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HEV infection in swine from Eastern Brazilian Amazon: Evidence of co-infection by different subtypes☆

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ABSTRACT

Hepatitis E virus (HEV) is a fecal-orally transmitted member of the genus *Hepevirus* that causes acute hepatitis in humans and is widely distributed throughout the world. Pigs have been reported as the main source of genotypes 3 and 4 infection to humans in non-endemic areas. To investigate HEV infection in pigs from different regions of Pará state (Eastern Brazilian Amazon), we performed serological and molecular analyses of serum, fecal and liver samples from 151 adult pigs slaughtered between April and October 2010 in slaughterhouses in the metropolitan region of Belém, Pará. Among the animals tested, 8.6% (13/151) were positive for anti-HEV IgG but not for anti-HEV IgM. HEV RNA was detected in 4.8% (22/453) of the samples analyzed and 9.9% (15/151) of the animals had at least one positive sample. Phylogenetic analysis showed that all sequences belonged to genotype 3 that were related to human isolates from other non-endemic regions, suggesting that the isolates had zoonotic potential. Subtypes 3c and 3f were simultaneously detected in some pigs, suggesting co-infection by more than one strain and/or the presence of a recombinant virus. These results constitute the first molecular and serologic evidence of swine HEV circulation in the Eastern Brazilian Amazon.

1. Introduction

Hepatitis E virus (HEV) is an RNA virus of the genus *Hepevirus* that causes acute hepatitis in humans and is transmitted through fecal-oral route [1]. In mammals, HEV strains have been classified into four genotypes (1–4) based on their genetic diversity; additionally, subtypes have been identified within each genotype group and among them genotype 3 shows the highest variability [2].

These genotypes show a characteristic geographic distribution: genotype 1 is considered endemic in Asia and northern Africa; outbreaks of genotype 2 have been reported in Mexico and central Africa; genotype 3 is found in North and South America, parts of Europe and Japan; and genotype 4 has been reported in China, Japan, Taiwan and Vietnam [3].

Genotypes 1 and 2 are epidemic, infecting only humans and exhibiting low genetic diversity (five and two subtypes, respectively) compared with genotypes 3 and 4 (ten and seven subtypes, respectively).

Genotypes 3 and 4 are described as zoonotic because they are found in areas where human and animal isolates of HEV shows strong genetic similarities [3]. Meng et al. were the first to identify swine HEV and phylogenetic analysis revealed that it was highly similar to human HEV isolate, suggesting a probable zoonotic infection [4].

Domestic pigs are reported to be the main source of genotypes 3 and 4 infection in humans living in non-endemic regions [5,6], but HEV has also been identified in wild boar [7,8], deer [7], mongoose [9], rats [10], rabbits [11], and there is also a similar avian virus [12,13].

In Brazil, swine HEV was isolated for the first time from fecal samples of pigs from the São Paulo state, in South-eastern Brazil. A phylogenetic analysis of these samples demonstrated similarities with genotype 3 [14]. HEV genotype 3 infections were also found in pigs and effluents from a pig slaughterhouse in Rio de Janeiro state, located at Southeastern Brazil [15,16].

Recently, the first human case of autochthonous hepatitis E in Brazil was identified in a patient with acute non-A–C hepatitis. The virus infecting this patient was identified as HEV genotype 3b, which indicated that the origin of the infection was probably zoonotic [16].

Studies on the occurrence of HEV infection in swine are still scarce in Brazil. Due to the zoonotic features of this infection, further studies on HEV epidemiology are needed. Therefore, the aim of this study was to investigate the prevalence of HEV infection in pigs at slaughterhouses in Pará state, Eastern Brazilian Amazon and to characterize the genotypes circulating in this region.

2. Material and methods

2.1. Samples

From April to October 2010, serum, feces and liver samples were collected from 151 slaughtered pigs (approximately six months old) from different regions of Pará state, Brazil. The samples were collected from 95 animals from the municipality of Bujarú slaughtered in an officially registered slaughterhouse located in the municipality of Santa Isabel do Pará. This slaughterhouse operates according to the sanitary inspection criteria established by the Agricultural Protection Agency of the state of Pará, which are mainly based on the visual inspection of the viscera and carcasses prior to the approval of the slaughter products for sale.

