Low prevalence of hepatitis E virus in the liver of Corsican pigs slaughtered after 12 months despite high antibody seroprevalence

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Low prevalence of hepatitis E virus in the liver of Corsican pigs slaughtered after 12 months despite high antibody seroprevalence

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Abstract

Hepatitis E virus (HEV) infection can be acute and benign or evolve to chronic hepatitis with rapid progression toward cirrhosis or liver failure in humans. Hence, hepatitis E (HE) disease is a major public health concern. In countries where pig populations are highly contaminated with HEV, human cases of HE are mainly foodborne, occurring frequently after consumption of raw or undercooked pork products or liver. Among factors associated to the presence of HEV in pork livers from intensive rearing systems, early slaughter (≤6 months) seems to be major. In Corsica, local pigs are raised in extensive farming systems and slaughtered after 12 months. To evaluate if slaughter of pigs over 12 months reduces the risk of HEV presence in livers, 1197 liver samples were randomly collected in 2 Corsican slaughterhouses. Presence of HEV RNA was detected in liver and HEV seroprevalence was determined in paired serum. The sampling included 1083 livers from animals between 12 and 48 months and 114 livers from animals <12 months. The samples were predominantly from semi-extensive and extensive farms ($n = 1154$). Estimated HEV seroprevalence was high, that is, >88%, and HEV RNA prevalence in adult pig livers (>12 months old) was low, that is, 0.18%. However, in livers from younger animals (<12 months), including piglets below 6 months old, 5.3% (6/114) of the samples were positive for HEV RNA. Sequences recovered from positive livers belonged to HEV genotype 3c and 3f. The presence of infectious HEV was confirmed in two livers by the detection of HEV replication in HepaRG cell cultures. Thus, this study demonstrates the low prevalence of HEV in livers of pigs over 12 months, even in farms with high HEV circulation. This observation may open new perspectives on the preferential use of livers from animals older than 12 months in raw pork liver products.

KEYWORDS
Corsica, hepatitis E virus, liver contamination, pig extensive breeding

1 | INTRODUCTION

ptHepatitis E virus (HEV) is a single-stranded positive sense RNA virus that belongs to the *Hepeviridae* family. Four main genotypes, belonging to the *Orthohepevirus A* species, are able to infect humans. Genotypes 1 (HEV-1) and 2 (HEV-2) are restricted to humans whereas genotypes 3 (HEV-3) and 4 (HEV-4) are zoonotic (Kamar et al., 2017). In endemic regions, with poor sanitation, HEV-1 and HEV-2 are transmitted between humans via the faecal-oral route by contaminated drinking water. In contrast, zoonotic HEV-3 is present worldwide and HEV-4 mainly in China and Southeast Asia. They are both maintained by animal species such as domestic pigs, wild boar, deer and rabbits (Pavio et al., 2017). Transmission to humans can occur after consumption of raw or undercooked meat products derived from the liver of infected animals. Other routes of transmission of the virus include the consumption of food contaminated with HEV via the environment (seafood, soft fruits, vegetable and water) or by direct contact with infected animals, mostly during hunting or farming practices (Doceul et al., 2016). In any case, foodborne transmission is considered to be the primary mode of HEV contamination (Pavio et al., 2021; Van Cauteren et al., 2017).

HEV is hyperendemic in human in several areas of France including Corsica (Mansuy et al., 2016). In this Mediterranean island, studies have reported seroprevalences of anti-HEV antibodies of 52% and 56% in the human population (Capai, Masse, et al., 2019; Capai et al., 2020). Risk factors such as the consumption of traditional Corsican liver sausages called Ficatelli and the practices of skinning and butchering have also been identified (Capai, Masse, et al., 2019). Interestingly, another study reported that a high percentage of Ficatelli (30%) contained HEV RNA (Pavio et al., 2014). The geographical origin of the livers used in these Ficatelli was not indicated; thus, a local origin remains hypothetical.

ptSwine are recognized as the main reservoir of zoonotic HEV. Anti-HEV antibodies and HEV RNA have been detected in domestic pigs worldwide (Pavio et al., 2017; Salines et al., 2017; Treagus et al., 2021). High seroprevalences ranging from 65% to 100% at the farm-level and from 20% to 93% at the individual level have been reported in different industrialized countries (Salines et al., 2017; Sooryanarain et al., 2020; Treagus et al., 2021). In France, an individual HEV seroprevalence of 31% and 60% has been found and 3%–4% of pork livers collected at slaughterhouses were found positive for HEV RNA (Feurer et al., 2018; Rose et al., 2011). In the literature, up to 43% of pig livers and up to 71% of sausages containing liver were shown to contain HEV RNA worldwide (Boxman et al., 2019; Harrison et al., 2021; Pavio et al., 2017; Treagus et al., 2021). HEV has also been detected in other pork products such as raw meat sausages, not supposed to contain pork liver (Boxman et al., 2020; Di Bartolo et al., 2012; Moor et al., 2018; Szabo et al., 2015). Pork livers or liver sausages, positive for HEV RNA, can contain HEV infectious particles as shown using experimental infection in pigs or cell cultures (Berto, Grierson, et al., 2013; Berto, Van der Poel, et al., 2013; Bouwknegt et al., 2007; Feagins et al., 2007, 2008; Takahashi et al., 2012).

In intensive farming system, pigs are usually slaughtered at 6–7 months of age, and the different factors associated with increased risk of finding HEV-positive livers are early slaughter, genetic background of pigs, lack of hygienic measures, and the origin of drinking water (Salines et al., 2017; Walachowski et al., 2014). Thus, age of pig when slaughtered is crucial regarding HEV liver contamination. Furthermore, one recent study has shown a lower HEV prevalence of 2.2% in heavy pigs (mean age 9 months old) than in lightweight pigs, 11.5% (mean age 4.5 months old) (Chelli et al., 2021). However, up to now, no study has addressed the presence of HEV in livers of pigs older than 12 months.

