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1 **Viral and antibody HEV prevalence in swine at slaughterhouse in Italy**

2

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16

1 **Abstract:**

2 Hepatitis E is an acute disease of humans caused by a small RNA virus, Hepatitis E virus (HEV). In
3 recent years, an increasing number of autochthonous human infections have been reported in
4 industrialized countries. Genotype 3 is the main HEV type circulating in swine, and is also reported
5 in sporadic cases of hepatitis E in humans worldwide. To date one serotype has been described. We
6 have conducted a survey to detect antibodies against HEV in 48 swine at a slaughterhouse in
7 Northern Italy, using ELISA test. Mean seroprevalence in the studied animal group was 87.%. Bile,
8 liver and feces from the 48 animals were also collected, and HEV RNA was detected by nested
9 reverse transcription-polymerase chain reaction, amplifying a fragment of the ORF2. HEV genome
10 was most frequently detected in bile samples (51.1%), followed by feces (33.3%) and liver (20.8%).
11 Thirty-one out of 48 studied pigs (64.6%) were positive for HEV RNA in at least one sample.
12 Overall, HEV RNA was found at a statistically higher rate in the 3-4 month-old than in 9-10 month-
13 old animals (95.0% vs. 42.9%). Genetic characterization of swine strains identified was performed
14 by sequencing and database alignment. Phylogenetic analysis on the nucleotide sequences from 16
15 positive PCR products indicated that all strains belonged to genotype 3, clustering in two branches
16 subtypes g3c and g3f.

17

18 *Keywords:* Hepatitis E Virus; Genotype 3, swine;-ELISA.

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20

1 **1. Introduction**

2 Hepatitis E is an important cause of enterically transmitted self-limiting non-A, non-B hepatitis in
3 humans (Purcell and Emerson, 2001). The etiological agent is a small non-enveloped positive sense
4 single-stranded RNA virus, the Hepatitis E virus (HEV), recently classified in the new *Hepevirus*
5 genus in the proposed family *Hepeviridae* (Emerson et al., 2004). The genome of HEV is
6 approximately 7.5 kb, and contains three open reading frames (ORFs) and short 5' and 3'
7 untranslated regions. ORF1 encodes non-structural proteins including an RNA-dependent RNA
8 polymerase, whereas ORF2 encodes the glycoprotein of the viral capsid, and ORF3 a small protein
9 of unknown function (Panda et al., 2007). Although one serotype is actually recognized, HEV is
10 classified in four different genotypes based on the nucleotide sequence, and 24 subtypes (Lu et al.,
11 2006), which show different geographical distributions.

12 Particularly, genotype 1 and 2 have been described in Asia, Africa and Mexico, and include
13 primarily human strains (Schlauder and Mushahwar, 2001) that can be further divided into five (a–
14 e) and two (a and b) subtypes, respectively. Conversely, genotype 3 and 4 strains circulate in Asian,
15 American and European developed countries, and have been detected in humans, pigs, and other
16 animal species. Genotype 3 and 4 can be divided into ten (a–j) and seven (a–g) subtypes (Lu et al.,
17 2006; Vasickova et al., 2009). A fifth HEV genotype has been suggested to infect rabbits (Zhao et
18 al., 2009), showing approximately 75% nucleotide divergence from established HEV types.

19 Hepatitis E is considered to be endemic in several developing countries, where it occurs as
20 outbreaks mainly transmitted via fecally contaminated drinking water (Emerson and Purcell, 2003).

21 In industrialized countries, HEV infection is sporadic and is often reported in patients with history
22 of travelling in endemic areas (Péron et al., 2006). However, in the last decade an increasing
23 number of sporadic human HEV cases without history of travelling have been reported in Japan,
24 USA and several European countries, including Italy (Zanetti et al., 1999; Buti et al., 2004; Borgen
25 et al., 2008; Dalton et al., 2008; Mansuy et al., 2009).

