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 sampling in the Brescia province, from January 2011 to December 2011, with the aim of collecting fresh stool specimens from different age groups of pigs, in order to monitor the circulation of HEV in swine. 

The samples were genotyped and all these samples 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 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).

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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 cytoskeleton-associated phosphoprotein (Meng et al., 2002). HEV has recently been classified as the prototype member in the Hepivirus genus, Hepivirusidae 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-limiting 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; Gardinalli 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 swine HEV were available in the GenBank database, belonging to G3 HEV.

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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 km² 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 finishing, 379 fattening farms, and that there are > 0.17 farms/km², this southern district was an area of high pig density (more than 1400 animals/km²) (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 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 MgCl₂, 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 TGC 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 GGG GAG CAG 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) | Breeding 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                    | WI (6)     |
| Total FF | 5           | 16,800                | 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), WI (1) |
| 17       | FA          | 4500                  | 10                    | 2                    | WI (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) showed 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 were 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
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 sFP 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 et al., 2013). No simultaneous infection of different strains was detected in positive samples.

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 | 98/95      | Spain/Netherlands    | Sewage/swine  | 1/3f           |
| BS-2    | BCN12/NLSw28      | AF490993/AF336292 | 98/95      | 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 | 94/91      | 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.
Traditions are strictly linked to the consumption of raw pork meat. This is particularly important because the virus is potentially pathogenic 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 plants (17) and the low number of analysed samples (183), this work evidenced the circulation of HEV subtypes 3e and 3f. Arch Virol 160:153-60.

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