Hepatitis E virus genotype 3 and sporadically also genotype 1 circulate in the population of Catalonia, Spain

Pilar Clemente-Casares, Jesus Rodriguez-Manzano and Rosina Girones

ABSTRACT

Autochthonous hepatitis E virus (HEV) strains have been described infecting populations of industrialized countries, previously considered as non-endemic areas. The HEV strains circulating in one of those areas in south-western Europe (Barcelona, Spain) have been studied by analysing amplicons obtained from HEV genomes identified in wastewater, biosolids and sludge. Six sewage and two biosolid HEV positive samples from urban wastewater treatment plants and two positive HEV sludge samples with animal contamination were analysed by cloning and sequencing of 10–12 clones per sample. The results proved the presence of HEV strains belonging to genotype 3 and also sporadically to genotype 1 in urban sewage and biosolids, showing the simultaneous circulation of diverse HEV strains in the human population of the studied area. Only HEV genotype 3 was identified in slaughterhouse sludge samples. The circulation of genotype 1 in industrialized areas may have further health implications since this genotype has been associated with important epidemics in developing areas. Contamination of food and water through their contact with sewage not properly treated and biosolids presenting HEV may represent a significant risk for human populations in relation to HEV even in industrialized areas.

Key words | biosolids, environmental contamination, hepatitis E, *Hepevirus*, sewage, sludge

Pilar Clemente-Casares
Jesus Rodriguez-Manzano
Rosina Girones (corresponding author)
Department of Microbiology,
Faculty of Biology,
University of Barcelona,
Avinguda Diagonal 645,
08028 Barcelona,
Spain
Tel.: +34-93-4021483
Fax: +34-93-4039047
E-mail: *rgirones@ub.edu*

Pilar Clemente-Casares
CRIB, University of Castilla-La Mancha,
Avenida Almansa 14,
02006 Albacete,
Spain

ABBREVIATIONS

cDNA	complementary deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DTT	dithiothreitol
ORF2	open reading frame 2
PBS	phosphate buffered saline
PCR	polymerase chain reaction
RNA	ribonucleic acid

INTRODUCTION

Hepatitis E virus (HEV) is an RNA virus responsible for more than 50% of acute hepatitis in developing countries (Yarbough 1999). In these areas, sporadic and epidemic acute hepatitis E are frequently identified, mainly due to faecally contaminated water (Emerson & Purcell 2003). Traditionally, industrialized countries have been considered non-endemic areas for this virus, as only sporadic cases related to imported strains were diagnosed (Bader *et al.* 1991; Dawson *et al.* 1992). However, several HEV isolates have recently been identified from patients with acute hepatitis who lived in developed areas and with no history of travelling to endemic areas (Schlauder *et al.* 1998, 1999; Worm *et al.* 1998; Zanetti *et al.* 1999; Pina *et al.* 2000; Takahashi *et al.* 2001, 2002; Mizuo *et al.* 2002). The HEV strains isolated from these cases, which belong to genotypes 3 and 4, can be considered autochthonous of these industrialized areas and seem to have a low pathogenic profile (Purcell & Emerson 2008). However, the arrival and circulation of genotypes 1 and 2 in these areas may have further health implications since these genotypes have been associated with important epidemics. During these

doi: 10.2166/wh.2009.120

epidemics, high mortality rates have been observed, especially in pregnant women (Balayan 1997).

Studying the presence of a pathogen in the sewage generated by a population provides a global view of the excretion, and therefore the prevalence of infection in this population. A previous study carried out in Barcelona revealed the presence of HEV strains in 43.5% of the sewage samples collected between 1994 and 2002 (Clemente-Casares *et al.* 2003). More than 20 autochthonous strains have been identified since then in urban sewage in Barcelona (Spain), where the seroprevalence in a population sample has recently been estimated as 7.3% (Buti *et al.* 2006). The sequences detected belonged to genotype 3 (Clemente-Casares *et al.* 2003; Bofill-Mas *et al.* 2006; Albinana-Gimenez *et al.* 2006).

Viruses present in wastewater effluents are discharged into aquatic environments. They also accumulate in the biosolids or sludge generated during the treatment of wastewater, which are sometimes applied to fields or used as fertilizers (Straub *et al.* 1993). Accidental contamination of food and water by these viruses represents a risk for human populations. In a study conducted in Barcelona (Bofill-Mas *et al.* 2006), HEV was detected in two out of five biosolid samples collected from an urban sewage treatment plant. Although most of the sequences found were very similar to others previously detected in Barcelona (genotype 3), genotype 1 HEV sequences were also identified.

HEV has also been detected infecting swine, deer, wild boars, mongooses and chickens (Meng *et al.* 1997; Haqshenas *et al.* 2001; Matsuda *et al.* 2003; Tei *et al.* 2003). The strains isolated from the mammals, which belong to genotypes 3 and 4, are very similar to those infecting humans, especially if they have been detected in the same area (Hsieh *et al.* 1998; Haqshenas *et al.* 2001). Indeed, transmission of HEV to humans due to the consumption of uncooked meat from deer and wild boars has already been proven (Matsuda *et al.* 2003; Tei *et al.* 2003). Several research works performed in Spain allowed the detection of naturally HEV infected pigs and also the presence of the virus in sewage and sludge generated in slaughterhouses (Pina *et al.* 2000; Clemente-Casares *et al.* 2003; Albinana-Gimenez *et al.* 2006; Seminati *et al.* 2008; Fernandez-Barredo *et al.* 2007). The arrival of those animal HEV strains in the environment can also represent a risk for human populations.