1 The overall mortality rate associated with Hepatitis E is generally low (<1%), except for
2 pregnant women, who may exhibit mortality rates up to 28% (Purcell and Emerson 2001;
3 Mushawar, 2008). Despite the low number of cases diagnosed in developed countries,
4 seroprevalence of HEV in humans can vary from 1% to 16% (Buti et al., 2006; Mansuy et al.,
5 2008). Several studies have shown that the pig farmers, swine veterinarians and other swine
6 workers, have an increased risk of HEV infection (Meng et al., 2002; Vulcano et al., 2007;
7 Bouwknegt et al., 2008). Moreover, recent investigations have shown that seropositive animals are
8 present in up to 97% of the pig herds (Rutjes et al., 2007; Seminati et al., 2008). These evidences,
9 together with the detection of anti-HEV antibodies in animals such as rodents (Favorov et al.,
10 2000), pigs, cows and goats (Wang et al., 2002) suggest that hepatitis E virus may also have a
11 zoonotic transmission and that animals can act as a reservoir.

12 The first animal strain of HEV was identified in a pig in the United States in 1997, and was
13 shown to be closely related to human HEV (Meng et al., 1997). Several data, involving sporadic
14 and clustered human cases of hepatitis E, support transmission of HEV to humans from swine, wild
15 boar and deer, in some cases related to consumption of raw meat. The genomic sequences of the
16 viruses identified in food or animals in these cases were in fact closely related to each other
17 (Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Li et al., 2005). Most importantly, in
18 Japan and in the US, 2% and 11% of commercial pig livers sold in grocery stores were positive for
19 swine HEV RNA (Yazaki et al., 2003; Feagins et al., 2007). In Italy, the first autochthonous human
20 case of hepatitis E was reported (Zanetti et al., 1999) in 1997, and the virus identified belonged to
21 genotype 3. A limited number of studies conducted on pigs in Northern Italy (Caprioli et al., 2007;
22 Di Bartolo et al., 2008) indicate that swine HEV circulates largely within pig herds in the country,
23 although consistent data on possible different prevalence of viral infection in pigs by age,
24 particularly among animals entering the pork food chain, are still unavailable.

25 This study was aimed to assess if HEV is present in swine at the slaughterhouse, and the
26 genetic correlation between viruses detected in Italian pigs with other strains circulating in Europe.

1 Anti-HEV serum antibody was determined by a commercial ELISA test adapted to detect swine
2 antibodies. Moreover, HEV RNA was determined in bile, liver and feces samples, and genetic
3 characterization of identified HEV swine strains was performed against existing sequences of
4 human and swine origin.

5 **2. Materials and methods**

6 *2.1. Pigs and samples*

7 Between November-December 2008, 46 sera, 48 livers, 48 stools and 45 bile samples were
8 collected in a slaughterhouse in Northern Italy from 48 clinically healthy pigs, belonging to five
9 different farms from the surrounding area. Twenty-eight animals were 9-10 month-old (160-170 kg
10 live body weight, usual age for slaughtering in Italy) and twenty were 3 month-old.

11 From each animal a bile sample was withdrawn with a sterile syringe (used once and then
12 discarded) through the gall-bladder wall, and a sample of gross intestine content was collected after
13 cutting the bowel with a sterile blade. Samples were stored at -80°C until processing. Fecal and bile
14 samples were suspended in 10% diethyl pyrocarbonate (DEPC) water, and were stored at -80°C
15 until analyzed. Pig livers were collected and stored immediately at -80°C. Serum samples were
16 obtained from intracardiac clot and stored at -20°C.

17 *2.2. RNA extraction and HEV Reverse transcription (RT)-Nested-PCR*

18 A 200 mg sample of each pig liver was homogenized using a Tissue Lyser (Qiagen, Hilden,
19 Germany). The obtained homogenate (around 0.75 ml) was centrifuged for 3 min at 13,000 rpm
20 twice, and the supernatant was collected and immediately subjected to RNA extraction using the
21 RNeasy Mini kit (Qiagen, Hilden, Germany), and eluted in 60 µl DEPC water, according to the
22 manufacturer's instructions.

23 One-hundred seventy microliters of 10% (W/vol) fecal and 10% (vol/vol) bile suspensions
24 were used for viral RNA extraction, using the RNeasy Mini kit (Qiagen, Hilden, Germany).

