

# Hepatitis E virus in Italy: molecular analysis of travel-related and autochthonous cases

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Human hepatitis E virus (HEV) is considered an emerging pathogen in industrialized countries. The aim of the present study was to contribute to the body of knowledge available on the molecular epidemiology of acute hepatitis E in Italy. Three sets of HEV-specific primers targeting the ORF1 and ORF2 were used to examine serum samples collected from acute hepatitis patients positive for anti-HEV IgG and/or IgM, between 2007 and 2010. Seventeen patients (39.5%) tested HEV RNA-positive: 12 infections, due to genotype 1, were associated with travel to endemic areas (Bangladesh, India and Pakistan), while five infections, due to genotype 3, were presumably autochthonous. Risk factors identified in this group included exposure to raw seafood, pork liver sausages and wild boar. Results from the present study confirm that human HEV infection in Italy is caused by different genotypes, depending on whether the infection is travel-related or autochthonous.

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## INTRODUCTION

Hepatitis E (HE) is an enterically transmitted acute viral hepatitis that occurs frequently in epidemic outbreaks and often spreads by way of faecally contaminated drinking water (Mushahwar, 2008). The causative agent is a non-enveloped RNA virus of the family *Hepeviridae*. Four major genotypes (G1–G4) and several subtypes within each genotype are known to infect humans. All hepatitis E virus (HEV) strains share one major serologically cross-reactive epitope, and, therefore, belong to the same serotype. While genotypes 1 and 2 are found exclusively in humans, genotypes 3 and 4 may also infect animals: swine, chickens, deer, mongooses and rabbits (Meng, 2010). The geographical distribution of HEV genotypes is complex and in continuous evolution. G1 consists of strains circulating in Africa and Asia (Egypt, Algeria, Morocco, Sudan and Chad; India, Pakistan, Nepal, Bangladesh and China); G2 is found in Mexico and in some African countries (Nigeria, Namibia, Chad and Sudan); G3 is widely distributed, mainly – but not exclusively – in the USA, Europe and Japan; G4 is restricted to India and East Asia (Pelosi & Clarke, 2008).

HE infection may vary in severity from asymptomatic to fulminant; case fatality rates range between 0.5 and 4% overall, but may reach 20–25% during pregnancy. HEV infection in endemic countries (Central and South-east

Asia, North and West Africa and Mexico) is mostly a waterborne disease, associated with large epidemics due to the contamination of water supplies with sewage. In these countries, HEV is responsible for over 50% of cases of acute viral hepatitis and seroprevalence ranges from 15 to 60% (Dalton *et al.*, 2007). In contrast, North America, Europe and Japan have traditionally been considered non-endemic for HEV. Acute infection is rarely diagnosed in these regions, and is largely confined to travellers returning from endemic areas (Lewis *et al.*, 2010a). Recently, however, an increasing number of autochthonous cases (non-travel-associated) have been reported in non-endemic countries. These cases are mostly due to HEV G3, which is common in swine livestock. Indeed, accumulating evidence indicates that HEV is a zoonosis, and that pigs, along with other animal species, are reservoirs (Pavio *et al.*, 2010).

Despite the scarcity of information on the occurrence of HEV in Italy, different authors have demonstrated a sustained circulation of the virus in this country (Cacopardo *et al.*, 1997; Grieco *et al.*, 2001; Romano *et al.*, 2011; Zanetti *et al.*, 1999). In a previous study, aimed at providing preliminary information on the occurrence of HEV through the molecular analysis of urban sewage samples (La Rosa *et al.*, 2010), we found a high proportion of HEV-positive sewage samples in Italy (16%, mostly G1, but also G3) evenly distributed across the entire country. The causes and public health implications of this finding are, as yet, unknown.

The objective of the present study was that of contributing to the information available on the molecular epidemiology

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FR751520–FR751544.

Supplementary tables are available with the online version of this paper.

of acute HE in Italian patients, using PCR amplification and phylogenetic analysis.

## RESULTS

Seventeen (39.5%) of the 43 serum samples tested were positive for HEV RNA by one or more of the PCR assays used (Table 1).

The broad-range methyltransferase (MTase) assay detected 11 positive samples – seven G1 and four G3. The G1-specific

RNA-dependent RNA polymerase (RdRp) assay confirmed the seven positive results and detected five additional G1-positive samples not identified with the first method. The capsid assay detected six positive samples, confirming five of the previously detected cases of HEV G1 and detecting one new case of HEV G3. We therefore detected a total of 12 G1- and five G3-positive samples by combining the three methods (Table 1).

Of the 12 patients positive for HEV G1, 10 were immigrants from Bangladesh, one was an immigrant from

**Table 1.** PCR results obtained from anti-HEV-positive clinical samples

All samples were positive for both anti-HEV IgG and anti-HEV IgM except E806, which was positive for anti-HEV IgG and negative for anti-HEV IgM. M, Male; F, female.