The samples from other 56 animals were collected at three illegal slaughterhouses in the municipality of Belém. These establishments were not legally registered with the official health regulatory agencies for this type of activity, and they typically slaughter animals from small-scale family farms for the direct sale of the slaughter products in open-air markets and stores in the city. These 56 animals were from the municipalities of Belém, Marituba, Santa Isabel do Pará, Castanhal and the Marajó Archipelago.

Serum, liver and feces samples were collected during the slaughter of each animal, and all the biological material was frozen at −70 °C until further analysis.

This study was approval by the Ethics Committee for Animal Research of the Evandro Chagas Institute Belém/Pará/Brazil (Approval No. 0019/2010/CEPAN/IEC/SVS/MS), and the procedures involved met all ethical and animal welfare requirements of this committee.

2.2. Serological detection

The presence of anti-HEV IgM and IgG were analyzed in all serum samples using the commercial indirect ELISA kit RecomWell HEV IgM and IgG (Mikrogen, Neuried, Germany) according to the manufacturer's instructions. Briefly, 10 μL serum samples were diluted in 1 mL buffer solution and 100 μL of this dilution, positive, negative and cutoff controls were applied to wells coated with ORF2 and ORF3 HEV proteins, incubated for 1 h at room temperature, washed four times (300 μL per well), incubated again with 100 μL anti-human peroxidase conjugate, washed, and incubated with 100 μL substrate solution. Absorbance values were measured with an automatic microplate reader (450 nm/650 nm) immediately after reaction blocking. Background threshold and antibody concentrations were calculated based on formulas according to the values of the positive, negative and cutoff controls provided in the commercial assay.

To confirm serological reactivity, serum samples with positive or inconclusive results using ELISA were subsequently tested with the commercial immunoblotting kit RecomLine HEV (Mikrogen, Neuried, Germany). Diluted serum samples were incubated with test strips loaded with purified HEV recombinant antigens and a color reaction in gray scale at the specific sites in test strips provides positive result.

In addition to the positive and negative controls supplied with the kits, internal positive controls were added (positive anti-HEV human and swine serum samples). All the positive and/or inconclusive samples were tested in duplicate and the serological tests were also validated according to the manufacturer's guidelines.

2.3. RNA extraction

HEV RNA was extracted from the 250 μL serum samples by the acid guanidinium thiocyanate/phenol/chloroform method [17] using the TRIzol LS reagent (Invitrogen, CA, USA) using the manufacturer's instructions.

Extraction of HEV RNA from the fecal samples was carried out by using RNeasy mini kits (QIAGEN, Hilden, Germany): fecal samples were prepared in a 10% solution diluted in PBS pH 7.4 and after vigorous shaking were incubated at 4 °C overnight. Samples were then centrifuged at 20,000 × g for 5 min and the supernatant was used to extraction with RNeasy mini kit according to the manufacturer's recommendations.

Viral RNA was also extracted from the liver samples using RNeasy mini kits (QIAGEN) following manufacturer's instructions.

2.4. Nested RT-PCR

Reverse transcription for cDNA synthesis was performed using random primers and the Moloney Murine Leukemia Virus Reverse Transcriptase (Invitrogen).

We used nested RT-PCR to amplify three regions of the HEV genome. Initially, two sets of primers that amplified fragments of ORF1 and ORF2 genes were used [18].

ORF1 primers were located at nucleotide positions 56–79 and 473–451, which corresponded to the methyltransferase coding region. The external primers (ConsORF1-s1: 5′-CTGGCATYACTACTGCYATTGAGC-3′ and ConsORF1-a1: 5′-CCATCRARRCAGTAAGTGCGGTC-3′) and internal primers (ConsORF1-s2: 5′-CTGCCYTKGCGAA-TGCTGTGG-3′ and ConsORF1-a2: 5′-GGCAGWRTACCA-RCGCTGAACATC-3′) amplified 418 and 287 bp fragments, respectively.

ORF2 primers corresponded to positions 6298–6321 and 6494–6470 of the Burmese prototype. The external primers (ConsORF2-s1: 5′-GACAGAATTRATTTCGTCGGCTGG-3′ and ConsORF2-a1: 5′-CTTGTTCRTGYTGGTTRTCATAATC-3′) amplified a 197 bp product in the first PCR, and the internal primers (ConsORF2-s2: 5′-GTYGTCTCRGCCAATGGCGAGC-3′ and ConsORF2-a2: 5′-GTTCRTGYTGGTTRTCATAATCCTG-3′) amplified a 145 bp product in the second PCR.