25 Extracted RNA was used immediately for a HEV-specific Reverse transcription (RT)-
26 nested-PCR using SuperScript One-Step RT-PCR with Platinum Taq (Invitrogen; Carlsbad, CA,

1 USA), and two degenerate primer sets targeting the ORF2 region encoding for the capsid protein.
2 External primer forward HEVORF2con-s1—5' GACAGAATTRATTTTCGTCGGCTGG-3' and
3 reverse HEVORF2con-a1—5' CTTGTTTCRTGYTGGTTRTCATAATC-3' were used for RT-PCR,
4 and for the nested-PCR step forward primer HEVORF2con-s2—5'
5 GTYGTCTCRGCCAATGGCGAGC-3' and HEVORF2con-a2—5'
6 GTTCRTGYTGGTTRTCATAATCCTG-3' inner primers were used, yielding a final product of
7 145 bp (Erker et al., 1999).

8 Briefly, 0.4 μ M of primers HEVORF2con-s1 and HEVORF2con-A1 and 3 μ l of extracted
9 RNA were used in RT-PCR in a final volume of 15 μ l. Reverse-transcription and PCR were
10 performed under following conditions: RT cycle at 45°C for 30 min, denaturation at 94°C for 2
11 min, and 40 cycles at 94°C for 45 s, 49°C for 45 s, and 72°C for 1 min, followed by a final
12 elongation step of 72°C for 7 min. For the second round nested PCR, the inner oligos
13 HEVORF2con-s2 and HEVORF2con-a2 (0.3 μ M) were used, with 2 μ l of DNA template obtained
14 from the first RT-PCR or 2 μ l a ten-fold dilution (amount of template corresponding to 10% of the
15 final volume), using AmpliTaq DNA polymerase (Applied Biosystems, ABI, Foster City, CA,
16 USA) according to the manufacturer's instruction. The PCR was performed as follows: 1 cycle at
17 94°C for 10 min, followed by 40 cycles at 94°C for 45 s, 50°C for 45 s, and 72°C for 1 min, and a
18 final elongation step of 72°C for 7 min.

19 To obtain a longer DNA fragment for sequencing, samples positive to the first protocol
20 described above were also analyzed using a second nested RT-PCR procedure with primer pairs
21 3156-3157 (first run) and 3158-3159 (nested-PCR) (Huang et al., 2002) amplifying a 320 bp region
22 in ORF2. The amount of RNA, DNA and the parameters for the second RT-PCR protocol were
23 similar to the first one, except that primers 3156-3157 (first run) and 3158-3159 were used.

24 Negative (DEPC water samples) and positive (a collection swine bile sample, previously
25 confirmed to be positive for HEV; Martelli et al., 2008) samples were included as controls in each
26 step: RNA extraction, first and second PCRs. All samples resulting negative were re-tested in

1 nested-PCR using a ten-fold dilution of the first PCR. Moreover, to evaluate if negative results were
2 due to inhibition, RNA resulted negative to PCR analysis were spiked with a positive control HEV
3 RNA and re-tested. All spiked RNA samples resulted positive, confirming the absence of inhibitors.

4 Amplified products were stained with ethidium bromide in a 2% agarose gel. To abate risks
5 of cross-contamination, each phase of diagnostic routine (from sample preparation throughout
6 nested PCR) was completed in separate rooms, inside biohazard cabinets or PCR hoods, and using
7 dedicated equipment, pipettes and disposables.

8 *2.3. Sequencing and phylogenetic analysis*

9 Positive DNA PCR products, obtained with the second nested-PCR protocol (see above), were
10 excised from agarose gels and purified using the Nucleospin Extract II (Macherey-Nagel GmbH &
11 Co., Düren, Germany) following manufacturer's instructions. Unfortunately, it was not possible to
12 sequence all samples due to the low amount of DNA. Sequence reactions were performed by
13 Macrogen Inc. (Seoul, Korea). Sequences obtained (Acc. no. HM769970; HM769971; HM769972;
14 HM769973; HM769974; HM769975; HM769976; HM769977; HM769978; HM769979;
15 HM769982; HM769980; HM769981; HM769983) were compared with those of HEV available in
16 NCBI GenBank (<http://www.ncbi.nlm.nih.gov>). Alignments were performed using DNASIS Max
17 software (Hitachi Software Engineering Company, Alameda, CA, USA). Phylogenetic analyses
18 were carried out with the Bionumerics software packages (Applied Maths, Kortrijk, Belgium), and
19 the dendrogram was obtained with the UPGMA method.