On the other hand, higher levels of HEV infection and antibody prevalence have been found in organic, extensive or open breeding farms in comparison to conventional, intensive or closed systems (Jori et al., 2016; Lopez-Lopez et al., 2018; Rutjes et al., 2014). Hence, the type of pig breeding systems has an impact on within-farm HEV infection pressure.

In Corsica, pigs are generally raised in semi-extensive or extensive farming systems and are slaughtered late (>12 months), in comparison to intensive systems (Relun et al., 2015). The detected seroprevalence of HEV in Corsican pigs ranges between 85% and 88% (Charrier et al., 2018; Jori et al., 2016) and HEV RNA was detected in 9.2% (85/919) faecal sample collected in 16 different farms across Corsica (Capai, Maestrini, et al., 2019). The detection rate of HEV in faecal samples seems to decrease dramatically after 6 months of age (Capai, Maestrini, et al., 2019). Because faecal shedding of HEV is related to productive liver infection, the decline in HEV detection in faces shall coincide with a low level of HEV prevalence in livers. Thus, with slaughter after 12 months it is likely that the risk of HEV presence in livers at the time of slaughter is very low. To evaluate the possible impact of the traditional Corsican farming system on the risk of HEV foodborne transmission from pig livers or food products containing pig liver, the prevalence of HEV RNA was determined in pig livers randomly collected at slaughterhouses. Positive samples were sequenced for subtyping and the presence of HEV infectious particles was tested using the HepaRG cell culture system.

2 | MATERIAL AND METHODS

2.1 | Sample and data collection

Animals' samples (liver and serum) were collected randomly on a given day of visit at slaughterhouse, during the pig slaughter season (from November year *n* to March year *n* + 1). Sample collection was repeated during four consecutive seasons (from 2017 to 2021). Samples were collected in two of the four official slaughterhouses of Corsica, one in 'Haute Corse' and the other in 'Corse du Sud'. For each animal sampled, data on age, farm location and type of farming system were recorded.

Corsica island can be divided into 14 different areas (micro-regions) according to 'Atlas agricole corse' edition 2015 (AGRESTE, 2015) that aggregate geographical areas according to natural boundaries of municipalities. The samples collected originated from 12 of the 14 micro-regions (Figure 1).

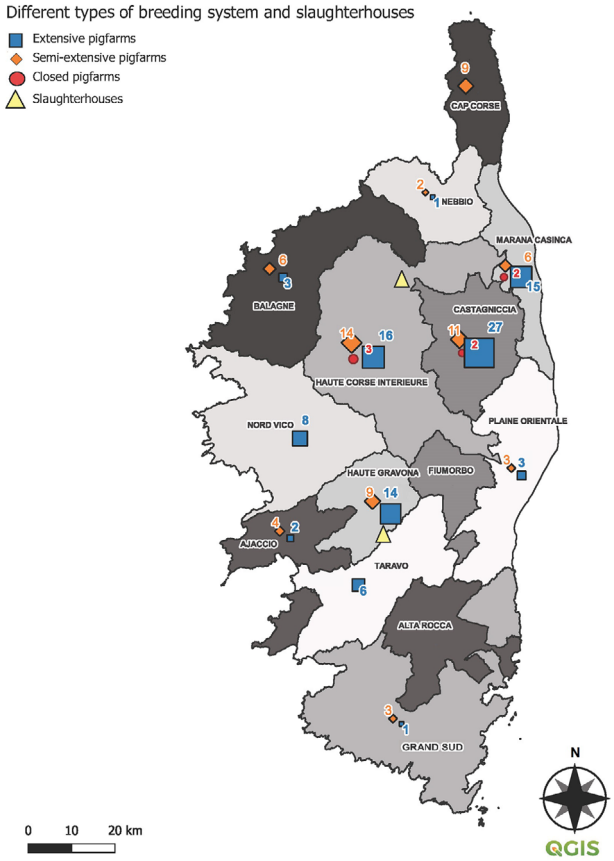

FIGURE 1 **Cartography of the different types of pig farms by micro-region and slaughterhouse location**. This cartography shows the different types of pig farms and their numbers by micro-regions. The blue squares, the orange diamonds and the red circles represent the extensive, semi-extensive and closed breeding system respectively. The yellow triangles correspond to the two slaughterhouses collected during the study. The numbers present on the figure correspond to the numbers of farms sampled by micro-regions. Cartography realized with the software QGIS

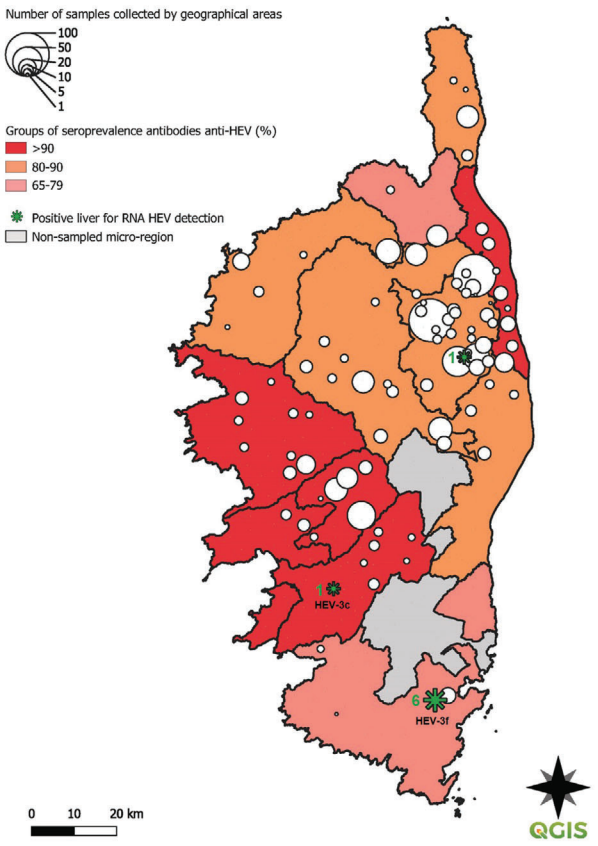

FIGURE 2 **Cartography of the seroprevalences by micro-region, samples collected and localization of positive liver for RNA HEV detection**. This map presents the different estimated seroprevalences according to a colour code from pink to red for the three following seroprevalence classes: 65%–79%, 80%–89% and greater than 90%. The grey areas correspond to the micro-regions not sampled. The white circles correspond to the number of samples collected by area and the size is proportional to this number. The green stars correspond to location where HEV RNAs were detected in livers. HEV subtypes (HEV-3c and HEV-3f) identified in positive livers are indicated. Cartography realized with the software QGIS