Patient ID	Age (years)	Gender	Year of isolation	Patient's country of origin	Possible risk factors	PCR results: assay (PCRs)			Genotype
						MTase (666/667)	RdRp (653/654)	Capsid (711/712)	
E704	26	M	2007	Italy	Eating raw seafood	+	–	–	G3
E804	30	M	2008	Bangladesh	Travel to Bangladesh	+	+	–	G1
E805	24	M	2008	Italy	Travel to India	+	+	–	G1
E806	40	M	2008	Bangladesh	Travel to Bangladesh	–	+	–	G1
E807	46	M	2008	Italy	Unknown	–	–	+	G3
809	34	M	2008	Bangladesh	Travel to Bangladesh	–	+	+*	G1
E-101	33	M	2009	Bangladesh	Travel to Bangladesh	+	+	+	G1
E-107	21	M	2009	Bangladesh	Travel to Bangladesh	–	+	–	G1
E-108	42	F	2009	Bangladesh	Travel to Bangladesh	–	+	–	G1
E-109	38	M	2009	Bangladesh	Travel to Bangladesh	+	+	–	G1
1001	24	M	2010	Pakistan	Travel to Pakistan	+	+	–	G1
1002	66	M	2010	Italy	Eating pork liver sausages	+	–	–	G3
1004	37	M	2010	Italy	Eating/ dissecting a wild boar	+	–	–	G3
1006	29	M	2010	Bangladesh	Travel to Bangladesh	+	+	+*	G1
1007	50	M	2010	Italy	Eating raw seafood	+	–	–	G3
1009	30	M	2010	Bangladesh	Travel to Bangladesh	+*	+*	+	G1
1016	26	M	2010	Bangladesh	Travel to Bangladesh	–	+	+	G1

\*Sequences not included in the phylogenetic trees due to poor quality.

Pakistan and one was Italian. All immigrants had returned from a trip to their country of origin shortly before the onset of symptoms. The Italian patient had returned from a recent trip to India. The five patients with G3 were all Italian, none of whom had travelled abroad recently nor had contact with recent travellers. Two of these patients reported having eaten raw seafood 2–4 weeks before the onset of symptoms; one patient reported having eaten dried pork liver sausages and another was a hunter of wild boar.

The course and outcome of clinical illness in patients developing travel-related HEV were different from those observed in patients developing autochthonous HEV. Patients from the first group were all hospitalized, for periods ranging from 3 to 15 days (median, 10 days), due to severe symptoms (jaundice, vomit and/or fever), and had very high liver enzyme, alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels (mean values 1790 and 1730, respectively). The second group of patients, on the other hand, showed less severe symptoms, lower ALT and AST levels (mean values 1146 IU l<sup>-1</sup> and 436 respectively), and did not require hospitalization. G1 infections were more likely to occur in September (6/12 patients), while no clear seasonality was observed for G3. Median age was 31 and 47.6 for patients with G1 and G3, respectively. Overall, the male:female ratio was 16:1.

The results of the phylogenetic study performed on the sequences obtained using the broad-range MTase assay are presented in Fig. 1. The tree was constructed by aligning the 10 sequences obtained from our amplification products and 44 HEV G1–G4 sequences from GenBank. The sequences clustered into four main groups, corresponding to genotypes 1–4. The G1 cluster comprises GenBank strains from Asia and Africa as well as the following sequences obtained in the present study: ID E101, E109, E804, E805 and 1001. The G3 cluster contained, intermixed in the tree, strains from humans and animals (pigs, wild boar, wild deer and mongoose) detected worldwide, as well as the following sequences obtained in the present study: ID E704, 1002, 1004 and 1007. These sequences, isolated from non-traveller patients, clustered together with European strains responsible for sporadic cases of autochthonous hepatitis (GenBank accession no. EU495148 from France, GenBank accession no. FJ956757 from Germany, GenBank accession no. AF195064 from Spain) as well as with swine and wild boar strains (GenBank accession no. EU723516 from Spain and GenBank accession no. FJ998008 from Germany) identified in European countries.

The results of the phylogenetic study performed on the sequences obtained using the G1-specific RdRp assay are presented in Fig. 2. This phylogenetic tree includes 11 sequences from the current study and 26 corresponding sequences from human and animal HEV G1–G4 obtained from GenBank. For a comparison between clinical and environmental strains, we also included 18 G1 sequences from urban sewage samples collected in Italy in 2008–2009

by our research group, and tested using the same molecular assay. The strains harboured by the cases presumably imported from Asia (Bangladesh, India and Pakistan) showed a high sequence identity (99–100%, 99% and 100%, respectively) with strains isolated in Sweden from patients returning from the same countries (GenBank accession nos HM641298, HM641284 and HM641271, respectively). Only one of our current clinical samples (ID 1001, from Pakistan) clustered with the sewage samples isolated in Italy in our 2008–2009 study (La Rosa *et al.*, 2010), and showed 100% identity with some of the environmental samples.

