Epidemiological and genetic evaluation of HEV in swine slaughtered in Sicily region (Italy)

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ABSTRACT

Hepatitis E virus (HEV) is the etiological agent of acute viral hepatitis, a disease transmitted by the oral-faecal route. In Europe, zoonotic transmission of HEV-3 genotype is associated with the consumption of raw or slightly cooked meat of pigs and wild boars that are considered the main reservoirs.

This work aims to assess the occurrence of swines' HEV RNA liver samples and rectal swabs slaughtered in Sicily using biomolecular methods. HEV-RNA was detected in 17.5% (21/120) liver samples analyzed and in 3.7% (3/81) rectal swabs examined. All positive samples were predicted as genotype 3 and subtype 3c (75%). These data suggest a potential HEV transmission to humans through close contact with pig breeders, veterinarians, slaughterhouse personnel, and pork meat product consumption. Moreover, there are few scientific data evaluating the HEV spread around pigs and humans in Sicily. Therefore, further studies are necessary to correlate humans with swine serotypes and to assess the HEV presence and persistence in food and the risk during the slaughtering process. These surveys allow to clarify the role of the swine species as a potential source of infection for other domestic or wild animals and humans and to establish possible control measures throughout the food chain.

Keywords

Zoonosis; Molecular methods; Epidemiological survey; Genotype 3; Phylogenetic analysis

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1.INTRODUCTION

Hepatitis E virus (HEV) is the etiological agent of acute viral hepatitis, a human disease caused by contaminated food or water consumption and transmitted by the faecal-oral route. In healthy people, it occurs as a self-limiting acute hepatitis that shows case fatality rates between 1% and 5% (Pavia et al., 2021). In pregnant women and immunocompromised individuals, HEV develops fulminant hepatic failure or chronic liver damage that reaches rates up to 20% (Doceul et al., 2016; Ricci et al., 2017). The burden of this infection worldwide is unknown, and it is estimated that, over the past decade, there were more than 21,000 acute clinical HEV cases with 28 deaths in the European Union (EU) (World Health Organization (WHO), 2021). However, this data may be underestimated because human HEV disease is not notified in all EU countries. Moreover, the latter have different viral surveillance systems which prevents the comparison of reported HEV cases.

HEV is a hepatotropic, small non-enveloped, and single-stranded RNA virus which belongs to the family *Hepeviridae*, genus *Orthohepevirus*, species *Orthohepevirus A*. The latter includes five different genotypes infecting humans but, of them, HEV-3 and HEV-4 were detected also in animals (pigs, wild boar, deer, cows, goats, rabbits) and considered zoonotic. In fact, sero-epidemic studies have shown a high prevalence of anti-HEV antibodies in the healthy population of many countries (Ricci et al., 2017), suggesting zoonotic transmission of HEV due to its presence in animals, such as wild boars and pigs, which are considered the reservoirs (Di Cola et al., 2021).

To date, HEV is recognized as an emerging foodborne zoonosis and in Europe, HEV-3 represents the most common genotype detected in humans and animals while HEV-4 has been detected in humans and less in pigs (Chelli et al., 2021). During the last decade, Canada, Europe, Japan, and the United States reported HEV cases often related to people traveling in HEV endemic areas but, to date, people with no history of traveling abroad tested positive for HEV too. In Europe, HEV-3 was reported as the predominant genotype, widespread in pork, wild boar, and deer (Dalton et al., 2016;

De Sabato et al., 2018; Ricci et al., 2017). It was responsible for sporadic and clustered human cases associated with the consumption of pigs and wild boars' meat used in food products such as liver, offal products, sausages etc. In particular, HEV 3c, 3e, and 3f subtypes have been reported in both animals and humans worldwide (De Sabato et al., 2020b). In Italy, the HEV-3f subtype is widespread among humans and pigs followed by HEV-3e while HEV-3c is the most frequent subtype detected in wild boars, but occasionally in pigs (De Sabato et al., 2020a; Pierini et al., 2021). The detection of identical HEV sequences in food and patients highlighted that the consumption of processed wild boar and pig meat could be related to a high risk of either HEV-3 or HEV-4 human infection (Di Cola et al., 2021).

