



High prevalence of hepatitis E virus in raw sewage in Southern Italy

Federica Di Profio^a, Irene Melegari^a, Andrea Palombieri^a, Vittorio Sarchese^a, Alessio Arbuatti^a, Paola Fruci^a, Fulvio Marsilio^a, Vito Martella^b, Barbara Di Martino^{a,*}

^a Faculty of Veterinary Medicine, Università degli Studi di Teramo, Italy

^b Department of Veterinary Medicine, Università Aldo Moro di Bari, Valenzano, Italy



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ABSTRACT

Hepatitis E virus (HEV) infections constitute a significant health problem worldwide. The burden of hepatitis E in Italy seems low when compared with other European countries. In recent years, improved surveillance activities in Italy have revealed marked geographical differences in HEV epidemiology, with some regions characterised by higher seroprevalence rates. Abruzzo Region (Southern Italy) is currently recognised as a high-risk area for HEV infection. In this study, we investigated the epidemiology of HEV in Teramo Province by monitoring four wastewater treatment plants (WWTPs). Out of 56 influent sewage specimens collected during 2016–2017, HEV RNA was detected in 13/56 (23.2%) sewage samples from all the four WWTPs. Upon sequence analysis of the partial ORF2 gene, four strains showed the highest nucleotide identity to Gt3 subtype c, being more closely related to other HEVs previously identified in human and animal hosts in Abruzzo. For one strain, sequence data were generated only for a short region of the ORF1 gene, revealing the highest identity to HEVs Gt3 of subtype f. Altogether, the findings of this study confirm that HEV largely circulates in the setting investigated.

1. Introduction

Hepatitis E virus (HEV) infection is currently recognised as a major health problem worldwide (WHO, 2018; <http://www.who.int/mediacentre/factsheets/fs280/en/>). HEV is a small non-enveloped virus classified in the family *Hepeviridae*, genus *Orthohepevirus*, species *Orthohepevirus A* (Purdy et al., 2017; Smith et al., 2014). Based on the full-length genome, HEV strains are classified into 8 genotypes (Gt1–Gt8) (Smith et al., 2014). Four major Gt (1–4) have been implicated in human infection/disease. Gt1 and Gt2 are endemic in tropical and subtropical regions of Africa and Asia and restricted to humans, where they are predominantly transmitted through the faecal–oral route, either indirectly through contaminated drinking water or food. Gt3 and Gt4 infect humans and animals and are responsible of autochthonous human hepatitis E in industrialized countries (Kamar et al., 2012). In the European countries, HEV caused more than 21,000 clinical cases between 2005–2015, mostly of which autochthonous, with HEV Gt3 subtypes c, e and f being the most prevalent (Aspinall et al., 2017). In Italy, the burden of hepatitis E is still unclear. In five years of national surveillance activities spanning 2012 to 2016, out of 5,057 hepatitis cases notified to SEIEVA (Integrated Epidemiological System for Acute Viral Hepatitis) only 169 were confirmed as hepatitis E, with a national annual incidence rate of 0.72 cases per 1,000,000 inhabitants. Upon

nucleotide sequence analysis of 65 molecularly positive sera, Gt3 HEV RNA was identified in 66.2% (43/65) of the samples, Gt1 in 32.3% (21/65) and Gt4 in 1.5% (Alfonsi et al., 2018). To better understand the HEV epidemiology in Italy, in the last few years several serological surveys have been performed, revealing prevalence rates ranging from 1.3% to 48.9%, with marked geographical variations (Lucarelli et al., 2016; Mauceri et al., 2017; Spada et al., 2018). The most interesting scenario has been found in Abruzzo Region (Southern Italy). The seroprevalence of anti-HEV IgG and IgM among blood donors from L'Aquila prefecture (Abruzzo Region) in 2014 was 48.9% and 0.6%, respectively. HEV Gt3 subtype c was detected in two (0.6%) donors (Lucarelli et al., 2016). A nationwide HEV survey conducted during 2015–2016 on 10,011 plasma samples from Italian blood donors reported overall IgG and IgM prevalence rates of 8.7% and 0.4%, respectively. The IgG prevalence of 8.7% represents one of the lowest rates in the European countries where nationwide data are available. Interestingly, in the Italian survey the highest seropositivity rate (22.8%) was detected in Abruzzo Region, with a peak of 31.6% in the prefecture of L'Aquila (Spada et al., 2018). In a different epidemiological investigation, conducted on a cohort of HEV-seronegative blood donors (L'Aquila survey 2014) (Lucarelli et al., 2016), an overall incidence of 2.1/100 person-years was reported, with an estimated participant's cumulative probability of becoming HEV infected of 6.5% at 4