The objective of this study is the further characterization of the diversity of HEV strains that may be in one wastewater or sludge/biosolid sample that can potentially contaminate water and the environment and to determine whether genotypes other than genotype 3 are present in the wastewater and sludge generated in an industrialized area.

MATERIALS AND METHODS

Urban wastewater treatment plant samples

Urban sewage samples and biosolids generated during sewage treatment were collected from the sewage network of Barcelona (Spain) during the years 2001, 2003, 2005 and 2007. Each sample was collected in a sterile container and kept at 4°C for less than 8 hours until the viral particles were concentrated in phosphate buffered saline (PBS pH 7.3). Positive HEV samples identified by seminested-PCR were selected (six sewage samples and two biosolid samples). A summary of the studied samples is presented in Table 1. The presence of HEV in sewage from Barcelona has been estimated in previous studies as 43.5% (Clemente-Casares *et al.* 2003). Six out of 34 HEV-typified sewage samples were selected for this study. Two HEV positive biosolids of four tested samples (Bofill-Mas *et al.* 2006) were also included.

Slaughterhouse samples

Sludge samples with contamination of animal origin were collected in a slaughterhouse located in Catalonia (Spain) in 2004 and 2006. This slaughterhouse dealt with bovine and, especially, porcine adult animals (more than 80% of the processed animals were pigs). The samples were collected in sterile containers and kept at 4°C for less than 8 hours prior to the concentration of the viral particles. Two positive HEV sludge samples (out of five tested by Albinana-Gimenez *et al.* (2006) and Hundesa *et al.* (2006)) were selected for the study.

Viruses

Faecal suspensions obtained from monkeys (*Macaca mulatta*) infected with the HEV BCN strain (10% in PBS

Table 1 | Typification and diversity of the HEV strains identified by cloning the amplicons obtained from the analysed samples

Sample	Type of sample	Sampling (year/month)	Sequences found	% Intra-sample similarity	Genotype
BCN8	Urban sewage	2001/April	3 (BCN8.1 to 3)	90–96	G3
BCN10	Urban sewage	2001/May	1 (BCN10)	–	G3
BCN25	Urban sewage	2003/March	1 (BCN25)	–	G3
BCN26	Urban sewage	2003/May	5 (BCN26.1 to 5)	94–99	G3
BCN23	Urban sewage	2005/February	1 (BCN23)	–	G1
BCN27	Urban sewage	2007/December	1 (BCN27)	–	G1
BBCN1	Biosolid*	2005/January	2 (BBCN1.1 & 2)	99	G3
BBCN2	Biosolid*	2005/February	3 (BBCN2.1 to 3)	98–99	G1
E5	Sludge†	2004/May	4 (E5, E5.2 to 4)	92–99	G3
E6	Sludge†	2006/February	8 (E6.1 to 8)	89–99	G3

*From an urban sewage treatment plant.
†From a slaughterhouse (more than 80% of the processed animals in this slaughterhouse were pigs).

7.3) were used as positive control for the polymerase chain reaction (PCR) analysis. This strain had previously been isolated from sewage from Barcelona although it is genetically similar to Indian HEV genotype 1 strains (Pina *et al.* 1998). The viral suspensions were stored at −80°C.

Concentration of viral particles from sewage samples

The recovery and concentration of viral particles were carried out as described in previous studies. The protocol for processing sewage samples is based on ultracentrifugations and elution in 0.25 N glycine buffer pH 9.5 (Puig *et al.* 1994). Briefly, 42 ml of sewage sample were ultracentrifuged (110,000 × *g* for 1 hour at 4°C). The sediment was eluted with 3.5 ml 0.25 N glycine buffer (pH 9.5) and kept on ice for 30 minutes. After the addition of 2 × PBS, the suspension was centrifuged at 12,000 × *g* for 15 minutes to separate any suspended solids. Viruses in the supernatant were pelleted by ultracentrifugation (110,000 × *g* for 1 h at 4°C), resuspended in 0.1 ml 1 × PBS and stored at −80°C.

Concentration of viral particles from biosolid samples

As described previously (Bofill-Mas *et al.* 2006), 40 g of the biosolid samples were eluted in 50 ml glycine buffer pH 9.5 and stirred for 1 hour. The pH was neutralized with NaOH 5 M and the sample was centrifuged for 45 minutes at 38,400 × *g* at 4°C. Viral particles contained in the supernatant were pelleted at 110,000 × *g* for 1 hour at 4°C and finally eluted in 200 μl PBS. The viral concentrate was stored at −80°C until nucleic acid extraction.

Concentration of viral particles from sludge samples

The sludge samples from the slaughterhouse were processed with the same protocol used for sewage but starting with the elution of 1 g of sludge in 3.5 ml of 0.25 N glycine buffer pH 9.5 (Albinana-Gimenez *et al.* 2006).

RNA extraction

Viral nucleic acids from the samples were extracted with a protocol described previously (Boom *et al.* 1990) using guanidinium isothiocyanate (GuSCN) and adsorption of the nucleic acids to silica particles as described.

