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Detection of hepatitis E virus RNA in rats caught in pig farms from Northern Italy

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Abstract

Hepatitis E virus (HEV) strains belonging to the *Orthohepevirus* genus are divided into four species (*A*–*D*). HEV strains included in the *Orthohepevirus A* species infect humans and several other mammals. Among them, the HEV-3 and HEV-4 genotypes are zoonotic and infect both humans and animals, of which, pigs and wild boar are the main reservoirs. Viruses belonging to the *Orthohepevirus C* species (HEV-C) have been considered to infect rats of different species and carnivores. Recently, two studies reported the detection of HEV-C1 (rat HEV) RNA in immunocompromised and immunocompetent patients, suggesting a possible transmission of rat HEV to humans. The role of rats and mice as reservoir of HEV and the potential zoonotic transmission is still poorly known and deserves further investigation. To this purpose, in this study, the presence of HEV RNA was investigated in the intestinal contents and liver samples from 47 Black rats (*Rattus rattus*) and 21 House mice (*Mus musculus*) captured in four pig farms in Northern Italy. The presence of both *Orthohepevirus A* and *C* was investigated by the real-rime RT-PCR specific for HEV-1 to HEV-4 genotypes of *Orthohepevirus A* species and by a broad spectrum hemi-nested RT-PCR capable of detecting different HEV species including rat HEV. The intestinal content from two Black rats resulted positive for HEV-C1 RNA and for HEV-3 RNA, respectively. None of the House mice was HEV RNA positive. Sequence analyses confirmed the detection of HEV-C1, genotype G1 and HEV-3 subtype e. The viral strain HEV-3e detected in the rat was identical to swine HEV strains detected in the same farm. Liver samples were negative for the detection of either rat HEV or HEV-3.

KEYWORDS
hepatitis E, hepatitis E virus, mouse, *Orthohepevirus A*, *Orthohepevirus C*, rat

1 | INTRODUCTION

Hepatitis E virus (HEV) is a ubiquitous pathogen detected in humans, wildlife (wild boar, deer) and domestic animals (pigs). The disease in humans is usually self-limiting, but can become chronic in immunosuppressed patients (Kamar, Izopet, & Dalton, 2013).

The HEV genome is constituted of a positive single-stranded RNA (ssRNA) of about 7.2 kb length, containing three open reading frames (ORFs): the ORF1 encodes the non-structural proteins, the ORF2 encodes the capsid protein and the ORF3 encodes a small multifunctional phosphoprotein (Cao & Meng, 2012).

The International Committee on Taxonomy of Viruses (ICTV) classifies HEV in the family of *Hepeviridae*, divided into the two genera *Piscihepevirus* and *Orthohepevirus*. The latter includes the *Orthohepevirus A* that, differently from the other species (*Orthohepevirus B*–*D*), infects both humans and animals (Purdy

et al., 2017). The *Orthohepevirus A* is subdivided into eight genotypes. HEV-1 and HEV-2 genotypes infect only humans by the faecal-oral route and are responsible for large waterborne outbreaks in developing countries. The genotypes HEV-3, HEV-4 and HEV-7 can cause hepatitis in patients following the foodborne zoonotic route of transmission. The HEV-3 and HEV-4 circulate in developed countries and are responsible for sporadic cases and small outbreaks (Ruggeri et al., 2013), mainly due to the intake of raw or undercooked liver sausages or meat from HEV-3 and HEV-4 infected animals (i.e., swine, wild boar, and deer) (Pavio , Doceul, Bagdassarian, & Johne, 2017). Genotype HEV-7 infects camel and was detected in a patient with chronic infection who regularly consumed camel milk, suggesting the zoonotic transmission of HEV-7 (Lee et al., 2016). The other genotypes included in the *Orthohepevirus A* species have been detected in animals only (Lee et al., 2016; Woo et al., 2016).