2.2 | Type of breeding system

Samples from the three types of breeding systems present in Corsica were included: 96 from free-range Extensive Farms (E-farms), 67 from free-range Semi-Extensive Farms (SE-farms) and 7 from indoor Closed Farms (C-farms) (Figure 1). The two free-range breeding systems, E- and SE-farms, are predominant in Corsica (Relun et al., 2015). In SE-farms, after weaning (around eight-week-old), piglets are placed in an area with older pigs. All animals are growing in large outdoor fenced parcels and share feeding and watering points. In the E-farms, post-weaning piglets feed outside the farm, in large natural areas with pastures and under oak and chestnut trees. In both systems, pigs live at least until their 12 months outdoor before being slaughtered. This specificity leads to a seasonality of the breeding and, consequently, of the slaughter periods (Relun et al., 2015). These types of farming are described in details in a previous study (Capai, Maestrini, et al., 2019). In C-farms, pigs follow a classical closed intensive breeding system and are slaughtered usually around 6–8 months and no later than 18 months. Some lightweight pigs (2- to 3-month-old piglets), intended for the production of fresh meat (Spit-Roasted Piglet), can also enter the food chain from E- and SE-farms.

2.3 | Serological analysis

The detection of anti-HEV antibodies was performed using the HEV ELISA 4.0v kit (MP Diagnostics, Illkirch, France) as described previously (Salines et al., 2015). This sandwich ELISA allows the detection of antibodies against HEV in serum samples. All IgG, IgM and IgA from various species are detected using recombinant antigen based on the ORF2.1 fragment, which is conserved between different HEV-1 to HEV-4 strains. The presence or absence of antibodies specific for HEV is determined by relating the mean optical density at 450 nm to the threshold that was defined as the mean for negative controls +0.3.

2.4 | Nucleic acid isolation

Fifty milligrams of liver tissues were homogenized in 800 μl PBS, using the Fast Prep 24 System (MP Biomedicals, Illkirch, France) in Lysis Matrix D tubes (MP Biomedicals, Illkirch, France). Three cycles of 45 s were performed with an incubation of 5 min, on ice, between each cycle.

RNA extraction from liver was performed, using magnetic bead-based separation technology, with the KingFisher™ Duo (Thermo Fisher Scientific, Courtaboeuf, France). Totals RNAs from sample lysate (200 μl) were extracted using the MagMAX core nucleic acid purification kit (Thermo Fisher Scientific, Courtaboeuf, France) and plastic consumables (96 deep well plate, 12-tip comb and 12-elution strip), according to the manufacturer's instructions and the KingFisher instrument guide. Total RNAs were eluted in 90 μl of RNAse free water.

2.5 | HEV RNA detection and sequencing

HEV RNA detection in liver samples was performed using a real-time quantitative RT-PCR as previously adapted from a method described by Jothikumar et al. (2006).

TaqMan RT-qPCR reactions were performed with the QuantiTec Probe RT-PCR kit (Qiagen, Hilden, Germany), according to the manufacturer's instruction. Amplification reactions were made with 2 μl of total RNAs, 0.25 mM reverse primer (5′-AGGGGTTGGTTGGATGAA-3′), 0.1 mM forward primer (5′-GGTGGTTTCTGGGGTGAC-3′) and 5 mM probe (FAM-TGATTCTCAGCCCTTCGC-MGB) as previously described (Garson et al., 2012). A LightCycler 480 apparatus (Roche, Basel, Switzerland) was used with the following cycles: 20 min at 50°C (reverse transcription), 15 min at 95°C (RT denaturation), 45 cycles (denaturation at 95°C for 10 s and annealing and elongation at 50°C for 45 s).

Standard quantification curve was obtained using in vitro transcribed RNAs from the plasmid pCDNA 3.1 ORF2-3 HEV (Barnaud et al., 2012). The crossing points were calculated and the number of HEV RNA copies per sample, was estimated with the standard curve. The limit of detection (LOD) of the applied system is five copies of HEV RNA in 2 μl of total RNAs extract, which corresponds to 22.5 HEV RNA copies per milligram of liver.

RNA extracts from HEV-positive samples were used for genotyping. Amplification by RT-nested PCR assay targeting the previously selected ORF2 region (nucleotides 5996 to 6343) was used (Bouquet et al., 2011; Cooper et al., 2005). After two consecutive PCR, expected final product was 348 bp and was sequenced by Sanger by Eurofins, Ebersberg, Germany.

2.6 | Sequence analysis and phylogeny

Sequences were obtained from four positive samples out of eight by amplifying a small fragment of HEV ORF2 (nucleotides 6070 to 6357 of the reference sequence HEV-3 EU360977). This genomic region, although short (approximately 290 nucleotides), is frequently used in phylogeny studies (Baylis et al., 2021) since it reflects the diversity of full-length HEV genomes. All sequences obtained (GenBank accession numbers OL504991 through OL504994) are of genotype 3. Sequences were aligned using Muscle (MEGA X) with reference sequences for genotype 3 subtyping published by Smith et al. (2020). Screening for high sequence identity was performed using the GenBank basic local alignment search tool (BLAST) to identify the 5 best hits. The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model. The tree with the highest log likelihood (−5,238,446) is shown in Figure 3. The percentage of trees in which the associated taxa clustered together is shown next to the branches (1000 replicates). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbour-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted in MEGA X.