In particular, pigs represent a major risk of HEV transmission to humans when their contaminated meat products are consumed raw or undercooked. There are several risk factors related to swine HEV infection such as the pigs’ age and, the type of farming. Firstly, a higher HEV-RNA prevalence has been detected in younger pigs (mean age: 4-5 months) than older (Di Bartolo et al., 2011). Secondly, different studies described conflicting data regarding HEV occurrence in extensive or intensive farming production (Lopez-Lopez et al., 2018). Moreover, professional exposure to infected animals such as wild boars or swine represents another important HEV transmission route. Several studies reported that breeders, veterinarians, and slaughterhouse personnel may be infected by HEV 1.5 times more than the general population suggesting an occupational risk (de Schryver et al., 2015; Mrzljak et al., 2021).

More studies are needed to evaluate the potential risk linked to the consumption of pork products and also to increase and improve the epidemiological surveillance information. These goals will be useful to better understand and reduce the zoonotic transmission of HEV caused by infected pigs.

To date, In Sicily, there aren't studies focusing on the HEV prevalence in swine and no human HEV cases have been reported since 2017, even if in Sicily region the pig meat products' consumption is one of the highest in Italy ("ISTAT", "SEIEVA").

Our study aimed to assess the HEV presence and genetic variability in liver samples and rectal swabs, of swine slaughtered, in the eastern area of the Sicilia region, to provide epidemiological data currently not available.

2. MATERIALS AND METHODS

2.1. Sampling

Between October 2021 and February 2022, 120 liver samples of pigs slaughtered in Sicily and only 81 rectal swabs from the same animals, were collected (Table 1). During the official veterinary control conducted by the local health authority, livers (30-40 cm^3) and rectal swabs were collected and transported under refrigerated conditions to the laboratory and stored at -80 °C until use.

2.2. Sample preparation and nucleic acid extraction

Virus extraction was performed following the method described by Di Pasquale et al. (2019) with appropriate modifications. Briefly, 1 g of liver was shredded with a sterile blade and was added to a tube containing 3.5 ml of TRIZOL Reagent (Life Technologies, Monza, Italy) and 1 sterile tungsten carbide bead (3mm diameter). Samples were homogenized for 5 min at 25 hz/s in Tissue Lyser II (Qiagen, GmbH, Hilden, Germany). After mechanical disruption of the livers, samples were incubated at Room Temperature (RT) for 15 min and centrifuged at 8000 x g for 20 min at 4 °C. Then the supernatant was added with 0.7 ml of chloroform and vortexed for 15 sec and incubated for 15 min at RT. The aqueous phase obtained by centrifugation at 8000 x g for 15 min at 4°C, was recovered and stored at – 80°C until use. In addition, the rectal swab samples were diluted in 1 ml of PBS and centrifuged at 15700g for 1 min; the homogenate was recovered and stored at -80°C until use.

2.3. Touchdown RT-PCR and Touchdown hemi-nested PCR

The touchdown RT-PCR (TD/RT-PCR) and Touchdown hemi-nested PCR assays were carried out as described in detail by Lorusso et al. (2022). Briefly, the RNA extract was retrotranscribed by RT-PCR touchdown (TD/RT-PCR) and subsequently amplified with Touchdown hemi-nested PCR (TD/hn-PCR) in order to increase sensitivity. The primers (HEV-FA228, HEV-R4598 and HEV-R4565) amplified a fragment of 346 bp belonging to RdRp of ORF1 (Drexler et al. 2012).

2.4. Detection of amplified products

TD/hn-PCR-amplified products were displayed by electrophoresis on 1.5% (w/v) agarose NA (Pharmacia, Uppsala, Sweden) gel in 1X TBE buffer containing 0.089 M Tris, 0.089 M boric acid, 0.002 M EDTA, pH 8.0 (USB, Cleveland, OH, USA), and stained with Green Gel Safe 10000X Nucleic Acid Stain (5 ll/100 ml) (Fisher Molecular Biology, USA). The Gene RulerTM 100 bp DNA Ladder molecular weight marker (MBI Fermentas, Vilnius, Lithuania) was used.

