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Endemic maintenance of human-related hepatitis E virus strains in synurbic wild boars, Barcelona Metropolitan Area, Spain

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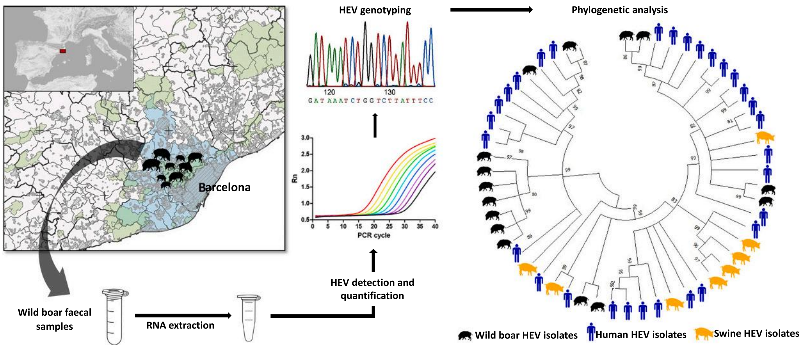
HIGHLIGHTS

- Hepatitis E virus (HEV) is an emerging global public health threat.
- Synurbic wild boars can carry and release HEV into urban environments.
- HEV was detected in piglets, juvenile and yearling wild boars, but not in adults.
- HEV strains infecting wild boars from Barcelona in 2016–2021 belonged to genotype 3.
- The wild boar HEV strains were closely related to those infecting BMA citizens.

GRAPHICAL ABSTRACT



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ABSTRACT

Hepatitis E virus (HEV), shared by humans, domestic animals, and wildlife, is an emerging global public health threat. Because wild boars are a major reservoir of HEV, the new zoonotic interfaces resulting from wild boar population increase and synurbization significantly contribute to increasing the risk of zoonotic transmission of HEV.

This study characterizes HEV strains of synurbic wild boars and assesses their relationship with sympatric human and domestic swine HEV strains. We analyzed the faeces of 312 synurbic wild boars collected from 2016

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Synurbization
Zoonosis

to 2021 in the Barcelona Metropolitan Area (BMA), where there is a high density of wild boars, and found 7 HEV-positive samples among those collected between 2019 and 2020. The molecular analysis of these isolates, along with 6 additional wild boar HEV isolates from a previous study, allowed us to establish a close phylogenetic relationship between these HEV strains and human HEV isolates from sympatric blood donors and domestic pigs from Catalonia. HEV-positive wild boar samples belonged to piglet, juvenile and yearling individuals, but not adults, indicating the endemic maintenance of HEV in the wild boar population of the BMA by naïve young individuals. All wild boar HEV isolates in this study classified within HEV genotype 3.

The results show, for the first time, a close molecular similarity between the HEV strains endemically maintained by the synurbic wild boars in the BMA and citizens from the same area and period. The data could also indicate that HEV infection presents a seasonal and interannual variability in wild boars of BMA. Further investigation is required to unveil the HEV transmission routes between synurbic wild boars and sympatric citizens. These findings can serve in other synurbic wildlife-human interfaces throughout the world.

1. Introduction

Hepatitis E virus (HEV) is a single-stranded RNA virus included in the Hepeviridae family, *Paslahepevirus* genus (Smith et al., 2014), and it is the causative agent of hepatitis E, an emerging global public health threat (Khuroo et al., 2016). HEV can cause epidemics, as well as sporadic cases related to foodborne zoonotic infections. Globally, one third of the human population lives in HEV endemic areas, with approximately 939 million humans having ever experienced HEV infection. Hepatitis E affects around 20 million people worldwide and causes approximately 50,000 human deaths every year (Li et al., 2020; Pallerla et al., 2020). In Europe and Asia, human HEV infection numbers have increased dramatically (Salvador et al., 2020).

