F. M. Ruggeri, 2011: Viral and antibody HEV prevalence in swine at slaughterhouse in Italy. *Veterinary microbiology,* 149, 330-338.

Erker, J. C., S. M. Desai and I. K. Mushahwar, 1999: Rapid detection of Hepatitis E virus RNA by reverse transcription-polymerase chain reaction using universal oligonucleotide primers. *Journal of virological methods,* 81, 109-113.

Forgach, P., N. Nowotny, K. Erdelyi, A. Boncz, J. Zentai, G. Szucs, G. Reuter and T. Bakonyi, 2010: Detection of hepatitis E virus in samples of animal origin collected in Hungary. *Veterinary microbiology,* 143, 106-116.

Garbuglia, A. R., P. Scognamiglio, N. Petrosillo, C. M. Mastroianni, P. Sordillo, D. Gentile, P. La Scala, E. Girardi and M. R. Capobianchi, 2013: Hepatitis e virus genotype 4 outbreak, Italy, 2011. *Emerging infectious diseases,* 19, 110-114.

Gyarmati, P., N. Mohammed, H. Norder, J. Blomberg, S. Belak and F. Widen, 2007: Universal detection of hepatitis E virus by two real-time PCR assays: TaqMan and Primer-Probe Energy Transfer. *Journal of virological methods,* 146, 226-235.

Huang, F. F., G. Haqshenas, D. K. Guenette, P. G. Halbur, S. K. Schommer, F. W. Pierson, T. E. Toth and X. J. Meng, 2002: Detection by reverse transcription-PCR and genetic characterization of field isolates of swine hepatitis E virus from pigs in different geographic regions of the United States. *Journal of clinical microbiology,* 40, 1326-1332.

Izopet, J., M. Dubois, S. Bertagnoli, S. Lhomme, S. Marchandeau, S. Boucher, N. Kamar, F. Abravanel and J. L. Guerin, 2012: Hepatitis E virus strains in rabbits and evidence of a closely related strain in humans, France. *Emerging infectious diseases,* 18, 1274-1281.

Johne, R., P. Dremsek, J. Reetz, G. Heckel, M. Hess and R. G. Ulrich, 2014: Hepeviridae: An expanding family of vertebrate viruses. *Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases,* 27C, 212-229.

Kamar, N., R. Bendall, F. Legrand-Abravanel, N. S. Xia, S. Ijaz, J. Izopet and H. R. Dalton, 2012: Hepatitis E. *Lancet,* 379, 2477-2488.

La Rosa, G., M. Muscillo, V. S. Vennarucci, A. R. Garbuglia, P. La Scala and M. R. Capobianchi, 2011: Hepatitis E virus in Italy: molecular analysis of travel-related and autochthonous cases. *The Journal of general virology,* 92, 1617-1626.

Martelli, F., A. Caprioli, M. Zengarini, A. Marata, C. Fiegna, I. Di Bartolo, F. M. Ruggeri, M. Delogu and F. Ostanello, 2008: Detection of hepatitis E virus (HEV) in a demographic managed wild boar (Sus scrofa scrofa) population in Italy. *Veterinary microbiology,* 126, 74-81.

Martinelli, N., E. Pavoni, D. Filogari, N. Ferrari, M. Chiari, E. Canelli and G. Lombardi, 2013: Hepatitis E Virus in Wild Boar in the Central Northern Part of Italy. *Transbound Emerg Dis*.

Matsuura, Y., M. Suzuki, K. Yoshimatsu, J. Arikawa, I. Takashima, M. Yokoyama, H. Igota, K. Yamauchi, S. Ishida, D. Fukui, G. Bando, M. Kosuge, H. Tsunemitsu, C. Koshimoto, K. Sakae, M. Chikahira, S. Ogawa, T. Miyamura, N. Takeda and T. C. Li, 2007: Prevalence of antibody to hepatitis E virus among wild sika deer, Cervus nippon, in Japan. *Archives of virology,* 152, 1375-1381.

Meng, X. J., 2013: Zoonotic and foodborne transmission of hepatitis E virus. *Seminars in liver disease,* 33, 41-49.