The samples were also tested using a third set of primers that amplified an additional ORF2 region. The external (3156NF: 5′-AATTATGCYCAGTAYCGRGTTG-3′ and 3157NR: 5′-CCCTTRTCYTGCTGMGCATTCTC-3′) and internal primers (3158NF: 5′-GTWATGCTYTGCATWCATGGCT-3′ and 3159NR: 5′-AGCCGACGAAATCAATTCTGTC-3′) used in the present study were described for the detection of HEV and amplified 731 and 348 bp products in the first and second PCRs, respectively [19,20].

All precautions and procedures suggested to avoid false positive results were strictly followed [21]. The extraction of nucleic acids, the PCR setup and especially the addition of first round PCR reaction to second round PCR were performed in completely separated rooms, when necessary inside biohazard cabinets or PCR hoods. Each stage was performed with separate micropipettes and all PCR procedures were carried out using aerosol filter tips. Other precautions such as frequent changing of gloves, working with reagents divided in aliquot, control of daily flow of personnel only from pre- to post-amplification areas and the use of UV-light and sodium hypochlorite solution to decontaminate surfaces were strictly observed in order to prevent cross-contamination.

Positive control (serum from a pig positive to HEV) to confirm the effectiveness of the reactions and negative controls (water) to check for contaminants were included in each run.

PCR reactions had a final volume of 50 μL and contained the following reagents: 35.3 μL DEPC-treated H_2O; 5 μL 10× PCR buffer; 1 μL dNTPs (10 mM) (Invitrogen); 1.5 μL $MgCl_2$ (50 mM) (Invitrogen); 20 pmol of each primer (forward and reverse); 1 U of Platinum Taq polymerase (Invitrogen) and 5 μL cDNA sample. In the second PCR, 5 μL of the first PCR product was added instead of cDNA.

PCR amplification cycles were: initial denaturation at 94 °C for 2 min, followed by 35 cycles of denaturation (94 °C for 30 s), annealing (50 °C for 30 s), extension (72 °C for 30 s) and a final extension at 72 °C for 5 min. The annealing temperature differed only for the 348 bp ORF2 primers (42 °C).

The products of the second PCR were labeled with SYBR dye (Invitrogen) and separated by electrophoresis on 1% agarose gel. Depending on the primers used, samples that exhibited sharp, intense bands with approximate sizes of 145, 287 or 348 base pairs were considered as positive.

2.5. Nucleotide sequencing and phylogenetic analysis

PCR products (ORF1 287 bp and ORF2 348 bp) from the second round reaction were purified using ExoSAP-IT PCR Clean-up Kit (GE Healthcare) and were then sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA, USA) according to the manufacturer's guidelines. Sequencing was carried out on an automated ABI 3500 DNA Sequencer (Applied Biosystems).

The quality of each electropherogram was evaluated using the Phred-Phrap software [22,23] and consensus sequences of forward and reverse sequences were obtained using CAP3 software available at the web page http://asparagin.cenargen.embrapa.br/phph/.

Sequences were aligned and edited using BioEdit (v. 7.0.8) and the integrated CLUSTAL W program [24]. HEV genotypes and subtypes were classified by phylogenetic reconstructions using published reference sequences from GenBank database (http://www.ncbi.nlm.nih.gov/).

Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.6.1 [25] under uncorrelated exponential relaxed molecular clock using the model of nucleotide substitution (GTR + G + I). The maximum clade credibility (MCC) tree was obtained from summarizing the 10,000 substitution trees and then it was removed 10% of burn-in using Tree Annotator v.1.6.1 [25].

Nucleotide sequence divergence was calculated using the program MEGA4 (Molecular Evolutionary Genetics Analysis) software version 4.0 [26].

3. Results

3.1. Serological detection

Anti-HEV IgG was detected using ELISA (background threshold = 0.3) in 4.6% (7/151) of the samples (mean absorbance = 0.643 ± 0.214). In addition, 3.9% (6/151) of the samples showed inconclusive result (mean absorbance = 0.308 ± 0.024). The average concentrations of the anti-HEV IgG antibodies were 42.1 ± 14.6 U/mL for the seven positive samples and 20.5 ± 1.6 U/mL for the six inconclusive samples. Anti-HEV IgM was not detected in any of the analyzed samples.

