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Identification of HEV in symptom-free migrants and environmental samples in Italy

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SUMMARY. Hepatitis E virus (HEV) is considered an emerging pathogen in industrialized countries. The occurrence of HEV genotypes in samples of faeces from asymptomatic migrants arriving on the coasts of South Italy and environmental samples was investigated. Analyses of sequences were used to compare human and environmental genotypes. A total of 40 stool specimens, 12 samples of untreated urban sewage, 12 samples of treated urban sewage and 12 samples of surface water were analysed. Viruses were concentrated from water samples by the tangential flow ultrafiltration technique. The presence of HEV RNA was detected by nested RT-PCR. Viral isolates were sequenced and phylogenetically characterized. Two (5%) of the 40 faecal samples tested were found to be positive for HEV RNA (G1 and G3 genotypes). The virus was detected in 25% (3/12) of the untreated sewage samples and 25% (3/12) of the surface water samples: all isolates belonged to G3 genotype. None of the treated sewage samples were found to be HEV RNA positive. The virus was detected in the faeces of two asymptomatic subjects, suggesting a potential role for symptom-free HEV carriers as a human reservoir. G3 HEV strains were detected in the untreated sewage, as observed in similar studies conducted in other European countries but differing from another study conducted in Italy recently. Moreover, our results show the first case of HEV isolated from fresh surface waters.

Keywords: Hepatitis E virus, phylogenetic analysis, symptom-free carriers, surface water, urban sewage.

INTRODUCTION

Hepatitis E virus (HEV) infection is an important cause of acute hepatitis in tropical and subtropical regions where the virus is endemic. In contrast, in the United States, Europe and the developed countries of the Asia-Pacific, hepatitis E is responsible for occasional cases of acute viral hepatitis. Initially, such cases were found to be related to travel to high-risk areas, but in recent years, several sporadic locally acquired (autochthonous) cases have been published from these areas [1].

The virus has a single-stranded, positive-sense RNA genome containing three open reading frames (ORF1, ORF2 and ORF3) [2]. Based on nucleotide sequence analysis, HEV has been divided into four genotypes, namely genotypes 1–4 [3]. The geographical distribution of HEV genotypes is complex and in continuous evolution [1,4].

HEV, which is shed in the faeces of infected individuals, has been detected in sewage samples, suggesting that HEV contamination may also be present in aquatic environments. Accumulating evidence indicates that hepatitis E is also a zoonotic disease and pigs and other animal species are reservoirs for HEV [5].

Molecular approaches to the characterization and epidemiological study of infectious diseases are important as they provide insight into the circulation of prevalent strains and the arrival of new strains from different geographic areas [4].

The presence of anti-HEV IgG antibody has generally been taken as evidence of prior exposure to HEV. The duration of persistence of circulating IgG anti-HEV antibodies remains unclear [3]. In developed countries, anti-HEV antibody prevalence rates ranging from 1% to above 20% have been reported [6,7]. These appear to be higher than those expected from the low rate of clinically evident hepatitis E disease in these areas.

Abbreviations: EAP, External antisense primer; ESP, External sense primer; HEV, Hepatitis E virus; IAP, Internal antisense primer; ISP, Internal sense primer; ORF1, Open reading fram 1; ORF2, Open reading fram 2; ORF3, Open reading fram 3; WTP, Wastewater treatment plant.

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In Italy, the prevalence of anti-HEV antibodies among healthy individuals has been found to be approximately 1% in northern and up to 5% in southern regions. [8,9,10].

In our previous study carried out in 2003 in South Italy, the prevalence of anti-HEV antibodies was found to be 2.9% in the general population, 0.7% in intravenous drug users, 4.3% in haemodialysed patients and 15% in migrants [11]. Since then, no further epidemiological studies have been conducted in this area.

The objective of this study was to characterize the HEV genotypes circulating among the migrant population of Italy and to acquire a better understanding of the dynamics of circulation of this virus among human beings and the various environmental matrices. To this end, we investigated the occurrence of HEV genotypes in samples of faeces of asymptomatic migrants arriving on the coasts of southern Italy and in samples of surface and sewage waters.

MATERIALS AND METHODS

Study Area

In Italy, there is an increased and continuous inflow of migrants from countries where hepatitis E is endemic. The Italian region of Southern Italy can be deemed a 'border region' because of its geographic position, it has to face daily arrivals of migrants. This may represent a risk of the new HEV strains being introduced in Italy.