HEV has been classified into eight genotypes (GTs) (Smith et al., 2016), four of which (GTs 1–4) can infect humans (Kamar et al., 2017). Although most human cases of hepatitis E are mild (Chandra et al., 2008), HEV infection is particularly severe in pregnant women, with mortality rates as high as 30 %, and can be vertically transmitted to infants (Meng, 2013). Moreover, GTs 3 and 4 can cause severe and chronic infection in immunocompromised individuals, such as human immunodeficiency virus (HIV)-infected patients and transplant recipients, who have an increased risk of rapidly progressive liver disease. HEV infection mainly causes acute viral hepatitis, which can be accompanied by anorexia, jaundice, nausea, fever, abdominal pain, and hepatomegaly (Chandra et al., 2008). HEV infection might also cause extrahepatic manifestations, such as neurological clinical signs, renal damage, pancreatitis, and hematologic complications (Debing et al., 2016). HEV-associated severe neurological manifestations, including neuralgic amyotrophy, sensory loss, Parsonage–Turner syndrome, and Guillain-Barré syndrome, have been observed in both acute and chronic HEV-infected patients (Debing et al., 2016; Mendoza-Lopez et al., 2020; Jha et al., 2021).

HEV GTs 3 and 4 can also infect domestic animals including swine (*Sus scrofa domesticus*), sheep (*Ovis aries*), goat (*Capra hircus*), camel (*Camelus* spp.), and yak (*Bos grunniens*), as well as wildlife such as wild boar (*Sus scrofa* ssp.), red deer (*Cervus elaphus*), roe deer (*Capreolus capreolus*) and Alpine ibex (*Capra ibex*) (Smith et al., 2014; Palombieri et al., 2020; Sridhar et al., 2018; Wang et al., 2019; Xu et al., 2014). These species contribute to HEV circulation and maintenance, and represent a serious zoonotic risk to humans through the consumption of HEV-infected food products of these species, such as raw or undercooked meat and milk-derived products (Dziedzinska et al., 2020; Li et al., 2016; Meng, 2010; Rivero-Juarez et al., 2017).

Blood-borne transmission of HEV has also been reported (Boxall et al., 2006; Dreier et al., 2018; Hewitt et al., 2014; Izopet et al., 2017; Riveiro-Barciela et al., 2017, 2018) which has raised medical concern and prompted the introduction of HEV screening tests in blood banks (Boland et al., 2019; Bes et al., 2022). Furthermore, pet animals, including dogs, cats, rabbits, and horses, have been proposed as accidental hosts of HEV (Li et al., 2020; Liang et al., 2014; Wang et al., 2017), indicating that interactions between wild animals and pets may also contribute to indirect zoonotic transmission of HEV.

Wild boar and feral pig populations are geographic and demographically increasing worldwide for decades (Acevedo et al., 2014; Bosch et al., 2012; Massei et al., 2011, 2015; Sáez-Royuela and Tellería, 1986; Snow et al., 2017). Such increase and spread is accompanied by wild boar synurbization (Cahill et al., 2012; Castillo-Contreras et al., 2018; Náhlik et al., 2017; Licoppe et al., 2013), which creates new wild boar-human interactions, leading to conflicts, risk of attacks, and zoonotic hazard (Cahill et al., 2012; González-Crespo et al., 2023a, 2023b; Kotulski and König, 2008; Meng and Lindsay, 2009; Schielke et al., 2009). Synurbization is a particular case of synanthropization, which is the adaptation of animal populations to anthropogenic conditions (Luniak, 2004). A representative case of wild boar synurbization can be observed at the Barcelona Metropolitan Area (BMA; Fig. 1), where the wild boar population density ranged from 5 to 15 individuals per km^2 during the period 2004–2022 (Cahill et al., 2012; Castillo-Contreras et al., 2018, 2021; Rosell et al., 2022; Conejero et al., 2019). These individuals constitute a genotypically-differentiated population that has partially become synanthropic (Hagemann et al., 2022). In addition, a high serological antibody prevalence against HEV (59 %) and a HEV RNA prevalence in serum (16 %) and in faeces (12 %) has been reported in this wild boar population (Wang et al., 2019). The risk of wild boar-human direct and indirect contact in the BMA has been modelled, unveiling a key role of faeces in the interspecific HEV zoonotic transmission (González-Crespo et al., 2023a, 2023b).