All thirteen samples (8.6%; 13/151) with reactivity (positive or inconclusive) to anti-HEV IgG showed positive results in the immunoblotting assay. These positive

Table 1
Molecular detection of HEV in pigs from the state of Pará using nested RT-PCR.

Pig identification	Sample type	Results of RT-PCR HEV		
		ORF2 (145 bp)	ORF1 (287 bp)	ORF2 (348 bp)
#025	Serum	−	+	−
	Feces	−	−	−
	Liver	−	−	−
#027	Serum	+	+	−
	Feces	+	+	+
	Liver	+	+	+
#028	Serum	−	−	−
	Feces	−	−	−
	Liver	−	+	−
#029	Serum	−	−	−
	Feces	+	+	+
	Liver	−	+	−
#030	Serum	−	−	−
	Feces	−	+	−
	Liver	−	−	−
#031	Serum	−	−	−
	Feces	−	+	−
	Liver	−	−	−
#032	Serum	−	−	−
	Feces	−	+	−
	Liver	−	−	−
#034	Serum	−	−	−
	Feces	−	+	+
	Liver	−	−	−
#035	Serum	−	+	+
	Feces	−	+	+
	Liver	−	+	−
#036	Serum	−	−	−
	Feces	−	−	−
	Liver	−	+	−
#037	Serum	−	−	−
	Feces	−	+	+
	Liver	−	−	−
#080	Serum	−	−	−
	Feces	−	+	−
	Liver	−	−	−
#125	Serum	+	−	+
	Feces	+	+	+
	Liver	+	+	+
#147	Serum	−	−	−
	Feces	−	+	−
	Liver	−	−	−
#150	Serum	−	−	−
	Feces	+	−	−
	Liver	−	−	−
Positive samples/total examined (%)		8/453 (1.76%)	20/453 (4.41%)	10/453 (2.2%)

samples were collected from different geographical origins: ten were from the municipality of Bujarú, two were from the municipality of Castanhal, and one was from the Marajó Archipelago.

3.2. HEV RNA detection and sequencing

Nested RT-PCR with three sets of primers was used to analyze serum, feces and liver samples (n = 453) from 151 pigs. HEV RNA was detected in fifteen animals (9.9%; 15/151), which had at least one positive sample by at least one set of primers.

Among 453 samples analyzed, HEV RNA was detected in 4.8% (22/453) with at least one set of primers. Feces were the samples where HEV RNA was more frequently detected (12/22), followed by liver (6/22) and serum (4/22).

HEV RNA was amplified using all the three sets of primers only in five samples and the primers generating the 287 bp product showed the highest sensibility to HEV RNA detection. The 287 bp ORF1 fragment were amplified in 20

(4.4%) samples (three serum, eleven feces and six liver); the 145 bp ORF2 fragment in eight (1.7%) samples (two serum, four feces and two liver) and the 348 bp ORF2 fragment was amplified in ten (2.2%) samples (two serum, six feces and two liver).

Only three pigs had HEV RNA detectable in all samples analyzed (serum, liver and feces); in two pigs HEV RNA was detected only in liver samples; viremia was present in four pigs and twelve were shedding virus in feces (Table 1).

HEV RNA positive pigs were from different origins: twelve were from the municipality of Bujarú, two were from Castanhal and one was from Marajó.

Only one pig (#027) with serological evidence of HEV infection (anti-HEV IgG positive) had HEV RNA detectable (7.7%; 1/13), which was amplified in its three samples.

All amplified samples were sequenced but only sequences from ORF1 (287 bp) and ORF2 (348 bp) were used for genotype/subtype classification by phylogenetic analysis. Due to the short length (145 bp) of the other ORF2 amplified fragment, these sequences were not used to this analysis, they were only submitted for analysis in BLAST (Basic Local Alignment Search Tool, http://www.ncbi.nlm.nih.gov/BLAST) to confirm similarity with HEV.

Sequences with good quality were obtained from fifteen samples of the ORF1 (287 bp) and from seven of the ORF2 (348 bp), that were selected and after used for genotype/subtype characterization.