* Corresponding author.

E-mail address: bdimartino@unite.it (B. Di Martino).

Table 1List of primer used in this study. Nucleotide position refers to the sequence of the Gt3 subtype c prototype strain WBGER27 (GenBank accession no. [FJ705359](#)).

Primers	Position	Sequence (5' to 3')	Sense	References
JHEV FW	5308-5325	GGTGGTTCTGGGGTGC	+	Jothikumar et al. (2006)
JHEV REV	5360-5377	AGGGGTTGGTGGATGAA	-	Jothikumar et al. (2006)
JHEV P	5331 -5348	FAM-TGATTCTCAGCCCTCGC-BHQ	+	Jothikumar et al. (2006)
HEVestFW	5712-5733	AAYTATGCWCAGTACCGGGTTG	+	Wang et al. (2000)
HEVestREV	6420-6443	CCCTTATCCTGCTGAGCATTCTC	-	Wang et al. (2000)
HEVintFW	5997-6018	GTYATGTYTGCATACATGGCT	+	Wang et al. (2000)
HEVintREV	6323-6344	AGCCGACGAAATYAAATTCTGTC	-	Wang et al. (2000)
HEVORF2con-s1	6323-6346	GACAGAATTRATTCTCGCGCTGG	+	Wang et al. (1999)
HEVORF2con-a1	6495-6519	CTTGTTCRTGTYTGGTTTCATAATC	-	Wang et al. (1999)
HEVORF2con-s2	6372-6393	GTYGTCRGCATGGCAGC	+	Wang et al. (1999)
HEVORF2con-a2	6492-6516	GTTCRTGTYTGGTTRTCATAATCTG	-	Wang et al. (1999)
HEVORF1/1679	37-64	CCAYCAGTTYATHAAGGCTCC	+	Fogeda et al. (2009)
HEVORF1/1680	365-381	TACCAVCGCTGRACRTC	-	Fogeda et al. (2009)
HEVORF1/1681	51-69	CTCCCTGGCRTYACWACTGC	+	Fogeda et al. (2009)
HEVORF2/1682	203-222	GGRTGRTTCAIARVACYTC	-	Fogeda et al. (2009)

Table 2

List of the HEV positive sewage samples collected at the four WWTPs assessed. Collection date, number of RNA copies/ml, qualitative RT-PCR results and identified subtype were reported for each sample.

Positive sample	WWTP	Collection date	qRT-PCR RNA copies/ml	Nested RT-PCR	Subtype
AG1/AG2	WWTP-1	Dec 2016	2.8 × 10 ³	NEG	n/d
AG3/AG4	WWTP-1	Dec 2016	5.8 × 10 ⁵	POS	3c
AF1/AF2	WWTP-1	Dec 2016	8.6 × 10 ³	POS	3c
6 A	WWTP-1	Jan 2017	1.3 × 10 ⁵	POS	3f
AP1/AP2	WWTP-1	Jan 2017	2.0 × 10 ⁴	NEG	n/d
AP3/AP4	WWTP-1	Jan 2017	7.9 × 10 ³	NEG	n/d
AP5/AP6	WWTP-1	Jan 2017	4.5 × 10 ³	NEG	n/d
PF1	WWTP-2	Feb 2017	2.6 × 10 ⁴	POS	3c
C7	WWTP-3	Feb 2017	1.7 × 10 ⁴	NEG	n/d
C1	WWTP-4	Feb 2017	5.2 × 10 ⁴	NEG	n/d
C4	WWTP-4	Feb 2017	1.8 × 10 ⁴	NEG	n/d
E1	WWTP-4	Mar 2017	3.0 × 10 ⁴	POS	3c
D6	WWTP-4	Mar 2017	6.1 × 10 ²	NEG	n/d