Image acquisition was performed with Gel DocTM EZ imager Bio-rad. Cycle sequencing was carried out using BigDye Terminator Cycle chemistry (Applied Biosystems, Foster City, California, US).

2.5. Data analysis

Sicilian consensus sequences were obtained using the Geneious software version 10.0.5 (Biomatters Ltd, New Zealand) and molecular data were combined with the dataset of sequences generated in Calabria region by Lorusso et al. (2022). The two datasets were merged and dereplicated to identify identical sequences (unique haplotypes) by using mothur v 1.45.3 (Schloss et al., 2009). Genotypes and subtypes of fragment of HEV sequences were predicted through the online HEVnet tool (Mulder et al., 2019). Molecular data, including the RNA-dependent RNA polymerase gene fragment sampled in the Italian regions from several hosts, were downloaded from GenBank (February 2022). Sequences were aligned using MAFFT (Katoh et al., 2018) and the alignment was manually checked using SeaView v.4.0 (Gouy et al., 2010). A Maximum Likelihood tree was built in FastTree (Price et al., 2010) using GTR gamma model and HEV-4 strain was used as an outgroup (AB197673). Visualization and annotation of the tree were performed in iTOL ("iTOL: Interactive Tree Of Life," n.d.) (Letunic and Bork, 2019). Matrices of pairwise p-distances (the number of differences divided by the total number of sites) were generated for nucleotide and protein sequences included in the tree using MEGA 11 (Tamura et al., 2021) and amino acid multialignment was generated and visualized using SeaView v.4.0 (Gouy et al., 2010). A map of sampling sites was built using leaflet R package ("leafletR: Interactive Web-Maps Based on the Leaflet JavaScript Library. R package version 0.4-0").

3. RESULTS

The HEV-RNA was detected in 17.5% of liver samples (21/120) and in 3.7% of rectal swabs (3/81) by the amplification of a 346 bp fragment. All pigs tested positive for HEV-RNA in rectal swabs gave a negative result in the liver samples of the same animals and vice versa (Table 1). The generation of unique sequences reduced the dataset from 24 sequences to 4 haplotypes (non-redundant unique sequences, hap_1, hap_5, hap_6, hap_7) (Table 2). The phylogenetic approach of the HEVnet tool identified all haplotypes belonged to the hepatitis E virus with high support (>90%) whereas prediction of the subtype HEV-3c was inferred with low support (about 70%) for two haplotypes (Hap_1 and Hap_5) and no subtype was inferred for Hap_6 and Hap_7 (Table 2). Evolutionary prediction by the HEVnet showed all haplotypes matched FJ705359 as the closest reference sequence that was previously found both in human and wild boar and in several geographical areas (France, Germany, Netherlands, Sweden, UK, Thailand, Canada). Based on a total of 103 sequences, after the removal of redundances, the tree-building included 87 nucleotide sequences of 291 bp (including the four haplotypes and the outgroup) (Figure 1). In the tree, our haplotypes were grouped together in a clade and the German reference sampled in wild boar (FJ705359) was confirmed to be the most related to the Sicilian haplotypes. Sister sequences to the clade also are represented by two references, one collected in Central Italy from wild boar (MT840360) by Aprea et al. (2020) and a second sampled in Bulgaria from swine (MZ519907) by Palombieri et al. (2021). Details of p-distances matrices and amino acid multi-alignment are available in the Supplementary Data file and Supp Fig 1.

4. DISCUSSION

The results achieved in our study highlight the HEV incidence in pigs (*Sus scrofa domesticus*) slaughtered in the Sicily region, especially in the eastern part of the island, with an overall HEV-3 RNA presence of 17.5% (21/120) in liver samples and 3.7% (3/81) in rectal swabs. These findings indicated that HEV infection is widely spread in Sicilian pigs, and they are consistent with data obtained in southern Italy where HEV was detected in 16.5% of pigs' liver samples (Chelli et al., 2021). However, the point prevalence value estimated in liver samples is higher if compared with previous studies in Italy detecting HEV-RNA between 0 and 5% (Di Bartolo et al., 2008; Mughini-Gras et al., 2017; Pavoni et al., 2015) and also, with recent European scientific works conducted in France, Slovenia and Spain where up to 2.8% of liver samples tested positive for HEV (Chelli et al., 2021; Di Bartolo et al., 2012). However, in Europe an higher prevalence was detected in Netherlands where up to 11% of swine liver samples tested positive for HEV-RNA (Boxman et al., 2022).