Detection of HEV by seminested RT-PCR

The HEV RNA was amplified by seminested RT-PCR with degenerated primers described by Erker *et al.* (1999) using a one-step RT-PCR (Albinana-Gimenez *et al.* 2006), followed by a second-round PCR. Five μl of the extracted nucleic acids and a tenfold dilution, corresponding to 2 and 0.2 ml of sewage, 100 mg and 10 mg of dry weight of biosolid and 50 mg and 5 mg of sludge, were analysed for each sample. Fragments of 198 and 148 bp-long (first-round and

second-round products respectively) were amplified within the ORF2 of the HEV genome.

The RT-PCR assay was performed using the OneStep RT-PCR kit from QIAGEN. Five μl of the extracted nucleic acids or a tenfold dilution were added to a total volume reaction of 50 μl containing 1 × OneStep QIAGEN Buffer, 2 μl of QIAGEN OneStep Enzyme Mix, 400 μM of each dNTP, 10 units of ribonuclease inhibitor (Applied Biosystems) and 25 pmol of each outer primer (HEVORF2con-a1 and HEVORF2con-s1). After 30 minutes at 50°C, the reaction was heated at 95°C for 15 minutes, followed by 35 cycles at 94°C for 20 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 1 minute. All amplifications were completed with a 10 minute, 72°C extension period.

The second-round PCR was carried out by adding 1 μl of the first-round product to a new batch of 50 μl PCR reaction mixture containing 1 × PCR Gold Buffer (Perkin-Elmer Roche), 1.5 mM $MgCl_2$, 10 nmol of each dNTP, 2 units of Ampli Taq Gold™ (Perkin-Elmer Roche) and 25 pmol of each nested primer (HEVORF2con-a1 and HEVORF2con-s2). The products were analysed by agarose gel electrophoresis using ethidium bromide as a stain.

Standard precautions were applied in all the manipulations in order to reduce the probability of sample contamination by amplified DNA molecules. Negative controls for the RT-PCR reaction and the extraction process were added in every assay and the positive amplifications were confirmed by sequencing analysis. External positive controls were used by adding viral particles to viral concentrates. All samples were also analysed for the presence of viral indicators of faecal contamination, human adenoviruses and porcine adenovirus, and no significant inhibition was observed.

Cloning of the amplicons

The amplicons obtained were purified using the QIAquick PCR purification Kit (QIAGEN, Inc.) and ligated into a pGEM®-T EasyVector (pGEM®-T EasyVector System II, Promega Co.) following the manufacturer's instructions. The ligation product was used to transform *Escherichia coli* JM109 competent cells (Promega Co.) or DH5α. Transformed bacterial clones were detected by blue/white screening. The insert from presumptive transformed clones was amplified by inoculation of some growth into a mix containing 1 × Reaction Buffer (160 mM $(NH_4)_2SO_4$, 670 mM Tris-HCl pH 8.8, Tween-20) (Bioron, GmbH, Germany), 2 mM $MgCl_2$, 2 units of Taq DNA polymerase (Bioron, GmbH, Germany), 10 nmol of each dNTP and 10 pmol of the T7 and SP6 primers (sequences contained in the vector). Following a denaturing step of 3 minutes at 94°C, 35 cycles at 94°C for 20 seconds, 55°C for 30 seconds and 72°C for 30 seconds were carried out. All amplifications were completed with a 10 minute, 72°C extension period. The products were analysed by agarose gel electrophoresis using ethidium bromide as a stain.

Sequence analysis

Ten to 12 clones per sample were sequenced. The purified DNA was sequenced with the ABI PRISM™ Dye Terminator Cycle Sequencing Ready Reaction kit version 3.1 with Ampli Taq® DNA polymerase FS (Applied Biosystems) following the manufacturer's instructions. The results were checked using the ABI PRISM 377 automated sequencer (Perkin-Elmer, Applied Biosystems). A fragment of 101 bp of each amplicon was compared with other HEV sequences present in the GenBank and the EMBL (European Molecular Biology Library) using the basic BLAST program of the NCBI (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/BLAST/, accessed 20 March 2009). Alignments of the sequences were carried out using the ClustalW program of the EBI (European Bioinformatics Institute of the EMBL, http://www.ebi.ac.uk/clustalw/, accessed 20 March 2009). Phylograms were generated by the UPGMA algorithm using the NEIGHBOR program. The robustness of the grouping was determined by bootstrap resampling of the multiple sequence alignments (1,000 sets) with the programs SEQBOOT, DNADIST, NEIGHBOR and CONSENSE. The output graphics of the trees were created with the TREEVIEW package, version 1.5 (Page 1996). Sequences present in the databases and used for phylogenetic studies are shown in Table 2. The GenBank accession numbers for the sequences reported in this paper are EU450839-EU450866 and EU729700. Accession numbers for BCN10, BCN23 and E5, previously reported, are

Table 2 | Nucleotide sequence accession numbers for hepatitis E virus strains used for phylogenetic analysis

Country	Strain*	Accession number
Barcelona, Spain	BCN	AF058684
	BCN3	AF490985
	BCN4	AF491003
	BCN5	AF490986
	BCN6	AF490987
	BCN7	AF490988
	BCN12	AF490993
	BCN16	AF490997
	VH1	AF491000
	VH2	AF491001
	VH3	AY540113
	VH4	AY540114
	VH5	AY540115
	Por1	AF491002
France	France	AY626042
Greece	Greece1	AF110391
	Greece2	AF110392
Italy	Italy	AF110390
Austria	Austria	AF279123
The Netherlands	SwNe	AF336292
Canada	SwCanada	AY115488
United States	USA1	AF060668
	USA2	AF060669
	SwUSA	AF082843
Japan	Japan1	AB074918
	Japan2	AB089824
	Japan3	AB220971
Mexico	Mexico	M74506
Pakistan	Pakistan	M80581
Burma	Burma	M73218
China	China1	D11092
	China2	AJ272108
	China3	AF082094
Chad	Chad	AY204877
Egypt	Egypt	AF051352
Morocco	Morocco	AY230202
India	India	DQ459342

*As named in Figure 1.