Clinical samples

A total of 40 stool specimens and 40 serum samples were collected on a voluntary basis in 2011 from migrants aged 18–47 years (mean, 27.5) housed at the holding centre in South Italy. The nationality of the migrants is shown in Table 1.

Migrant holding centres in Italy provide accommodation and assistance to non-EU citizens on their arrival in the country. Their stay in the centres is limited to the time necessary to establish their identity and determine their legal status in Italian territory. The current system of migrant centres was established by Italian Law [12] and is designed to provide the refugees with:

- cultural mediation;
- information on Italian immigration law and
- health care.

With regard to healthcare services, we proceeded on a voluntary basis and complied with the principles laid down in the Declaration of Helsinki.

Table 1 A total of 40 clinical samples were collected on a voluntary basis in 2011

Number of subjects	Geographical origin	Sex (M\F)	Mean age	(Max–Min)
7	Kosovo	4/3	30.1	40–19
7	Bangladesh	6/1	25.5	35–19
10	Pakistan	5/5	29.1	47–18
4	Turkey	2/2	25.7	31–18
10	Tunisie	7/3	28.1	35–18
2	Libya	0/2	26.5	28–25
Total 40		24/16	27.5	47–18

At the time of the study, none of the subjects showed clinical symptoms associated with acute hepatitis. All of them had normal aminotransferase levels and were seronegative for IgM and IgG anti-HEV antibodies (HEV IgG and IgM ELISA Genelabs Diagnostics, Singapore). They were all negative for hepatitis A, B and C markers (commercially available immunoassays). It was not possible to carry out any follow-up studies of the subjects examined. The faecal specimens were stored at –20 °C until being tested by molecular biology techniques. The faecal samples (0.5–1 mL) were added to approx. 5 mL of 0.89% NaCl, centrifuged for 20 min at 3000 rpm and filtered using a 0.22-μm filter.

Nucleic acid extraction

RNA was extracted from samples using the QIAamp Viral RNA kit (QIAGEN AG, Basel, Switzerland) in accordance with the manufacturer's instructions. Viral RNA was eluted in 60 μL of elution buffer.

Environmental samples

Environmental monitoring was carried out in a region of South Italy, in untreated, treated urban sewage and surface waters from the Castellana-Trafili channel, which collects runoff from grazing land and discharge from the treatment plant (Fig. 1).

The Otranto's WTP (wastewater treatment plant) has a load capacity of 15.341 equivalent inhabitants and performs primary, secondary and chlorine treatments [13]. This WTP collects also the 'migrant centre' wastewater.

The environmental monitoring was performed monthly from January 2011 to December 2011. In total, 12 one-litre grabbed samples of urban sewage taken from the sewage treatment plant before being treated, 12 ten-litre samples of urban treated sewage taken from the same sewage treatment plant after being treated with chlorine and 12 ten-litre samples of surface water taken from the Castellana-Trafili channel were examined. The samples (one for each matrix) were placed in sterile containers, transported to the laboratory at + 4 °C and processed by ultrafiltration within 12 h of collection.

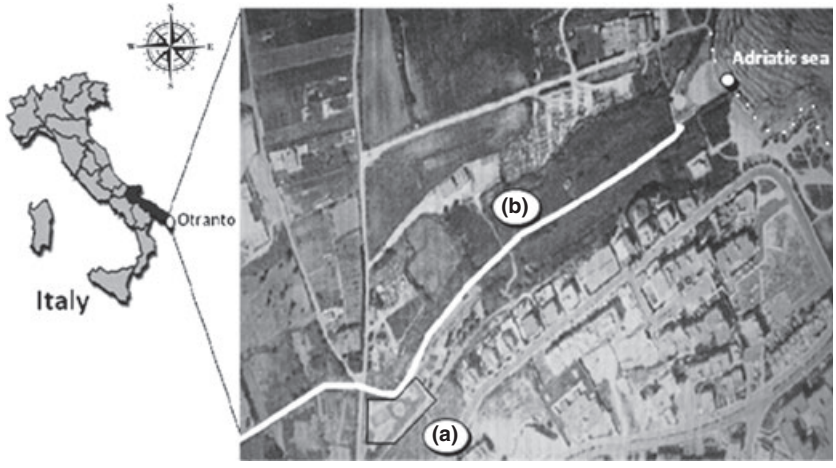

Fig. 1 Sampling locations for detection of HEV in Otranto (Lecce, Italy). (a) Sewage treatment plant (b) Castellana-Trafili channel (surface waters).