In our study, liver and fecal samples were collected from the same pigs but no one animal tested positive for HEV-RNA in both sample types. This occurrence could be related to liver sampling errors. The acute phase of infection is generally referred as to the start of HEV shedding in feces. Based on the infection dose and route, HEV shedding in pigs can last from 7 to 50 days (Meester et al., 2021; Salines et al., 2017). However HEV RNA could be detected in the liver, bile, and other organs after the end of shedding and viremia and consequently, pigs can transmit HEV to humans if infected organs are consumed raw or slightly cooked (Meester et al., 2021).

Our study describes an HEV-RNA incidence in rectal swabs in line with results obtained by Chelli et al. (2021) and Di Martino et al. (2010) where HEV-RNA was detected in 1.9% and 7.3% of Italian pigs' faecal samples respectively. Nevertheless, these outcomes are lower than data detected in other Italian studies that presented a higher HEV-RNA prevalence in swine rectal swabs' reaching percentages from 24.8 up to 100% (Chelli et al., 2021). Our results could be justified considering that most positive samples came from the same farms areas as described in Table 1. Moreover, the type of farm (intensive or extensive) could have also influenced the exposure of animals to the virus, increasing HEV infection in these species (Lopez-Lopez et al., 2018). Unfortunately, this information was not available and future studies are needed to search for a correlation about the type of pig farming with the major risk to be infected for swine and professional workers. Furthermore, in our study, all pigs were 9- to 12-month-old and, consequently, it was impossible to correlate the HEV positivity trends with the pigs' age.

Generally, HEV has been detected at high rates in animals of different age groups in Italian slaughterhouses and the HEV-3f subtype has been the most frequent in humans and pigs, followed by the HEV-3e, HEV-3c (De Sabato et al., 2020b). On the other hand, in our study, 18/24 (75%) HEV3 positive liver samples were predicted as subtype 3c with bootstrap ≥70%, even if the fragment employed for the molecular analysis was short. This evidence suggests that subtype 3c is potentially dominant in pigs around the Sicilian territory. Moreover, based on the results obtained in our study, it is interesting to note that haplotype 1 was detected in pigs breaded in different eastern Sicilian farms representing one of the most widely spread haplotype. Furthermore, previous study (Lorusso et al., 2022) identified the same haplotype also in liver samples of Calabrian wild boars where, in the same way, it represented the main haplotype circulating. This evidence could suggest direct transmission between wild animals and pigs raised on semi-free farms and/or environmental contamination, as described by Montagnaro et al. (2015) and Wu et al. (2011) reporting environmental interaction between wild boars and swine.

The limitation of sampling areas and the number of farms in which samples were taken does not allow a complete evaluation of HEV prevalence in the Sicily region and a wider sampling involving all the Sicilian provinces, will be necessary to carry out an epidemiological characterization and define the riskiest areas. Furthermore, this study suggests that workers operating in the swine food chain could be infected by the zoonotic HEV strains during industrial and domestic slaughtering processes. Thus, the slaughterhouse could represent an epidemiological focal point to determine HEV transmission in a geographical area. It might also support national analysis to establish risk management measures.

Furthermore, more studies are needed to search for a correlation between swine and human HEV serotypes to confirm and define the role played by this species as a reservoir and potential source of infection for both domestic and wild animals, and not least for humans.

5. CONCLUSION

Our study highlights the high circulation of HEV among pigs slaughtered in the eastern part of Sicily; this data could be extended to the whole region involving all Sicilian sampling areas and the Italian peninsula. Therefore, further investigation necessary to understand the circulation of HEV genotypes between the pigs farmed and consequently to establish swine HEV prevalence and the real risk of infection for personnel operating in the swine food chain. The Competent Authorities should enforce the risk assessment systems to establish measures for the improvement of public health. Moreover, they could implement biosafety rules during the swine farming and slaughtering process to contain the risk of HEV infection.