AF490991 (Clemente-Casares *et al.* 2003), DQ400354 and DQ400356 (Albinana-Gimenez *et al.* 2006), respectively.

RESULTS

Six samples of urban sewage, two biosolid samples and two samples of sludge from a slaughterhouse, positive for HEV, were studied by cloning and sequencing of 10–12 clones per sample in order to obtain information on the potential diversity of HEV strains excreted in the population and that may be accumulated in the generated residues. One hundred and six clones were sequenced. The results are summarized in Table 1.

As expected, most of the samples presented HEV genotype 3 strains. Four urban sewage samples (BCN10, BCN23, BCN25 and BCN27) showed just one sequence among all the analysed clones: BCN23 and BCN27 typified as genotype 1. Three different sequences were found from sample BCN8 and five from BCN26. The results obtained from the two biosolid samples collected in the urban sewage treatment plant showed two sequences from BBCN1 and three from BBCN2, BBCN2.1 to BBCN2.3 belonging to genotype 1. More diversity was identified in the sludge samples collected in the slaughterhouse, as four and eight sequences were detected from E5 and E6, respectively. Differences among sequences detected from the same sample ranged from 1% to 11% (Table 1). When comparing different samples, the biggest differences were observed between samples containing sequences belonging to different genotypes: that is, differences between sequences from BCN8 and BBCN2 ranged from 22.8% to 24.8%, and 19.8% to 25.7% between BBCN2 and E6. In contrast, similarities between genotype 3 sequences from samples with human and animal contamination were as high as 98.0% (between BCN26.1 and E5.3).

As expected, genotype 3 was the most prevalent genotype in the area (74 clones), although genotype 1 was also detected. The phylogenetic analysis of the sequences obtained in the study is shown in Figure 1. All the sequences detected from samples BCN8, BCN10, BCN25, BCN26, BBCN1, E5 and E6 belonged to genotype 3. They were very similar or even 100% identical in the studied fragment to other isolates previously