3.3. Phylogenetic analysis

The phylogenetic analyses of fifteen sequences from the ORF1 5′-terminal region shows that all isolates grouped with HEV genotype 3 reference sequences. Among these, fourteen were much more closely related with subtype 3c, whereas the other sequence (#035) grouped with sequences of subtype 3f (Fig. 1). A calculation of the divergence among the fifteen ORF1 sequences indicated that they had a nucleotide identity ranging from 78% to 100%.

The topology of phylogenetic tree based on the ORF2 5′-terminal sequences also showed that only HEV genotype 3 circulated among infected pigs from Pará state (Fig. 2). In this analysis, three sequences grouped with subtype 3c and the other four with subtype 3f. These seven samples had a nucleotide identity ranging from 93% to 100%.

Two pigs (#034 and #035) showed different subtypes in the same sample (feces): for these cases, when ORF1 sequence was analyzed the strain was classified as subtype 3c and based on results of the ORF2 sequence analysis, the samples were classified as subtype 3f (Figs. 1 and 2). The pig #035 also showed different subtypes in different samples analyzed: ORF1 sequences of the strain isolated from feces and liver samples were classified as 3c, but the ORF1 sequence of the strain isolated from serum sample was 3f (Fig. 1). Table 2 summarizes these findings.

4. Discussion

In this study, we showed that HEV is circulating among pigs from different areas of the Pará state, located in Eastern Brazilian Amazon, North region of the country. We

Table 2
Divergent HEV subtypes found in samples from pigs in the state of Pará.

Animal #	Sample	HEV subtypes identified by phylogenetic analysis	
		ORF1	ORF2
#034	Feces	3c	3f
#035	Serum	3f	3f
	Feces	3c	3f
	Liver	3c	ND

ND = not determined.

found that among pigs from this region 9.9% (15/151) were currently infected with HEV (HEV RNA positive); these findings confirm the circulation of HEV among pigs in Brazil, where pigs with HEV infection also has been reported in other three different regions of the country [14,15,27].

The prevalence of anti-HEV IgG found among pigs from Pará state was lower (8.6%; 13/151) than that previously reported among pigs from other Brazilian regions: in pigs from Mato Grosso state a high (81.2%) seroprevalence of anti-HEV IgG was found [28], whereas in the Rio de Janeiro state, at southeastern region, the seroprevalence was 24.3% [29]. Differences in prevalence of serological markers of HEV infection (anti-HEV IgG/IgM) around the world have been related to specificity and sensibility of the commercially available serological assays and this may explain the differences seen in HEV seroprevalence results among pigs from different regions of Brazil [30]. Moreover, some studies have been show that HEV prevalence is directly associated with differences in the hygiene and sanitary management of the rearing facilities [31,32].

Likewise, the failure to detect anti-HEV IgM in the present study may also be related to the sensitivity and specificity of the test used for the serological diagnosis [33], but can also be due to the brief period while this immunoglobulin is detected during the infection cycle [34]. Anti-HEV IgM antibody levels appear to be age-dependent, with the highest proportion found in young pigs (up to three months old) in response to their first contact with the virus, which usually occurs soon after weaning [20,35–38]. The samples analyzed in the present study were obtained from pigs at slaughter age (approximately six months), what may have led to the failure in detecting IgM antibodies.

Interestingly, in the present study, HEV RNA was more frequently detected among pigs without serological evidence of HEV infection: among fifteen pigs with positive PCR, only one (pig #027) had detectable anti-HEV IgG. In this pig, anti-HEV IgG was detected at low levels (25.6 U/mL) and HEV RNA was detected in its serum, feces and liver samples. Considering the possibility that the virus can be maintained among infected populations, this animal may represents a case of re-infection [37].

