Detection and molecular characterisation of swine Hepatitis E virus in Brescia province, Italy

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Abstract

Hepatitis E virus (HEV) is an important public health concern in many developing countries and it occurs in sporadic forms in industrialized areas. With the discovery of swine HEV in pigs, which is genetically closely related to human HEV, hepatitis E is considered to be a zoonotic disease. To investigate the circulation of HEV within a distinct area of Lombardy region (Northern Italy), 17 pig farms were subjected to monitoring study by collection of fresh stool samples each represented by ground-pooled specimens. In particular, three distinct types of breeding farms were focused, represented by farrow to weaning, farrow to finish and fattening farms, respectively. Epidemiological data confirm that in Europe the seroprevalence in pigs, more than 9 month of age, ranges from 51.4 to 75%, while in 3-9 months fatteners is about 38%. In France and Italy, the positivity among farms is respectively 30 and 97.4% and the seroprevalence in Italy is 50.2%. Since HEV viremia was typically observed in the early period of life in swine, faeces were collected in boxes containing weaning pigs. For the study, 183 stool samples were collected and amplifications were performed with universal primers specific for the ORF2 region of genome. Twenty-eight samples resulted positive to HEV RNA and genotyping demonstrated that they were closely related to HEV strains belonging to genotype 3 and circulating in Europe. Comparison with reference strains from GenBank excluded their similarity to genotype 1, 2 or 4 confirming that genotype 3 strains are circulating in Europe. Since it was demonstrated that swine act as a reservoir for HEV, and since many strains into HEV genotype 3 share a strong molecular similarity to human HEV, it was important to detect the presence of HEV in a restricted area with a very high density of pigs.

Introduction

Hepatitis E virus (HEV) is a not enveloped, single stranded, positive-sense RNA virus. The genomic RNA is about 7.5 kb and contains three open reading frames (ORFs): ORF1 is predicted to encode non-structural proteins, ORF2 encodes the putative capsid protein, and ORF3 encodes a cytosckeleton-associated phosphoprotein (Meng *et al.,* 2002). HEV has recently been classified as the prototype member in the *Hepevirus* genus, *Hepeviridae* family. Although HEV strains belong to a single serotype, they show a considerable genetic diversity, according to time and place of isolation (Aggarwal and Naik, 2009); there exist at least four major genotypes (G1 to G4): type 1 (Asian strains, also detected in Europe associated with travellers, type 2 (Mexican and African strains), type 3 (strains from sporadic human cases in industrialized countries, also found in swine, wild boar, and sika deer). Type 4 strains, from human sporadic cases in East Asia, were also detected in Italy in humans (Garbuglia *et al*., 2013) and in pigs (Monne *et al*., 2015).

HEV is transmitted primarily by the faecal-oral route through contaminated water, and is the causative agent of hepatitis E, a self-limited enterically transmitted, non-A, non-B hepatitis in humans. Sporadic cases of hepatitis E have been documented in the United States and Europe mostly referred to travellers or pig handlers and veterinarians (Van Cuyck *et al.,* 2005). Hepatitis E is a zoonotic disease as HEV was found in pigs (Martinelli *et al.,* 2011; Gardinali *et al.,* 2012), wild boar (Takahashi *et al.,* 2004; Martinelli *et al.,* 2015; Caruso *et al.,* 2015), and since HEV infection was described among people who had eaten uncooked-infected deer meat (Tei *et al.,* 2003) and raw pork liver sausages (Renou *et al.,* 2014). Further observations confirming the association between pig liver or uncooked meat consumption, wild boar, or deer, and hepatitis E were reported also in Europe (Ruggeri *et al*., 2013b). The transmission to humans through food by the ingestion of infected meat products is not much probable when the virus is inactivated by the process of cooking, even if it has been described that swine HEV could have been transmitted to human beings after the consumption of fried and grilled pig liver (Yazaki *et al*., 2003).

