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1 **Detection of Hepatitis E virus (HEV) in a demographic managed wild boar**  
2 **(*Sus scrofa scrofa*) population in Italy**

3

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1    **Abstract**

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

17

18   **Keywords:** Hepatitis E virus (HEV); wild boar; Nested-RT-PCR

## 1 **1. Introduction**

2

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

10 The causative agent of the disease is the Hepatitis E virus (HEV), a small non-  
11 enveloped RNA virus classified as Hepevirus genus within the *Hepeviridae*  
12 family (Mayo, 2004). HEV isolates have been so far classified into four major  
13 genotypes. The majority of infections occurring in Asia and Africa are caused by  
14 genotype 1, whereas genotype 2 prevails in Mexico and Nigeria (Emerson and  
15 Purcell, 2003). In industrialized countries, where until few years ago the infection  
16 was considered non-endemic, only strains belonging to genotype 3 and 4 have  
17 been detected in persons without a history of recent travel to HEV endemic  
18 regions (Aggarwal and Krawczynski, 2000; Yoo et al., 2001; Emerson and  
19 Purcell, 2003). Genotype 3 prevails in USA and Europe, while genotype 4 is  
20 mainly distributed in China, Taiwan, Japan, Indonesia and Vietnam (Hsieh et al.,  
21 1999; Banks et al., 2004b; Zheng et al., 2006). Although hepatitis E is a sporadic  
22 disease in countries with good health-care conditions, the seroprevalence rate  
23 among healthy individuals can be rather high (Emerson and Purcell, 2003)

1       The first animal HEV strain was characterized in pigs in USA in 1997 (Meng  
2 et al., 1997). Since then, several other strains have been described in pigs  
3 worldwide, virtually all belonging to genotype 3 and 4 (Lu et al., 2006) except  
4 one genotype 1 strain recently detected in Cambodia (Caron et al., 2006). In  
5 particular, swine strains have been demonstrated to have a high sequence  
6 homologies to autochthonous human strains, suggesting that swine can represent a  
7 reservoir of the infection and that zoonotic transmission of HEV may play a  
8 relevant role in industrialized countries (Meng et al., 1998; Zanetti et al., 1999;  
9 Van Der Poel et al., 2001; Clemente-Casares et al., 2003; Buti et al., 2004). In this  
10 regard, several studies have reported that in people who work in contact with  
11 swine such as pig farmers, veterinarians and slaughterhouse workers, the HEV  
12 seroprevalence rate can be higher than that reported in normal control populations  
13 (Hsieh et al., 1999; Drobeniuc et al., 2001; Meng et al., 2002; Withers et al.,  
14 2002).

15       The first direct evidence of food-borne transmission of HEV to humans from  
16 animal meat was reported in Japan in 2003 (Tei et al., 2003), where hepatitis E  
17 cases occurred in association with the consumption of undercooked contaminated  
18 *Sika* deer meat. In this case, the HEV strain isolated from the patients was  
19 identical to that detected in the left-over contaminated deer meat (Tei et al., 2003).  
20 The possibility of foodborne zoonotic transmission of HEV is further supported  
21 by other studies in which the relation between consumption of meat or organs  
22 from pigs (Yazaki et al., 2003) or from wild boars (Matsuda et al., 2003; Sonoda  
23 et al., 2004; Takahashi et al., 2004; Tamada et al., 2004; Kitajima et al., 2004; Li

1 et al., 2005; Masuda et al., 2005; Nishizawa et al., 2005) and the clinical disease  
2 has been demonstrated. The disease can be now considered an emerging food-  
3 borne zoonosis.

4 To date, studies to evaluate the presence of HEV in wild boars or wild pigs  
5 have been conducted in Australia (Chandler et al., 1999) and Japan (Matsuda et  
6 al., 2003; Yazaki et al., 2003; Sonoda et al., 2004; Takahashi et al., 2004; Tamada  
7 et al., 2004; Li et al., 2005; Masuda et al., 2005; Nishizawa et al., 2005).  
8 However, the study in Australia was performed on wild hogs, while the Japanese  
9 investigation concerned the 2 wild boars subspecies *Sus scrofa leucomyxtas* and  
10 *Sus scrofa riukiuanus* (Watanobe et al., 1999) which are present in that country  
11 but are phylogenetically different from European wild boars subspecies. To our  
12 knowledge, only minimal information on the circulation and the prevalence of  
13 HEV in European wild boars is presently available (de Deus et al., 2007b), despite  
14 several reports have already confirmed a wide circulation of HEV among  
15 domestic pigs in many European countries (Pina et al., 2000; Van Der Poel et al.,  
16 2001; Clemente-Casares et al., 2003; Banks et al., 2004a; Fernandez-Barredo et  
17 al., 2006; de Deus et al., 2007a; Caprioli et al., in press).