This study aims to molecularly characterize the HEV strains in wild boars from the BMA and assess their phylogenetic relationship with sympatric human and domestic swine HEV strains, to carry out the first eco-epidemiological study of HEV in the wild boar-human host community of the BMA.

2. Material and methods

2.1. Study area

The BMA is formed by 36 municipalities spreading over 636 km^2 and populated by 3.2 million people. It includes the Collserola Natural Park, an 8,000-hectares periurban Mediterranean forest, surrounded by urban areas (Fig. 1). Wild boars are hunted and consumed in the Controlled Hunting Zone of Collserola (Fig. 1A) (Rosell et al., 2022; Conejero et al., 2019), which supposes a risk of zoonotic HEV transmission to humans through ingestion of infected wild boar meat (Rivero-Juarez et al., 2017). Additionally, the new interface between synurbic wild boars and humans both in Collserola and the periurban areas of the BMA, resulting in interactions such as direct anthropic feeding or garbage consumption by wild boars (Castillo-Contreras et al., 2021; Conejero et al., 2019), creates new possibilities for bidirectional direct and/or indirect transmission of HEV between wild boars and humans (Wang et al., 2019; Pavio et al., 2017).

2.2. Sample collection

Faecal samples were obtained from wild boars captured from

October 2016 to January 2021 in the BMA (Table 1). The wild boars were either hunted in the Controlled Game Area of Collserola or live-captured using cage-traps, corral-traps, drop-nets and/or tele-anaesthesia (Barasona et al., 2013; Conejero et al., 2022; Torres-Blas et al., 2020) in urban and peri-urban areas of the BMA (Fig. 1). Age was estimated in all the wild boars through tooth eruption and replacement up to three years (Boitani and Mattei, 1992; Matschke, 1967) and wear patterns beyond three years (Potel, 1979). Age class was assigned as follows: piglets (up to 6 months), juveniles (between 6 and 12 months), yearlings (between 12 and 24 months) or adults (over 24 months) (Castillo-Contreras et al., 2021). Since both hunting and live-capture are included within the regular management plan of this species in the BMA, no animal ethic committee permit was required nor issued. The faecal samples were collected directly from the rectum, placed in RNAlater (RNA Stabilization Reagent, Qiagen), and stored at −80 °C until analysis.

Due to the high risk posed by HEV to blood transfusion safety, the Banc de Sang i Teixits (BST) introduced a routine screening test for HEV in 2017.

2.3. HEV RNA detection by RT-qPCR assay and sequencing

The faecal samples preserved in RNAlater were homogenized and clarified by centrifugation at 8000 rpm for 3 min. Total RNA from the faecal suspensions was isolated using the QIAamp® Viral RNA (Qiagen) according to the manufacturer's protocol. The HEV ORF2 6026-6435 and 6367-6776 regions were amplified for all the wild boar samples by nested RT-PCR (RT-nPCR) assay as previously described (Bes et al., 2022; Smith et al., 2012), using SuperScript Vilo® (Thermo Fisher) and Quantitect Multiplex (QIAGEN). The amplified products were purified using the QIAquick® PCR Purification Kit (QIAGEN). Sanger sequencing of the ORF2 amplicons was performed at Macrogen®. The HEV RNA of the positive samples was quantified by RT-qPCR as previously described (Bes et al., 2022), using HEV-specific primers and FAM/MGB probe targeting the ORF3 region (Slot et al., 2013). To increase sample size, six positive samples from a previous study (Wang et al., 2019) were amplified using the methodology described above.