20 *2.4. ELISA commercial kit*

21 Swine sera (diluted 1:20) were tested by the commercial ELISA BioChain kit (Hayward, CA,
22 USA), intended for detection of anti-HEV IgG antibodies in human sera, with the following
23 modifications. For the analysis of porcine samples, the secondary antibody was replaced with a
24 Horseradish peroxidase-labeled mouse anti-swine IgG antibody (SIGMA, St. Louis, MO, USA),
25 diluted 1:10,000. The ELISA cut-off was established following this formula: Cut off Value (COV)
26 = 0.1 + the mean OD of negative controls. Test sera were considered as positive if OD450 was

1 greater than the COV value, as indicated by manufacturer. Three sera from SPF pigs were used as
2 negative controls, and a serum from an experimentally infected swine (kindly provided by Dr.
3 Nicole Pavio, ANSES-ENVA-INRA, Maisons-Alfort, France; Rose et al., 2010) was used as
4 positive control in all experiments.

5 2.5. Statistical analysis

6 To identify a possible correlation between the HEV prevalence and anti-HEV seroprevalence and
7 the age of animals, swine were subdivided in two categories (young animals: 3-4 months of age and
8 adult animals: 9-10 months of age). All statistical analyses were performed using the software SPSS
9 12.0.0 (SPSS Inc., Chicago, IL, USA).

10 3. Results

11 3.1. HEV detection by RT-PCR

12 HEV RNA was detected in at least one sample collected from 31 out of 48 pigs examined (64.6%),
13 based on results obtained with the first testing nested RT-PCR protocol amplifying a 145 nt stretch
14 of the ORF2 (Erker et al., 1999). The results of HEV detection in the feces, bile and liver are
15 summarized in Table 1, with respect to the age of swine. Bile was the type of sample most
16 frequently found positive for HEV RNA (23/45), followed by feces (16/48) and livers (10/48). HEV
17 RNA was detected in both pigs of 3-4 (19/20) and 9-10 (12/28) months of age, although to different
18 rates. In younger swine, the HEV RNA prevalence in bile or feces was significantly higher than in
19 fattening pigs ($\chi^2 = 3.94$, $p=0.047$; $\chi^2 = 15.47$, $p=0.001$). Conversely, no statistically significant
20 differences ($p > 0.05$) were observed in the HEV prevalence in liver samples between age classes.

21 Considering the detection of HEV RNA in at least one sample, a significantly higher HEV
22 prevalence ($\chi^2 = 13.87$; $p=0.001$) was altogether observed in swine of 3-4 months, with 19 positives
23 out of 20 tested (95.0%), than in adult animals (9-10 months), in which the prevalence was 42.9%
24 (12 positives out of 28 tested).

1 Moreover, 17 animals resulted positive for more than one sample (Table 1). In one animal,
2 HEV RNA was detected in all three specimens tested, whereas nine pigs were positive for bile and
3 feces, five for bile and liver, and two for liver and feces.

4 To obtain a longer stretch of DNA to be analyzed by sequencing, a subset of 33 samples (14 bile, 12
5 feces and 7 liver), positive at the first analysis, were further analyzed using a second nested-RT-
6 PCR protocol (Huang et al., 2002), that amplifies a 320 nt ORF2 fragment. In 18 cases out of 33
7 samples, positivity was confirmed by both PCR tests.

8 *3.2. Seroprevalence of swine HEV antibodies*

9 Forty out of 46 swine sera tested (87 %) resulted positive by a commercial ELISA test adapted to
10 detect swine IgG antibody. Swine polyclonal sera were used as positive and negative controls (see
11 Materials and Methods). No statistically significant differences ($p > 0.05$) in the HEV
12 seroprevalence were detected between age classes. The seroprevalence was slightly higher in swine
13 of 9-10 months (25 positive/27 total; 92.6%), than in animals of 3-4 months, in which the
14 seroprevalence was 78.9% (15/19). The proportion of HEV RNA positive bile, feces and liver
15 samples did not differ significantly ($p > 0.05$) between seronegative and seropositive pigs.

16 Twenty-five of the 40 (62.5%) seropositive pigs were also positive for HEV genome in bile,
17 liver and/or feces, whereas fifteen (37.5%) were negative for the presence of viral RNA in any
18 sample (Table 2).