The *Orthohepevirus C* species is divided into HEV-C1 (rat HEV) infecting rodents and eulipotyphlids (musk shrew), HEV-C2 infecting ferret and mink, and the recently proposed HEV-C3 and HEV-C4 detected in Chevrier's Field Mouse (*Apodemus chevrieri*) and Père David's vole (*Eothenomys melanogaster*) in China (Smith et al., 2014; Wang et al., 2018). HEV strains belonging to *Orthohepevirus C* have been detected in Europe and in the USA in Black rat (*Rattus rattus*) and Norway rat (*Rattus norvegicus*), and also in other species (*Rattus flavipectus*, *Rattus rattoides*) in Asian countries (Favorov, Kosoy, Tsarev, Childs, & Margolis, 2000; Johne et al., 2010a; Johne et al., 2010b; Purcell et al., 2011; Johne et al., 2012; Kanai et al., 2012; Lack, Volk, & Van den Bussche, 2012; Li et al., 2013a; Mulyanto et al., 2014; Widen et al., 2014; Ryll et al., 2017; Simanavicius et al., 2018). Recently, HEV-C RNA detection has also been reported in common voles (*Microtus arvalis*) in Hungary (Kurucz et al., 2019). Several studies have reported the detection of IgG and IgM against HEV in Norway rat and Black rat with seroprevalence ranging between 24% and 37% depending on the studies and up to 94% in the USA (Kabrane-Lazizi et al., 1999; Favorov et al., 2000; Purcell et al., 2011; Johne et al., 2012; Li et al., 2013b; Mulyanto et al., 2014; Simanavicius et al., 2018), confirming the wide circulation of HEV in commensal synanthropic *Rattus* spp.

Zoonotic and inter-species transmission of rat HEV strains is still controversial. In rhesus monkeys (Purcell et al., 2011) and pigs (Cossaboom et al., 2012) experimental infections with rat HEV strains have been unsuccessful. However, two studies reported the presence of antibodies against HEV-C1 antigen in healthy forestry workers in Germany, and the presence of IgG and IgM against HEV-C1 antigen in sera of patients presenting acute hepatitis, suggesting that HEV-C1 can infect humans (Dremsek et al., 2012; Shimizu et al., 2016). The data were further supported by two recent studies that reported infections caused by rat HEV strains in a transplant recipient with persistent hepatitis and in an immunocompromised patient with severe acute hepatitis (Sridhar et al., 2018; Andonov et al., 2019).

Impacts

- Detection of rat HEV RNA in the intestinal content of a rat captured in a pig farm.
- One strain detected from a rat intestinal sample was classified into the HEV-3 zoonotic genotype.
- Identical HEV-3e strains were detected in faeces of pigs and rat in the same farm.

The other relevant sanitary aspect of rat as HEV carrier is the possibility of interspecies transmission of HEV-3 between pigs, wild boar, humans and rats. Only three studies reported the detection of HEV-3 in rats, in the USA, Japan and Belgium (Kanai et al., 2012; Lack et al., 2012; Ryll et al., 2017). The presence of HEV-3 RNA has been also reported in a House mouse captured in a pig farm. However, no information was available on the HEV infection status of animals in the farm (Grierson, Rabie, Lambert, Choudhury, & Smith, 2018).

In the present study, the occurrence of HEV RNA of *Orthohepevirus A* and *C* was determined in paired intestinal contents and liver samples from 47 Black rats and from 21 House mice caught within four pig farms in Northern Italy.

2 | MATERIALS AND METHODS

2.1 | Samples collection

Between June and July 2015, 47 Black rats were caught in three different pig farms, located in Northern Italy. In September 2018, 21 House mice were caught in another farm located in the same geographical area. The rodents were caught using commercial traps during routine activities of pest control. An approved veterinarian trapped the animals following ethical rules.

During necroscopy, liver and intestinal contents were sampled and stored at −80°C. The sampling size consisted of 20 animals from two farms, allowing the detection of HEV presence within the rodents population when the expected prevalence at least 11%, with a 90% probability. From the third farm a limited number of animals were captured (n = 7); in this case design prevalence is 30%.

In 2018 in one of the investigated farm, 20 pool faecal samples were collected from pen floor housing weaners. One-hundred mg of faecal intestinal contents and pig faecal samples were re-suspended in 1 ml of distilled water (10% w/v) and subjected to the nucleic acid extraction.

2.2 | RNA extraction

Total RNA was extracted from 140 µl of 10% faecal suspensions or 100 mg of liver samples from 47 rats, 21 mice and 20 pig faecal pools using the RNeasy Mini Kit (Qiagen) and eluted in 60 µl of RNase-free water. The RNA was stored at −80°C until use.