Twelve (7.9%; 12/151) seropositive animals in which HEV RNA was not detected appear to represent cases of previous infection. Although HEV infection may occur in any age, it has been reported that it occurred more frequently among pigs aging from 3 to 4 months and when most of these animal reach slaughter age, they had already
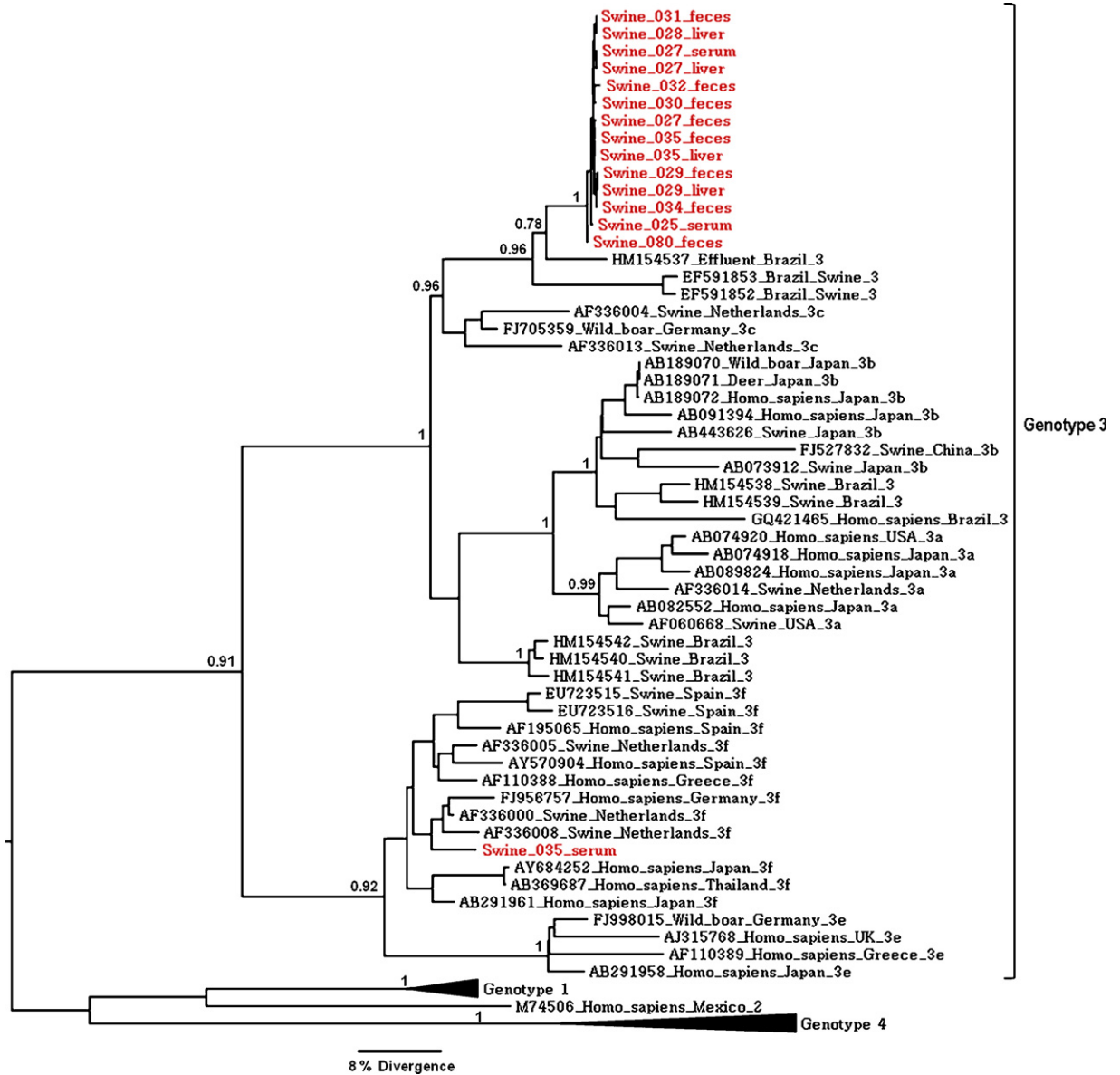
Fig. 1. Maximum clade credibility (MCC) tree estimated by Bayesian analysis of HEV sequences from the 5′ terminal region of ORF1 (266 nt). Sequences characterized in this study (*n* = 15) are highlighted in red and sequences obtained from GenBank (*n* = 75) are identified by their accession number followed by the source, geographic origin and genotype/subtype of the isolated virus. The collapsed clades correspond to sequences of HEV genotypes 1 and 3. The values of posterior probability are shown above their respective key nodes.

developed anti-HEV and the presence of the virus is not detected in blood, liver or fecal samples [39].