The human and swine HEV isolates from industrialized countries are genetically clustered together in the same genotype (either G3 or G4) (Pavio *et al.,* 2010; Ruggeri *et al.,* 2013b). Veterinarians, slaughterhouse workers and pig handlers, show an increased prevalence of anti-HEV antibodies, suggesting a potential pig-to-human HEV transmission (Di Bartolo *et al.,* 2011; Carpentier *et al.,* 2012). In Italy, in the last two decades, 5 sequences on 22 autochthonous (not related to travels abroad) HEV sporadic cases were genotyped and all these strains were G3 HEV (Zanetti *et al*., 1999; Romanò *et al*., 2011).

In swine, the natural route(s) for HEV transmission remain unknown, even if repeated direct daily contact among pigs confined in the same pen may enhance the spread of the virus. Moreover, it was shown that swine HEV viremia and faecal virus shedding generally occur in pigs of 2 to 4 months of age (Cooper *et al.,* 2005; Vasickova *et al.,* 2009). However, the prevalence of HEV in pig faeces may be more various (Di Bartolo *et al.,* 2011; Martinelli *et al.,* 2011).

Nevertheless, people such as breeders, slaughterers and veterinarians could be at risk of infection, as already demonstrated (Meng *et al.,* 2002; Ruggeri *et al.,* 2013a). Recent virological surveys in Europe detected HEV in high sample proportions (often >40%) from apparently healthy animals at slaughterhouses, next to entering the pork production chain and being commercialized (Ruggeri *et al*., 2013b).

In Germany 42.7% to 50.3% pig sera were found to be anti-HEV IgG-positive. While 38.4% of fatteners (age between 3 and 9 months) exhibited HEV-specific antibodies, 51.4% of sows age older than 9 months exhibited anti-HEV antibodies. Fatteners kept in Southern Germany had a significantly higher HEV IgG prevalence compared to fatteners in the Northern federal states (Krumbholz *et al.,* 2013; Dremsek *et al.,* 2013). In France, 30% of the sampled farms resulted positive for HEV detection, while seroprevalence in slaughter-age pigs at the farm level reached almost 75% (Walachowski *et al.,* 2014). In Italy, 97.43% of farms and 50.21% of swine sera resulted positive for anti-HEV IgG antibodies, confiming that HEV is widespread in pigs in Italy and

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might be endemic on most farms (Martinelli *et al.,* 2011).

Since the Province of Brescia (Lombardy Region, Northern Italy) has an intense breeding activity of pigs, and since there are scarce informations about HEV in local farms, the aim of this study was to collect preliminary data referring to the prevalence of HEV among different types of breeding farms.

Materials and Methods

Sample collection

Since this survey was intended to obtain preliminary data about the possible circulation of HEV among local pig farms, the herd types were selected based on the willingness of farmers. Also, no specific sampling criteria were selected for the collection of faecal specimens. The considered geographic area was limited to about 240 km^2 in the southern side of the province of Brescia, (Northern Italy). Since in the province of Brescia there are more than 1,400,000 heads, with 224 farrow to weaning, 105 farrow to finish, 379 fattening farms, and that there are > 0.17 farms/km^2, this southern district was an area of high pig density (more than 1400 animals/km^2) (Zanardi *et al*., 2007; Bellini *et al*., 2007). One hundred eighty three faecal samples were collected from pigs 2-4 month-old, housed in 17 herds (7 farrow to weaning, 5 farrow to finish and 5 fattening farms, respectively). The number of animals ranged from 400 to 4500 per herd. The average number of sows in farms with >1000 animals was 250. Acquired pigs in fattening farms were 20-45 kg (live weight) and 3 months old. Each sample weighted 100 g, and was the result of a random collection of fresh stool harvested in five different points from the ground of pens containing 20-25 young animals each. The number of faecal samples by herd is reported in Table 1, and the average value of collections is 10 per farm.