2.7 | HepaRG infections with liver samples

HepaRG cell culture: Human HepaRG™ cells were grown as previously described (Pellerin et al., 2021). Briefly, HepaRG were seeded into 6-well plates (2×10^5 cells/well) and maintained in growth medium for 2 weeks. Medium with 1.2% DMSO was replaced for two extra weeks for cell differentiation into hepatocytes prior to infection. Then the medium was renewed every 2–3 days.

Inoculum preparation: livers found positive for HEV RNA by RT-qPCR were homogenized using a blender with two volumes of PBS (20 g/40 ml). Samples were first centrifuged at 4000 g for 20 min. Then supernatants were centrifuged a second time at 10,000 g during 3 min. To prepare viral suspension for cell infection, supernatants were 10-fold diluted in cell medium and passed through a 0.45 and then a 0.22 μm filters.

Cell infection: Liver suspensions were diluted in growth medium to a final volume of 1 ml (1/2 dilution) and cells were infected with 1 e+06 copies of HEV RNA/ml. After 24 h, HEV inoculum was removed and cells were washed three times in PBS before adding 2 ml of growth medium. Every 2–3 days, one-half of the culture medium was replaced with fresh growth medium. Infections were maintained up to 121 days. Cells were not passaged during the duration of the infection. Supernatants from infected cells were regularly collected for HEV RNA detection by RT-qPCR. Since the viral titres were stable from day 20 until day 121, only the first 56 days are shown in Figure 4.

2.8 | Statistical analyses and cartography

The seroprevalences of anti-HEV antibodies in pig sera samples were calculated by age groups, type of breeding system, year of collection and geographic areas. For each seroprevalence, the two-sided 95% CI

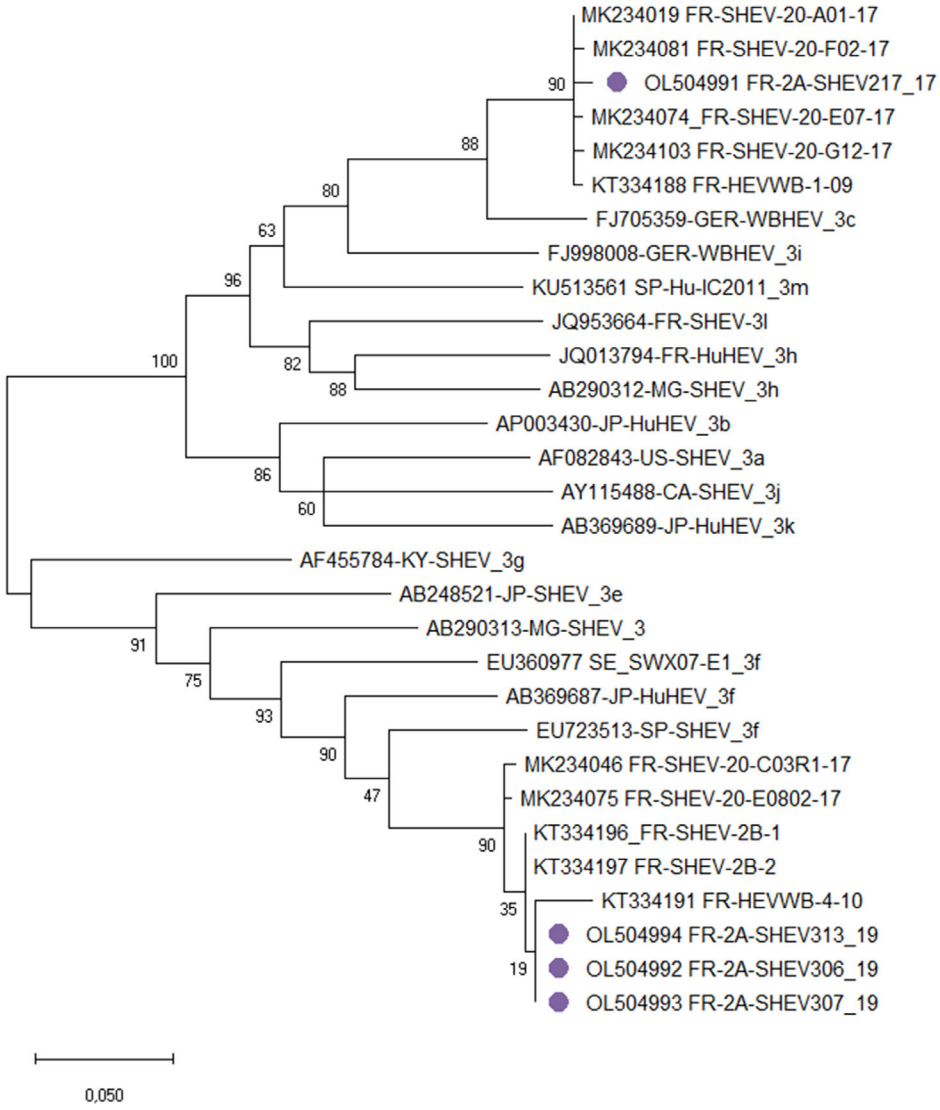

FIGURE 3 **Phylogenetic tree of partial HEV ORF2 sequences**. Phylogenetic tree with amplified HEV sequences in positive livers (purple dots). Partial ORF2 sequences were aligned using Muscle (MEGA X) with the reference sequences, for genotype 3 subtyping, published by Smith et al 2020 (Smith et al., 2020). The 5 closest sequences were searched with the GenBank basic local alignment tool (BLAST). The tree with the highest log likelihood (−52384.46) is shown. The percentage of trees in which the associated taxa clustered together is shown next to the branches (1000 replicates). The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. Evolutionary analyses were conducted with MEGA X

was calculated. Categorical variables were expressed as the number of cases (percentages). Frequencies were compared using the χ^2 test or Fisher's exact test ($p < .05$). Odds ratios (ORs), including their 95% CIs, were calculated for the logistic regression models. The multivariate logistic regression analysis included variables that were related to outcome variables in the univariate analysis with a p value < .2.