Fourteen pigs were found with a positive PCR in the absence of HEV serum antibodies. HEV replication without serological evidence of HEV infection in pigs has been related to recent infection when HEV RNA was detected in liver or bile. On the other hand, when it was detected only in stools, it was suggested a possible transient intestinal presence of the virus without replication after ingestion [35]. This pattern was found in eight pigs in this study. Among seronegative pigs, HEV RNA was more frequently detected in fecal samples, followed by liver and serum.

The pigs that had HEV RNA detectable only in feces may be cases of transient intestinal presence of the virus. Nevertheless, it is reasonable to consider that HEV antibodies were in low levels and the serological assay used was not sensitive enough for their detection. In fact, a recent study reported low levels of ELISA optical density values for anti-HEV IgG among most of the studied pigs, independently of the life cycle period evaluated: 2, 8, 18 or 22–29 weeks [20]. These authors also reported that in all sampling, especially at the third (18 weeks) and final sampling (slaughter), HEV RNA was more frequently detected in feces than in serum [20]. This finding agrees with the knowledge that fecal virus
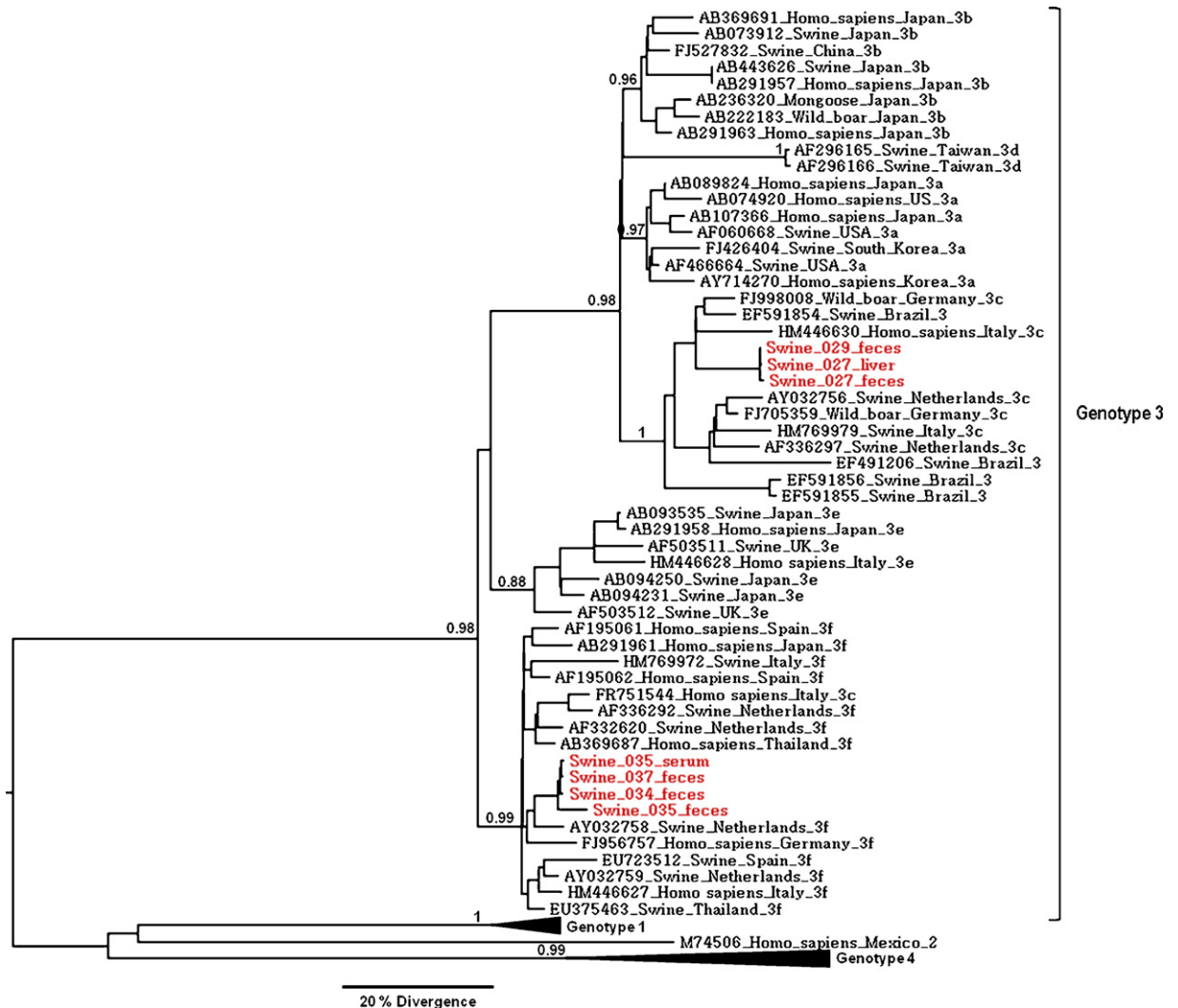
Fig. 2. Maximum clade credibility (MCC) tree estimated by Bayesian analysis of HEV sequences from the 5′ terminal region of ORF2 (346 nt). Sequences characterized in this study ($n = 7$) are highlighted in red and sequences obtained from GenBank ($n = 94$) are identified by their accession number followed by the source, geographic origin and genotype/subtype of the isolated virus. The collapsed clades correspond to sequences of HEV genotypes 1 and 3. The values of posterior probability are shown above their respective key nodes.