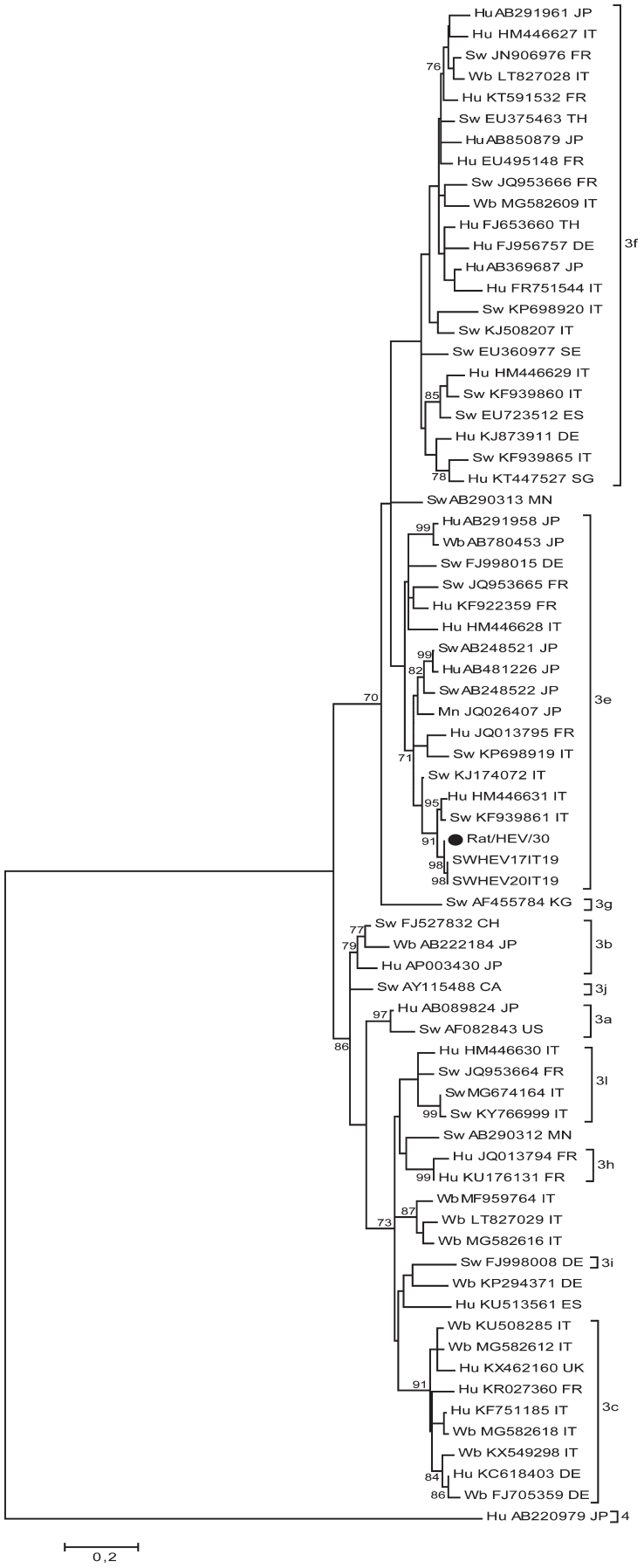


FIGURE 1 Maximum likelihood phylogenetic tree based on 328nt fragment within the HEV ORF2 region of 72 HEV-3 strains from human, swine and wild boar including HEV-3 reference strains. The HEV-4 strain was used as outgroup. The Rat/HEV/30 Italian strain is indicated by a black circle. Bootstraps values >70 are indicated at their respective nodes. Representative porcine, human, wild boar and monkey strains are included. Each entry includes host (Hu, human; Mn, monkey; Sw, swine; Wb, wild boar), accession number and countries origin of strains (CA, Canada; CN, China; DE, Germany; ES, Spain; FR, France; IT, Italy; JP, Japan; KG, Kyrgyzstan; MN, Mongolia; SE, Sweden; SG, Singapore; TH, Thailand; UK, The United Kingdom of Great Britain; US, The United States of America)

2.3 | HEV genome detection

The RNA extracted from faecal and liver samples was tested by real-time reverse transcription PCR (real-time RT-PCR) for the broad range detection of HEV-1 to HEV-4 genotypes (Jothikumar, Cromeans, Robertson, Meng, & Hill, 2006) using the RNA Ultrasense One-Step Quantitative RT-PCR System (Life Technologies). The sample positive by the real-time RT-PCR was analysed by a nested RT-PCR detecting HEV-3 and amplifying a 493 nt region within ORF2 (Boxman et al., 2017).

