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Detection of Hepatitis E virus (HEV) in a demographic managed wild boar population in Italy

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Abstract

Hepatitis E virus (HEV) is the causative agent of hepatitis E. Swine and human HEV strains are genetically related, suggesting the occurrence of zoonotic transmission. Recently, in Japan, cases of food-borne HEV transmission have been described in people after consuming raw or undercooked meat from wild boars or pigs. Although swine HEV strains have been detected in pig herds in many European countries, only minimal information is presently available about the circulation and the prevalence of HEV in wild boars in Europe. In this study, we investigated the presence of HEV in a demographic managed wild boar population in Italy. Detection of HEV RNA was accomplished using a nested reverse-transcription-polymerase chain reaction on bile samples from 88 shot animals. HEV RNA was detected in 22 out of 88 animals tested (25%). Phylogenetic analysis on the nucleotide sequences obtained from ten positive PCR products indicated that only one HEV strain was circulating in the wild boar population considered, and that this strain was closer to human and swine HEV strains circulating in Europe than to wild boar Japanese strains.

Keywords: Hepatitis E virus (HEV); wild boar; Nested-RT-PCR
1. Introduction

Hepatitis E is a human viral disease with clinical and morphological features of acute hepatitis. The infection represents an important public health concern in many developing countries, where it is primarily transmitted by fecal–oral route through contaminated water and food (Emerson and Purcell, 2003), and is often responsible for epidemic outbreaks. In most affected people the course of the disease is mild, except for pregnant women in which mortality rate can reach 20% (Aggarwal and Krawczynski, 2000).

The causative agent of the disease is the Hepatitis E virus (HEV), a small non-enveloped RNA virus classified as Hepevirus genus within the *Hepeviridae* family (Mayo, 2004). HEV isolates have been so far classified into four major genotypes. The majority of infections occurring in Asia and Africa are caused by genotype 1, whereas genotype 2 prevails in Mexico and Nigeria (Emerson and Purcell, 2003). In industrialized countries, where until few years ago the infection was considered non-endemic, only strains belonging to genotype 3 and 4 have been detected in persons without a history of recent travel to HEV endemic regions (Aggarwal and Krawczynski, 2000; Yoo et al., 2001; Emerson and Purcell, 2003). Genotype 3 prevails in USA and Europe, while genotype 4 is mainly distributed in China, Taiwan, Japan, Indonesia and Vietnam (Hsieh et al., 1999; Banks et al., 2004b; Zheng et al., 2006). Although hepatitis E is a sporadic disease in countries with good health-care conditions, the seroprevalence rate among healthy individuals can be rather high (Emerson and Purcell, 2003).
The first animal HEV strain was characterized in pigs in USA in 1997 (Meng et al., 1997). Since then, several other strains have been described in pigs worldwide, virtually all belonging to genotype 3 and 4 (Lu et al., 2006) except one genotype 1 strain recently detected in Cambodia (Caron et al., 2006). In particular, swine strains have been demonstrated to have a high sequence homologies to autochthonous human strains, suggesting that swine can represent a reservoir of the infection and that zoonotic transmission of HEV may play a relevant role in industrialized countries (Meng et al., 1998; Zanetti et al., 1999; Van Der Poel et al., 2001; Clemente-Casares et al., 2003; Buti et al., 2004). In this regard, several studies have reported that in people who work in contact with swine such as pig farmers, veterinarians and slaughterhouse workers, the HEV seroprevalence rate can be higher than that reported in normal control populations (Hsieh et al., 1999; Drobeniuc et al., 2001; Meng et al., 2002; Withers et al., 2002).

The first direct evidence of food-borne transmission of HEV to humans from animal meat was reported in Japan in 2003 (Tei et al., 2003), where hepatitis E cases occurred in association with the consumption of undercooked contaminated Sika deer meat. In this case, the HEV strain isolated from the patients was identical to that detected in the left-over contaminated deer meat (Tei et al., 2003). The possibility of foodborne zoonotic transmission of HEV is further supported by other studies in which the relation between consumption of meat or organs from pigs (Yazaki et al., 2003) or from wild boars (Matsuda et al., 2003; Sonoda et al., 2004; Takahashi et al., 2004; Tamada et al., 2004; Kitajima et al., 2004; Li...
et al., 2005; Masuda et al., 2005; Nishizawa et al., 2005) and the clinical disease
has been demonstrated. The disease can be now considered an emerging food-
borne zoonosis.