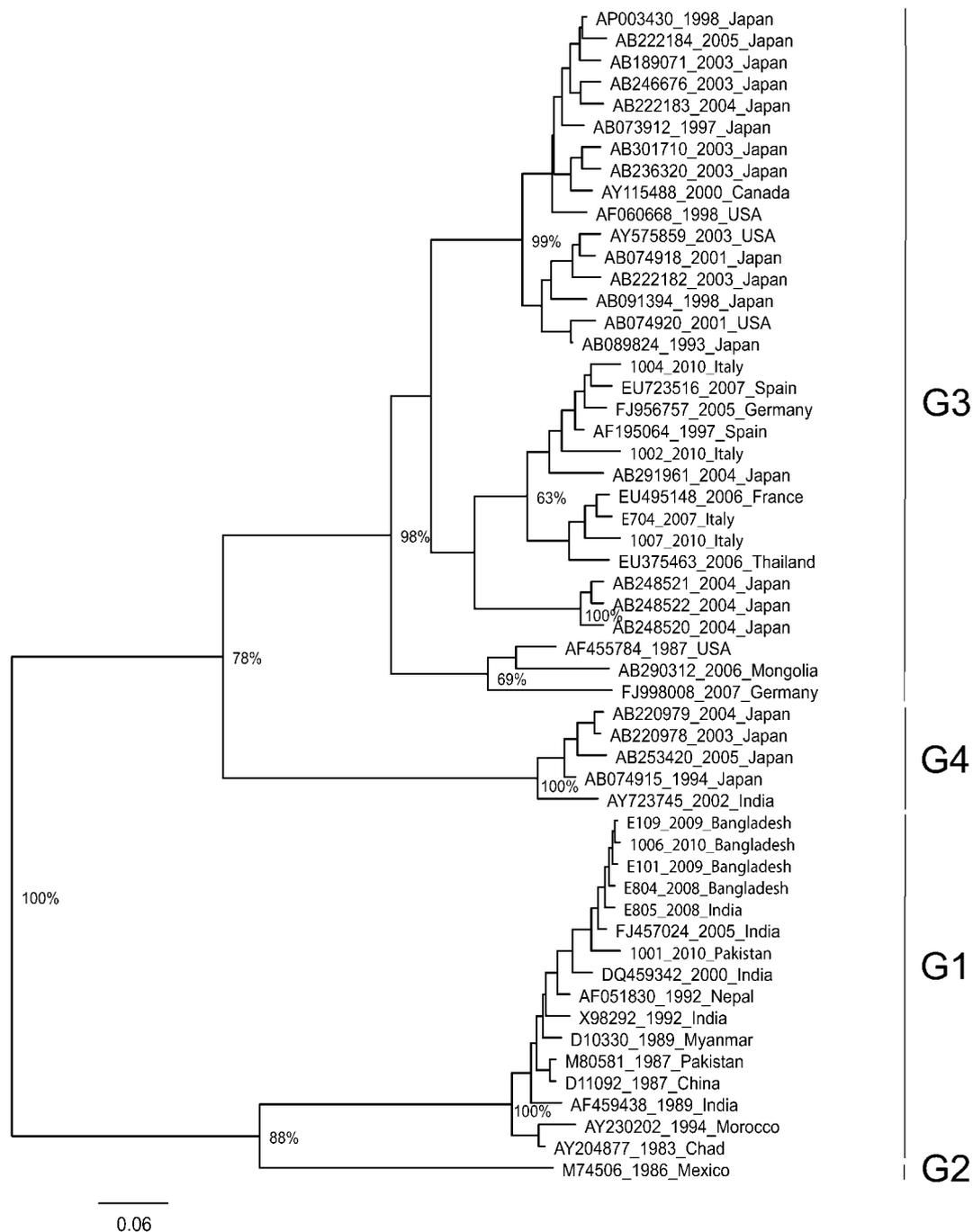
The mean genetic distance between environmental sequences and all other clinical samples analysed in the present study was 0.108 (Tamura–Nei genetic distance – number of base substitutions per site from averaging over all sequence pairs between groups – calculated by using the MEGA5 software).