Concentration of viruses from untreated sewage, treated sewage and channel (surface) waters

Viruses were concentrated from water samples by the tangential flow ultrafiltration technique using polypropylene membranes with a 10-KDa molecular weight cut-off and a pore size of 0.001–0.01 μm. These filters have been used in previous studies to concentrate viruses present in water, particularly enteroviruses and bacteriophages, with a sensitivity of 1 viral particle/ mL of water sample analysed [14, 15]. To eliminate particulate matter, the samples were prefiltered with 12-μm polypropylene membranes. The water samples were concentrated in two consecutive steps using firstly a MAXIFLEX 25 ECO filtration system (Schleicher & Schuell, Dassel, Germany) equipped with a polysulfone membrane with a surface area of 0.1 m^2 and secondly an ULTRAMINIFLEX filtration system (Schleicher & Schuell) equipped with a membrane with a smaller surface area (0.0024 m^2).The concentrated samples, with a volume of about 40 mL, were decontaminated with chloroform (1:10) before being analysed by biomolecular techniques.

Nucleic acid extraction from environmental samples

Viral RNA was extracted from 140 μL of concentrated samples using the 'QIAamp Viral RNA mini kit' (QIAGEN AG, Basel, Switzerland) following the manufacturer's instructions. The final RNA pellet was resuspended in 60 μL of buffer.

Detection of HEV RNA and genotyping of HEV

Ten microlitres of RNA from the environmental and faecal samples was retro-transcribed and amplified by reverse transcriptase (RT)-nested polymerase chain reaction (PCR).

Two sets of primers were used for detecting a portion of the HEV RdRp gene by nested RT-PCR, as reported previously [16,17]. The first round of amplification was performed in a total of 100 μL of reaction by adding 50 μL of cDNA to 50 μL reaction mixture containing 10 × PCR buffer, 25 mmol/l $MgCl_2$, 2 mmol/l dNTP, 10 μmol/l external primer [ESP 3880-3905 (external sense primer) 5′-ACATTTGAATTATCTGACATTGTGCA-3′; EAP 4930-4955 (external antisense primer) 5′-ACACACATCTGAGCTACATTCGTGAG-3′], 2 U of Taq polymerase (New England Biolabs, MA) and water. The second round of amplification was performed by adding 10 μL of the first PCR product to 90 μL of reaction mixture containing 10 × PCR buffer, 25 mmol/l $MgCl_2$, 2 mmol/l dNTP, 10 μmol/l internal primers [ISP 4147-4170 (internal sense primer) 5′-GACGTGTCCAGGATCACCTTCTTC-3′; IAP 4682–4705 (internal antisense primer) 5′-ACTCACTGCAAAGCACTATCGAAT-3′], 2 U of Taq polymerase and water. All the RT-PCRs were subjected to 1.5% agarose gel electrophoresis to confirm the positive samples.

Sequence analysis of HEV isolates

Based on the findings of Zhai *et al.* [17], the RdRp region in the RNA-dependent RNA polymerase domain was chosen for phylogenetic analysis. Evolutionary trees based on the RdRp region correlate well with those based on the complete genome. The purified products were sequenced on both strands using the Big Dye Terminator v. 1.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA) with a PRISM 3100 Genetic Analyzer (Applied Biosystems). A phylogenetic tree was constructed and genetic distance calculated. A BLAST search was carried out to confirm the identity of the strains (http://www.ncbi.nlm.nih.gov/blast). Bioinformatic analysis included all of the nucleotide sequences obtained here, as well as 10 prototype sequences obtained from GenBank (http://www.ncbi.nlm.nih.gov/). An avian HEV was used as an outgroup. A neighbour-joining algorithm was implemented for construction of a phylogenetic tree using MEGA 4.0.2 [18]. The evolutionary distances were computed using the maximum composite likelihood method. To confirm the reliability of the pairwise comparison and phylogenetic tree analysis, bootstrap

resampling was carried out on 1000 data sets. Multiple sequence alignment was conducted using ClustalW software (http://www.ebi.ac.uk/clustalw).

Nucleotide sequences accession numbers

All of the sequences determined in this study were deposited in the GenBank database under accession no. JX898212 (SEWAGE 1) JX898213 (SEWAGE 2), JX898214 (SEWAGE 3), JX898215 (SURFACEWATER 1), JX898216 (SURFACEWATER 2), JX898217 (SURFACEWATER 3), JX898218 (HUMAN STOOL SAMPLE 1) and JX898219 (HUMAN STOOL SAMPLE 2).