The prevalence of HEV RNA in pig liver samples was calculated for the overall samples and among adults (pigs older than 12 months). All statistical analyses were performed using the R program (http://www.r-proje ct.org).

The two maps (Figures 1 and 2) were built with the software Qgis (https://www.qgis.org/fr/site/). The first map depicts the geographical origin of the samples within the 14 different areas (micro-regions) of Corsica, with the number and type of breeding systems collected and the location of the 2 slaughterhouses. The second map shows the seroprevalences estimated with the number of samples included per location and the localization of HEV RNA positive livers with their HEV subtypes.

3 | RESULTS

3.1 | Sample characteristics

Overall, 1197 pig livers and 1238 serum samples (including paired sera with livers) were collected. The age distribution of the pig samples collected during the four successive slaughter seasons is described in Table 1. Twenty-one liver and serum samples from piglets (<6 months;

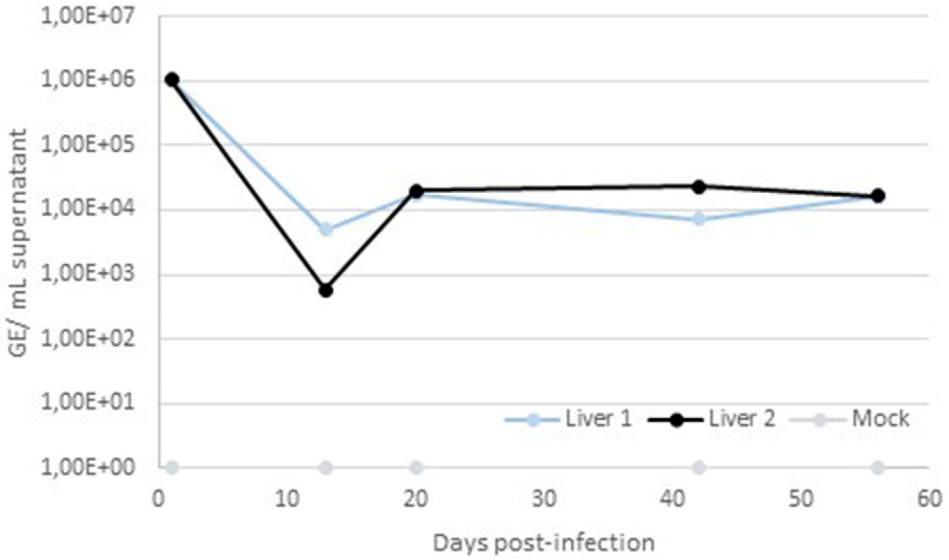

FIGURE 4 **Infection of HepaRG cells with two different HEV positive livers from piglets.** Two HEV RNA positive livers were homogenized to infect HeparRG cell cultures. Viral production was monitored in the supernatants by RT-qPCR. Liver 1 corresponds to the sequence OL504992 with anti-HEV antibodies present in the matched serum. Liver 2 corresponds to sequence OL504994 with anti-HEV antibodies absent in the matched serum. Half of the culture medium was renewed 3 times a week

1.7%), 93 livers and 94 sera from 6- 12-old-month pigs (7.6%), 891 livers and 928 sera from 12- to 24-month-old pigs (75.0%) and 192 livers and 195 sera for pigs older than 24 months (15.7%) were analysed (Table 1, Supplementary Table S1). The breeding systems were in majority E-frams (n = 96) and SE-farms (n = 67), with few C-farms (n = 7) (Figure 1). Samples originated from 12 of the 14 official geographic micro-regions of Corsica (Figure 1).

3.2 | Seroprevalence of anti-HEV antibodies

The overall HEV seroprevalence, among the 1238 pigs sampled, was 88.53% [86.62–90.25] (1096/1238) (Table 2).

Seroprevalence values grouped according to the different variables (age groups, type of breeding systems and geographic areas) are presented in Table 2. The seroprevalences by age groups ranged significantly between 47.6% among 1- to 6-month-old pigs and 90.3% among the older pigs (24 months) (p = 2e-05); ORs increased with age (5.79 [2.10–16.42] for 6–12 months group to 10.19 [3.84–27.68] for animals >24 months). The type of breeding systems is significantly associated with the detection of anti-HEV antibodies (p = 1e-05). In C-Farms, the estimated seroprevalence was 81.8% and in E-Farms 92. 7%. The results of HEV seroprevalence emanated from almost all Corsican micro-regions (12/14) (Figure 2). Sample sizes per area varied between 22 and 335 and HEV seroprevalence ranged between 66.7% (NEBBIO, North-Est) and 94.12% (TARAVO South-Centre-West) (p = 7e-04) (Table 2, Figure 2). The multivariate logistic regression model (Table 2) showed that increasing age and the type of breeding systems (E-farms) are factors associated with the presence of anti-HEV antibodies in pig sera (p = 8e-04 and 1 e-04, respectively).

3.3 | Prevalence of RNA HEV in pig livers

The overall prevalence of HEV RNA in pig livers was 0.67% (8/1197) and 0.17% (2/1176) among adult pigs (>6 months) or 0.18% (2/1083) among old pigs (>12 months) (Table 3). In contrast, in young animals, between 1 and 6 months, HEV prevalence was very high 28.57% (6/21). No positive sample was detected in the 93 samples from the 6- to 12-month-old animals category nor in the 192 samples from older animals (>24 months). Two samples were positive among 891 (0.22%) in the 12–24 months category (Table 3). In the different types of breeding systems, C-farms, SE-farms and E-farms, prevalence of HEV positive livers were of none, 1.2% and 0.31%, respectively.