19 Two out of 6 (33.3%) seronegative animals were negative for the presence of viral RNA in
20 all samples, whereas the remaining four (66.7%) pigs were positive for HEV genome in their bile,
21 liver and/or feces (Table 2).

22 For two animals sera samples could not be collected and these were not included into this
23 comparison.

24 *3.3. Sequencing and phylogenetic analysis*

25 Partial sequences (320 nt ORF2 fragment) of the capsid protein gene were obtained from 14

1 samples (5 from bile, 5 feces and 4 liver) and deposited into NCBI GenBank (Acc. no. HM769970;
2 HM769971; HM769972; HM769973; HM769974; HM769975; HM769976; HM769977;
3 HM769978; HM769979; HM769982; HM769980; HM769981; HM769983). HEV sequences were
4 obtained from both liver and bile samples in one case, and from bile and stools from three pigs. In
5 these cases, a nucleotide identity of 100% between the HEV sequences obtained from different
6 specimens of the same pig was found. Following sequencing and comparative analysis with all the 4
7 known genotypes of HEV, all sequences were found to belong to genotype g3, as reported
8 previously for porcine and human HEV from Europe, and clustered in either subtype c or f (Figure
9 1). The two clusters, g3c and g3f, showed nucleotide identity of 79% one to each other. The 8 HEV
10 strains forming the g3c cluster showed 98.9-100% nucleotide identity one to each other (Acc. no.
11 HM769970; HM769971; HM769973; HM769979; HM769980; HM769981; HM769982;
12 HM769983). This branch shared 81.7-92% nucleotide identity with other g3c HEV sequences
13 available in NCBI GenBank. With respect to other Italian swine HEV strains, the present g3c HEV
14 strains showed up to 100% nucleotide identity with strain HEVSwBO88IT/06 previously described
15 by Martelli et al. in 2010 (Acc. no. GU369940).

16 The second cluster, enclosing 6 HEV sequences of genotype 3f shared 88.4% nucleotide
17 identity with other genotype 3f strains sequences available in NCBI GenBank. This cluster
18 displayed an 80% nucleotide identity with earlier swine HEV strains described in Italy (Martelli et
19 al., 2010; Acc. no. GU178998-99). The present 3f HEV strains showed a nucleotide identity one to
20 each other ranging between 94% and 100% (Acc. no. HM769972; HM769974; HM769975;
21 HM769976; HM769977; HM769978). Seventeen additional sequences were obtained from 12 fecal
22 and 5 liver samples, using the 121 bp fragments obtained with a different nested RT-PCR protocol
23 (Erker et al., 1999); also this sequence group confirmed that all HEV strains belonged to g3,
24 subtype c and f. Identity to swine HEV strains previously described in Italy was 88.5% (Acc. no.
25 EF682083) to 100% (Di Bartolo et al., 2008; Acc. no. EF681107), for strains belonging to subtype
26 f. Strains belonging to subtype c shared 80.9% nucleotide identity with a swine HEV strain recently

1 reported in Italy (Acc. no. GQ223721.1; Di Martino et al., 2010) (data not shown). Due to their
2 short length, the 121 bp DNA amplicons obtained by the con-a2/con-s2 nested-RT-PCR were not
3 included into comparative phylogenetic analysis.

4 **4. Discussion**

5 In the last decade, several cases of human hepatitis E potentially transmitted through swine-human
6 contact or related to consumption of raw meat from infected deer, pig or wild boar have been
7 reported (Matsuda et al., 2003; Tei et al., 2003; Yazaki et al., 2003; Li et al., 2005). Due to the lack
8 of conventional cell culture systems to isolate the virus, diagnosis relies on molecular detection of
9 genomic HEV RNA in clinical samples, or food or environmental matrices. Although sensitive,
10 molecular approaches are limited by the knowledge that HEV viremia and shedding have usually
11 short duration (Peralta et al., 2009; Kanai et al., 2010). Furthermore, in pigs HEV infection is
12 asymptomatic, making it impossible to target peak infection phases for optimal test sensitivity.

13 The present study was planned to assess the presence of HEV infection in pigs at
14 slaughterhouse, and it complements information from previous investigations conducted in Italy
15 showing extensive viral circulation in breeding farms. Also, it further confirms that despite some
16 differences in infection rates between ages, the farmed swine retains its susceptibility to HEV at any
17 age, and that a remarkable proportion of pigs shed virus even at the latest breeding stages when they
18 are transferred to slaughter, that in Italy normally involves also animals of 9 months of age and
19 more.