RESULTS

Presence of HEV in human stool samples

Forty samples from patients not exhibiting symptoms of acute hepatitis (24 men, 16 women), aged between 18 and 47 (mean, 27.5), were tested for HEV RNA. All subjects were originally from endemic areas or had been in endemic areas in the 3 months before arriving in Italy and all denied being in epidemiologically high-risk groups for parenteral hepatitis. None of them had worked with pigs.

Two (5%) of the 40 faecal samples tested were found to be positive for HEV RNA by the PCR assays. The phylogenetic study revealed two genotypes, G1 and G3. The G1-positive patient was a migrant from Pakistan, while the G3-positive patient was a migrant from Bangladesh.

Presence of HEV in environmental samples

16.7% (6/36) of the environmental samples examined were HEV RNA positive.

In detail, the virus was detected in 25% (3/12) of the untreated sewage samples and 25% (3/12) of the channel (surface) water samples. All the environmental strains belonged to genotype G3. No trace of HEV was found in any of the samples taken from the treated sewage discharged by the plant.

Phylogenetic analysis

Phylogenetic comparison of the RdRp region of HEV isolates showed similarity between the HEV strains detected in the untreated sewage and a faecal sample, while there was no similarity between the strains of the untreated sewage and those of the surface waters.

Figure 2 shows the genetic relationships between the HEV sequences obtained in this study and prototype sequences obtained from GenBank. The HEV strain tree can be divided into two clusters. Cluster 1 included G3 strains belonging to the untreated sewage samples and a clinical sample. The untreated sewage samples had 82–90% identity with reference strains EU407822, FJ982328 and

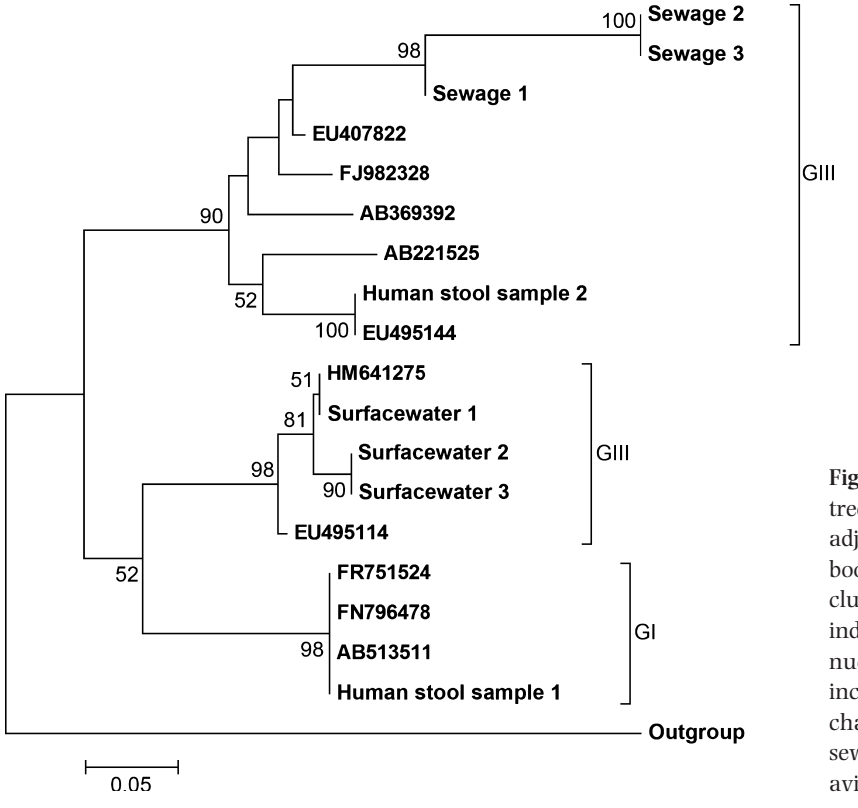

Fig. 2 Neighbour-joining phylogenetic tree based on the RdRp domain. Numbers adjacent to nodes represent percentage bootstrap support (1000 replicates) for clusters to right of node. The scale bar indicates a genetic distance of 0.05% nucleotide substitution per position. Tree includes strains isolated in waters from channel sampling site (surface water), sewage and human stool samples. An avian HEV was used as an outgroup.