years after enrolment ([Marcantonio et al., 2019](#)). Of interest, one of the newly infected blood donors resulted positive for HEV RNA Gt3c, adding further evidence on the circulation of this subtype in the area assessed.

Taken together these findings suggest that Abruzzo, chiefly L'Aquila Prefecture, is a high-risk area for HEV infections. Environmental investigations are an important tool of HEV surveillance, especially in geographical areas considered at high risk, as they provide a direct measurement of virus presence in the ecosystem. Herein, to draw a more complete picture of HEV epidemiology in Abruzzo Region, HEV circulation was monitored in untreated sewage samples collected from four wastewater treatment plants (WWTPs) in the province of Teramo.

2. Materials and methods

A total of 56 influent sewage specimens were collected from

December 2016 to March 2017. Daily flows in the four WWTPs (WWTP localities: 1-Alba Adriatica, 2-Martinsicuro, 3-Villa Pavone, 4-Sant'Atto) range from 5,000 to 18,000 cubic meters, with a design capacity from 15,000 to 100,000 population equivalents. For each plant, sample collection was carried out by swabbing different surface points of the separation grids (influent bar screens and grit chambers) used for primary wastewater treatment. Each sewage sample, diluted in phosphate-buffered saline (0.15 M, pH 7.2) to prepare a total of 1 ml, was seeded with 100 µl of feline calicivirus strain F9 (ATCC® VR-782™) at the final titer of 4 × 10⁶ TCID50/ml in order to evaluate the RNA extraction efficiency and the possible presence of inhibitors. Total RNA was extracted by using the TRIzol LS (Invitrogen, Ltd, Paisley, UK) procedure and analysed by HEV-specific real-time reverse transcription PCR (qRT-PCR), targeting a conserved 68 nucleotide (nt) region of *ORF3* gene, as previously described ([Jothikumar et al., 2006](#)). An HEV plasmid was constructed by cloning the 68 bp *ORF3* fragment of a wild boar strain (HEV/WB/P6-15/ITA, accession no. [KU508285](#)) ([Di Profio et al., 2016](#)) into the Topo TA cloning vector (Invitrogen, Ltd, Milan, Italy). Tenfold serial dilutions of the plasmid ranging from 10² to 10⁷ copies per reaction were used in each PCR run. The first WHO international standard for HEV RNA (code 6329/10) was used to standardize the system. A standard curve was generated from the copy number and corresponding cycle threshold (*C_t*) value. The viral titer was calculated as RNA copies per millilitre (ml) of sewage sample. Viral RNA quantification was performed using the SuperScript III platinum OneStep Quantitative RT-PCR system (Invitrogen Ltd, Milan, Italy) in a 25 µl volume comprising 5 µl of extracted RNA and 20 µl of master mix. Primers and TaqMan probe were used at concentrations of 200 and 100 nM, respectively. Amplification for sequencing was attempted on all the samples containing quantifiable HEV RNA by using three different nested RT-PCR protocols (indicated as strategy A, B, C) ([Fogeda et al., 2009; Wang et al., 1999, 2000](#)) targeting highly conserved regions of *ORF1* or *ORF2* genes within the species *Orthohepevirus A*. Primers and the probe sequences used in this study are listed in [Table 1](#).