20 In fact, nested RT-PCR testing in this study revealed that 64.6% (31 out of 48 examined
21 pigs) were overall positive for the presence of HEV genome, although as many as 95% (19 out of
22 20) of young pigs (3-4 months of age) resulted to be infected compared to 42.9% (12/28) of adults
23 (9-10 months of age). Similar findings have been recently reported in several European countries,
24 including Italy (Di Bartolo et al., 2008) where HEV positive animals were detected in all age groups
25 (Fernandez-Barredo et al., 2006; de Deus et al., 2007; Leblanc et al., 2007; McCreary et al., 2008;
26 Breum et al., 2010). The higher prevalence detected in younger animals compared to older animals

1 is in line with the hypothesis that, as it occurs for other infectious diseases, young animals are more
2 susceptible also to HEV infection, due to loss of maternal immunity (Meng et al., 1997; Kanai et al.,
3 2010) or to an incomplete or short-lasting protective immunity, permitting continuous re-infection
4 (Fernandez-Barredo et al., 2006).

5 The novelty of the present study was the simultaneous detection of swine HEV genome in
6 both tissues (liver), and excretions (bile and feces) of the same naturally infected pig. The results
7 obtained showed that the bile was the most frequently positive sample where to detect the virus
8 (23/45), followed by fecal samples (16/48) and livers (10/48).

9 Bile has been previously indicated as the sample where HEV can be detected more easily,
10 for longer periods and more frequently than in liver, feces and serum (Halbur et al., 2001; de Deus
11 et al., 2007). This is probably due to the fact that liver is the main site of virus replication, and
12 although liver infection may be focal, thus reducing the chance of collecting positive tissue samples
13 for diagnostics, viral progeny is in all cases accumulating in the bile (Williams et al., 2001; de Deus
14 et al., 2007).

15 The higher prevalence (64.6%) of genotype 3 HEV infections detected in this study,
16 compared to that observed among Italian pigs previously (5.9%, Caprioli et al., 2007; 42%, Di
17 Bartolo et al., 2008; 7.3%, Di Martino et al., 2010; 29.9%, Martelli et al., 2010) can be due at least
18 in part to multiple types of samples tested from the same animal, increasing the sensitivity of HEV
19 RNA detection. As described previously, the choice of the samples may in fact be crucial for
20 successful molecular diagnosis (de Deus et al., 2007; Vasickova et al., 2009). Also the RNA
21 detection method used could be crucial. In fact, our results confirm a different sensitivity of
22 different methods, suggesting that the use of several RT-PCR protocols may increase sensitivity of
23 HEV RNA detection (Vasickova et al., 2009). In our study, not all of the positive samples detected
24 with a first protocol, amplifying a 120 nt genome fragment, could be confirmed with a different
25 method that amplifies a longer genome fragment (Huang et al., 2002) although this latter resulted
26 useful for a more detailed comparison of HEV strain nucleotide sequences.

1 As an aid to diagnostics and for epidemiological studies on HEV infection prevalence,
2 several immunologic tests have been developed for antibody detection in serum, but available
3 diagnostic kits are intended for human use, and are based on HEV genotype 1 or 2 peptides.
4 Although recent studies suggest that genotype 3 antigen derived from swine HEV may be a better
5 candidate for testing HEV serology in pigs (Rose et al., 2010), only one serotype has been described
6 for HEV (Lu et al., 2006), and differences between ORF2 proteins of human and swine origins do
7 not seem to be relevant (Arankalle et al., 2007). Commercial ELISA tests for humans can therefore
8 be used for animal sera, provided a suitable secondary antibody specific for the animal
9 immunoglobulin is used. With a similar approach, we have tested swine sera in the present study.