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TABLES

TABLE 1. Liver and rectal swab samples of pigs tested positive for HEV-RNA and belonged to different areas of the Sicily region

Sampling areas[a]	**HEV pos./total no. of livers**	**HEV pos./total no. of rectal swab**	**Age**
Santa Venerina (CT)	6/21	0	9 – 12 months
Giarre (CT)	0/6	0	9 – 12 months
Piedimento Etneo (CT)	3/12	0	9 – 12 months
Modica (RG)	3/18[b]	3/18[b]	9 – 12 months
Barcellona Pozzo Di Gotto (ME)	6/15	0/15	9 – 12 months
Santa Maria di Licodia (CT)	0/9	0/9	9 – 12 months
Maniace (CT)	3/18	0/18	9 – 12 months
Belpasso (CT)	0/3	0/3	9 – 12 months
Bronte (CT)	0/3	0/3	9 – 12 months
Francia	0/15[c]	0/15[c]	9 – 12 months
HEV POS. TOT. NO.	21/120	3/ 81	

a Every sampling area includes one farm except Modica and Piedimento Etneo presenting two farms each.

b Positive liver samples are not from the same pig as rectal swabs.

c Farmed in France and slaughtered in Sicily.

TABLE 2. Dataset's summary of liver and rectal swab samples of pigs tested positive for HEV-RNA and belonged to different areas of the Sicily region

Samples with identical HEV nucleotide sequences (haplotypes), the genotype and subtypes' assignments inferred by HEVnet, were reported for each haplotype.

Haplotype	**Samples**		**Genotype**	**Subtype**	**Reference**
	Liver ID[a]	**Rectal swabs ID**[a]			
Hap_1 **15 positive samples**	MODICA_2, MODICA_3 MODICA_4 SANTA_VENERINA_1, SANTA_VENERINA_2, SANTA_VENERINA_3, MANIACE_5 MANIACE_6 MANIACE_7 GOTTO_1 GOTTO_2 GOTTO_6 GOTTO_9 GOTTO_13 GOTTO_15		HEV3	HEV3c	Germany – Sus scrofa (FJ705359)
Hap_5 **3 positive samples**		MODICA_1S MODICA_3S MODICA_6S	HEV3	HEV3c	Germany – Sus scrofa (FJ705359)
Hap_6 **3 positive samples**	SANTA_VENERINA_5 SANTA_VENERINA_6 SANTA_VENERINA_7		HEV3	Could not assign	Germany – Sus scrofa (FJ705359)
Hap_7 **3 positive samples**	PIEDIMENTO_ETNEO_2 PIEDIMENTO_ETNEO_7 nPIEDIMENTO_ETNEO_8		HEV3	Could not assign	Germany – Sus scrofa (FJ705359)

a: The ID was generated using the municipality of pigs' farming with a progressive number.