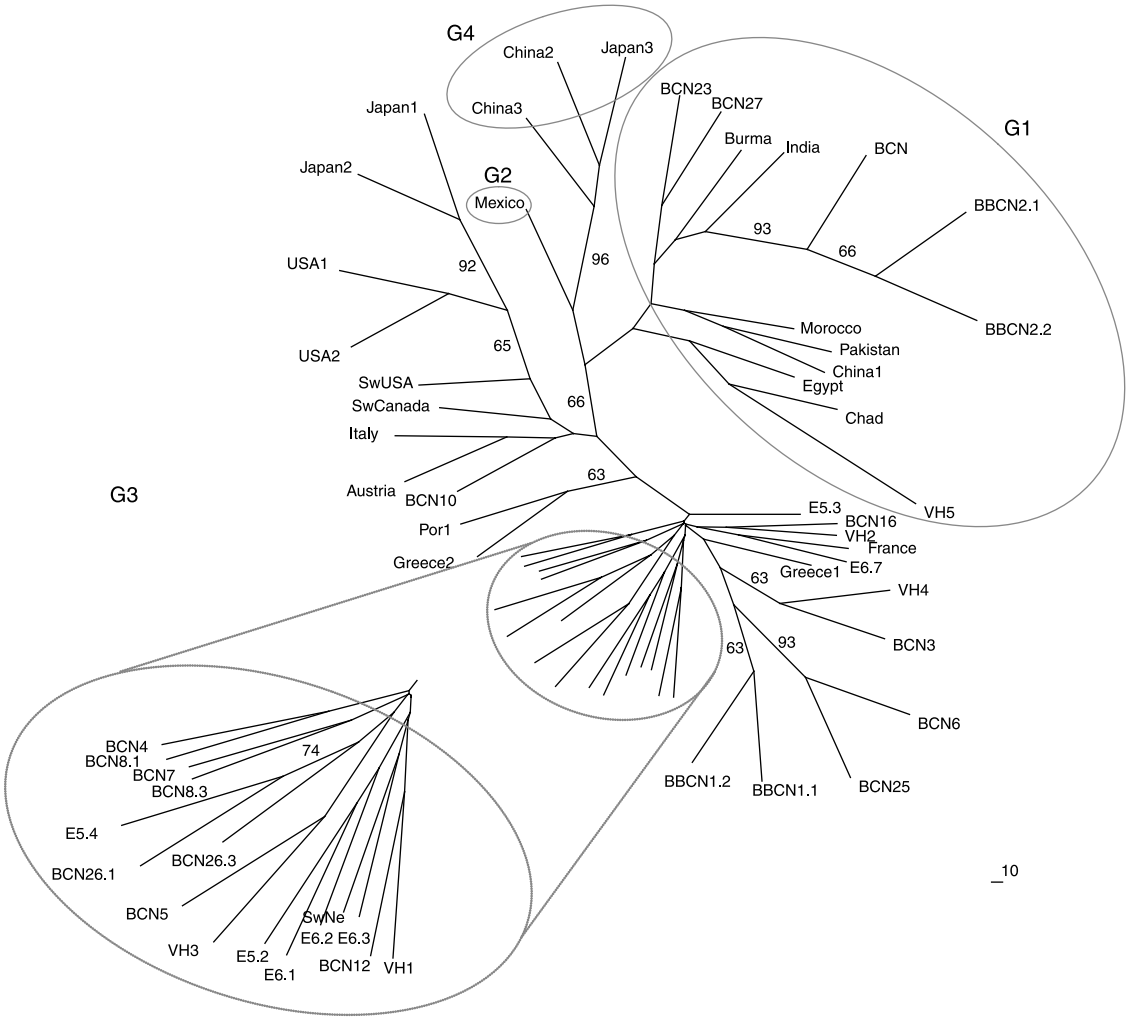

Figure 1 | Unrooted phylogenetic tree depicting relationships over 101 nucleotides within ORF2 among representative HEV strains reported in this study and others isolates from genotype 1, genotype 2, genotype 3 and genotype 4. The internal node numbers represent bootstrap values (1,000 replicates) expressed as the percentage of all trees. Only values greater than 60 are represented. GenBank accession numbers of the sequences used for phylogenetic analysis can be found in Table 2.

detected in Barcelona from sewage and clinical serum samples of hepatitis E cases. BCN23, for example, was 100% identical in the sequenced region to BCN6, detected 2 years before.

Three samples (BCN23, BCN27 and BBCN2) showed sequences belonging to genotype 1. When comparing sequences from BCN23 and BBCN2, collected in the same wastewater treatment plant and during the same month, the identity was only about 92.1–93.1%. BBCN2.1 to BBCN2.3 were 98–100% identical to a strain from South Africa (strain RSA-1) and 98–99% identical to the BCN strain, detected from sewage in Barcelona and previously considered as imported as it clustered within genotype 1 (Pina *et al.* 1998). BCN27 was 97% identical in the studied fragment to BCN23. They were highly similar to the isolate BurmaEpi-1-3 from Myanmar (95.0% and 97%, respectively) and to one from India (DQ459342) (95.0% and 96.0%, respectively). The nucleotide accession numbers of the HEV strains used for the phylogenetic analysis are shown in Table 2.

While genotypes 3 and 1 were found in sewage and biosolid samples with human faecal contamination, sludge containing animal faecal contamination only exhibited sequences belonging to genotype 3 as expected. Those animal HEV sequences were very similar to other European isolates, independently of their human or animal origin;

E6.1 presented the highest identity (97.0%) with the isolate NLSW28 (from swine in the Netherlands) and with BCN12 and BCN9 (from urban sewage from Barcelona). Similar cases are observed with E6.4, E6.8 or E5.2.

DISCUSSION

The variability of HEV sequences observed in this study suggests that diverse HEV strains are commonly circulating in the population of the studied area. According to the high number of sewage samples containing HEV found over previous studies in Barcelona (Clemente-Casares *et al.* 2003) and the seroprevalence values reported by Buti *et al.* 2006 (7.3%), HEV infections in humans (mostly subclinical infections) are probably more prevalent than expected. The low number of identified acute hepatitis E cases in the area (Pina *et al.* 2000; Buti *et al.* 2004) and the short excretion period described for HEV infections would suggest that the frequent detection of HEV in urban sewage must be related to the presence of abundant subclinical infections with excretion of HEV viral particles by the population. The underestimation of the prevalence of HEV infection may be related to multiple reasons including the existence of subclinical infections, the reduction of anti-HEV IgG levels after several years of initial exposure in the population or the lack of antibody response (Buti *et al.* 2004, 2006). The seroprevalence found in Catalonia by Buti *et al.* (2006) is an intermediate value compared with other values reported among blood donors in recent studies conducted in other industrialized areas. According to Bouwknegt *et al.* (2007) seroprevalence in this group is about 2.0% in The Netherlands, while in south-west England or south-west France the seroprevalence is around 16% (Dalton *et al.* 2008; Mansuy *et al.* 2008). However, higher values can be found in endemic areas such as China (43%) or Egypt (45.2%) (Abdelhady *et al.* 1998; Li *et al.* 2006).

It has been confirmed in the study that the predominant genotype in Barcelona is genotype 3, as nearly all the sequences obtained from samples collected in this area belonged to this genotype (Figure 1). In fact, about 92% of the HEV typified samples collected in Barcelona in different studies belonged to genotype 3 and 8% to genotype 1 (Clemente-Casares *et al.* 2003; Albinana-Gimenez *et al.* 2006; Bofill-Mas *et al.* 2006). However, it is significant that HEV genotype 1 was also detected in BCN23 and BBCN2 collected in 2005 and BCN27 collected in 2007. Only genotype 3 was detected from samples with animal origin. The sequences with animal origin identified in the study were highly similar to those with human origin in the same area as previously reported (Clemente-Casares *et al.* 2003). With the exception of BCN26, a higher number of different sequences were obtained from samples with animal contamination than from those with human contamination.