All the amplicons were excised from agarose gel and purified with the QIAquick Gel Extraction Kit (Qiagen, Milan, Italy). Sequencing was

Table 3

Molecular prevalence of HEV in four WWTPs in province of Teramo (Abruzzo, Italy).

Methods	WWTP-1 100000 ab/eq Positive/Total (%)	WWTP-2 100000 ab/eq Positive/Total (%)	WWTP-3 42000 ab/eq Positive/Total (%)	WWTP-4 12000 ab/eq Positive/Total (%)	TOTAL Positive/Total (%)
qRT-PCR	7/12 (58.3%)	1/16 (6.25%)	1/8 (12.5%)	4/20 (20%)	13/56 (23.2%)
Nested RT-PCR A	0/12 (0%)	1/16 (6.25%)	0/8 (0%)	1/20 (5%)	2/56 (3.6%)
Nested RT-PCR B	2/12 (16.6%)	0/16 (0%)	0/8 (0%)	0/20 (0%)	2/56 (3.6%)
Nested RT-PCR C	1/12 (8.3%)	0/16 (0%)	0/8 (0%)	0/20 (0%)	1/56 (1.8%)

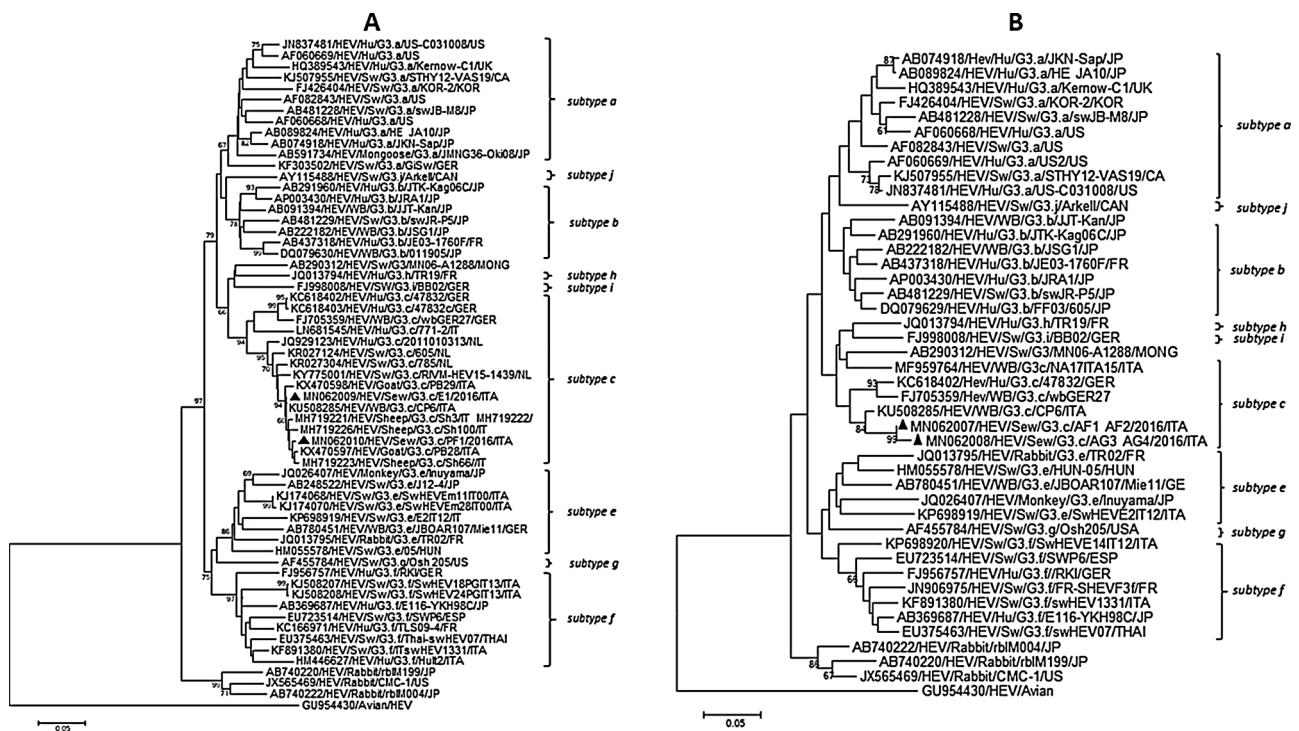


Fig. 1. (A) Phylogenetic tree constructed on the 0.3 kb of ORF2 gene of the two sewage HEV strains amplified with the nested RT-PCR protocol indicated in this study as strategy A. (B) Tree constructed on a fragment of 146 bp portion