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

1

## 2 **2. Materials and methods**

3

### 4 **2.1. Wild boar population**

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

16

### 17 **2.2. Sample collection**

18 Eighty-eight apparently healthy wild boars shot between March and September  
19 2006 were selected for the study. For each animal, age, sex, length of the body  
20 (from the tip of the snout to the base of the tail) and weight were evaluated and  
21 recorded. Animals were aged by the evaluation of tooth eruption and replacement  
22 patterns (Vicente et al., 2004); their ages ranged between 4 and 37 months, and  
23 most of them (83/88) were of over 6 months of age. Wild boars of this age were



1 deliberately selected because they represent those usually intended for human  
2 consumption. During the slaughtering process, a bile sample was withdrawn from  
3 each animal with a sterile syringe (used once and then discarded) through the gall-  
4 bladder wall, and stored at -80°C until processing.

5

### 6 **2.3. RNA extraction and HEV RT-Nested-PCR**

7 Each bile sample was diluted 1:10 in DEPC water. Total RNA was extracted  
8 from 140 µl of solution using a QiaAmp viral RNA kit (Qiagen, Hilden, Germany)  
9 according to the manufacturer's instructions. RNA reverse transcription (RT) and  
10 first PCR reaction were conducted using a Superscript III One-step RT-PCR  
11 System with Platinum Taq DNA polymerase (Invitrogen, Carlsbad, California,  
12 USA) according to the manufacturer's instructions. The RT-PCR reaction was  
13 conducted in a ICycler (Bio-Rad, Hemel Hempstead, UK) thermal cycler under  
14 the following conditions: 45°C for 30 minutes for RT, 94°C for 2 minutes for the  
15 initial cDNA denaturation, followed by 39 cycles of denaturation at 94°C for 1  
16 minute, annealing at 49°C for 90 seconds, elongation at 72°C for 1 minute, and a  
17 final elongation at 72°C for 5 minutes. Nested PCR was conducted using a  
18 recombinant Taq DNA polymerase (Fermentas, Burlington, Ontario, Canada)  
19 according to the manufacturer's instructions, following the subsequent thermal  
20 conditions: initial denaturation at 95°C for 3 minutes followed by 39 cycles of  
21 denaturation at 94°C for 45 seconds, annealing at 49°C for 1 minute, elongation at  
22 72°C for 2 minutes, and a final elongation at 72°C for 7 minutes. For RT-PCR and  
23 Nested PCR, sets of degenerate primers, HEVORF2con-a1/HEVORF2con-S1 and

1 HEVORF2con-a2/HEVORF2con-S2, amplifying a 145 bp region of the HEV  
2 open reading frame 2 (ORF2) were used (Erker et al., 1999). At each stage of the  
3 reaction (extraction, first round and second round PCR amplification) a negative  
4 (DEPC water) and a positive control (swine HEV positive bile, kindly provided  
5 by Dr. Marga Martin, Universitat Autònoma di Barcelona) were used. To further  
6 minimize the possibility of cross-contamination, strict anti-contamination  
7 procedures, including the use of separate rooms, safety hoods, frequent  
8 discharging of gloves and accurate cleaning of surfaces and materials were  
9 implemented. Amplified products were visualized in a 2% agarose gel stained  
10 with ethidium bromide.

11

#### 12 ***2.4. Sequencing and phylogenetic analysis***

13 To further confirm the identity of the strains detected by Nested-PCR, a  
14 nucleotide sequence analysis was performed on 10 Nested-PCR positive products  
15 corresponding to a fraction of sampled animals of different age and sex classes.

16 Nested-RT-PCR products of the expected size (145 bp) were excised from a  
17 2% agarose gel, purified with a High Pure PCR Product Purification Kit (Roche,  
18 Indianapolis, USA), and sequenced using the PCR primers with the BigDye  
19 Terminator Cycle Sequencing Ready Reaction Kit version 3.1 (Perkin Elmer,  
20 Applied Biosystems, Foster City, CA), in an automated sequencer (ABI Prism 310  
21 DNA sequencer, Applied Biosystems, Foster City, CA). The sequences obtained  
22 were assembled and aligned with other swine, human and wild boar HEV  
23 sequences present in NCBI GenBank (<http://www.ncbi.nlm.nih.gov/>), using the