2.4. Phylogenetic analysis

The phylogenetic analysis included the wild boar HEV sequences obtained between October 2016 and January 2021, as well as the six HEV isolates obtained in a previous study (Wang et al., 2019). Additionally, we incorporated HEV sequences from domestic pigs in Catalonia, which were registered in GenBank (EU723512-16), and human HEV sequences obtained through routine HEV screening at the Catalan Blood Bank from April 2017 to June 2020 (Bes et al., 2022). The reference sequences for HEV subtypes also were obtained from GenBank (Smith et al., 2016). Two phylogenetic trees were constructed by Neighbour-Joining Method (NJ), using the MEGA software version 11. The first phylogenetic tree was based on the alignment of the 633 bp sequences of two of ORF2 (Fig. 2). However, due to the unavailability of such extended sequences for all human HEV isolates, a second phylogenetic tree was constructed using a shorter region of ORF2 (349 bp sequences) (Fig. 3). The HEV sequences obtained from wild boars in the BMA have been deposited in GenBank (OR488603-OR488615).

3. Results

3.1. Incidence of HEV infection among wild boars from the BMA

Seven (2.2 %) wild boar faecal samples were positive by RT-nPCR among 312 samples obtained between October 2016 and January 2021. All the positive samples belonged to five juveniles, one piglet and

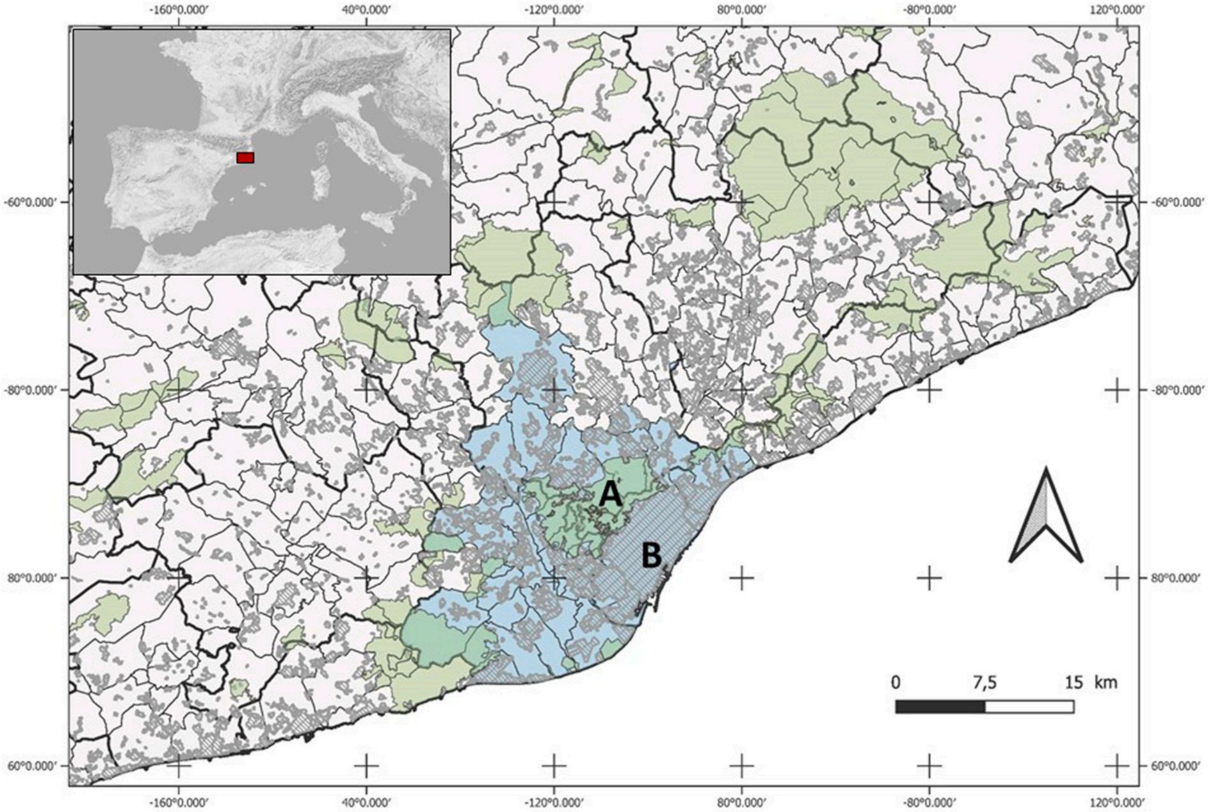


Fig. 1. Urban areas (grey) and natural protected areas (green) in and around the Metropolitan Area of Barcelona (blue), including Collserola Natural Park (A) and the city of Barcelona (B).