at the 3' end of ORF2 of two additional HEV strains amplified with the primers sets referred as strategy B. Both the trees were generated using the Neighbor-joining method and the p-distance model supplying a statistical support with bootstrapping of 1000 replicates. The scale bar indicates nucleotide substitutions per site. Black triangles indicate the HEV sequences detected in this study. Abbreviations: Hu, human; Sew, sewage; Sw, swine; WB, wild boar.

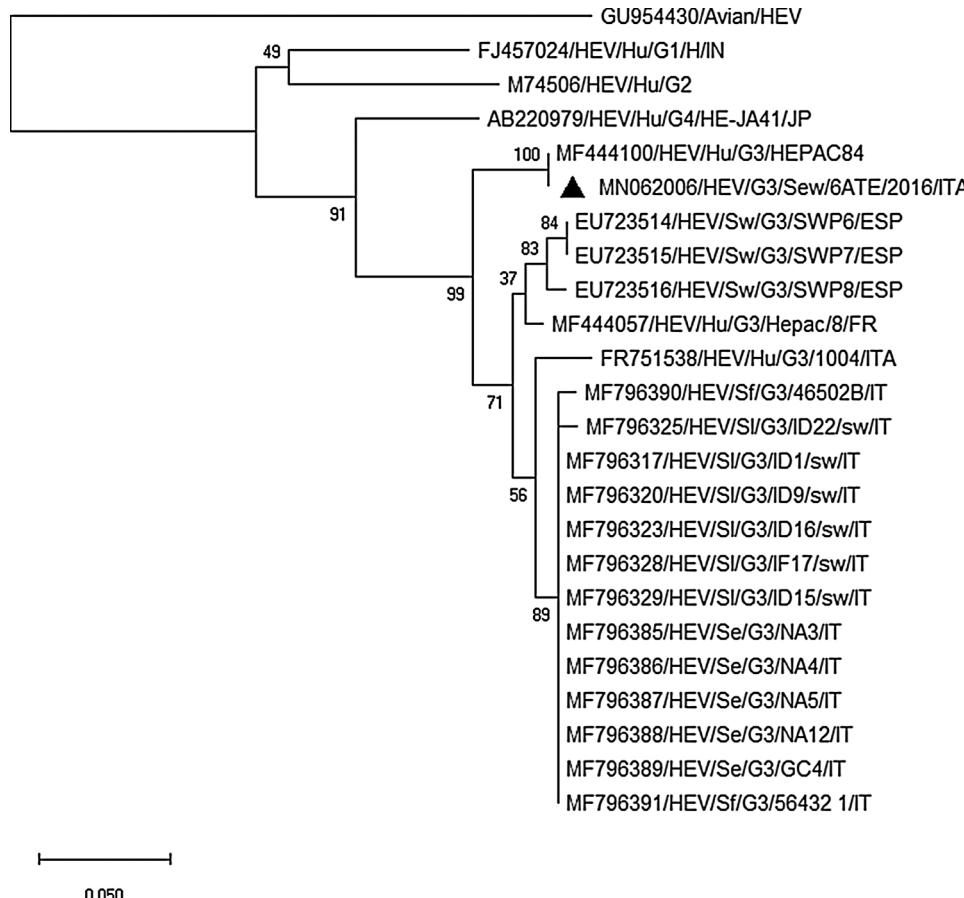


Fig. 2. Neighbor-joining phylogenetic tree constructed on the 172 bp region of the ORF1 of one sewage HEV strains amplified with the nested RT-PCR protocol indicated in this study as strategy C. The tree was generated using the p-distance model supplying a statistical support with bootstrapping of 1000 replicates. Black triangles indicate the HEV sequence detected in this study. Abbreviations: Hu, human; Sew, sewage; Sw, swine; Sl, slurry; Se, seawater; Sf, shellfish.