To date, studies to evaluate the presence of HEV in wild boars or wild pigs
have been conducted in Australia (Chandler et al., 1999) and Japan (Matsuda et
al., 2003; Yazaki et al., 2003; Sonoda et al., 2004; Takahashi et al., 2004; Tamada
et al., 2004; Li et al., 2005; Masuda et al., 2005; Nishizawa et al., 2005).  
However, the study in Australia was performed on wild hogs, while the Japanese
investigation concerned the 2 wild boars subspecies *Sus scrofa leucomyxtas* and
*Sus scrofa riukiuanus* (Watanobe et al., 1999) which are present in that country
but are phylogenetically different from European wild boars subspecies. To our
knowledge, only minimal information on the circulation and the prevalence of
HEV in European wild boars is presently available (de Deus et al., 2007b), despite
several reports have already confirmed a wide circulation of HEV among
domestic pigs in many European countries (Pina et al., 2000; Van Der Poel et al.,
2001; Clemente-Casares et al., 2003; Banks et al., 2004a; Fernandez-Barredo et
al., 2006; de Deus et al., 2007a; Caprioli et al., in press).

In this study we evaluated the prevalence of HEV in a wild boar population in
Northern Italy. Genetic characterization of identified wild boar strains was
performed and the sequences obtained were compared with existing sequences of
human, swine and wild boar origin.
2. Materials and methods

2.1. Wild boar population

Sampling was performed on a wild boar (Sus scrofa scrofa) population living in a Regional Park (Gessi Bolognesi, 48.15 Km²) located in the Emilia-Romagna Region (North-East of Italy). In 2001, the density of wild boars in the area was approximately 18 animals/Km². Since then, in agreement with the park regulation, a demographic control program was applied using a simultaneous technique of selective shooting and trapping with cages. A sub-population of tagged animals structured by age and sex was constantly maintained and monitored. During the period of the study, the density of the wild boar population was between 2 animals/Km² (pre-reproductive period) and 5.5 animals/Km² (post-reproductive period). The boar density was estimated by considering the number of culled animals and the frequency of captured and recaptured tagged individuals.

2.2. Sample collection

Eighty-eight apparently healthy wild boars shot between March and September 2006 were selected for the study. For each animal, age, sex, length of the body (from the tip of the snout to the base of the tail) and weight were evaluated and recorded. Animals were aged by the evaluation of tooth eruption and replacement patterns (Vicente et al., 2004); their ages ranged between 4 and 37 months, and most of them (83/88) were of over 6 months of age. Wild boars of this age were
deliberately selected because they represent those usually intended for human consumption. During the slaughtering process, a bile sample was withdrawn from each animal with a sterile syringe (used once and then discarded) through the gall-bladder wall, and stored at -80°C until processing.

2.3. RNA extraction and HEV RT-Nested-PCR

Each bile sample was diluted 1:10 in DEPC water. Total RNA was extracted from 140 µl of solution using a QiaAmp viral RNA kit (Qiagen, Hiden, Germany) according to the manufacturer’s instructions. RNA reverse transcription (RT) and first PCR reaction were conducted using a Superscript III One-step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California, USA) according to the manufacturer’s instructions. The RT-PCR reaction was conducted in a ICycler (Bio-Rad, Hemel Hempstead, UK) thermal cycler under the following conditions: 45°C for 30 minutes for RT, 94°C for 2 minutes for the initial cDNA denaturation, followed by 39 cycles of denaturation at 94°C for 1 minute, annealing at 49°C for 90 seconds, elongation at 72°C for 1 minute, and a final elongation at 72°C for 5 minutes. Nested PCR was conducted using a recombinant Taq DNA polymerase (Fermentas, Burlington, Ontario, Canada) according to the manufacturer’s instructions, following the subsequent thermal conditions: initial denaturation at 95°C for 3 minutes followed by 39 cycles of denaturation at 94°C for 45 seconds, annealing at 49°C for 1 minute, elongation at 72°C for 2 minutes, and a final elongation at 72°C for 7 minutes. For RT-PCR and Nested PCR, sets of degenerate primers, HEVORF2con-a1/HEVORF2con-S1 and
HEVORF2con-a2/HEVORF2con-S2, amplifying a 145 bp region of the HEV open reading frame 2 (ORF2) were used (Erker et al., 1999). At each stage of the reaction (extraction, first round and second round PCR amplification) a negative (DEPC water) and a positive control (swine HEV positive bile, kindly provided by Dr. Marga Martin, Universitat Autònoma di Barcelona) were used. To further minimize the possibility of cross-contamination, strict anti-contamination procedures, including the use of separate rooms, safety hoods, frequent discharging of gloves and accurate cleaning of surfaces and materials were implemented. Amplified products were visualized in a 2% agarose gel stained with ethidium bromide.