The RNA samples were further analysed by an end-point broad spectrum pan-HEV hemi-nested RT-PCR, amplifying a 330nt fragment in the RNA dependent RNA polymerase region (RdRp, ORF1). The first round RT-PCR was performed by using the HEV-cs/HEV-casN primer pair, and the hemi-nested PCR was carried out using the HEV-csN/HEV-casN primer combination as described by Lack et al. (2012).

The RNA from 20 pig pool faecal samples was analysed by the broad range real-time RT-PCR (Jothikumar et al., 2006), and two positive samples were subjected to the above described nested RT-PCR to amplify 493 nt region within ORF2 (Boxman et al., 2017) subjected to sequence.

PCR products were separated on a 1% agarose gel, stained with GelRed Nucleic Acid Gel Stain (Biotium) and visualised under UV light.

2.4 | Nucleotide sequencing

PCR products were purified by Exonuclease I and Shrimp Alkaline Phosphatase reactions (ExoSAP-IT, Affymetrix, USB), incubating at 37°C for 30 min, followed by 15 min at 80°C. Nucleotide sequencing of amplicons was performed by Eurofins Genomics.

2.5 | Phylogenetic analysis

The nucleotide sequences were analysed and edited using the Bionumerics software V.6.5 (Applied Maths) and deposited in GenBank under the accession number Rat/HEV/18, KX844624; Rat/HEV/30, MK689363; SWHEV17IT19, MK689361 and SWHEV20IT19, MK689362. Sequence similarity searches were performed using the nBLAST server on the NCBI GenBank database (http://www.ncbi.nlm.nih.gov/genbank/index.html). The maximum likelihood (ML) tree was drawn with the MEGA7 software (www.megasoftwares.com) using the general time reversible model with a gamma distribution and invariant sites as suggested by the MEGA7 model test, and 1,000 bootstrap replicates. The sequence datasets used to assign the strain to genotypes G1, G2 and G3 of HEV-C1 and to the HEV-3 subtypes are based on the reference strains for HEV classification indicated by Mulyanto et al. (2014) and Smith et al. (2016), respectively.

3 | RESULTS

Paired intestinal content and liver samples collected from 47 Black rats, and 21 House mice were tested for the presence of HEV RNA. A real-time RT-PCR for detection of the genotypes HEV-1 to HEV-4 of *Orthohepevirus A* and an end-point pan-HEV hemi-nested RT-PCR, amplifying RdRp fragment, were performed. None of the mice (21) was positive for HEV either in liver or in faecal samples.