AB369392, sequences derived from humans and animals (oysters and pigs) detected in Asia. The human stool sample 2 had 86–95% identity with the human strains AB221525 and EU495144, identified in Japan and France, respectively. Cluster 2 can also be subdivided into two subclusters: the first included the G3 strains from the channel water samples and the second included only one G1 strain from a faecal sample. The first subcluster had 90–96% identity with the reference strains HM641275 and EU495114, identified in humans in European countries. In the second subcluster, the faecal sample clustered and showed a high sequence identity (96–100%) with the sewage samples FN796478 and FR751524 isolated in Italy [19] and with the human strain AB513511 isolated in Pakistan.

DISCUSSION

Hepatitis E virus (HEV) is considered an emerging pathogen in industrialized countries. The virus is generally diagnosed in travellers returning from areas where the disease is endemic, but autochthonous cases have also been documented, indicating that a reservoir of HEV may exist [20,21].

The transmission of infection is normally via the oral-faecal route. Research conducted on samples of faeces, untreated urban sewage and surface waters is thus useful for a good understanding of the circulation dynamics of the virus. Information on the duration of faecal excretion of viral particles by infected individuals is also crucial to understanding the role of HEV reservoirs.

According to some authors [22], the presence of HEV RNA is detectable in stool or serum samples exclusively during the phases of clinical symptoms and biochemical hepatitis. However, the possibility that there may be asymptomatic carriers of HEV has been hypothesized by other authors [23], and our data are consistent with this. Indeed, we detected the virus in the faeces of two asymptomatic subjects with normal transaminase values and without acute antibody response. We suggest a potential role for symptom-free HEV carriers as a human reservoir. Given the way that migrant holding centres are organized, it was not possible to perform any follow-up on the two subjects. Therefore, it cannot be excluded that the two inmates of the centre subsequently manifested the clinical symptoms of hepatitis E, with the appearance of the relative antibodies. In any case, our results demonstrate that the virus can be passed in the faeces of carriers before the onset of the disease's acute phase.

In our study, two different genotypes – G1 and G3 – were detected in faecal samples provided by migrants from Pakistan and Bangladesh, respectively. HEV is known to be endemic in these two countries. While G3 is one of the most frequently encountered genotypes among autochthonous cases documented in Italy and the rest of Europe, until a few years ago, G1 had been detected only in endemic areas associated with significant epidemics. More recently, it has also been detected in Italy in migrants [20]. Therefore, our data are consistent with what has been reported in the literature, which for some years now has noted the presence of G1 in Italy and other European countries [24].

Sequence analysis of the two HEV strains detected in the faecal samples of two non-European citizens returned two distinct clusters. The first, containing the G3 genotype detected in the sample provided by a Bangladeshi migrant showed 81–91% identity with two human strains identified in Japan and France. The second cluster, containing the G1 genotype isolated from the sample of a Pakistani migrant, showed 100% sequence identity with a human strain characteristic of the subject's endemic region of origin. It also clustered very closely with a sewage sample isolated in Italy [19] and with a human strain isolated in Italy from another Pakistani migrant [20]. This finding further supports the epidemiological link between HEV G1 and migration from endemic areas.

Our results revealed a high proportion of HEV-positive environmental samples. Genotype 3, autochthonous to industrialized countries, was found in all environmental samples. This is consistent with what has been found in similar studies conducted in other European countries (Spain and France), as well as in the United States, but differs from another study conducted in Italy recently in which it was above all genotype 1 that was identified [19, 25–27].

While the presence of HEV in wastewaters has also been found in Italy by other authors [19, 28], it has never been detected in surface waters in Italy before. Our results thus represent the first case of HEV isolated from fresh surface waters. Although the detection of virions by molecular methods does not necessarily entail the presence of infectious particles, the presence of HEV RNA in waters can be considered a parameter of faecal contamination, indicating the existence of a possible public health risk.

No sample of treated sewage was found to be HEV RNA positive. Therefore, the WTP was seen to be highly effective in the elimination of viral contamination.

The high frequency of positive samples recorded for the surface waters suggests that it receives untreated sewage.

The circulation of HEV in Italy may be favoured by the arrival of numerous non-Europeans from countries where the disease is endemic or where there are poor standards of health and hygiene, or by people returning from visits to countries at risk.

Constant epidemiological surveillance is thus required, to gather information on the occurrence and diversity of the strains circulating in Italy and plan adequate prevention measures. Further studies are required to gain a better understanding of viral persistence in asymptomatic subjects. Information on the duration of faecal excretion of viral particles by infected individuals is also crucial to understand the pathogenesis and transmission dynamics of this disease.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest with respect to this manuscript.

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