The results of the phylogenetic study performed on the sequences obtained using the capsid-specific PCR are shown in Fig. 3. In this tree, only G1 and G3 strains were included, with three HEV G1 sequences and one HEV G3 from the present study. In addition, G1 and G3 sequences, along with their respective subtype characterizations, were retrieved from GenBank and included in the tree. Our G1 sequences, all from Bangladeshi patients, clustered in subtype 1a, and showed a high sequence identity (98–99%) with G1 sequences detected in Italy, in 2008–2009, in patients with a history of travel to Bangladesh (GenBank accession nos HM446596 and HM446600). The only G3 capsid sequence obtained in the present study was of subtype 3f and showed the highest similarity (94% nucleotide identity) with the NLSW28 strain (GenBank accession no. AF336292), a swine HEV identified in The Netherlands. The 3f cluster also included strains isolated in Italy from patients with no history of travelling abroad (GenBank accession nos HM446627 and HM446629), as well as swine strains isolated in Italy in 2008 (GenBank accession nos HM769972, HM769974, and HM769976) (Di Bartolo *et al.*, 2011).

The evolutionary distances in the three different regions for G1 and G3 were computed using the Tamura–Nei method in the MEGA5 software. G3 sequences showed a higher genetic variability than G1 sequences in all analysed regions (see Supplementary Table S2, available in JGV Online).

## DISCUSSION

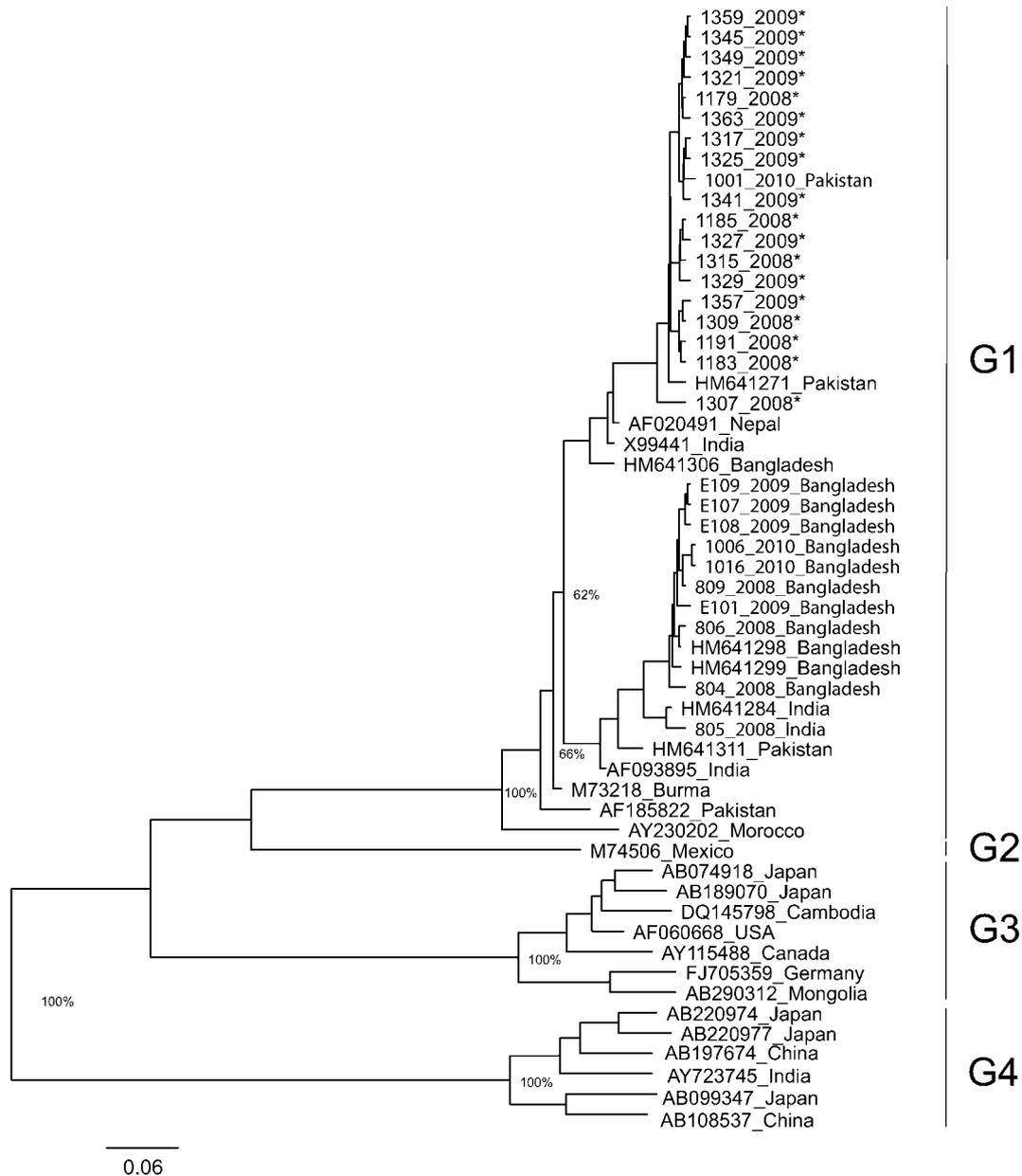
Human HEV-related acute liver disease exhibits two distinct epidemiological patterns (Aggarwal, 2011; Pavio *et al.*, 2010). In non-industrialized countries, where it causes large outbreaks with significant rates of morbidity and death, HEV infection represents a major public health concern. In industrialized countries, on the other hand, the disease is non-endemic, usually confined to travellers returning from endemic regions, and infections are more