The majority of positive samples (75%; n = 6/8) were from 2- to 3-month-old piglets. These six piglets were from the same pig SE-farm. The two other positive livers were from older pigs (12 months and 15 months) coming from two different E-farms (Table 3). HEV RNA copy numbers per gram of liver ranged from 1.78 e+04 to 4.67 e+08, with the highest quantities detected in livers from piglets (Table 3). HEV RNA was detected in the paired serum samples from all 2- to 3-month-old piglets, with levels ranging from 2.01 e+04 to 1.01 e+06, but not in older animals (Table 3). The two adult pigs and half of the piglets (3/6) had anti-HEV antibodies (Table 3).

3.4 | HEV sequences analysis and phylogeny

From the eight HEV-positive liver samples, four partial ORF2 genetic sequences were obtained. One sequence was isolated from one pig over 12 months in 2017 (Genbank accession number OL504991) and the 3 others from the 2- to 3-month-young piglets, from the same

herd, sampled in 2019 (GenBank accession number OL504992 to OL504994). All sequences belong to HEV genotype 3 (HEV-3). Using reference sequences for HEV-3 subtyping, one sequence is assigned to HEV-3c (OL504991) and the 3 others (OL504992 to OL504994) to HEV-3f (Figure 3). These last 3 have more that 99% identity in nucleotides between each other and belong to animals living in the same farm. For each sequence, the 5 best hits were searched using the BLAST tool in GenBank. All sequences clustered with Corsican sequences previously published (Figure 3). The sequence OL504991 (HEV-3c) has more than 98% identity in nucleotides with other swine HEV sequences from Corsica (MK234019, MK234081, MK234074, MK234003) isolated in 2017 and one wild boar sequence from Corsica (KT334188) isolated in 2009 (Figure 3). The sequences OL504992 to OL504994 (HEV-3f) have near 99% identity in nucleotides with other swine HEV sequences from Corsica isolated in 2009 (KT334196 and KT334197) or in 2017 (MK234046 and MK234075) and with a wild boar sequence isolated in 2010 (KT334191) (Figure 3).

3.5 | Infection of HepaRG cells with HEV positive liver

To determine if the presence of HEV RNA in liver samples corresponds to infectious virus, we tested the ability of two positive HEV livers from piglets (GenBank accession number OL504992 and OL504994) to induce an HEV replication in the human hepatic cell line HepaRG. These two liver samples were chosen for their high HEV RNA load (>4 e+08 HEV RNA copies/g) and were issued from piglets with (OL504994) or without (OL504992) anti-HEV antibodies. After sample preparation, HepaRG were infected and the presence of viral RNA was monitored in the supernatant by RT-qPCR for up to 56 days (Figure 4). From day 20 post-infection, an increase in viral RNA was detected in supernatant of both cultures and maintained for up to 56 days (Figure 4). Therefore, HEV RNA positive livers collected from piglets contain infectious HEV.

4 | DISCUSSION

Pigs are considered the major reservoir of zoonotic HEV worldwide and consumption of pork product is strongly associated to hepatitis E in humans (Pavio et al., 2021). One control method to reduce the burden of HEV in pork products is to decrease the number of infected animals entering the food chain. A major factor influencing the presence of HEV in pig livers is the age of pig at slaughter (Salines et al., 2017).

In Corsica, pigs are raised in extensive (E-) and semi-extensive (SE-) farms and are slaughtered after 12 months. Pig slaughtering is seasonal, occurring between the end of November and mid-March. Every season, around 12,808 pigs are slaughtered, from 550 farms with a majority of SE-farms (Direction régionale de l'alimentation de l'agriculture et de la forêt de Corse, 2021; Relun et al., 2015). In the present study, pig livers and serum samples were collected during four successive seasons, covering two of the four slaughterhouses of the island. Pigs randomly selected were mostly (90%) older than 12

TABLE 1 Age distribution of pigs included for liver and serum during the four seasons of collect

Age groups	1–6 months				6–12 months				12–24 months				>24 months				Overall (N)	
	Liver		Serum		Liver		Serum		Liver		Serum		Liver		Serum			
Year of collect	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	*n*	%	Liver	Serum
2017–2018	7	2.6	7	2.6	14	5.1	14	5.1	242	88.3	253	92.4	0	0.0	0	0.0	**263**	**274**
2018–2019	12	4	12	4	36	12.3	37	12.4	181	62	187	62.5	62	21.2	63	21.1	**291**	**299**
2019–2020	2	0.4	2	0.4	41	7.9	41	7.9	361	72.5	381	73.6	94	18.2	94	18.2	**498**	**518**
2020–2021	0	0.0	0	0.0	2	1.4	2	1.4	107	72.8	107	72.8	36	24.8	38	25.9	**145**	**147**
Overall	**21**	**1.7**	**21**	**1.7**	**93**	**7.6**	**94**	**7.6**	**891**	**74.4**	**928**	**75.0**	**192**	**16.0**	**195**	**15.7**	**1197**	**1238**

TABLE 2 Anti-HEV seroprevalences by age groups, type of breeding and geographic areas. Univariate and multivariate analysis