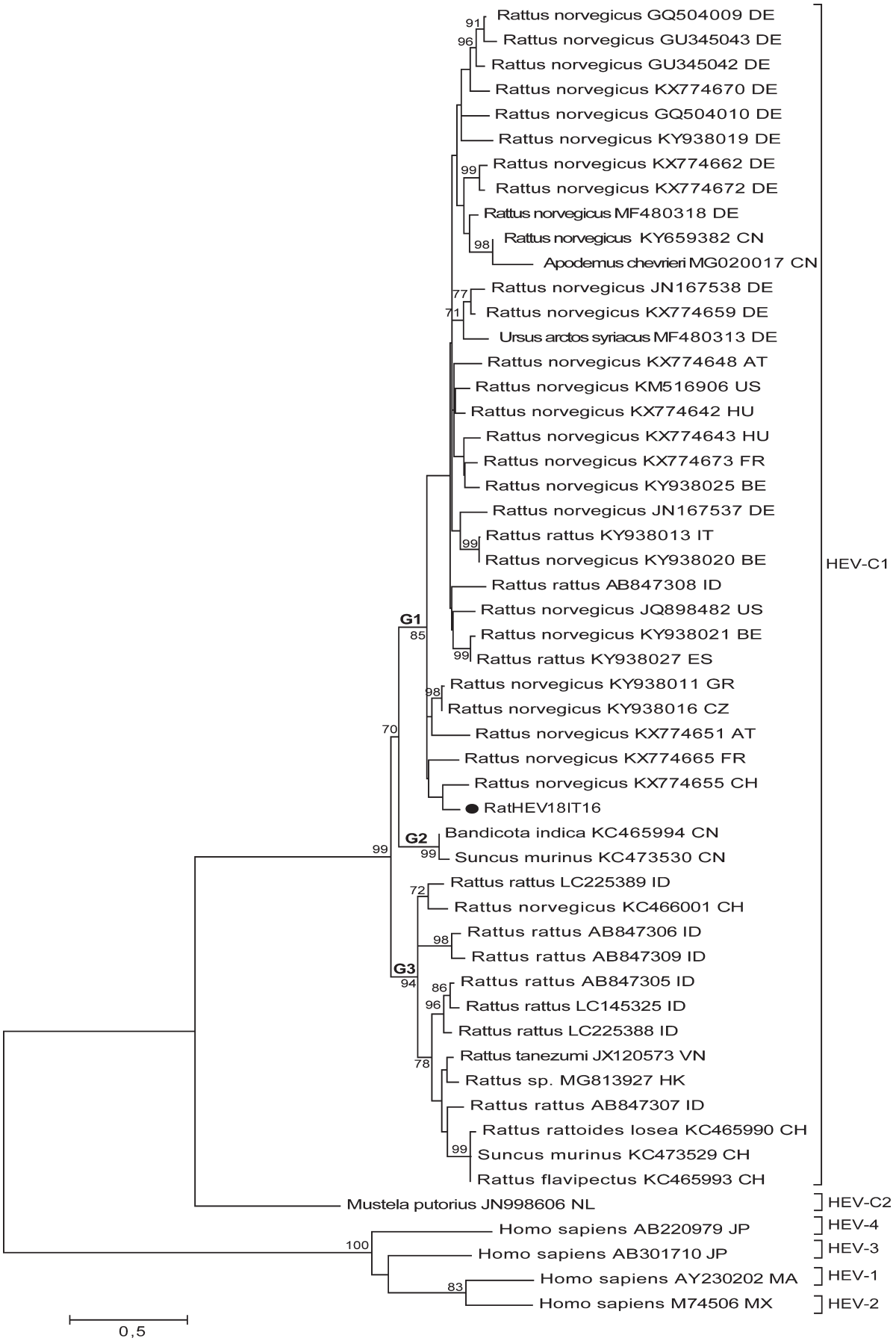
One rat intestinal content (Rat/HEV/18) out of 47 (2%) was positive for HEV RNA only by the pan-HEV hemi-nested RT-PCR. The intestinal content of a different animal (Rat/HEV/30) was positive only by real-time RT-PCR for HEV-1 to HEV-4 (2%). Liver samples were negative.

The rats positive for HEV RNA were both adult female, with a mean weight of 190 gr, and were sampled in two different months (June and July 2015) from two pig farms. The Rat/HEV/18 was retrieved from a farm where it was not possible to obtain pig samples, and no information on the presence of HEV in the farm is available. Conversely, the Rat/HEV/30 was caught in a pig farm that was investigated in 2018 for the presence of the zoonotic HEV-3 and HEV-4. Analyses on 20 pig pool faecal samples confirmed the presence of HEV by real-time RT-PCR in nine samples. Sequence analyses on the ORF2 fragments were performed on two HEV positive pig samples and matched with HEV-3.

The ORF2 (493 nt) sequence of Rat/HEV/30 clustered with other HEV-3 genotype sequences. The HEV-3 strains from pigs (SWHEV17IT19, SWHEV20IT19) and the rat shared 99.0% nucleotide identity (nt. id.) and 100.0% amino acid identity (aa. id.). The phylogenetic tree (Figure 1), built using the HEV-3 ORF2 sequences, showed the clustering of Rat/HEV/30, SWHEV17IT19 and SWHEV20IT19 within the HEV-3e subtype group, displaying the 89.0% nt. id. with the HEV-3e subtype prototype strain swJ8-5 (AB248521) (92.0% aa. id.), suggesting the assignment of Rat/HEV/30 to the HEV-3e subtype. The three Italian strains formed a supported monophyletic cluster with two additional Italian strains: one human strain (HuIt6, HM446631) (93.0% nt. id., 96.0% aa. id.) detected in 2009 and one swine strain (HEV/13RS366-16, KF939861) (94.0% nt. id., 97.0% aa. id.) detected in 2012.