**Fig. 1.** Phylogenetic analysis in ORF1 (broad-range MTase assay, PCR 667). Maximum clade credibility tree based on results from the MTase assay generated using Bayesian Markov Chain Monte Carlo (MCMC) analysis. HEV GenBank sequences are cited by their respective accession numbers followed by the geographical origin of the strain. Study sequences are given in bold and are cited as follows: ID-year of isolation-geographical origin of the strain, deduced from the patient's travel history. The GenBank accession numbers of the sequences included are shown on the tree. Posterior probability values greater than 60 % are shown at the main nodes of the trees. Bar indicates the number of nucleotide substitutions per site.

often foodborne than waterborne (Teo, 2007). Recently, enhanced surveillance has detected a growing number of sporadic cases in patients with no travel history in many European countries, including Italy, USA and Japan. In

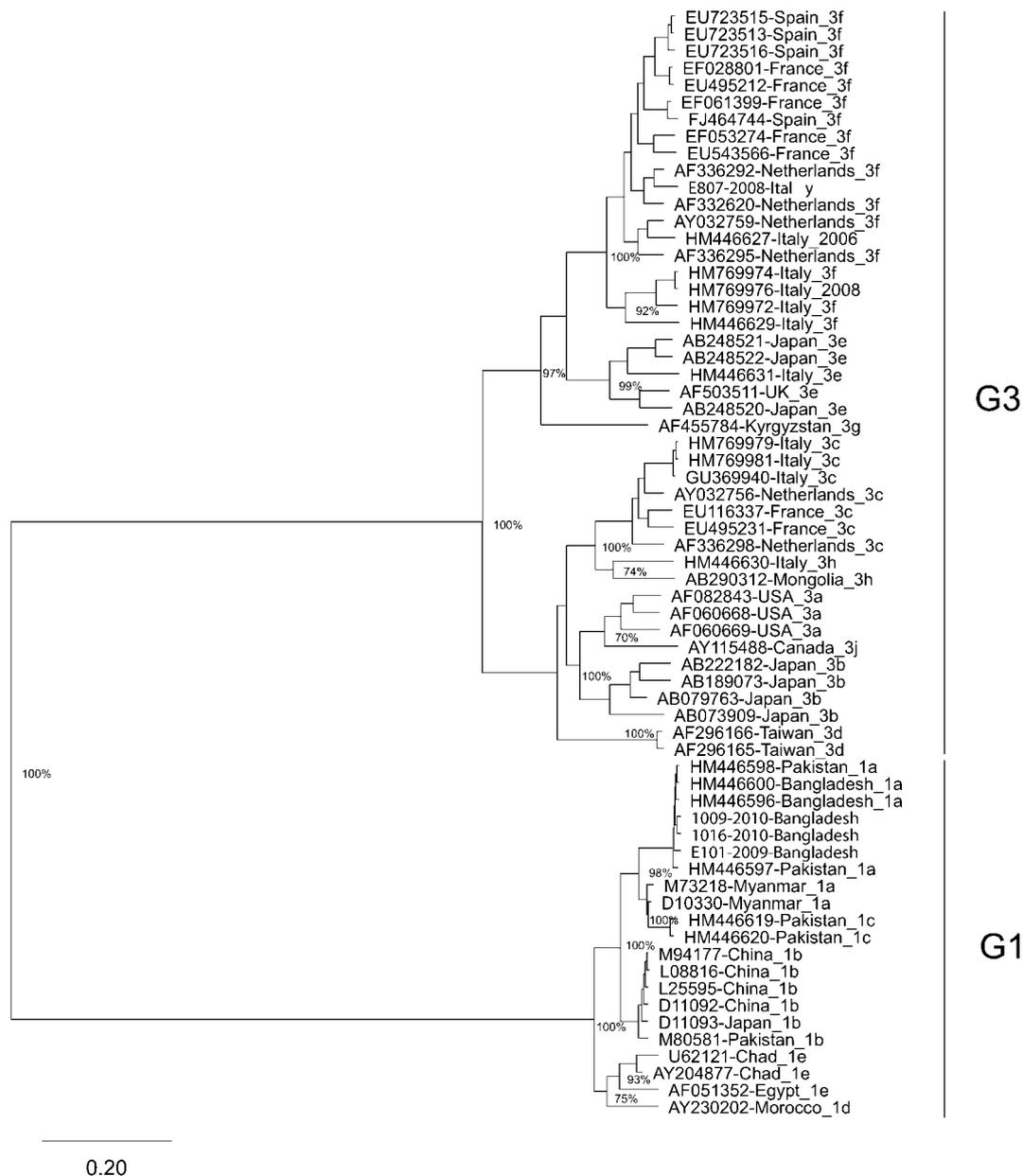
Italy, different authors have demonstrated an appreciable frequency of HEV infections, generally diagnosed in travellers returning from endemic areas. Autochthonous (non-travel-related) cases, however, have also been documented, pointing