Table 1
Year, age and sex distribution of the wild boar samples from the MAB analyzed for hepatitis E virus RNA. The successfully sequenced samples are indicated between parentheses.

Year	Piglet			Juvenile			Yearling			Adult			All ages		
	Female	Male	**Subtotal**	Female	Male	**Subtotal**	Female	Male	**Subtotal**	Female	Male	**Subtotal**	Female	Male	**Total**
2016	0	0	**0**	0	0	**0**	1	3	**4**	8	8	**16**	9	11	**20**
2017	0	1	**1**	0	0	**0**	5	10	**15**	29	17	**46**	34	28	**62**
2018	0	0	**0**	0	0	**0**	1	1	**2**	18	5	**23**	19	6	**25**
2019	4	2	**6**	5 (1)	8 (1)	**13 (2)**	25	10	**35**	38	14	**52**	72 (1)	34 (1)	**106 (2)**
2020	6	7 (1)	**13 (1)**	8 (2)	11 (1)	**19 (3)**	11	14 (1)	**25 (1)**	20	17	**37**	45 (2)	49 (3)	**94 (5)**
2021	0	0	**0**	1	0	**1**	1	2	**3**	1	0	**1**	3	2	**5**
Total	**10**	**10 (1)**	**20 (1)**	**14 (3)**	**19 (2)**	**33 (5)**	**44**	**40 (1)**	**84 (1)**	**114**	**61**	**175**	**182 (3)**	**130 (4)**	**312 (7)**

one yearling captured between January 2019 and December 2020 (Table 1).

The mean of HEV RNA quantification of positive samples by Real Time RT-qPCR was $6.75 \times 10^7 \pm 13.40 \times 10^7$ viral particles for g of faeces (Table 2).

A total of 170 blood donations from throughout Catalonia, tested in the Blood and Tissue Bank of Barcelona between November 2017 and April 2020, were positive for HEV RNA (Bes et al., 2022). The more similar human HEV isolates where added in the phylogenetic analysis.

3.2. Phylogenetic analysis of wild boar HEV isolates

Both phylogenetic analyses similarly identified all the wild boar sequences as HEV GT3 (Figs. 2 and 3). Six wild boar HEV sequences belonged to subtype 3f, six to subtype 3 h/i and one belonged to subtype 3c. The subtype 3f sequences formed two clades: one of them constituted by four wild boar sequences from 2020 (SS20028, SS20029, SS20162, SS20164) and other clade with two sequences from 2015 and 2016 (SS15189, SS16045). Both clades included HEV strains from Catalan blood donors. The six wild boar HEV sequences of the h/i clade showed little variability in the two phylogenetic trees, despite including HEV sequences extending from 2015 to 2020. The wild boar HEV 3c isolate shared significant sequence similarity with HEV 3c strains circulating among wild boars in Germany and human blood donors in the BMA. Although the domestic pig HEV strains from Catalonia belonged to HEV 3f subtype, all of them were far from the wild boar 3f sequences (Figs. 2 and 3).