Although the methods applied in this study allow the detection of a diversity of strains present in one specific sample, some limitations must be considered. The use of an amplification method can potentially lead to the introduction of changes related to the error of the polymerase activity. Although this cannot be completely ruled out, when comparing the obtained sequences with others present in the databases, nucleotide changes were mainly observed in the third position of the codons. In fact, all the isolates obtained presented the aa sequence expected for each genotype and the sequencing of the HEV strain used as a positive control produced an identical sequence after being amplified and sequenced in the diverse studies. It must also be considered that the sequences analysed by cloning amplicons probably represent only some of the most abundant HEV strains of that sample. The diversity of HEV strains present in one sample may be still underestimated considering the potential preferential amplification of some sequences over others by PCR or the study of a limited number of clones. Some sequences present originally in a sample may not be represented among the analysed clones. Thus, the presence of more genotype 1 sequences in the tested samples that would be present in lower numbers among other genotype 3 strains cannot be ruled out.

Genotype 3 infections presumably associated with water have been recently described in Europe. In France, 15 patients were identified having as a risk factor to acquire an HEV infection through the consumption of water either directly from a spring or private well or vegetables from a vegetable garden, irrigated with water from a river or a private well (Renou *et al.* 2008). A study conducted in the Netherlands found one patient whose HEV strain was also

isolated from a ditch near the house 3 months after the onset of illness. This ditch received sewage effluent from a leakage in the household septic tank (Borgen *et al.* 2008). HEV has also been detected contaminating river waters. Genotype 3 sequences were isolated from the river Maas in the Netherlands (Borgen *et al.* 2008) and from two bivalve samples harvested in a Japanese river (Li *et al.* 2007). According to the evidence, environmental isolates in industrialized areas usually correspond to autochthonous sequences of HEV genotype 3, which seems to be less virulent than genotypes 1 and 2 (Purcell & Emerson 2008). However, the identification of genotype 1 sequences in sewage or solid waste in these areas suggests the possibility of sporadic environmental contamination by this genotype, responsible for large epidemics of hepatitis E in developing areas (Emerson & Purcell 2003). Although improved treatment of water and sewage has become the best defence against enterically transmitted diseases, the methods currently applied are usually less effective in removing viruses than enteric bacteria (Rao & Melnick 1986; Bofill-Mas *et al.* 2006). Moreover, those excreted strains accumulate in biosolid/sludge during waste treatment. Thus, suitable disposal and treatment of the sludge/biosolids generated also seems essential. Special attention must be paid when genotype 1 infections without history of recent travel are detected, especially if any association to uncontrolled water sources is pointed out.

CONCLUSIONS

This study provides evidence that diverse HEV strains are commonly infecting humans and pigs in the studied area. Although identified strains infecting pigs have been typified in all samples as genotype 3, sewage and biosolids from urban sewage treatment plants may contain not only diverse HEV genotype 3 strains but also sporadically HEV genotype 1. The circulation of genotype 1 in industrialized areas may have further health implications since this genotype has been associated with important epidemics with high mortality rates, especially in pregnant women. Contamination of food and water through their contact with sewage not properly treated and biosolids presenting HEV may represent a significant risk for human populations even in industrialized areas. Thus, suitable disposal and treatment of the sewage and sludge/biosolids generated is important whether the source of contamination is of human or animal origin.

ACKNOWLEDGEMENTS

This work was partially supported by the Ministerio de Educación y Ciencia of the Spanish Government (project AGL2005-07776-C03-02), the Xarxa de Referència de Biotecnologia de Catalunya and the Grup de Recerca Consolidat de la Generalitat de Catalunya (2005SGR00592). We thank the Serveis Científico-Tècnics of the University of Barcelona for their efficient sequencing services.

REFERENCES

Abdelhady, S. I., Al-Din, M. S. & El-Din, M. E. 1998 A high hepatitis E virus seroprevalence among unpaid blood donors and haemodialysis patients in Egypt. *J. Egypt Public Health Assoc.* **73**, 165–179.

Albinana-Gimenez, N., Clemente-Casares, P., Bofill-Mas, S., Hundesa, A., Ribas, F. & Girones, R. 2006 Distribution of human polyomaviruses, adenoviruses, and hepatitis E virus in the environment and in a drinking-water treatment plant. *Environ. Sci. Technol.* **40**, 7416–7422.

Bader, T. F., Krawczynski, K., Polish, L. B. & Favorov, M. O. 1991 Hepatitis E in a U.S. traveler to Mexico. *N. Engl. J. Med.* **325**, 1659.

Balayan, M. S. 1997 Epidemiology of hepatitis E virus infection. *J. Viral Hepat.* **4**, 155–165.

Bofill-Mas, S., Albinana-Gimenez, N., Clemente-Casares, P., Hundesa, A., Rodriguez-Manzano, J., Allard, A., Calvo, M. & Girones, R. 2006 Quantification and stability of human adenoviruses and polyomavirus JCPyV in wastewater matrices. *Appl. Environ. Microbiol.* **72**, 7894–7896.

Boom, R., Sol, S. J., Salimans, M., Jansen, C. L., Werthein-van Dillen, P. M. & van der Noordaa, J. 1990 Rapid and simple method for purification of nucleic acids. *J. Clin. Microbiol.* **28**, 495–503.