**Fig. 2.** Phylogenetic analysis in the ORF1 (G1-specific RdRp assay, PCR 654). Maximum clade credibility tree based on results from the RdRp assay generated using Bayesian MCMC analysis. HEV GenBank sequences are cited by their respective accession numbers followed by the geographical origin of the strain. Study sequences are given in bold and are cited as follows: ID-year of isolation-geographical origin of the strain, deduced from the patient's travel history. Sequences from urban-sewage samples are marked with an asterisk and cited as ID-year of isolation. The GenBank accession numbers of the sequences included in this analysis are shown on the tree. Posterior probability values greater than 60 % are shown at the main nodes of the trees. Bar indicates the number of nucleotide substitutions per site.

to a possible autochthonous circulation of the virus in human populations, sustained by the presence of animal HEV reservoir(s) (Cacopardo *et al.*, 1997; Schlauder *et al.*, 1999; Zanetti *et al.*, 1999). The first autochthonous HEV strain in Italy was discovered in 1999 (Zanetti *et al.*, 1999). Recently, a long-term prospective study showed that most cases of HEV in this country are travel-related

and caused by G1, while autochthonous cases are caused by G3 (Romanò *et al.*, 2011). Our group recently found a high proportion of HEV-positive samples (16%) in a molecular screening of raw sewage samples from 11 wastewater-treatment plants in Italy, mostly of the travel-related G1, but also of the autochthonous kind G3 (La Rosa *et al.*, 2010).



**Fig. 3.** Phylogenetic analysis in the ORF2 (broad-range capsid assay, PCR 712). Maximum clade credibility tree based on results from the capsid assay generated using Bayesian MCMC analysis. HEV GenBank sequences are cited by their respective accession numbers followed by geographical origin of the strain and subtype classification. Study sequences are given in bold and are cited as follows: ID-year of isolation-geographical origin of the strain, deduced from the patient's travel history. Subtypes within each genotype are also indicated. Posterior probability values greater than 60 % are shown at the main nodes of the trees. Bar indicates the number of nucleotide substitutions per site.

In this study, we aimed at contributing to the body of knowledge on the molecular epidemiology of acute HE in Italy. Using HEV-specific primers, we analysed 43 serum samples collected, between 2007 and 2010, from patients with acute hepatitis and IgG and/or IgM anti-HEV antibodies.

To maximize HEV-RNA detection, we used three different PCR methods: initially, we employed a broad-range assay

in the MTase gene (ORF1), known to detect all HEV genotypes. A G1-specific assay in the RdRp (ORF1) was later used to confirm the results obtained in the MTase region. During the preparation of the present manuscript, a long-term prospective study was published (Romanò *et al.*, 2011) on travel-related and autochthonous HEV in Italy. The authors of this study submitted several new G1 and G3 HEV sequences to GenBank, obtained using a broad-range

assay in the ORF2 region, able to determine both HEV genotypes and subtypes. This allowed us to compare our samples to others collected in Italy. We therefore screened our collection with the PCR assay in the capsid region.

About 40 % of the samples were positive for HEV-RNA by one or more of the above assays: 12 were positive for G1 and five for G3. Several of our HEV-infected patients were, nevertheless, HEV-RNA negative. In these cases, viraemia may have subsided relatively early, before the patients in question presented to the hospital (Huang *et al.*, 2010). RNA is detectable in blood from as early as 2 weeks before and for 2–4 weeks after the onset of symptoms (Aggarwal *et al.*, 2000); IgM antibodies can persist from 2 to 6 months, while anti-HEV IgG appears soon after IgM, and persists for a longer period of time (Pelosi & Clarke, 2008). One of the cases (ID E806) was rather atypical, in that it showed HEV RNA and IgG antibody in the absence of specific anti-IgM. This could be attributed either to a decline of anti-HEV IgM to undetectable levels before the disappearance of HEV RNA, or to a false-negative result due to the low sensitivity of the ELISA method in the detection of IgM, as described by other authors (Legrand-Abravanel *et al.*, 2009; Herremans *et al.*, 2007; Renou *et al.*, 2009).

The high male:female ratio observed is consistent with previously published data (Panda *et al.*, 2007). In non-endemic industrialized countries, the fact that travel-related HEV is found mostly in men is probably due, at least in part, to the fact that both immigration and tourism to exotic regions of the world are much more frequent among males than females. The median age for G3 patients in this study was higher than that for G1. In Europe, HEV G3 is generally reported to occur in men and older people more frequently than in women and younger patients; on the other hand, in highly endemic countries clinical infection is more frequent among adolescents and young adults (Lewis *et al.*, 2010b).