Viral RNA extraction

Stools were clarified with sterile RNase-free water 1:10 w/v and viral RNA was extracted using a commercial kit with silica membranes (Nucleospin RNA II kit; Macherey, Nagel, Germany). Briefly, 100 µL of faecal suspension were added to 350 µL of a guanidinium isothiocyanate-buffer and 3.5 µL of β-mercaptoethanol for the lysis of cells. Then, 350 µL of 70% ethanol were added, and the suspension was centrifuged 1 minute at 11,000 x g. Contaminating DNAwas removed by a DNase I solution directly applied onto the silica membranes (95 µL of a 10% DNase solution) for 15 minutes at room temperature. Three washing steps with two different buffers removed salts, metabolites and PCR inhibitors. Pure RNA was finally eluted with 60 µL of RNase-free-water, centrifugating at 11,000 x g for 1 min.

Real-time nested polymerase chain reaction

Viral RNA was reverse transcribed with random primers into cDNA, and a Nested PCR was performed, following a protocol previously described (Erker *et al.,* 1999). Since the viral genome was extracted from stool, cDNA was amplified both undiluted and 1:10 diluted, to avoid inhibitions to the PCR.

Degenerate primers detected all HEV strains targeting the ORF2 region of genome, also with significant sequence variations.

For the reaction, 1.5 mM $MgCl_2$, 0.1 mM each dNTPs, and 0.5 µM each primers were employed. 0.02 U/µL AmpliTaq DNA Polymerase (Life Technologies -USA) was used. The primers for the first PCR were ORF2-S1 (5'-GAC AGA ATT RAT TTC GTC GGC TGG-3') and ORF2-A1 (5'-CTT GTT CRT GYT GGT TRT CAT AAT C-3'), while for the Nested PCR were ORF2-S2 (5'-GTY GTC TCR GCC AAT GGC GAG C-3') and ORF2-A2 (5'-GTT CRT GYT GGT TRT CAT AAT CCT G-3'). PCRs were carried out in 0.2-mL microcentrifuge tubes with 55 µL of PCR-master-mix and 5 µL of template cDNA and tubes were then placed in an automated thermal cycler (Gene Amp PCR System 9700; Life Technologies, Carlsbad, CA, USA). PCR and Nested PCR reactions, were processed with an initial inactivation step at 95°C for 3 min, followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 30 sec, and elongation at 72°C for 1 min. Final elongation step was 72°C for 10 min; PCR products were separated by agarose gel electrophoresis (2.5%) in 1X TAE buffer [(0.04 M Tris-acetate, 0.001 M EDTA (pH 8.00)], stained with ethidium bromide. Images were visualized on UV light and reproduced by a digital camera with Kodak 1D 3.6 program (expected PCR product: 145 bp).

Nucleotide sequencing and phylogenetic analysis

PCR products were purified with QIAquick® Gel Extraction Kit (Qiagen, Valencia, CA, USA) and then cycle sequenced on both strands of ORF2 PCR by BigDye® Terminator Cycle Sequencing kit (v1.1; Applied Biosystems, Carlsbad, CA, USA) using the same primers as used for nested PCR amplification. Sequence reactions were separated on ABI3130 genetic analyzer (Applied Biosystems). Sequences were assembled using SeqMan (Lasergene package; DNAStar Inc., Madison, WI, USA) and

Table 1. Distribution of Hepatitis E virus strains per different herd types.

Herd (n.)	Herd (type)	Breeded animals (n.)	Samples collected (n.)	Positive samples (n.)	Strains (n.)
1	FW	2000	10	-	
2	FW	1000	22	-	
3	FW	2700	13	-	
4	FW	1200	11	2	N1 (1), HUN-E113/VH1 (1)
5	FW	2000	10	-	
6	FW	1800	10	2	BCN-5 (2)
7	FW	3600	10	-	
Total FW	7	14,300	86	4	
8	FF	1150	13	4	NLSw28/BCN-12 (4)
9	FF	800	6	-	
10	FF	1200	10	1	NLSw28/BCN-12 (1)
11	FF	3000	10	-	
12	FF	400	10	6	W1 (6)
Total FF	5	16,900	49	11	
13	FA	500	10	2	BCN10 (2)
14	FA	3300	10	4	HUN-E113/VH1 (4)
15	FA	1038	10	-	
16	FA	2800	8	5	NLSw28/BCN12 (4), W1 (1)
17	FA	4500	10	2	W1 (2)
Total FA	5	12,138	48	13	
Total	17	43,338	183	28	