4. Discussion

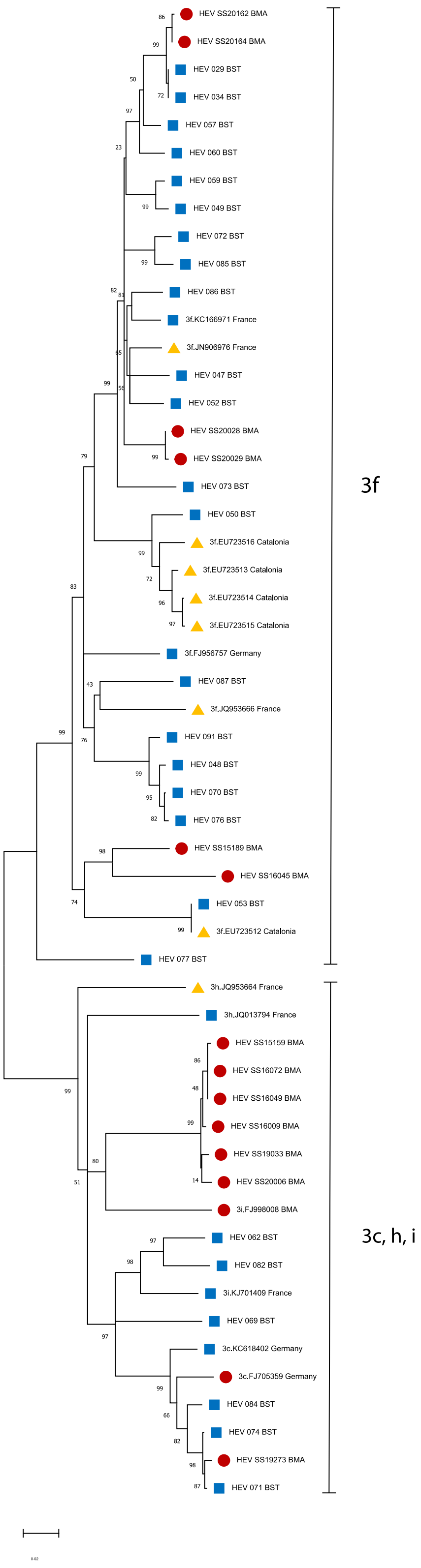
The environmental changes and human activity have triggered an increase and expansion of wild boar populations in several European countries (Schielke et al., 2009; de Deus et al., 2008; Adlhoch et al., 2009; Martinelli et al., 2015). The urban sprawl of the last decades and the changes in the forest landscape has favored of wild boar expansion into urban areas due to access to anthropogenic food resources. This expansion increases the interactions between citizens and infected animals.

Zoonotic HEV transmission from wild boars to humans can occur either directly, through meat consumption (Rivero-Juarez et al., 2017; Pavio et al., 2017, 2016; Tamada et al., 2004), or indirectly, through environments contaminated with faeces of HEV-infected wild boars or mediated by other animals (González-Crespo et al., 2023b; Meng and Lindsay, 2009; Pavio et al., 2017; Kuno et al., 2003). Evidence of the susceptibility of domestic dogs and cats to HEV infection reinforces the potential role of pets in the zoonotic transmission of HEV (Li et al., 2020).

Such direct and indirect transmission routes of HEV account for a higher occupational risk for butchers, slaughterhouse workers, veterinarians, farmers, hunters, and forestry workers (Pavio et al., 2017; Mrzljak et al., 2021). The increased occupation of urban spaces by wild boars in search of food resources, such as rummage garbage and direct feeding by humans, can intensify the expansion of the virus. Moreover, HEV circulation in wild boar populations, along with increased interactions between citizens and infected animals, may have contributed to the emergence of this zoonotic infection in urban areas. This closer contact not only increase the potential risk of HEV transmission to occupational workers but also to global human population (González-Crespo et al., 2023a, 2023b; Conejero et al., 2019; Stillfried et al., 2017). However, further studies are needed to ascertain the interspecific HEV transmission routes from wild boars to citizens and vice versa in the BMA. Specifically, the presence of HEV both in the meat of wild boar hunted in the BMA and aimed for human consumption, on the one hand, and in the water and soil of public recreational areas, on the other hand, should be assessed as potential foodborne and environmental sources of infection from wild boars to humans, respectively (Rivero-Juarez et al., 2017; Baez et al., 2017; Parashar et al., 2011; Takuissu et al., 2022; Van Der Poel, 2014). Similarly, potential HEV sources for wild boars through rubbish and wastewater should also be assessed to gain a comprehensive insight of the epidemiological scenario in urban and peri-urban environments involving humans and wild boars, such as the BMA. In these contexts, pets such as dogs and cats could also bridge HEV transmission between wild boars and humans (Li et al., 2020; Caballero-Gómez et al., 2022; Lyoo et al., 2019; Tsachev et al., 2023; Veronesi et al., 2021) and should also deserve further investigation.