The fact that G1 infections were more likely to be diagnosed in September (50 % of patients) may be partly attributable to the fact that many return to Italy in this period after a summer spent in the country of origin.

Clinical symptoms varied from mild to more severe hepatitis requiring hospitalization (up to 15 days). Patients with G3 tended to suffer less severe symptoms, had lower ALT levels and required no hospitalization. This can lead to an underestimation of the occurrence of G3 infections. On the other hand, patients with G1 were all hospitalized due to severe symptoms. These findings are in agreement with studies indicating that the HEV genotype is linked to the severity of liver disease (Mizuo *et al.*, 2005). The countries from which our HEV cases seem to have been 'imported' are Bangladesh, Pakistan and India. In Bangladesh HEV is endemic, with seroprevalence rates ranging from 27 to 60 % (Labrique *et al.*, 2009). In Pakistan, HEV remains highly endemic, mainly affecting the adult population (Alavian *et al.*, 2009). In India, HEV

accounts for 30–70 % of acute sporadic hepatitis, and the anti-HEV seroprevalence rate in the general population is as high as 35 % (Mathur *et al.*, 2001). Most travellers from industrialized countries are not immune to HEV and therefore may be at risk for acute HEV infection when travelling to endemic areas. Immigrants to industrialized countries from endemic areas could have gradually lost humoral protection and therefore be exposed to infection when returning to their country of origin.

Two of our HEV G3-positive patients who had not travelled outside Italy reported having eaten raw seafood 2–4 weeks before the onset of symptoms. Shellfish-associated HE has been documented in the USA and Europe (Renou *et al.*, 2008; Sadler *et al.*, 2006). The recovery from sewage not only of HEV genome (Clemente-Casares *et al.*, 2009; Rodriguez-Manzano *et al.*, 2010), but also of viable, infectious HEV virions (Pina *et al.*, 2000), suggests that sewage-contaminated waters may cause both waterborne and foodborne HEV infections. Most HEV strains isolated from sewage in Europe have been classified as G1. Shellfish and other kinds of seafood are, therefore, liable to be contaminated with this genotype, which could represent a potential risk for autochthonous HEV infection in humans. Further studies are needed to determine the genotypes and infectivity of HEV strains in the natural environment and in shellfish.

Due to the high degree of sequence similarity between HEV strains from deer, domesticated pigs and humans, these animals are thought to play a role in the transmission of HEV. A third patient reported having eaten dried pork liver sausages; the ingestion of raw pork liver sausage has been linked to autochthonous human HE in a recent case-control study (Pavio *et al.*, 2010).

Wild boar is known to be an important reservoir of HEV in Europe (Kaba *et al.*, 2010; Schielke *et al.*, 2009). A fourth patient was a hunter, who may have been infected during the dissection of a wild boar or through the ingestion of its meat. Our results confirm that contaminated food products, if consumed raw or undercooked, could potentially transmit the virus to humans.

The phylogenetic tree obtained in the MTase region shows four main clusters, corresponding to genotypes 1–4. G3 strains detected in this study were intermixed with other G3 strains isolated worldwide from humans and animals (pigs, wild boar, wild deer and mongoose), and were related to swine strains previously identified in Europe. In European countries, swine and wild boar represent a vast reservoir of HEV G3. In Italy, HEV strains are widespread among both symptomatic and asymptomatic pigs (Di Bartolo *et al.*, 2011; Martelli *et al.*, 2010; Masia *et al.*, 2009), with prevalence ranging from 5.9 to 42 % (Caprioli *et al.*, 2007; Di Bartolo *et al.*, 2008).

The phylogenetic analysis performed on RdRp PCR products showed that G1 strains detected in this study from patients upon their return to Italy from Pakistan,

Bangladesh and India clustered very closely with strains originating from the same endemic regions (sequence identity 99–100%). This finding further supports the epidemiological link between HEV G1 and travel history to endemic areas. The environmental HEV G1 strains detected in our previous investigation (La Rosa *et al.*, 2010) clustered together in a distinct clade of G1 sequences, showing relatively little similarity to clinical strains, with the exception of a single clinical sample (ID 1001). The reason for the different clustering of environmental and clinical isolates is difficult to explain and implies the need for further investigation.

The phylogenetic tree in the capsid region showed that G1 sequences, all from Bangladeshi patients, were of subtype 1a, and grouped with G1 sequences detected in Italy in patients who travelled to Bangladesh (Fig. 3). The single G3 sequence, detected in 2008, was of subtype 3f, and showed similarity with Italian strains isolated from patients with no history of travelling abroad, and also with swine strains isolated in Italy in 2008 (from 92 to 100% predicted amino acid identity). The genetic relatedness between HEV isolates obtained from humans and those obtained from swine in the same geographical regions suggest possible zoonotic as well as foodborne transmission.