FW, farrow to weaning; FF, farrow to finish; FA, fattening. In the last column, the reference strains (and their number) with higher similarity to the sequence obtained in positive samples.

aligned by using the Clustal W programme. Sequences were subjected to BLAST search at http://www.ncbi.nlm.nih.gov/pubmed. Phylogenetic analysis was performed using as references HEV sequences belonging to genotype 1 to 4 according to the currently most accepted HEV classification proposed by Lu *et al.* (2006). Unrooted tree was generated using the distance-based Neighbor-Joining method determined on the base of the model selection function implemented within the MEGA 5 software. Bootstrap values were calculated on 1000 replicates of the alignment.

Results

Among the 183 stool samples tested, each represented by ground-pooled specimens, 28 (15.3 %) were positive for the HEV RNA. Positive samples were found in 4 of the 5 fattening (FA) farms, in 3 of the 5 farrows to finish (FF) herds, and in 2 of the 7 farrows to weaning (FW) herds (Table 1). Blast search of partial ORF 2 sequences obtained, revealed that HEV strains of the present study could be clustered in at least 6 7 groups based on the % percentage of nucleotide identity towards HEV strains retrieved from GenBank (Table 2). In particular, group 1 (5 sequences) was 94% identical to swine HEV strain NLSw28 (AF336292) and 91-92% similar to BCN12 strain (AF490993). Group 2 (4 sequences) showed 97% identity to BCN12 strain (AF490993) and 95% to NLSw28 (AF336292). Group 3 (9 sequences) shared 92% similarity with W1 strain (AF490998); Group 4 (2 sequences) shared 98% similarity with BCN5 strain (AF490986) and 94-95% with NLSw28 (AF336292); group 5 (2 sequences) shared 94% similarity with N1 strain (AF490999) and 92% to HUN-E113 (EF530669); group 6 (2 sequences) was 94% similar to BCN10 strain (AF490991); group 7 (4 sequences) showed 94% nucleotide identity with strain HUN-E113 (EF530669) and 91% similarity with VH1 strain (AF401000).

Sequences similar to BCN-12, NLSw28 and W1 strain derived from viruses circulating among farrow to finish pig farms, while those related to N1, VH1, HUN-E113 and BCN-5 strain were from HEV detected in farrow to weaning pig farms. HEV sequences similar to BCN10, BCN12, W1, NLSw28, HUN-E113 and VH1 strain were recovered in fattening farms (Table 1). Phylogenetic analysis (Figure 1) conducted comparing the sequences of the present work to HEV reference sequences (according to the classification proposed by Lu *et al.*, 2006), revealed that all the sequences could be placed in different branches within Genotype 3. It also confirmed the presence of 7 main clusters within genotype 3 and excluded any relation to genotype 1, 2 and 4. In particular, sequences belonging to group 1, 2 and 4 grouped together with reference sequence of genotype 3f. Sequences of group 5 were more related to genotype 3c sequences; group 6 sequences clustered among genotype 3 h. As for sequences of groups 3 and 7 further subtyping was not possible. This was probably due to