The RdRp sequence of Rat/HEV/18 matched in the NCBI database with other *Orthohepevirus C* strains sequences. The highest nucleotide and amino acid identities were observed (87.0% nt. id., 98.0% aa. id.) with respect to the rat strain MVZ201020 (JQ898482) previously collected in the USA and with other strains belonging to

FIGURE 2 Maximum likelihood phylogenetic tree reconstruction based on 282nt fragment within the HEV RdRp region (ORF1) of 49 HEV-C1 strains. The ferret HEV-C2 and HEV-1 to HEV-4 genotype strains are used as outgroup. The Rat/HEV/18 Italian strain is indicated by a black circle. Bootstraps values >70 are indicated at their respective nodes. Each entry includes host species, accession number and countries origin of strains (AT, Austria; BE, Belgium; CN, China; CZ, Czech Republic; DE, Germany; ES, Spain; FR, France; GR, Greece; HK, Hong Kong; HU, Hungary; ID, Indonesia; IT, Italy; JP, Japan; MA, Morocco; MX, Mexico; NL, The Netherlands; US, The United States of America; VN, Viet Nam)


Rattus norvegicus GQ504009 DE
Rattus norvegicus GU345043 DE
Rattus norvegicus GU345042 DE
Rattus norvegicus KX774670 DE
Rattus norvegicus GQ504010 DE
Rattus norvegicus KY938019 DE
Rattus norvegicus KX774662 DE
Rattus norvegicus KX774672 DE
Rattus norvegicus MF480318 DE
Rattus norvegicus KY659382 CN
Apodemus chevrieri MG020017 CN
Rattus norvegicus JN167538 DE
Rattus norvegicus KX774659 DE
Ursus arctos syriacus MF480313 DE
Rattus norvegicus KX774648 AT
Rattus norvegicus KM516906 US
Rattus norvegicus KX774642 HU
Rattus norvegicus KX774643 HU
Rattus norvegicus KX774673 FR
Rattus norvegicus KY938025 BE
Rattus norvegicus JN167537 DE
Rattus rattus KY938013 IT
Rattus norvegicus KY938020 BE
Rattus rattus AB847308 ID
Rattus norvegicus JQ898482 US
Rattus norvegicus KY938021 BE
Rattus rattus KY938027 ES
Rattus norvegicus KY938011 GR
Rattus norvegicus KY938016 CZ
Rattus norvegicus KX774651 AT
Rattus norvegicus KX774665 FR
Rattus norvegicus KX774655 CH
RatHEV18IT16
Bandicota indica KC465994 CN
Suncus murinus KC473530 CN
Rattus rattus LC225389 ID
Rattus norvegicus KC466001 CH
Rattus rattus AB847306 ID
Rattus rattus AB847309 ID
Rattus rattus AB847305 ID
Rattus rattus LC145325 ID
Rattus rattus LC225388 ID
Rattus tanezumi JX120573 VN
Rattus sp. MG813927 HK
Rattus rattus AB847307 ID
Rattus rattoides losea KC465990 CH
Suncus murinus KC473529 CH
Rattus flavipectus KC465993 CH
Mustela putorius JN998606 NL
Homo sapiens AB220979 JP
Homo sapiens AB301710 JP
Homo sapiens AY230202 MA
Homo sapiens M74506 MX
G1
G2
G3
HEV-C1
HEV-C2
HEV-4
HEV-3
HEV-1
HEV-2
0,5

Orthohepevirus C species. Low nucleotide and amino acid sequence similarities were observed with genotypes HEV-3 and HEV-4 (59.0%–62.0% nt. id., 64.0%–70.0% aa. id.).

The phylogenetic tree (Figure 2) showed the clustering of Rat/HEV/18 strain with the other rat HEV strains classified into HEV-C1 and within the G1 genotype group. The Italian strain showed 85.5% nucleotide sequence identity and 92.5% amino acid identity with the HEV-C1 genotype G1 reference strain rat/R63/DEU/2009 (GU345042). The differences observed in the nucleotide sequence of Rat/HEV/18 with respect to other strains from rat, ranged between 13.0% and 17.0% (Figure 2). Overall, the Italian strain Rat/HEV/18 was assigned to HEV-C1, revealing a strict clustering with other rat HEV genotype G1 strains detected worldwide, and being separate from the ferret HEV-C2 strain (JN998606).