2.4. Sequencing and phylogenetic analysis

To further confirm the identity of the strains detected by Nested-PCR, a nucleotide sequence analysis was performed on 10 Nested-PCR positive products corresponding to a fraction of sampled animals of different age and sex classes. Nested-RT-PCR products of the expected size (145 bp) were excised from a 2% agarose gel, purified with a High Pure PCR Product Purification Kit (Roche, Indianapolis, USA), and sequenced using the PCR primers with the BigDye Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Perkin Elmer, Applied Biosystems, Foster City, CA), in an automated sequencer (ABI Prism 310 DNA sequencer, Applied Biosystems, Foster City, CA). The sequences obtained were assembled and aligned with other swine, human and wild boar HEV sequences present in NCBI GenBank (http://www.ncbi.nlm.nih.gov/), using the
DNASIS Max software (Hitachi Software Engineering Company, Alameda, CA, USA) and an avian HEV (GenBank accession no. AY535004) as out-group. The HEV wild boar sequences used were all Japanese (GenBank accession no.: DQ079630; AB189070; AB222184), because no nucleotide or deduced amino acid sequences of European wild boar strains were available in NCBI GenBank. Dendrogram was drawn with the Bionumerics software packages (Applied Maths, Kortrijk, Belgium) using Unweighted Pair Group Method, with Arithmetic averages (UPGMA). GenBank accession numbers for HEV genome sequences investigated in this study are as follows: i. Italian wild boar HEV strain: wbITBO06/9, EF681108; ii. Italian swine HEV strains: MO/9_3/06/IT, EF681107; MO/36_4/06/IT, EF682083; HEVBO/01, EF681109; HEVPI/01, EF681110; iii. Italian human HEV strain: AF110390; iv. European swine HEV strain: AF336292; v. European human HEV strains: AY940427.1 and DQ200292; vi. European sewage strain: AF490994; vii. US swine strain: AY575857.

2.5. Statistical analysis
To identify a possible correlation between the HEV prevalence and the age of animals, wild boars were subdivided in three categories (< 12; 12-24; > 24 months of age) as previously reported (Vicente et al., 2004).
To evaluate the possible effects of the infection on the biometric characteristics of the animals, weight and body length of the HEV positive animals were compared to those of the negative animals of the same age and sex.
Kolmogorov – Smirnov test for goodness of adaptation was used to verify distribution normality. On the basis of the results of Kolmogorov – Smirnov test, Student’s $t$ test was used to compare quantitative data. Categorical data were analyzed with chi-square test. Data were analyzed with the SPSS software for Windows 12.0 (SPSS Inc., Chicago, USA).

Prevalence of HEV by age and sex classes was calculated with a 95% confidence interval (CI).

### 3. Results

HEV genome was detected in 22 of the 88 bile samples tested (25%, 95% CI 18.6-32.6). Prevalence rates determined for the whole population and for animals of different age and sex classes are reported in Table 1. No statistically significant differences ($P>0.05$) in the HEV prevalence were detected between sex and age classes. Biometric characteristics (weight and length of the body) of the infected animals were not statistically different from those of the non-infected animals within the same age and sex classes.