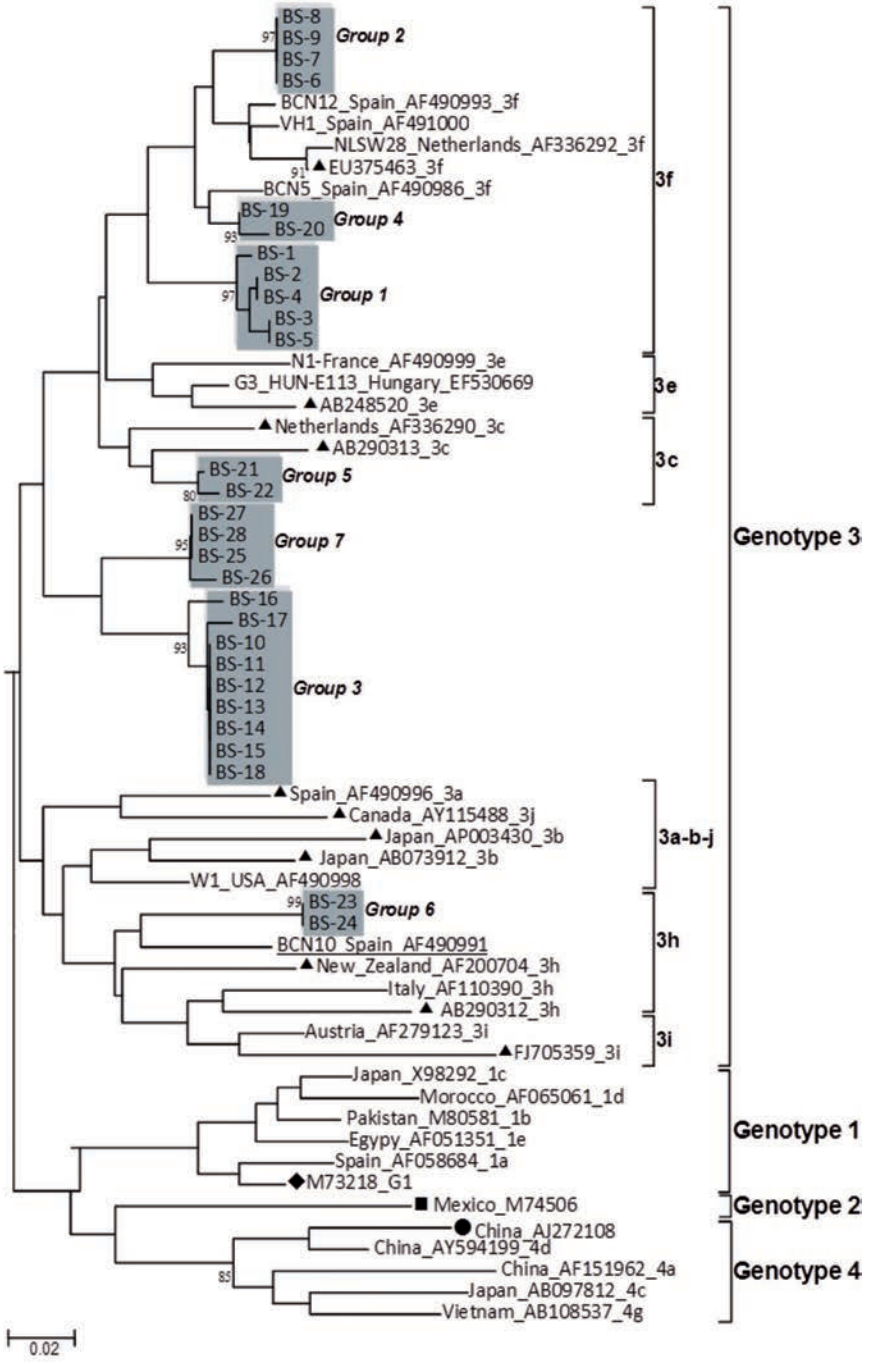

Figure 1. Phylogenetic relationship over a 122-bp fragment within ORF2 among 28 Hepatitis E virus strains of this study and reference sequences belonging to HEV genotype and subtype, according to Lu *et al.* (2006) classification. Genotype I (♦); genotype II (■); genotype IV (●). Within genotype III, reference sequences referred to each known subtype are marked as ▲. Hepatitis E virus sequences belonging to groups 1 to 7 are highlighted in grey boxes. Sequence AF110390 represents a human sporadic case in Italy (Zanetti *et al.*, 1999). Unrooted tree was constructed using the Neighbor-Joining method (MEGA 5). Bootstrap values represent the percentage of 1000 trees supporting the clustering; only values above 70% are shown.

the existence of possible inconsistencies within current HEV classification especially when applied to the analysis of partial ORF2 region (Oliveira-Filho *et. al.,* 2013). No simultaneous infection of different strains was detected in positive samples.