1 DNASIS Max software (Hitachi Software Engineering Company, Alameda, CA,  
2 USA) and an avian HEV (GenBank accession no. AY535004) as out-group. The  
3 HEV wild boar sequences used were all Japanese (GenBank accession no.:  
4 DQ079630; AB189070; AB222184), because no nucleotide or deduced amino  
5 acid sequences of European wild boar strains were available in NCBI GenBank.  
6 Dendrogram was drawn with the Bionumerics software packages (Applied Maths,  
7 Kortrijk, Belgium) using Unweighted Pair Group Method, with Arithmetic  
8 averages (UPGMA). GenBank accession numbers for HEV genome sequences  
9 investigated in this study are as follows: i. Italian wild boar HEV strain:  
10 wbITBO06/9, EF681108; ii. Italian swine HEV strains: MO/9\_3/06/IT,  
11 EF681107; MO/36\_4/06/IT, EF682083; HEVBO/01, EF681109; HEVPI/01,  
12 EF681110; iii. Italian human HEV strain: AF110390; iv. European swine HEV  
13 strain: AF336292; v. European human HEV strains: AY940427.1 and DQ200292;  
14 vi. European sewage strain: AF490994; vii. US swine strain: AY575857.

15

## 16 **2.5. Statistical analysis**

17 To identify a possible correlation between the HEV prevalence and the age of  
18 animals, wild boars were subdivided in three categories (< 12; 12-24; > 24 months  
19 of age) as previously reported (Vicente et al., 2004).

20 To evaluate the possible effects of the infection on the biometric characteristics  
21 of the animals, weight and body length of the HEV positive animals were  
22 compared to those of the negative animals of the same age and sex.

1 Kolmogorov – Smirnov test for goodness of adaptation was used to verify  
2 distribution normality. On the basis of the results of Kolmogorov – Smirnov test,  
3 Student's *t* test was used to compare quantitative data. Categorical data were  
4 analyzed with chi-square test. Data were analyzed with the SPSS software for  
5 Windows 12.0 (SPSS Inc., Chicago, USA).

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

8

### 9 **3. Results**

10 HEV genome was detected in 22 of the 88 bile samples tested (25%, 95% CI  
11 18.6-32.6). Prevalence rates determined for the whole population and for animals  
12 of different age and sex classes are reported in Table 1. No statistically significant  
13 differences ( $P>0.05$ ) in the HEV prevalence were detected between sex and age  
14 classes. Biometric characteristics (weight and length of the body) of the infected  
15 animals were not statistically different from those of the non-infected animals  
16 within the same age and sex classes.

17 The sequence alignment of the 10 Nested-PCR positive samples analyzed  
18 demonstrated that all samples contained an identical HEV sequence. As shown in  
19 the dendrogram with the analyzed sequences (Fig. 1), the Italian wild boar strain  
20 sequence (wbITBO06) belonged to genotype 3, as other swine and human  
21 European indigenous HEV strains. In particular, the Italian wild boar strain was  
22 related (92% identity) to a sewage HEV strain from an industrialized area of  
23 Spain (AF490994) (Clemente-Casares et al., 2003), to a swine strain (AF336292)

1 detected in The Netherlands (92.2% identity) (Van Der Poel et al., 2001) and to  
2 two Italian swine strains (MO/36\_4/IT/06 and MO/9\_3/IT/06; 91.0% and 92.2%  
3 identity, respectively) detected in 2005 in a pig farm located in Northern Italy  
4 (unpublished data). The degree of identity was lower (83-86%) when our strain  
5 was compared to two other swine strains detected in Italy in 2004 (HEVPI/01 e  
6 HEVBO01) (Caprioli et al., in press), and to an autochthons Italian human strain  
7 (It1) described in Italy (83.1%) in 1999 (Zanetti et al., 1999). Moreover  
8 wbITBO06/9 showed only a 66 to 86% nucleotide identity with wild boar HEV  
9 strains detected in Japan (DQ079630; AB222184, AB189070).

10

#### 11 **4. Discussion**

12 This study represents the first report on the presence of HEV in wild boars in  
13 Italy, and confirms that HEV actively circulates in European wild boar (*Sus scrofa*  
14 *scrofa*) populations (de Deus et al., 2007b), and not only in subspecies of the  
15 Asian lineages (*Sus scrofa leucomysta* and *Sus scrofa riukiuanus*) (Kitajima et al.,  
16 2004; Sonoda et al., 2004; Masuda et al., 2005).

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

19 In our study, 25 per cent of the animals tested positive for HEV RNA. This  
20 prevalence is generally higher than that reported in other studies on wild boars.  
21 Sonoda et al. (2004) reported a prevalence of 2.4% in a sample of 41 animals,  
22 while Nishizawa et al. (2005) of 2.3% among 89 animals. Kitajima et al. (2004)  
23 reported a higher prevalence (42.8%), but this study was performed on only 7

1 animals. The different prevalence of HEV infection detected in our study may  
2 indicate a truly higher circulation of HEV in the investigated wild boar  
3 population, although differences due to the type of specimen and the PCR method  
4 chosen cannot be excluded. In previous reports, detection of HEV was performed  
5 on either serum and/or liver samples (Choi and Chae, 2003; Banks et al., 2004a),  
6 whereas we examined bile samples, that have been recently reported to be the  
7 most reliable specimen for the detection of HEV in pigs (de Deus et al., 2007a). In  
8 addition, differences in the HEV prevalence might be also related to a different  
9 infectivity of the HEV strains or to differences in the biology and ecology of the  
10 wild boar populations considered (genetics of the animals, density of the  
11 population, environmental characteristics, etc.). In this regard, it is of interest to  
12 notice that the only other study conducted on European wild boars (de Deus et al.,  
13 2007b) also revealed a prevalence of viremic animals in the order of 20%.