		Serology IgG anti-HEV (sera)s			Univariate analysis		Multivariate analysis
Modalities	**Variables**	***n***	***N***	**% [CI 95]**	***p* Value**	**OR [CI 95]**	***p* Value**
Age groups	1–6 months	10	21	47.62 [25.71–70.22]	**2 e-05**	reference	**8 e-04**
	6–12 months	79	94	84.04 [75.05–90.78]		**5.79 [2.10–16.42]**	
	12–24 months	831	928	89.55 [87.39–91.44]		**9.42 [3.88–23.18]**	
	>24 months	176	195	90.26 [85.21–94.03]		**10.19 [3.84–27.68]**	
Type of breeding system	Closed	36	44	81.82 [67.28–91.80]	**1 e-05**	Reference	**1 e-04**
	Semi-extensive	435	520	83.65 [80.19–86.73]		1.14 [0.48–2.42]	
	Extensive	625	674	92.73 [90.50–94.57]		**2.83 [1.17–6.16]**	
Geographic areas	BALAGNE	59	70	84.29 [73.62–91.88]	**7 e-04**	1.05 [0.5–2.33]	Non-significant
	CAP	39	45	86.67 [73.21–94.95]		1.27 [0.52–3.59]	
	CASTAGNICCIA	324	361	89.75 [86.15–92.68]		**1.71 [1.01–2.9]**	
	GRAND AJACCIO	27	29	93.10 [77.23–99.15]		2.64 [0.73–16.97]	
	GRAND SUD	17	22	77.27 [54.63–92.18]		0.67 [0.24–2.16]	
	HAUTE CORSE INTERIEURE	143	171	83.63 [77.21–88.85]		Reference	
	HAUTE GRAVONA	120	128	93.75 [88.06–97.26]		**2.94 [1.35–7.12]**	
	MARANA CASINCA	159	171	92.98 [88.06–96.32]		**2.59 [1.3–5.48]**	
	NEBBIO	22	33	66.67 [48.17–82.04]		**0.39 [0.17–0.92]**	
	NORD VICO	81	87	93.10 [85.59–97.43]		**2.64 [1.12–7.31]**	
	PLAINE ORIENTALE	58	67	86.57 [76.02–93.67]		1.26 [0.58–2.98]	
	TARAVO	32	34	94.12 [80.32–99.28]		3.13 [0.88–20.02]	
	Not determined	15	20	75.00 [50.89–91.34]		/	
Overall	**1096**	**1238**	**88.53 [86.62–90.25]**				

Significance of bold values indicates $p < .05$.

TABLE 3 Description of pigs positive for HEV RNA in the liver

	Number of pigs positive for HEV RNA	Number of samples	Prevalence of RNA HEV % [CI 95]
1–6 months	6	21	28.57 [11.28–52.17]
6–12 months	0	93	0.00 [0.00–0.039]
12–24 months	2	891	0.22 [0.03–0.81]
>24 months	0	192	0.00 [0.00–1.90]
Overall	8	1197	0.67 [0.29–1.31]
Among adult pigs (>6 months)	2	1176	0.17 [0.02–0.61]
Among older pigs (>12 months)	2	1083	0.18 [0.02–0.66]
Closed	0	43	0.00 [0.00–8.22]
Semi-extensive	6	501	1.20 [0.44–2.59]
Extensive	2	653	0.31 [0.03–1.10]

Season of collect	Type of breeding	Area	Age	Anti-HEV antibodies	Viral load serum	Viral load liver	Genbank accession number
2017–2018	Extensive	TARAVO	>12 months	Yes	nd	1.53 e+05	OL504991
2018—2019	Extensive	CASTAGNICCIA	15 months	Yes	nd	4.07 e+05	None
2018—2019	Semi-extensive	GRAND SUD	2–3 months	No	5.93 e+05	2.99 e+05	None
				No	6.80 e+04	2.33 e+08	–
				No	1.01 e+06	4.67 e+08	OL504992*
				Yes	2.01 e+04	1.78 e+04	None
				Yes	2.86 e+05	7.92 e+05	OL504993
				Yes	5.56 e+05	4.75 e+08	OL504994*

nd: not detected; None: no sequence obtained; –: no ORF2 RT nested-PCR performed.
*Liver samples used for cell culture: liver 1: OL504992 and liver 2: OL504994.

months of age (1083/1197), originating from 163 E-or SE-farms and 7 C-farms, located in 12 out of 14 micro-regions of Corsica. Our sampling is representative of the Corsican pig population slaughtered. We had previously shown that HEV circulates strongly in the pig population in Corsica and we confirm here a high HEV seroprevalence of 88.5% (1096/1238) with an age-related increase (47.6% before 6 months of age, 84% after 6 months of age, and 89.5% after 12 months of age). These results are in agreement with our previous observations in 2013, where 88% of Corsican pigs had anti-HEV antibodies (182/206), with similar differences between young and adult animals (42% vs. 93%) (Jori et al., 2016). This result underlines the stable enzootic presence of HEV in pigs in Corsica for several years and provides new data on HEV seroprevalence in pigs older than 12 months of age, raised in extensive free-range farms.

Overall HEV seroprevalence in Corsican pigs is high (>80%) but we observed an impact of the pig breeding systems on HEV seroprevalence. High HEV seroprevalence was associated with E- farming systems (92.7%) in comparison to C (81.8%) and SE systems (83.7%). In other studies, farms typology is not often described, but most of them correspond to intensive type (Salines et al., 2017; Sooryanarain et al., 2020). In two published studies, the impact of the farming systems on HEV seroprevalence was estimated in animals around 6 months old, from organic farms, free-range or conventional farms (Rutjes et al., 2014) or extensive versus intensive farms (Lopez-Lopez et al., 2018). Both studies found higher HEV seroprevalence in open breeding systems as in E-farms from Corsica. It can be hypothesized that in extensive herds, higher frequency of contact between animals of different age groups favours a wider dissemination of HEV. Environmental contamination contributes to HEV exposure within farms (Andraud et al., 2013). Thus, a greater exposure to faecal matter in outdoor environment, with limited cleaning, certainly increases the rate of transmission to a larger number of individuals. HEV is secreted in faeces as a non-enveloped virus and is believed to be resistant for several months (Wolff et al., 2022).

The samples collected originated from 12 different micro-regions in Corsica. These regions are based on an official classification of the Island into 14 regions (AGRESTE, 2015). The univariate analysis, suggested a region effect that was not confirmed by multivariate analysis. Farms density and practices may vary between regions and level of contact between animals from different neighbouring farms could be important and need further investigations.