10 Not unexpectedly, presence of serum antibody directed at HEV involved overall a higher
11 proportion of pigs, forty out of 46 swine sera tested (87%), compared to detection of virus genome
12 (64.6%). The seroprevalence rate in 3-4 months old pigs was 78.9%, reaching 92.6% in 9-10
13 months old pigs. As described by others (de Deus et al., 2008; Seminati et al., 2008), seroprevalence
14 increases with age, as a consequence of repeated contact with the virus. In our study, the 2 different
15 age groups examined showed only a moderate difference in seropositivity, which increased up to
16 92.6% in the older animal group (9-10 months), indicating that pigs aged 3-4 months have already
17 been largely exposed to the circulating virus sustaining high antibody prevalence within the herd.
18 The proportion of HEV RNA positive stools and livers did not differ markedly between pigs with
19 presence or absence of anti-viral serum antibody. In fact, HEV seropositive and seronegative pigs
20 had a positive PCR in 32.5% (13/40) and 33.3% (2/6) of fecal samples, respectively. Bigger yet not
21 statistically significant difference were found for liver and bile samples, which contained HEV
22 RNA in 17.5% (7/40) vs. 50.0% (3/6) and in 51.4 % (19/37) vs. 33.3% (2/6), respectively, for
23 seropositive compared to seronegative pigs. The proportion of RNA positive bile samples in
24 seronegative animals (33.3%) was identical to that determined for stool (33.3%) and higher for liver
25 (50%). In line with the concept that replicating in the liver, before its release into the gut, HEV is
26 accumulated in the gallbladder, where it may likely reach higher concentrations than in other

1 matrices, a remarkably higher number of seropositive pigs exhibited presence of HEV RNA in the
2 bile (51.4%). It is possible that these latter pigs were undergoing a more productive phase of HEV
3 infection in their liver at the time of sampling, consequently exhibiting an enhanced serum antibody
4 production and viral titer in the blood. This is however hard to demonstrate, as both molecular and
5 immunological methods for HEV may have a limited sensitivity, particularly in the pig, and because
6 presence of RNA does not necessarily correlate with the infectious titer of virus. Overall, when
7 applicable, bile testing for virus RNA may represent the most sensitive indication of productive
8 infection.

9 Four pigs proved HEV RNA-positive in the absence of measurable serum antibodies. HEV
10 RNA was detected in fecal samples from 2 of these animals, in bile from 2 animals and in liver
11 from 3. These samples were tested repeatedly and resulted consistently positive for HEV, and at
12 least in the 3 cases with HEV RNA in liver and/or bile, the antibody seronegativity is likely to
13 indicate a recent viral infection not yet resulted in a detectable immune response to HEV, although
14 a possible false negative result of serological test may not be ignored. In the remaining case, with
15 only positive HEV stools, these possibly reflect ingestion and transient intestinal presence of the
16 virus, without any further replication.

17 Another finding of this study is the observation that different HEV strains circulate among
18 Italian pigs, as provided by sequence analysis of a 320 nt ORF2 fragment. All strains detected
19 belonged to g3, which is the only genotype found thus far in Europe from either pigs or humans
20 with endemic hepatitis E (Mansuy et al., 2004; Ijaz et al., 2005; Dalton et al., 2007; Fogeda et al.,
21 2009; Norder et al., 2009; Brost et al., 2010). The ORF2 fragment sequences obtained clustered in
22 two main branches, corresponding to subtype f (6/14 cases tested) and c (8 cases). The two clusters,
23 g3f and g3c, showed nucleotide identity of 79% one to each other. Subtype f is the genotype most
24 commonly found in pigs and humans in European countries (Fogeda et al., 2009; Kaba et al., 2009;
25 Rutjes et al., 2009) including Italy; in our previous studies g3f HEV strains have been detected in
26 pigs and wild boars (Caprioli et al., 2007; Di Bartolo et al., 2008; Martelli et al., 2008; Martelli et

1 al., 2010). More recently, spreading of g3c in European pigs has been reported (Rutjes et al., 2009)
2 including detection of this subtype in pigs in Italy (Di Martino et al., 2010; Martelli et al., 2010). In
3 Italy, the actual prevalence of hepatitis E in humans is not known, although an earliest human case
4 of autochthonous HEV was described in the remote 1999 (Zanetti et al., 1999). This HEV strain,
5 sequenced in a short stretch of the ORF2, was found to belong to genotype 3c, and comparison with
6 the same region of the swine strains reported in this study yields 83.9% nucleotide identity. We
7 have also shown that HEV sequences obtained from different types of specimen from a same
8 animal shared a nucleotide identity of 100%, suggesting that a single HEV strain was replicating in
9 each animal infected, or was at least overgrowing other possible coinfecting strains.