Borgen, K., Herremans, T., Duizer, E., Vennema, H., Rutjes, S., Bosman, A., de Roda Husman, A. M. & Koopmans, M. 2008 Non-travel related hepatitis E virus genotype 3 infections in The Netherlands; a case series 2004–2006. *BMC Infect. Dis.* **8**(8), 61.

Bouwknegt, M., Engel, B., Herremans, M. M., Widdowson, M. A., Worm, H. C., Koopmans, M. P., Frankena, K., de Roda Husman, A. M., De Jong, M. C. & Van Der Poel, W. H. 2007

Bayesian estimation of hepatitis E virus seroprevalence for populations with different exposure levels to swine in The Netherlands. *Epidemiol. Infect.* **136**(4), 567–576.

Buti, M., Clemente-Casares, P., Jardi, R., Formiga-Cruz, M., Schaper, M., Valdes, A., Rodriguez-Frias, F., Esteban, E. & Girones, R. 2004 Sporadic cases of acute autochthonous hepatitis E in Spain. *J. Hepatol.* **41**, 126–131.

Buti, M., Domínguez, A., Plans, P., Jardi, R., Schaper, M., Espunes, J., Cardenosa, N., Rodriguez-Frias, F., Esteban, R., Plasencia, A. & Salleras, L. 2006 Community-based seroepidemiological survey of HEV infection in Catalonia, Spain. *Clin. Vaccine Immunol.* **13**(12), 1328–1332.

Clemente-Casares, P., Pina, S., Buti, M., Jardi, R. & Girones, R. 2003 Hepatitis E virus epidemiology in industrialized countries. *Emerg. Infect. Dis.* **9**, 448–455.

Dalton, H. R., Stableforth, W., Thurairajah, P., Hazeldine, S., Remnarace, R., Usama, W., Farrington, L., Hamad, N., Sieberhagen, C., Ellis, V., Mitchell, J., Hussaini, S. H., Banks, M., Ijaz, S. & Bendall, R. P. 2008 Autochthonous hepatitis E in Southwest England: natural history, complications and seasonal variation, and hepatitis E virus IgG seroprevalence in blood donors, the elderly and patients with chronic liver disease. *Eur. J. Gastroenterol. Hepatol.* **20**(8), 784–790.

Dawson, G. J., Mushahwar, I. K., Chau, K. H. & Gitnick, G. L. 1992 Detection of long-lasting antibody to hepatitis E virus in a US traveler to Pakistan. *Lancet* **340**, 426–427.

Emerson, S. U. & Purcell, R. H. 2003 Hepatitis E virus. *Rev. Med. Virol.* **13**, 145–154.

Erker, J. C., Desai, S. M. & Mushahwar, I. K. 1999 Rapid detection of hepatitis E virus RNA by reverse transcription-polymerase chain reaction using universal oligonucleotide primers. *J. Virol. Methods* **81**, 109–113.

Fernandez-Barredo, S., Galiana, C., Garcia, A., Gomez-Munoz, M. T., Vega, S., Rodríguez-Iglesias, M. A. & Perez-Gracia, M. T. 2007 Prevalence and genetic characterization of hepatitis E virus in paired samples of feces and serum from naturally infected pigs. *Can. J. Vet. Res.* **71**, 236–240.

Haqshenas, G., Shivaprasad, H. L., Woolcock, P. R., Read, D. H. & Meng, X. J. 2001 Genetic identification and characterization of a novel virus related to human hepatitis E virus from chickens with hepatitis-splenomegaly syndrome in the United States. *J. Gen. Virol.* **82**, 2449–2462.

Hsieh, S. Y., Yang, P. Y., Ho, Y. P., Chu, C. M. & Liaw, Y. F. 1998 Identification of a novel strain of hepatitis E virus responsible for sporadic acute hepatitis in Taiwan. *J. Med. Virol.* **55**, 300–304.

Hundesa, A., Maluquer de Motes, C., Bofill-Mas, S., Albinana-Gimenez, N. & Girones, R. 2006 Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. *Appl. Environ. Microbiol.* **72**(12), 7886–7893.

Li, R. C., Ge, S. X., Li, Y. P., Zheng, Y. J., Nong, Y., Guo, Q. S., Zhang, J., Ng, M. H. & Xia, N. S. 2006 Seroprevalence of hepatitis E virus infection, rural southern People's Republic of China. *Emerg. Infect. Dis.* **12**(11), 1682–1688.

Li, T. C., Miyamura, T. & Takeda, N. 2007 Short report: detection of hepatitis E virus RNA from the bivalve Yamato-Shijimi (*Corbicula jponica*) in Japan. *Am. J. Trop. Med. Hyg.* **76**(1), 170–172.

Mansuy, J. M., Legrand-Abravanel, F., Calot, J. P., Peron, J. M., Alric, L., Agudo, S., Rech, H., Destruel, F. & Izopet, J. 2008 High prevalence of anti-hepatitis E virus antibodies in blood donors from South West France. *J. Med. Virol.* **80**, 289–293.

Matsuda, H., Okada, K., Takahashi, K. & Mishiro, S. 2003 Severe hepatitis E virus infection after ingestion of uncooked liver from a wild boar. *J. Infect. Dis.* **188**, 944.