The phylogenetic analysis allowed assessing the relationship between human and wild boar HEV isolates, showing a high degree of similarity between the strains identified in both species. This high similarity suggests that wild boars might be a source of human HEV infections in the metropolitan area. The 3f HEV subtype is the most common in Europe (Himmelsbach et al., 2018) and in our study had a prevalence of 46 % among infected wild boars. This subtype is also the most prevalent among Catalan blood donors (Bes et al., 2022). The other HEV subtypes detected (3 h/i and 3c) have also been identified in both blood donors and wild boars from the BMA. Some blood donor HEV strains were more similar to wild boar HEV isolates than to domestic pig HEV strains (Figs. 2 and 3). In addition, wild boar and domestic pig HEV isolates formed different clades (Figs. 2, 3), indicating a variety of transmission routes of HEV infection. However, further studies are needed to safely verify the HEV transmission from wild boars to BMA citizens.

Seropositivity against HEV has been reported to increase with age in wild boar populations (Wu et al., 2022), which could explain the absence of HEV RNA positivity in adult wild boars (Table 1). The maintenance of endemic infections in wildlife populations by naïve young age classes has been repeatedly reported, with adults having healed from the disease (Giacometti et al., 2002; Naranjo et al., 2008; López-Olvera et al., 2024). Thus, the lack of detection of HEV RNA in adult wild boars suggests that HEV is endemic in the wild boar population from the BMA and that young animals are exposed to HEV infection in early life, whereas adult animals are protected from reinfection. Consequently, the increase in susceptible individuals due to the birth season would lead to a seasonal pattern in HEV infection in wild boar populations, as previously described (Rivero-Juarez et al.,



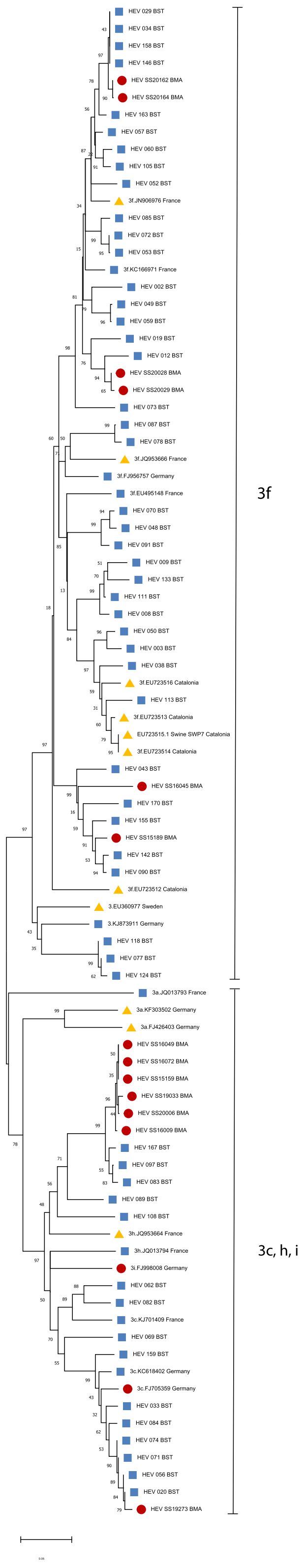


Fig. 2. Phylogeny inferred by neighbour-joining method (NJ) obtained by an alignment of 349 nucleotides, covering part of the ORF2 gene of HEV. The phylogenetic tree includes HEV strain isolates from wild boars (marked by red circles), along with domestic pigs (marked by orange triangles) and human HEV isolates (marked by blue squares). Bootstraps shown at the internal nodes represent the probability of branch formation.