carried out using BigDye Terminator Cycle chemistry (Applied Biosystems, Foster City, California, US). Sequence and phylogenetics analyses were performed with Geneious version 10.2.4. software (Biomatters Ltd., Auckland, New Zealand). Phylogenetic analysis was performed using the Neighbor-joining method, the p-distance supplying a statistical support with bootstrapping of 1000 replicates.

3. Results

Molecular screening by qRT-PCR detected HEV RNA in 13/56 (23.2%) sewage samples, with viral loads ranging from 6.1×10^2 to 5.8×10^5 RNA copies/ml (cut-off Ct 38.00). The 13 positive specimens were identified from all the four tested wastewater treatment plants. When re-testing the thirteen positive samples by conventional RT-PCR, a total of 5 samples yielded a band of expected size with at least one of the three nested RT-PCR assays used in the study (Table 2). Of these, two samples collected from WWTP-2 and WWTP-4 were found positive with the strategy A, amplifying a region of 348 bp of the *ORF2* gene (Wang et al., 2000). Two additional specimens (WWTP-1) resulted positive with the protocol B, targeting a 146 bp (Wang et al., 1999) portion at the 3' end of *ORF2* gene, whilst one sample (WWTP-1) was amplified using the primer sets targeting a 172 bp region (strategy C) of the *ORF1* (Fogeda et al., 2009) (Table 3). The sequences obtained in this study were deposited in GenBank under accession numbers MN062006-MN062010. Upon sequence analysis, all the five strains showed the highest nt identity to HEV Gt3 (88.8–97.8%), whilst identities to Gt1, Gt2 and Gt4 were 76.0–77.0% nt. The partial *ORF2* genes (MN062007-10) were compared to reference and prototype sequences of known HEV Gt3 subtypes (Smith et al., 2016) and used to generate a Neighbour-joining tree for each of the two regions obtained (Fig. 1A and B). In the tree (Fig. 1A), the HEV strains (MN062009-10) detected in this study clustered within the Gt3 subtype c (93.0–98.0% nt identities), together with other HEVs previously identified in humans and animals (Boxman et al., 2017; Lhomme et al., 2015; Smith et al., 2015), included sequences detected in a healthy blood donor (Lucarelli et al., 2016), in a wild boar, in goat and sheep in Abruzzo Region (Di Martino et al., 2016; Di Profio et al., 2016; Sarchese et al., 2019). A similar tree topology was obtained when the tree was inferred with a shorter region located at the 3' end of *ORF2* (Fig. 1B). In our analysis, only one sample (6ATE/2016/ITA) was amplified with the primer sets targeting a fragment of the *ORF1* gene. Based on inspection of the tree, the HEV strain (MN062006) clustered along with Gt3 sequences (Fig. 2). The highest genetic correlation was found to Gt3 classified within the subtype f (92.0–95.0% nt identities), whilst identities to the other subtypes were 86.5–89.0% nt.