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

17 Positive animals were detected in each age classes, including juveniles of 4  
18 months of age, indicating that infection can occur at least starting from this age.  
19 The presence of HEV RNA in animals older than 24 months extends previous  
20 studies findings (Sonoda et al., 2004; Nishizawa et al., 2005) reporting HEV  
21 infection in wild boars of approximately two years of age. These data are in  
22 contrast with the results of most of the studies conducted on domestic swine,  
23 which indicate that infection mainly occurs in animals of 3 to 5 months of age, has

1 a short duration, and is generally self limiting (Meng et al., 1997; Meng et al.,  
2 1998).

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

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

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

1 analyzed. This might mean that the selective pressure on HEV in wild boar  
2 populations can be probably low.

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

22 Besides boar hunters, also people assigned to the density-control program in  
23 the Regional Park area should be considered a high risk category for contracting



1 the infection because of routine handling of live animals and carcasses during  
2 routine activity.

3

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1 **Figure and table legends**

2

3

4 Table 1

5 Prevalence of HEV infection by age and sex class

6

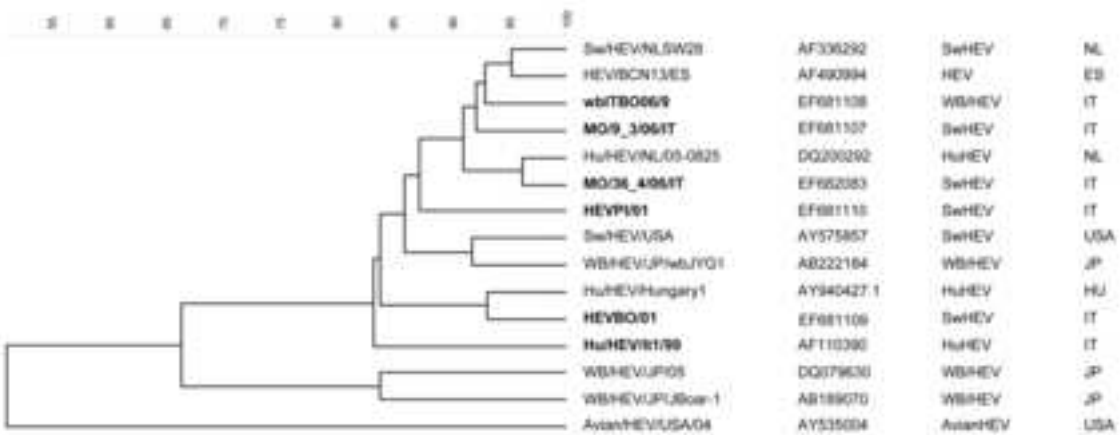
7

8 Fig. 1

9 Dendrogram constructed by neighbour-joining method including nucleotide  
10 sequences from human (HuHEV), swine (SwHEV) and wild boar (WBHEV)  
11 HEV stains identified in different countries (as indicated). GenBank accession no.  
12 are also reported. An avian HEV strain (AY535004) was used as out-group. The  
13 Italian wild boar strain (**wbITBOO6/9**), the four Italian swine HEV strains  
14 (**MO/9\_3/06/IT**, **MO/36\_4/06/IT**, **HEVBO/01** and **HEVPI/01**) and the human  
15 Italian strain are in bold.



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Sex class	Age class	Estimated total			Estimated prevalence in the whole	
		population	Total examined	HEV positive	HEV prevalence	population (95% CI)
All animals	<12 months	172	23	8	34.8	20.4 - 51.7
	13-24 months	87	53	11	20.8	14.9 - 27.6
	> 24 months	20	12	3	25.0	15.0 - 40.0
	Total	279	88	22	25.0	18.6 - 32.6
Male	<12 months	74	8	4	50.0	25.7 - 74.3
	13-24 months	39	20	3	15.0	7.7 - 28.2
	> 24 months	6	6	2	33.3	-
	Total	119	34	9	26.5	16.8 - 38.7
Female	<12 months	98	15	4	26.7	12.2 - 46.9
	13-24 months	51	33	8	24.2	17.7 - 33.3
	> 24 months	11	6	1	16.7	9.1 - 36.4
	Total	160	54	13	24.1	16.3 - 33.7