Meng, X. J., Purcell, R. H., Halburg, P. G., Lehman, J. R., Webb, D. M., Tsareva, T. S., Haynes, J. S., Thacker, B. J. & Emerson, S. U. 1997 A novel virus in swine is closely related to the human hepatitis E virus. *Proc. Natl Acad. Sci. USA* **94**, 9860–9865.

Mizuo, H., Suzuki, K., Takikawa, Y., Sugai, Y., Tokita, H., Akahane, Y., Itoh, K., Gotanda, Y., Takahashi, M., Nishizawa, T. & Okamoto, H. 2002 Polyphyletic strains of hepatitis E virus are responsible for sporadic cases of acute hepatitis in Japan. *J. Clin. Microbiol.* **40**(9), 3209–3218.

Page, R. M. 1996 TREEVIEW: an application to display phylogenetic trees on personal computers. *Comput. Appl. Biosci.* **12**, 357–358.

Pina, S., Jofre, J., Emerson, S. U., Purcell, R. H. & Girones, R. 1998 Characterization of a strain of infectious hepatitis E virus isolated from sewage in an area where hepatitis E is not endemic. *Appl. Environ. Microbiol.* **64**, 4485–4488.

Pina, S., Buti, M., Cotrina, M., Piella, J. & Girones, R. 2000 VHE identified in serum from humans with acute hepatitis and in sewage of animal origin in Spain. *J. Hepatol.* **33**, 826–833.

Puig, M., Jofre, J., Lucena, F., Allard, A., Wadell, G. & Girones, R. 1994 Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification. *Appl. Environ. Microbiol.* **60**, 2963–2970.

Purcell, R. H. & Emerson, S. U. 2008 Hepatitis E: an emerging awareness of an old disease. *J. Hepatol.* **48**, 494–503.

Rao, V. C. & Melnick, J. L. 1986 Environmental virology. In *Aspects of Microbiology 13* (eds. J. A. Cole, C. J. Knowles & D. Schlessinger). American Society for Microbiology, Washington, DC.

Renou, C., Moreau, X., Pariente, A., Cadranel, J. F., Penaranda, G., Hardwigsen, J., Nicand, E., Izopet, J., Maringe, E., Morin, T., Arotcarena, R., Bourliere, M., Botreau, Y., Carenco, P., Causse, X., Conmbet, F., Gerolami, R., Igual, J.P., Halfon, P., Imbert, Y., Heluwaert, F., Louvel, D., Martin, T., Muller, P., Oules, V., Payen, J. L., Peron, J. M., Picon, M., Poncin, E., Rifflet, H., Rosa, I., Rossi, V., Pavio, N. & L'ANGH 2008 A national survey of acute hepatitis E in France. *Aliment. Pharmacol. Ther.* **27**(11), 1086–1093.

Schlauder, G. G., Dawson, G. J., Erker, J. C., Kwo, P. Y., Knigge, M. F., Smalley, D. L., Desai, S. M. & Mushahwar, I. K. 1998 The sequence and phylogenetic analysis of a novel hepatitis E

virus isolated from a patient with acute hepatitis reported in the United States. *J. Gen. Virol.* **79**, 447–456.

Schlauder, G. S., Desai, S. M., Zanetti, A. R., Tassopoolos, N. C. & Mushahwar, I. K. 1999 Novel hepatitis E virus (VHE) isolates from Europe: evidence for additional genotypes of VHE. *J. Med. Virol.* **57**, 243–251.

Seminati, C., Mateu, E., Peralta, B., de Deus, N. & Martin, M. 2008 Distribution of hepatitis E virus infection and its prevalence in pigs on commercial farms in Spain. *Vet. J.* **175**, 130–132.

Straub, T. M., Pepper, I. L. & Gerba, C. P. 1993 Hazards from pathogenic microorganisms in land-disposed sewage sludge. *Rev. Environ. Contam. Toxicol.* **132**, 55–91.

Takahashi, K., Iwata, K., Watanabe, N., Hatahara, T., Ohta, Y., Baba, K. & Mishiro, S. 2001 Full-genome nucleotide sequence of a hepatitis E virus strain that may be indigenous to Japan. *Virology* **287**, 9–12.

Takahashi, M., Nishizawa, T., Yoshikawa, A., Sato, S., Isoda, N., Kenichi, I., Sugano, K. & Okamoto, H. 2002 Identification of two distinct genotypes of hepatitis E virus in a Japanese patient with acute hepatitis who had not travelled abroad. *J. Gen. Virol.* **83**, 1931–1940.

Tei, S., Kitajima, N., Takahashi, K. & Mishiro, S. 2003 Zoonotic transmission of hepatitis E virus from deer to human beings. *Lancet* **362**, 371–373.

Worm, H. C., Wurzer, H. & Frösner, G. 1998 Sporadic hepatitis E in Austria. *N. Engl. J. Med.* **339**, 1554–1555.

Yarbough, P. O. 1999 Hepatitis E virus: advances in HEV biology and HEV vaccine approaches. *Intervirology* **42**, 179–184.

Zanetti, A. R., Schlauder, G. G., Ramano, L., Tanzi, E., Fabris, P., Dawson, G. J. & Mushahwar, I. K. 1999 Hepatitis E virus in patients with acute non-A-C hepatitis in Italy. *J. Med. Virol.* **57**, 356–360.

First received 30 October 2008; accepted in revised form 9 January 2009. Available online July 2009