4. Discussion

The presence of HEV RNA in untreated sewage has been documented in Italy with rates ranging from 4.7% to 16.7% (Alfonsi et al., 2018; Iaconelli et al., 2015; Idolò et al., 2013; La Fauci et al., 2010; La Rosa et al., 2010). In the present survey, molecular screening of sewage samples collected from four WWTPs covering over the 75% of Teramo Province, revealed the highest prevalence rate (23.2%; 13/56) ever reported in Italy. These findings confirm the circulation of HEV Gt3 in the investigated geographical area, which likely reflects the epidemiological situation of the local population. A large HEV surveillance (La Rosa et al., 2010) based on WWTPs of 11 Italian regions, revealed the presence of HEV RNA in 19 of 118 sewage samples (16%). Upon sequence analysis of the *ORF1* gene, 18 samples were characterised as HEV Gt1, and 1 sample as Gt3. In a more recent survey (Alfonsi et al., 2018) based on 53 WWTPs, HEV RNA was detected in 9 of 16 Italian regions with an overall prevalence rate of 5.6% (38/679). One third (13/38) of the positive environmental samples were characterised as HEV Gt1, while 25 (65.8%) were characterised as Gt3. The detection of either Gt1 or Gt3 in raw sewage seems to mirror the circulation of HEV

genotypes in the Italian population, in which viruses from Gt1 are responsible for the majority of the hepatitis cases associated with travel to highly endemic areas, whereas Gt3 strains are more often associated with local infections of zoonotic origin (Alfonsi et al., 2018). In our study, we could identify only Gt3 strains in the monitored WWTPs. Furthermore, four HEV Gt3 strains displayed the highest genetic correlation with HEV Gt3 subtype c, previously detected in pigs, wild-boars, small ruminants and humans in Abruzzo Region (Aprea et al., 2018; Di Martino et al., 2010, 2016; Di Profio et al., 2016; Lucarelli et al., 2016; Marcantonio et al., 2019; Sarchese et al., 2019). Altogether, our data reinforce the hypothesis that the high seroprevalence observed locally in humans in Abruzzo Region (Lucarelli et al., 2016; Marcantonio et al., 2019; Spada et al., 2018) are mainly due to autochthonous infections of zoonotic origin. A major limit of our investigation was the low number of HEV-positive sewage samples amplified by qualitative RT-PCR, likely due to the low sensitivity of the molecular strategies we used, greatly affected by the genetic diversity of HEV viruses, currently classified into at least eight genotypes and several subtypes.

Indeed, despite the use of two broad-spectrum methods based on the *ORF2* gene (Wang et al., 1999, 2000), out of thirteen positive qRT-PCR sewage samples, only four (RNA copies/ml ranging from 8.6×10^3 to 5.8×10^5) were detected by qualitative RT-PCR and none of the protocol was able to detect all the qRT-PCR positive samples. Likewise, the *ORF1* primer set (Fogeda et al., 2009) was able to amplify only one sample (1.3×10^5 RNA copies/ml), from which a Gt3 HEV strain of subtype f was identified. Of interest, although this subtype has been frequently detected in Italy in humans, in pigs and in wild boars (Di Bartolo et al., 2011; Di Pasquale et al., 2019; Romanò et al., 2011), it has never been reported in Abruzzo. Unfortunately, in spite of the several attempts, we are not able to amplify additional genomic regions and to characterise better this HEV strain.

5. Conclusions

In summary, our study provided evidence for a high diffusion of HEV in the investigated geographical settings suggesting that HEV infection could represent a public health problem in local population. There is concern in national and local health bodies that the burden of HEV infection in Italy is largely underestimated (Alfonsi et al., 2018). Therefore, it will be crucial to improve the epidemiological surveillance of hepatitis E-related cases with the inclusion of HEV in the diagnostic algorithm of hepatitis. An integrated environmental/animal/food surveillance could be useful to provide a more complete picture on the pathways of infections in order to establish adequate control measures, including those to protect environment from sewage contamination. In addition, the development and/or refinements of molecular diagnostic methods are pivotal to gather precious information on the epidemiology of HEV subtypes circulating in the eco-system.

Declaration of Competing Interest

All Authors declare that there are no financial or other relationships that might lead to a conflict of interest. All authors have seen and approved the manuscript and have contributed significantly to the work.

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