Discussion

The aim of this study was to estimate the prevalence of HEV-positive farms in a limited area in the southern side of the province of Brescia (Lombardy Region, Northern Italy). Monitoring data showed 28 positive samples (15.3%) confirmed as swine HEV strains by sequencing the PCR products, corresponding to a partial region of the ORF2. All positive samples were characterized as belonging to the HEV G3, due to their high similarity to G3 strains previously detected in Spain, Netherlands and Hungary (Clemente-Casares *et al.,* 2003; van der Poel *et al.,* 2001; Reuter *et al.,* 2009). Sequences similar to N1, HUN-E113, VH1, and BCN-5 strains derived from viruses present in FW pig farms, while those related to NLSw28, BCN-12 and W1 derived from viruses circulating among sFF pig farms. HEV sequences similar to BCN10, and the other previously cited strains (except for BCN-5), were collected in FA herds (Table 1). Higher diversity in positive samples was detected in 2/7 (28.6%) FW farms, while the prevalence of positive FF farms was 3/5 (60%). Four FA farms out of 5 (80%) resulted positive for HEV-PCR detection. All categories of farms resulted positive for HEV, but preliminary data did not permit to assign a statistically significant difference between them. However, FA system seemed to be linked to a higher diversity of HEV strain types and to a higher percentage of positive farms; this could be due to the diverse origins of the 3-3.5 months old introduced piglets (domestic and foreign), contributing to enhance the difference of strains and the HEV infection rate.

The total 15.3% value of positive samples (28/183), indicates that HEV was actually circulating among the swine population in the province of Brescia, and that results were not sporadic. This percentage is lower than that previously reported for individual faecal swabs in 3-4 months old weaners (Di Bartolo *et al.,* 2008), and for faeces at slaughterhouse in Italy (Di Bartolo *et al.,* 2011, 2012) (33% and 41% respectively), but are closely related to the European average (27%). However, data reported in our study is referred to a small geographic area, even if with a very high density of pigs' population.

Hepatitis E infection in humans is typically associated with endemic areas (Asia, Africa and South America), but HEV has also been isolated in patients with acute infections in countries where HEV is not endemic (Lin *et al.,* 2014). G3 and G4 circulate among humans in Europe and G3 is widespread in pigs, including Italy and other European countries (Di

Table 2. Sequences of the 28 positive field samples of the present work subjected to Blast search.