As for the sensitivity of the different assay used in this study, the RdRp assay displayed a higher sensitivity than the MTase assay. The capsid assay was found to be less sensitive than either of the other two for the detection of both G1 and G3, but was able to detect an additional G3-positive sample, indicating the usefulness of a combination of PCR assays to increase sensitivity for viruses showing considerable genetic diversity.

In conclusion, the results from the present study show that, in Italy, most cases of acute HEV are travel-related, in agreement with previous clinical and environmental studies. Increasing international trade, tourism and migration, including from countries endemic to HEV, and possibly changing environmental conditions, are likely to influence the global epidemiology of HEV. Sporadic cases of autochthonous HEV of probable zoonotic origin have also been documented. This phenomenon should be further studied and its impact on human health evaluated.

In this context, epidemiological and molecular-epidemiological surveillance projects can help trace patterns of spread and guide prevention strategies.

## METHODS

**Patients.** A total of 540 serum samples were collected, between January 2007 and September 2010, from patients presenting at the National Institute for Infectious Diseases (INMI) L. Spallanzani Hospital in Rome with suspected acute viral hepatitis. The diagnosis of acute HE was made on the basis of clinical presentation, raised transaminases and positive anti-HEV IgM and/or IgG antibodies, as determined by KHE3IW and KHE2IW immunoassays, respectively (Radim, Pomezia, Italy). One hundred and twenty-four sera were

anti-HEV IgG positive and 120 were reactive for anti-HEV IgM (one was positive only for IgG and five were positive only for IgM). Forty-three serum samples were available for HEV RNA detection. The following information was collected from the patients' hospital charts, where available: age, sex, symptoms and diagnosis on admission, exposure history (food, water, travel, activities and recent contact with infected individuals).

**HEV RNA detection and genotyping.** RNA extraction from sera was performed on MDX Biorobot (Qiagen). DNA and RNA internal controls were used to check for the presence of PCR-inhibiting factors in the eluates as previously described (Bartolini *et al.*, 2007). Eluted samples (50 µl) were stored in aliquots at –80 °C until use.

Samples were analysed using three sets of primers designed for HEV detection: an assay targeting the MTase gene in the ORF1 (MTase assay, PCR 666/667), an assay targeting the RdRp gene in the ORF1 (RdRp assay, PCR 653/654); and an assay targeting the capsid gene in the ORF2 (capsid assay, PCR 711/712). Broad-range genotyping primers targeting the MTase gene were developed by Fogeda and collaborators after a full-length alignment of human and animal HEV genome sequences deposited in the GenBank database (Fogeda *et al.*, 2009). The RdRp assay is specific for G1; it was originally described by McCaustland *et al.* (1991) and used by our group with some modification in a previous study for the molecular screening of raw-sewage samples (La Rosa *et al.*, 2010). The capsid gene was amplified using broad-range primers developed by Shrestha *et al.* (2003), with one modification consisting of the use of a single degenerate primer instead of a combination of three different forward primers. This assay is able to determine HEV genotypes as well as subtypes. The list of primers and PCRs used in this study is shown in Supplementary Table S1 (available in JGV Online).

Five microlitres of the extracted RNA and 22 pmol of each primer were used in a final mixture of 50 µl using the One-Step RT-PCR kit (Qiagen). After the first round of PCR amplification (40 cycles), one-tenth of the volume of the first PCR product was used for the second PCR assay (30 cycles) at the annealing temperatures shown in Supplementary Table S1. PCR products were purified by QIAquick PCR purification kit (Qiagen) labelled with Big Dye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems) and directly sequenced with a capillary automatic sequencer (3130xl Genetic Analyzer; Applied Biosystems) using the above PCR primers. All sequences were deposited in the GenBank database under accession nos FR751520–FR751544.

Estimates of mean evolutionary divergence (number of base substitutions per site) over all sequence pairs and within groups and subgroups were calculated by using the Tamura–Nei model in MEGA5. Results are shown in Supplementary Table S2.

The BEAST package version 1.6.1 ([http://beast.bio.ed.ac.uk/Main\\_Page](http://beast.bio.ed.ac.uk/Main_Page)) was used for Bayesian MCMC analysis of molecular sequences. For each dataset, the best-fit model of nucleotide substitution was determined using MEGA5. BEAST was used under the TN93 model, until all parameters converged with high effective sample size values (30–50 million generations, the first 10% discarded as burn-in, sampling every 1000th generation). The convergence of parameters during the MCMC was inspected with Tracer v.1.4. A single consensus tree (posterior probability >0.999) was produced with TreeAnnotator and viewed in FigTree v.1.3.1.

Poor quality sequences (four, see Table 1) were excluded from the phylogenetic analysis.

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