Fig. 3. Phylogeny inferred by neighbour-joining method (NJ) obtained by an alignment of 633 nucleotides, covering part of the ORF2 gene of HEV. The phylogenetic tree includes HEV strain isolates from wild boars (marked by red circles), along with domestic pigs (marked by orange triangles) and human HEV isolates (marked by blue squares). Bootstraps shown at the internal nodes represent the probability of branch formation.

Table 2
Demographic characteristics and faecal viral load of HEV-infected wild boars.

Identification	Sex	Age	Year	HEV IU/g faeces[a] (mean ± SD)
SS15159	Female	Juvenile	2015	$8.78 \times 10^{6} \pm 0.25 \times 10^{6}$
SS15189	Female	Piglet	2015	$1.80 \times 10^{7} \pm 0.05 \times 10^{7}$
SS16009	Female	Juvenile	2016	$5.49 \times 10^{5} \pm 0.96 \times 10^{5}$
SS16045	Female	Juvenile	2016	$3.86 \times 10^{7} \pm 0.17 \times 10^{7}$
SS16049	Female	Juvenile	2016	$9.46 \times 10^{7} \pm 0.34 \times 10^{7}$
SS16072	Male	Juvenile	2016	$1.21 \times 10^{7} \pm 0.09 \times 10^{7}$
SS19033	Male	Juvenile	2019	$3.94 \times 10^{5} \pm 0.02 \times 10^{5}$
SS19273	Female	Juvenile	2019	$4.16 \times 10^{5} \pm 0.28 \times 10^{5}$
SS20006	Male	Piglet	2020	$7.72 \times 10^{5} \pm 0.47 \times 10^{5}$
SS20028	Female	Juvenile	2020	$3.34 \times 10^{5} \pm 0.12 \times 10^{5}$
SS20029	Male	Juvenile	2020	$3.86 \times 10^{5} \pm 0.10 \times 10^{5}$
SS20162	Female	Juvenile	2020	$1.09 \times 10^{5} \pm 0.10 \times 10^{5}$
SS20164	Male	Yearling	2020	$4.90 \times 10^{5} \pm 0.78 \times 10^{5}$

[a] The faecal viral load is provided as HEV International Units (IU) per gram of faeces, as quantified through a calibration curve based on the first World Health Organization International Standard for HEV RNA (Paul-Ehrlich-Institut [PEI] code 6329/10).

2019). Beyond this seasonal pattern, the variability observed in HEV infection among wild boars suggests the presence of interannual cycles. The concentration of viral particles in the faecal samples varied among individuals (Table 2), but it was relatively higher than the viral loads reported by Di Pasquale et al. (2019).

To summarize, our results demonstrate, for the first time, a close molecular similarity between HEV strains from wild boars and humans from the same urban area during the same time period. The results also show the need to implement integrated urban and peri-urban wildlife population and health surveillance and monitoring programs as previously described and recommended (Cardoso et al., 2021; Barroso et al., 2023). Since wild boar synurbization is a global, increasing, and expanding phenomenon, the findings of this study should be useful for developing and establishing HEV monitoring, surveillance, and eventually control programs in both BMA and other urban areas throughout the world.

CRediT authorship contribution statement

Laia Ruiz-Ponsell: Writing – original draft, Investigation, Formal analysis. **Abir Monastiri:** Writing – review & editing, Investigation. **Marc López-Roig:** Writing – review & editing, Investigation. **Sílvia Sauleda:** Writing – review & editing, Investigation. **Marta Bes:** Writing – review & editing, Investigation. **Gregorio Mentaberre:** Writing – review & editing, Investigation. **María Escobar-González:** Writing – review & editing, Investigation. **Maria I. Costafreda:** Writing – original draft, Validation, Supervision, Investigation, Formal analysis, Data curation, Conceptualization. **Jorge R. López-Olvera:** Writing – review & editing, Resources, Investigation, Funding acquisition, Conceptualization. **Jordi Serra-Cobo:** Writing – original draft, Investigation, Funding acquisition, Formal analysis, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Data availability

Data will be made available on request.

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