FIGURES

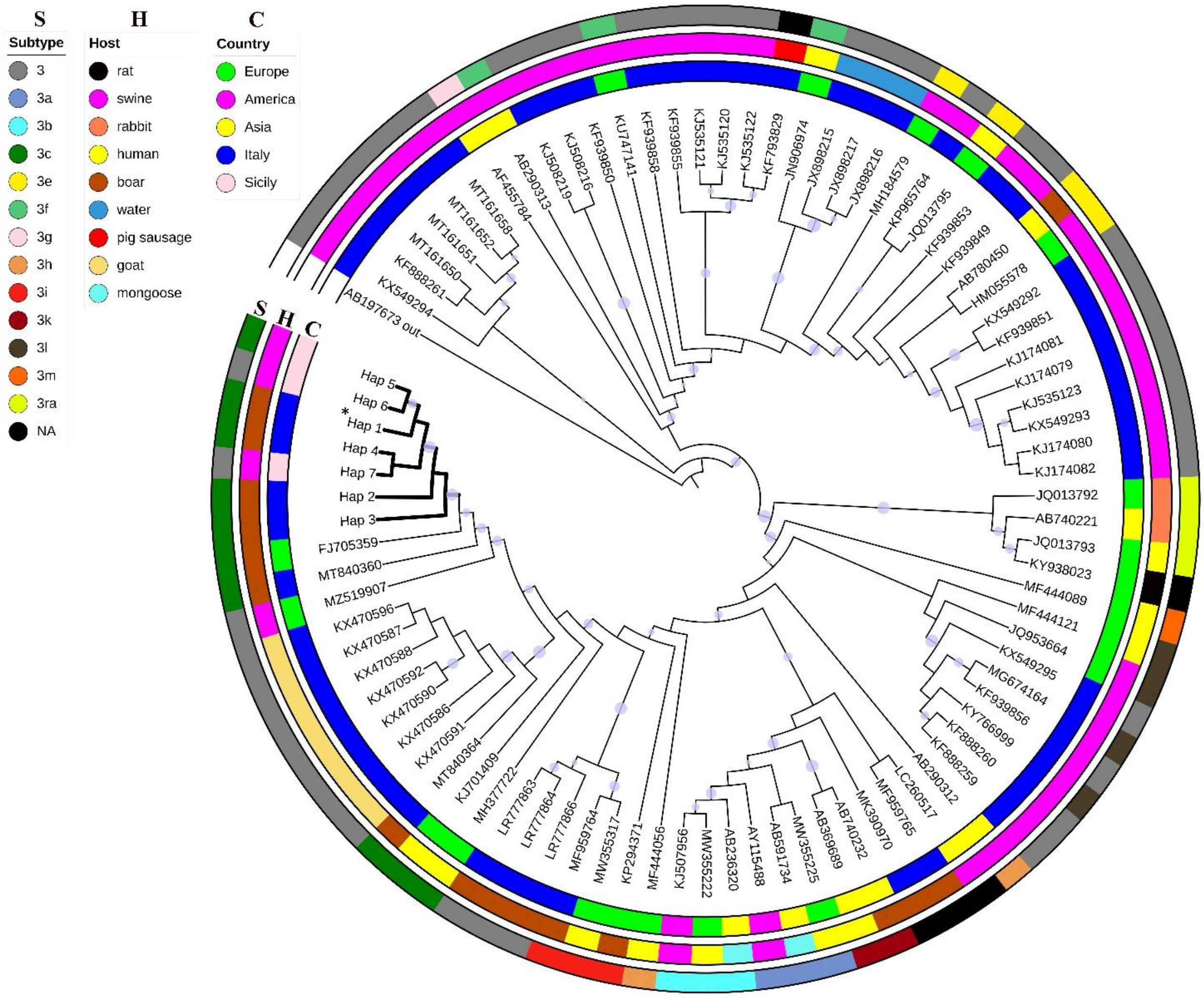

FIGURE 1. Maximum Likelihood tree of HEV 3 genotype using the RNA-dependent RNA polymerase.

The tree included our four haplotypes of 291 bp (Hap 1; Hap 5; Hap 6; Hap 7) detected in Sicily, three haplotypes (Hap 2; Hap 3; Hap 4) detected in Calabria region (Lorusso et al., 2022) and all samples available from other Italian regions and sources. Representative nucleotide sequences of other geographic areas were also included, and one HEV-4 strain was used as outgroup (AB197673). Bootstraps >70% were showed as symbols (dots) proportional to the values. Metadata associated to nucleotide sequences were showed by three colour code panels: Subtype (S), Host (H) and Country (C).

*Haplotype 1 was detected also in pigs.