10 The results obtained in this study are important for addressing risks for public health, since
11 HEV has been detected at high level (64.6%) in apparently healthy animals at slaughterhouse, next
12 to entering the pork production chain and being commercialized. The risk of HEV transmission due
13 to consumption of raw meat and due to contact with pigs has already been highlighted in other
14 countries, as HEV RNA was detected in pig liver at grocery stores in Japan, The Netherlands and
15 USA (Yazaki et al., 2003; Bouwknegt et al., 2007; Feagins et al., 2007). Raw liver is not widely
16 consumed, although eating of fresh liver sausage (Figatellu) has recently been associated with an
17 upsurge of hepatitis E cases in France (Colson et al., 2010). Limited information is available on
18 HEV presence in pig muscle, where virus detection is made harder than in liver by lower virus
19 concentration (Williams et al., 2001; Bouwknegt et al., 2009). Unfortunately, in this study muscle
20 was not available. Since presence of HEV in muscle can be expected to be much lower than in liver,
21 this issue should better be resolved in studies involving high numbers of animals. In addition, HEV
22 RNA was detected in slaughterhouse workers in UK and Spain (Dalton et al., 2007; Galiana et al.,
23 2008), suggesting that HEV transmission from pigs to humans during slaughtering may also occur.
24 Also in Italy, a recent serological study has shown that contact with pigs may increase the risk of
25 infection for workers (Vulcano et al., 2007).

26

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Figure 1. Dendrogram, drawn using UPGMA and an avian HEV strain (United States, accession no. AY535004) as outgroup, was based on partial nucleotide sequence of the ORF2 fragment. GenBank accession no., origin, country, and genotype are reported for all strains; for strains identified in this study the sample origin is also indicated. Strains identified in this study are indicated with symbol ♦ and in **boldface type**.

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Table 1. Molecular detection of HEV in bile, feces and liver from swine, by age of individual

Matrix	HEV positive/tested samples (%)					
	Young (3-4 months)		Adult (9-10 months)		Total	
Bile, feces and liver	0/20	(0.0)	1/28	(3.6)	1/48	(2.1)
Bile and feces	9/20	(45.0)	0/28	(0.0)	9/48	(18.8)
Bile and liver	2/20	(10.0)	3/28	(10.7)	5/48	(10.4)
Feces and liver	2/20	(10.0)	0/28	(0.0)	2/48	(4.2)
Bile only	2/20	(10.0)	6/28	(21.4)	8/48	(16.7)
Feces only	2/20	(10.0)	2/28	(7.1)	4/48	(8.3)
Liver only	2/20	(10.0)	0/28	(0.0)	2/48	(4.2)
Total bile ^a	13/19	(68.4)	10/26	(38.5)	23/45	(51.1)
Total feces	13/20	(65.0)	3/28	(10.7)	16/48	(33.3)
Total liver	6/20	(30.0)	4/28	(14.3)	10/48	(20.8)
Pigs positive in at least one sample	19/20	(95.0)	12/28	(42.9)	31/48	(64.6)
Pigs negative in any samples	1/20	(5.0)	16/28	(57.1)	17/48	(35.4)

^a For 3 of 48 animals, bile were not available

Table 2. Molecular detection of HEV RNA in different samples from HEV seropositive and seronegative pigs.

	HEV RNA positive samples			(No. of pigs)
	Bile	Liver	Feces	
HEV Seropositive	-	-	-	(15 ^a)
	+	-	-	(7)
	+	+	-	(3)
	+	+	+	(1)
	+	-	+	(8)
	-	-	+	(3)
	-	+	+	(1)
	-	+	-	(2)
Total	19	7	13	(40)
HEV Seronegative	-	-	-	(2)
	+	-	-	(0)
	+	+	-	(2)
	+	+	+	(0)
	+	-	+	(0)
	-	-	+	(1)
	-	+	+	(1)
	-	+	-	(0)
Total	2	3	2	(6)
Grand total	21^b	10	15	(46 ^c)

^a Figures in parentheses indicate the numbers of pigs exhibiting the pattern of HEV RNA positivity in the corresponding line.

^b Figures in bold indicate the numbers of samples resulted positive for HEV RNA, by group of animals.

^c Two pigs of the total 48 investigated in this study were not included, because their sera were unavailable for HEV antibody determination.