The sequence alignment of the 10 Nested-PCR positive samples analyzed demonstrated that all samples contained an identical HEV sequence. As shown in the dendrogram with the analyzed sequences (Fig. 1), the Italian wild boar strain sequence (wbITBO06) belonged to genotype 3, as other swine and human European indigenous HEV strains. In particular, the Italian wild boar strain was related (92% identity) to a sewage HEV strain from an industrialized area of Spain (AF490994) (Clemente-Casares et al., 2003), to a swine strain (AF336292)
detected in The Netherlands (92.2% identity) (Van Der Poel et al., 2001) and to two Italian swine strains (MO/36_4/IT/06 and MO/9_3/IT/06; 91.0% and 92.2% identity, respectively) detected in 2005 in a pig farm located in Northern Italy (unpublished data). The degree of identity was lower (83-86%) when our strain was compared to two other swine strains detected in Italy in 2004 (HEVPI/01 e HEVBO01) (Caprioli et al., in press), and to an autochthons Italian human strain (It1) described in Italy (83.1%) in 1999 (Zanetti et al., 1999). Moreover wbITBO06/9 showed only a 66 to 86% nucleotide identity with wild boar HEV strains detected in Japan (DQ079630; AB222184, AB189070).

4. Discussion

This study represents the first report on the presence of HEV in wild boars in Italy, and confirms that HEV actively circulates in European wild boar (Sus scrofa) populations (de Deus et al., 2007b), and not only in subspecies of the Asian lineages (Sus scrofa leucomysta and Sus scrofa riukiuanus) (Kitajima et al., 2004; Sonoda et al., 2004; Masuda et al., 2005).

Our results also confirm that wild boars, together with domestic pigs, may also represent an important animal reservoir of HEV infection.

In our study, 25 per cent of the animals tested positive for HEV RNA. This prevalence is generally higher than that reported in other studies on wild boars. Sonoda et al. (2004) reported a prevalence of 2.4% in a sample of 41 animals, while Nishizawa et al. (2005) of 2.3% among 89 animals. Kitajima et al. (2004) reported a higher prevalence (42.8%), but this study was performed on only 7
animals. The different prevalence of HEV infection detected in our study may indicate a truly higher circulation of HEV in the investigated wild boar population, although differences due to the type of specimen and the PCR method chosen cannot be excluded. In previous reports, detection of HEV was performed on either serum and/or liver samples (Choi and Chae, 2003; Banks et al., 2004a), whereas we examined bile samples, that have been recently reported to be the most reliable specimen for the detection of HEV in pigs (de Deus et al., 2007a). In addition, differences in the HEV prevalence might be also related to a different infectivity of the HEV strains or to differences in the biology and ecology of the wild boar populations considered (genetics of the animals, density of the population, environmental characteristics, etc.). In this regard, it is of interest to notice that the only other study conducted on European wild boars (de Deus et al., 2007b) also revealed a prevalence of viremic animals in the order of 20%.

Concerning the possible risk factors associated with the infection, no statistically significant differences (P>0.05) in the HEV prevalence were detected considering sex and age classes.

Positive animals were detected in each age classes, including juveniles of 4 months of age, indicating that infection can occur at least starting from this age. The presence of HEV RNA in animals older than 24 months extends previous studies findings (Sonoda et al., 2004; Nishizawa et al., 2005) reporting HEV infection in wild boars of approximately two years of age. These data are in contrast with the results of most of the studies conducted on domestic swine, which indicate that infection mainly occurs in animals of 3 to 5 months of age, has
a short duration, and is generally self limiting (Meng et al., 1997; Meng et al.,
1998).

These differences might suggest that infection in wild boars can become
chronic, possibly sustained by an incompletely protective immunity, or that there
is continuous re-infection favored by a short-lasting immunity. Neither can we
rule out the possibility that the virus strain identified may have found a naïve
population, infecting all animals independent of the age.

Biometric characteristics (weight and length of the body) of the infected
animals were not statistically different than those of uninfected animals belonging
to the same age and sex classes. These results, together with the fact that the wild
boars examined appeared clinically healthy, raise the hypothesis that also in wild
boars, as in domestic pigs (Meng et al., 1998), HEV infection may be subclinical.
Further studies will be necessary to better evaluate dynamic and clinical and
pathological effects of the infection in wild boars.

Comparison of the nucleotide sequences obtained from 10 positive selected
samples showed that they were all identical. Even though the nucleotide region
compared is only 97 bp long, this region is usually not conserved, and is
commonly used to differentiate HEV strains. Our findings therefore suggest that
only one HEV strain was probably circulating through wild boars in the Gessi
Bolognesi Regional-Park at the time of sampling. This result may further suggest
that the introduction or the selection of new HEV strains into the examined wild
boar population is an un-frequent event, and that the identified HEV strain did not
recently experience marked evolutionary changing, at least in the genome region.
analyzed. This might mean that the selective pressure on HEV in wild boar populations can be probably low.