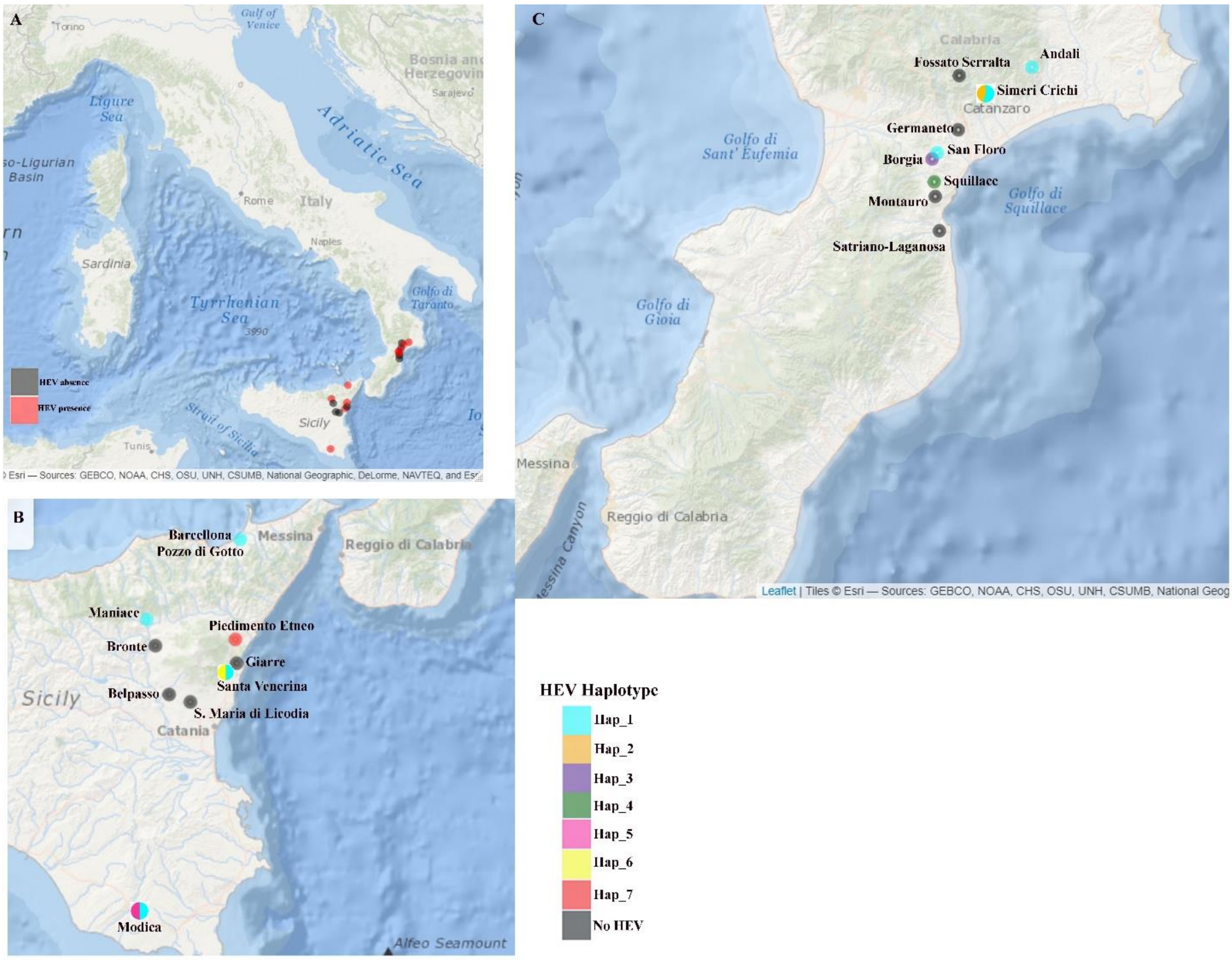

FIGURE 2.

A) Samples' georeferentiation and HEV results.

The map shows the georeferentiation of liver and rectal swabs' samples collected in Calabria and Sicily's municipalities. The HEV occurrence was also described: the red dots represent the areas where HEV was detected; grey dots represent areas with HEV absence.

B) Samples' map and haplotypes detected in Sicily region.

Map of sampling and corresponding haplotypes found in each municipality. Grey dots indicate sites where HEV was not detected.

C) Samples’ map and haplotypes detected in Calabrian region.

Map of sampling and corresponding haplotypes found in each municipality. Grey dots indicate sites where HEV was not detected.

Supplementary Interactive Plot Data (CSV)

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Supplementary Interactive Plot Data (CSV)

Supplementary data 1.xlsx

Conflict of Interest and Authorship Conformation Form

Please check the following as appropriate:

X All authors have participated in (a) conception and design, or analysis and interpretation of the data; (b) drafting the article or revising it critically for important intellectual content; and (c) approval of the final version.

X This manuscript has not been submitted to, nor is under review at, another journal or other publishing venue.

X The authors have no affiliation with any organization with a direct or indirect financial interest in the subject matter discussed in the manuscript

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