Sample	Strain ID	Accession number	% Identity	Origin of HEV	Source of HEV	Group/genotype
BS-1	BCN12/NLSw28	AF490993/AF336292	94/92	Spain/Netherlands	Sewage/swine	1/3f
BS-2	BCN12/NLSw28	AF490993/AF336292	94/92	Spain/Netherlands	Sewage/swine	1/3f
BS-3	BCN12/NLSw28	AF490993/AF336292	94/91	Spain/Netherlands	Sewage/swine	1/3f
BS-4	BCN12/NLSw28	AF490993/AF336292	94/92	Spain/Netherlands	Sewage/swine	1/3f
BS-5	BCN12/NLSw28	AF490993/AF336292	94/91	Spain/Netherlands	Sewage/swine	1/3f
BS-6	BCN12/NLSw28	AF490993/AF336292	97/95	Spain/Netherlands	Sewage/swine	2/3f
BS-7	BCN12/NLSw28	AF490993/AF336292	97/95	Spain/Netherlands	Sewage/swine	2/3f
BS-8	BCN12/NLSw28	AF490993/AF336292	97/95	Spain/Netherlands	Sewage/swine	2/3f
BS-9	BCN12/NLSw28	AF490993/AF336292	97/95	Spain/Netherlands	Sewage/swine	2/3f
BS-10	W1	AF490998	92	USA	Sewage	3/3
BS-11	W1	AF490998	92	USA	Sewage	3/3
BS-12	W1	AF490998	92	USA	Sewage	3/3
BS-13	W1	AF490998	92	USA	Sewage	3/3
BS-14	W1	AF490998	92	USA	Sewage	3/3
BS-15	W1	AF490998	92	USA	Sewage	3/3
BS-16	W1	AF490998	92	USA	Sewage	3/3
BS-17	W1	AF490998	92	USA	Sewage	3/3
BS-18	W1	AF490998	92	USA	Sewage	3/3
BS-19	BCN5/NLSw28	AF490986/AF336292	98/95	Spain/Netherlands	Sewage/swine	4/3f
BS-20	BCN5/NLSw28	AF490986/AF336292	98/94	Spain/Netherlands	Sewage/swine	4/3f
BS-21	N1/HUN-E113	AF490999/EF530669	94/92	France/Hungary	Sewage/human	5/3c
BS-22	N1/HUN-E113	AF490999/EF530669	94/92	France/Hungary	Sewage/human	5/3c
BS-23	BCN10	AF490991	92	Spain	Sewage	6/3h
BS-24	BCN10	AF490991	92	Spain	Sewage	6/3h
BS-25	HUN-E113/VH1	EF530669/AF491000	94/91	Hungary/Spain	Human	7/3
BS-26	HUN-E113/VH1	EF530669/AF491000	94/91	Hungary/Spain	Human	7/3
BS-27	HUN-E113/VH1	EF530669/AF491000	94/91	Hungary/Spain	Human	7/3
BS-28	HUN-E113/VH1	EF530669/AF491000	94/91	Hungary/Spain	Human	7/3

HEV, Hepatitis E virus; Based on blast scores, sequences could be distinct into 7 groups (1 to 7) showed in the column Group/genotype together with the assigned HEV genotype and subtype when possible.

Bartolo *et al.,* 2008; Berto *et al.,* 2012a, 2012b). The increasing cases of sporadic human HEV infections in countries of non-endemicity and the considerable rate of anti-HEV prevalence in sera (30-40%) (Candido *et al.,* 2012; Rapicetta *et al.,* 2013) indicates that humans are at risk of infection more than estimated. Moreover, human RNA sequences appear to be closely related to the swine RNA detected in the same countries (Purdy and Khudyakov, 2010).

As being HEV infection asymptomatic in pigs, particular attention could be paid to the production and the processing of pork meat, because the virus is potentially pathogen for human consumers. This is particularly important in those Italian regions where alimentary traditions are strictly linked to the consumption of raw pork meat. The HEV survival in some typical Italian swine-products, such as salami and sausages needs to be evaluated either during seasoning; in fact, recent studies confirmed that HEV present in pork liver sausage was still infectious, highlighting the actual risk for consumers (Berto *et al.,* 2012a, 2012b, 2013; Renou *et al.,* 2014).

Conclusions

In spite of the few considered breeding plants (17) and the low number of analysed samples (183), this work evidenced the circulation of HEV in Italian pig population, even in a restricted area. The data obtained could be considered indicative as the starting point for a deeper study about a hypothetical correlation between the HEV spreading in swine population and sporadic cases of hepatitis E in humans. This fact was already discussed in a recent report from European Food Safety Authority biohazard experts (EFSA, 2011) who underscored an urgent need for integrated studies on HEV circulation, performing farm to table integrated risk assessment. The correct understanding of the impact of the breeding system (farrow to weaning, farrow to finish, and fattening) on HEV epidemiology, as described above, needs further study.

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