To date, no study has specifically addressed the prevalence of HEV in pork livers from animals over 12 months. In the present work, 90% of pigs were over 12 months (1083/1197) and the prevalence of HEV RNA in pork livers was 0.18%. This value increased to 0.67% when including animals below 12 months ($n = 114$). This prevalence is very low compared to the 3%–4% reported in continental France (Feurer et al., 2018; Rose et al., 2011) or the 1%–43% found in other studies on pig livers from intensive farms slaughtered around 6 months (Treagus et al., 2021). Thus, late slaughter is likely to reduce the risk of HEV infection of pork livers entering into the food chain. A recent study, conducted at slaughterhouse, shows a decrease of HEV RNA prevalence to 2.2% in heavy pigs (mean age 9 months old) compared to 11.5% in lightweight pigs (mean age 4.5 months old). Type of farm was not indicated (Chelli et al., 2021). Here we show a lower HEV RNA prevalence in animals over 12 months (0.18%). This result is also in agreement with previous data obtained in Corsica. In the study by Capai, Maestrini et al. (2019), faecal samples from young pigs (<6 months old) or adult pigs (>6 months old) were collected in sixteen Corsican pig farms. A low HEV prevalence (1.6% [0.3–4.7]) was found in adult pigs (>6 months old) ($n = 185$), with the positive samples ($n = 3$) belonging to the 6–8 months subgroup ($n = 47$), suggesting a decrease of HEV infection after 8 months. In the study by Jori et al. (2016), only 24 pig liver samples were tested for the presence of HEV RNA. The samples were not divided into age groups, but the two livers found positive for HEV were from animals younger than 6 months of age. Therefore, the livers of animals younger than 6 months of age are more likely to contain HEV.

The low prevalence of HEV observed in Corsican pig livers may appear in contradiction with the high proportion of HEV-positive Ficatelli (30%), previously found in Corsica (Pavio et al., 2014); however, according to the Regional Office of Agriculture (ODARC), the market for cured meats in Corsica, such as Ficatelli, is characterized by a strong demand, higher than the supply (Office du Développement Agricole et Rural de Corse, 2018). As a result, industrial manufacturers have been established in Corsica in the past decades, with an economic model based on the import of raw material (carcasses) from intensive pig farms in continental France or Europe, cheaper than the local Corsican raw material. Hence, pig livers from these origins are more likely to be HEV positive, as pigs are slaughtered at the age of 6 months. Hence, it could explain the high prevalence of HEV RNA (30%) revealed in the study of Pavio et al. (2014), which was primarily based on samples from these types of industrial manufacturers.

Two HEV RNA-positive liver samples come from two pigs older than 12 months carrying anti-HEV antibodies. With the information available, it is not possible to know if the pigs were chronically infected or if they could have been re-infected close to slaughtering. It is known that other porcine viruses, with immunomodulatory effects, such as Porcine Reproductive and Respiratory Syndrome Virus or Porcine circovirus-2, can affect HEV course of infection leading to long-term secretion (Salines, Andraud, et al., 2019; Salines, Dumarest, et al., 2019; Salines et al., 2020). In human, HEV re-infection is suspected (Abravanel et al., 2014), thus it may occur in animals as well with possible decrease of anti-HEV immunity during time. The six other positive livers samples were from 2- to 3-month-old piglets, which is consistent with the observation that HEV acute infection occur in young animal after weaning (Andraud et al., 2013; Capai, Maestrini, et al., 2019; Salines, Dumarest, et al., 2019; Salines et al., 2017).

HEV sequences found in the positive pork livers were of HEV-3c and HEV-3f, which is consistent with previous studies in Corsica in 2013 and 2019 (Capai, Maestrini, et al., 2019; Pavio et al., 2016). The closest sequences are from swine faecal samples collected in 2013 (KT334196-97) and 2017 (MK234075-46) suggesting that in Corsica, HEV strains circulating within herds are quite stable over time. A high level of stability has been also reported in Sweden where a unique HEV sequence was observed within a herd during 2 years (Wang et al., 2019). A similar pattern of stability was also observed in wild boar

sequences isolated in 2009 and 2010 (KT334188, KT334191), suggesting that they are present in Corsica for nearly 10 years (Jori et al., 2016). The sequence similarity between pig and wild boar HEVs is in favour of virus spillover between the two populations (Pavio et al., 2016). According to our previous data in Corsica, the prevalence is generally twofold higher in domestic pig populations than in wild boar populations (Jori et al., 2016). Similarly, we have found that wild boars hunted in areas where there is cohabitation with domestic pigs' populations infected with HEV were more likely to be seropositive to HEV than wild boars hunted in areas with no pig farms nearby (Charrier et al., 2018). This suggests that although HEV transmission can occur between both populations in any direction, it is more likely to occur from domestic pigs to wild boars than the other way around (from wild boars to domestic pigs).

The presence of infectious HEV in pig livers entering the food chain is a key point of exposure risk for humans. To confirm this potential risk, two of the HEV RNA positive livers from piglets were used to inoculate HepaRG cells that are permissive to HEV infection. With these two livers, productive infections were obtained, confirming the initial presence of infectious virus in the livers collected at slaughterhouse. One of the livers was from an animal with anti-HEV antibodies (Liver 2) while the other animal did not (Liver 1). The presence of anti-HEV antibodies did not appear to have any effect on HEV infectivity since both cultures were positive. HEV particles in the liver are quasi-enveloped, and it is suggested that they are not sensitive to neutralization by antibodies (Nagashima et al., 2017).

5 | CONCLUSION

Our study shows that in extensive pig farming systems, HEV circulates highly but late slaughter of pigs is likely to reduce the number of HEV contaminated livers entering the food chain. Thus, the manufacture of food products containing raw pork liver, which are at high risk for HEV, would significantly reduce the risk of contamination for consumers, if pigs were slaughtered at an older age (>12 months). The exclusive use of local pork livers for the preparation of Corsican products, by all manufacturers, should reduce the prevalence of HEV RNA in Ficatelli.

Consumption of safe food products is one of the most important engagements of every public policy worldwide. Thus, increasing the age of animal at slaughter in the context of extensive farming contributes to reduce the risk of HEV infection. While other studies propose vaccination of humans and pigs to limit the HEV risk (Ji et al., 2021), our observation may open new perspectives on swine production in order to reduce the risk of consumer exposure to HEV.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted in the journal's author guidelines page, have been adhered to. Samples used in this study were from animals slaughtered according to the French regulation. Ethical approval was not required.

CONFLICT OF INTEREST

All authors have no actual or potential conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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