Monne, I., L. Ceglie, D. I. M. G, A. Natale, S. Zamprogna, A. Morreale, E. Rampazzo, G. Cattoli and L. Bonfanti, 2014: Hepatitis E virus genotype 4 in a pig farm, Italy, 2013. *Epidemiology and infection*, 1-5.

Nicoloso, S., Viliani, M, Mattioli, S, Riga F., 2010: Analisi consultiva stagioni faunistico-venatorie dal 2005-06 al 2009-10 e programma annuale operativo di gestione del cervo 2010-2011. . Available at: http://agricoltura.regione.emilia-romagna.it/caccia/temi/ungulati/documenti-ungulati/acater-centrale/relazioni-consuntive-dal-2005-6-al-2009-10-e-programma-annuale-operativo-2010-2011/view

Pavio, N., X. J. Meng and C. Renou, 2010: Zoonotic hepatitis E: animal reservoirs and emerging risks. *Vet Res,* 41, 46.

Ponterio, E., I. Di Bartolo, G. Orru, M. Liciardi, F. Ostanello and F. M. Ruggeri, 2014: Detection of serum antibodies to hepatitis E virus in domestic pigs in Italy using a recombinant swine HEV capsid protein. *BMC veterinary research,* 10, 133.

Romano, L., S. Paladini, C. Tagliacarne, M. Canuti, S. Bianchi and A. R. Zanetti, 2011: Hepatitis E in Italy: a long-term prospective study. *Journal of hepatology,* 54, 34-40.

Ruggeri, F. M., I. Di Bartolo, E. Ponterio, G. Angeloni, M. Trevisani and F. Ostanello, 2013: Zoonotic transmission of hepatitis E virus in industrialized countries. *The new microbiologica,* 36, 331-344.

Rutjes, S. A., F. Lodder-Verschoor, W. J. Lodder, J. van der Giessen, H. Reesink, M. Bouwknegt and A. M. de Roda Husman, 2010: Seroprevalence and molecular detection of hepatitis E virus in wild boar and red deer in The Netherlands. *Journal of virological methods,* 168, 197-206.

Scobie, L., V. Padler-Karavani, S. Le Bas-Bernardet, C. Crossan, J. Blaha, M. Matouskova, R. D. Hector, E. Cozzi, B. Vanhove, B. Charreau, G. Blancho, L. Bourdais, M. Tallacchini, J. M. Ribes, H. Yu, X. Chen, J. Kracikova, L. Broz, J. Hejnar, P. Vesely, Y. Takeuchi, A. Varki and J. P. Soulillou, 2013: Long-Term IgG Response to Porcine Neu5Gc Antigens without Transmission of PERV in Burn Patients Treated with Porcine Skin Xenografts. *J Immunol,* 191, 2907-2915.

Smith, D. B., P. Simmonds, S. Jameel, S. U. Emerson, T. J. Harrison, X. J. Meng, H. Okamoto, W. H. Van der Poel and M. A. Purdy, 2014: Consensus proposals for classification of the family Hepeviridae. *The Journal of general virology,* 95, 2223-2232.

Sonoda, H., M. Abe, T. Sugimoto, Y. Sato, M. Bando, E. Fukui, H. Mizuo, M. Takahashi, T. Nishizawa and H. Okamoto, 2004: Prevalence of hepatitis E virus (HEV) Infection in wild boars and deer and genetic identification of a genotype 3 HEV from a boar in Japan. *Journal of clinical microbiology,* 42, 5371-5374.

Tamura, K., G. Stecher, D. Peterson, A. Filipski and S. Kumar, 2013: MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular biology and evolution,* 30, 2725-2729.

Tei, S., N. Kitajima, K. Takahashi and S. Mishiro, 2003: Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet,* 362, 371-373.

Zakhour, M., H. Maalouf, I. Di Bartolo, L. Haugarreau, F. S. Le Guyader, N. Ruvoen-Clouet, J. C. Le Saux, F. M. Ruggeri, M. Pommepuy and J. Le Pendu, 2010: Bovine norovirus: carbohydrate ligand, environmental contamination, and potential cross-species transmission via oysters. *Applied and environmental microbiology,* 76, 6404-6411.

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Fig. 1. Phylogenetic tree based on the 290 bp sequences of the ORF2 fragment. Representative porcine, humans and deer strains are included. Each entry includes host (Hu: human, Sw: swine, Wwb: 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

352

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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