The phylogenetic analysis also showed that the Italian wild boar strain was genetically closer to human and swine HEV strains circulating in Europe than to wild boar strains characterized in Japan. This result is similar to previous reports demonstrating that human and swine HEV strains from the same geographic area (Europe) are most often closely related than with strains with a same origin identified in distant areas (Van Der Poel et al., 2001). To date, no other European wild boar HEV sequences are present in GenBank, therefore we cannot argue about a possible spread of wbITBO06 strain throughout Europe. However, our preliminary results suggest that there may be a geographical clustering of HEV strains. These findings, together with the observation that HEV infection may be subclinical and can be present also in animals at an age in which they are commonly hunted to be eaten, are of concern because of the possible risk of transmission of HEV to human beings by either contact with infected boars or ingestion of contaminated undercooked meat or organs. In this respect, the presence of 25 per cent HEV positive bile samples implies that at least wild boar liver can represent an organ at risk for zoonotic transmission. Although we did not collect information about the contamination of other organs or meat, it cannot be totally excluded that during the slaughtering process small amounts of bile might cross-contaminate other edible parts of the carcass.

Besides boar hunters, also people assigned to the density-control program in the Regional Park area should be considered a high risk category for contracting
the infection because of routine handling of live animals and carcasses during
routine activity.

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Figure and table legends

Table 1
Prevalence of HEV infection by age and sex class

Fig. 1
Dendrogram constructed by neighbour-joining method including nucleotide sequences from human (HuHEV), swine (SwHEV) and wild boar (WBHEV) HEV strains identified in different countries (as indicated). GenBank accession no. are also reported. An avian HEV strain (AY535004) was used as out-group. The Italian wild boar strain (wbITBOO6/9), the four Italian swine HEV strains (MO/9_3/06/IT, MO/36_4/06/IT, HEVBO/01 and HEVPI/01) and the human Italian strain are in bold.
<table>
<thead>
<tr>
<th>Sex class</th>
<th>Age class</th>
<th>Estimated total population</th>
<th>Total examined</th>
<th>HEV positive</th>
<th>HEV prevalence</th>
<th>Estimated prevalence in the whole population (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>All animals</td>
<td>&lt;12 months</td>
<td>172</td>
<td>23</td>
<td>8</td>
<td>34.8</td>
<td>20.4 - 51.7</td>
</tr>
<tr>
<td></td>
<td>13-24 months</td>
<td>87</td>
<td>53</td>
<td>11</td>
<td>20.8</td>
<td>14.9 - 27.6</td>
</tr>
<tr>
<td></td>
<td>&gt; 24 months</td>
<td>20</td>
<td>12</td>
<td>3</td>
<td>25.0</td>
<td>15.0 - 40.0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>279</td>
<td>88</td>
<td>22</td>
<td>25.0</td>
<td>18.6 - 32.6</td>
</tr>
<tr>
<td>Male</td>
<td>&lt;12 months</td>
<td>74</td>
<td>8</td>
<td>4</td>
<td>50.0</td>
<td>25.7 - 74.3</td>
</tr>
<tr>
<td></td>
<td>13-24 months</td>
<td>39</td>
<td>20</td>
<td>3</td>
<td>15.0</td>
<td>7.7 - 28.2</td>
</tr>
<tr>
<td></td>
<td>&gt; 24 months</td>
<td>6</td>
<td>6</td>
<td>2</td>
<td>33.3</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>119</td>
<td>34</td>
<td>9</td>
<td>26.5</td>
<td>16.8 - 38.7</td>
</tr>
<tr>
<td>Female</td>
<td>&lt;12 months</td>
<td>98</td>
<td>15</td>
<td>4</td>
<td>26.7</td>
<td>12.2 - 46.9</td>
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<tr>
<td></td>
<td>13-24 months</td>
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<td>33</td>
<td>8</td>
<td>24.2</td>
<td>17.7 - 33.3</td>
</tr>
<tr>
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<td>&gt; 24 months</td>
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<td>1</td>
<td>16.7</td>
<td>9.1 - 36.4</td>
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<td></td>
<td>Total</td>
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<td>24.1</td>
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