4 | DISCUSSION

The role of rats and mice in the epidemiology of HEV is still unknown. They are both synanthropic mammals and their ubiquitous presence in urban environments and in rural areas where pigs are farmed, make them an important target for HEV investigation. The present study was conducted to evaluate the possible circulation of rat HEV and zoonotic HEV-3 in rats and mice caught in swine farms.

The 21 mice sampled from a pig farm were negative for the presence of HEV RNA. We analysed a limited number of mice but the result is in line with previous studies, which showed mice as a scarce vector for HEV, considering both HEV-C1 and HEV-3 (Grierson et al., 2018; Kurucz et al., 2019). However, it is of concern that in a recent paper BALB/c mice were susceptible to infection with rabbit and swine HEV-3 strains through inoculation via gavage and contact-exposure (Huang et al., 2009; Sun et al., 2018). The mice susceptibility to HEV-3 is still controversially discussed suggesting that the role of mice as carrier of HEV should be further investigated.

The intestinal contents of Rat/HEV/18 and Rat/HEV/30 out of 47 Black rats analysed were found positive for rat HEV-C1 genotype G1 and HEV-3 RNAs, respectively. Conversely, the liver sampled from both animals was negative.

The Italian strain Rat/HEV/18 was classified as HEV-C1, genotype G1, although the analysis was performed on a short segment of the ORF1. In Europe, studies conducted on Norway rats and Black rats living in urban areas, reported the detection of HEV-C1 with prevalence ranging between 0.3% and 27.2% (Johne et al., 2010a; Johne et al., 2012; Wolf et al., 2013; Widen et al., 2014; Woo et al., 2016; Spahr et al., 2017; Simanavicius et al., 2018). In our study, we reported a low presence 2% of rat HEV RNA in rat intestinal contents. In addition, the phylogenetic analysis of Rat/HEV/18, detected in Black rats showed that it clustered together with the other rat HEV strains detected in Norway rats, confirming previous findings that rat HEV can infect different rat species indiscriminately (Lack et al., 2012; Simanavicius et al., 2018). The detection of HEV in the liver would also be expected in the presence of active replication, as the liver is the main organ of viral replication (Nan & Zhang, 2016).

Only the intestinal content from Rat/HEV/30 resulted positive for HEV-3 by real-time RT-PCR and by nested RT-PCR amplifying a fragment within the ORF2. The missing detection of HEV-3 in liver suggests the absence of infection in rats. A similar result was observed in the UK and in Japan. The HEV-3 strains detected in mice in the UK and in rats in Japan were identical to swine HEV strains circulating in the country (Grierson et al., 2018) and in the pig from the farm where animals were caught (Kanai et al., 2012). In the UK, HEV-3 RNAs were detected in faeces but not in the liver of mice (Grierson et al., 2018). In the absence of a clear evidence of HEV-3 replication in these animals, the presence of HEV-3 in the faecal content may represent the consequence of an ingestion of faeces containing the virus, as confirmed by the detection of the same viral strain in the faecal samples from swine collected on the same farm.

The finding of intestinal sample positive for HEV-3 in rat in swine farms and the circulation of rats through the pig pens poses the question of possible transmission of HEV strains between the two species. However, the rat HEV is more prevalent than HEV-3 in rats in Europe (Ryll et al., 2017). Rats and pigs can easily be exposed to each other faeces, where a high level of the virus could be released. Faeces could contaminate environment representing a source of contamination for pigs but also for pig workers although the detection of RNA is not sufficient to state that the virus is infectious (Mughini-Gras et al., 2017; Huang, Huang, Wagner, Chen, & Lu, 2019). Since rats and mice are synanthropic and can have a role on human infections, as reported by the detection of rat HEV strains in patients affected by acute hepatitis (Sridhar et al., 2018; Andonov et al., 2019), further investigation is required.

In conclusion, molecular surveillance and a phylogenetic approach may help clarify the epidemiology of rat HEV in nature and the role of biosecurity measures and rat and pest control procedures.

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CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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