shedding may persist longer than viremia [5,34]. Moreover, several studies had reported that detection of HEV RNA in the liver samples was less frequent than in feces or bile [5]. The latter is the sample where HEV RNA has been found more frequently [35,40]. It is possible that in the present study seronegative animals with positive PCR only in feces had also HEV RNA detectable in their bile, but we cannot confirm this because it was not collected.

Seronegative pigs with positive PCR may represent cases of HEV re-infection, although in some cases a more prolonged infection might have occurred, as suggested by other authors [31].

HEV strains characterized in this study were classified as genotype 3, the genotype that was also reported from pigs and humans living in countries with sporadic cases of HEV infection [1,4,18,41–50]. HEV genotype 3 was also previously described in Brazil infecting pigs from other regions

of the country [14,15,27] and involved in a human infection case [16].

A phylogenetic analysis of fifteen HEV ORF1 sequences indicated that fourteen strains belonged to subtype 3c and one sequence belonged to subtype 3f. This subtype 3f sequence was obtained from a strain isolated from serum sample of pig #035 and exhibited 22% nucleotide divergence from the other ORF1 sequences from the strains isolated from feces and liver samples obtained of the same animal. This result suggests the occurrence of co-infection by more than one subtype of the virus in a single animal, with one strain circulating in the blood stream and the other constrained to the liver and gastrointestinal system. This result is probably due the presence of multiple HEV variants circulating in the farm.

HEV subtypes 3c and 3f have also been identified conjointly in slaughtered pigs from Italy, however, the

sequences obtained from multiple samples taken from the same animal were identical, i.e., they did not group into different subtypes [35], as was found in pig #035 case. Although they did not detect cases of co-infection with multiple viral subtypes, the aforementioned authors did not rule out this possibility. Coexistence of more than one swine HEV subtype from the same genotype circulating in the environment appears to be relatively common [31,39,40]. Co-infection can also occur in humans; a mixed infection with HEV genotypes 3 and 4 was observed in a patient with acute hepatitis E in Japan [51].

The phylogeny of the seven ORF2 sequences indicated that three samples belonged to subtype 3c and four to the subtype 3f. Lu and collaborators [2] suggested that the classification of a HEV strain tends to remain constant even when different regions of the genome are analyzed. Therefore, the detection of ORF1 and ORF2 sequences belonging to different subtypes in pigs #034 and #035 supports the hypothesis that these pigs were co-infected with multiple subtypes, but we cannot rule out the existence of a recombinant virus.

The recombination event may occur as a result of co-infection or superinfection of the same host with two or more strains [52]. The occurrence of inter- and intra-genotype recombination among human and pig HEV strains have been reported [52–54]. It is possible that recombination can lead to the emergence of more virulent variants of the virus, especially if it happens within the capsid coding gene (ORF2) [53].

The presence of HEV infection in pigs at slaughter age confirms previous evidences that these animals may be a source of infection not only for pig farm workers, but also to slaughterhouse workers [43,55,56]. Moreover, HEV infected pigs at slaughter age also represent a risk of human infection by consumption of raw or undercooked meat from these animals [41,57–59]. For that reason, the detection of HEV in pigs from Pará reinforces the need for further studies to evaluate the pathogenicity of these isolates and the risk factors for transmission to humans, especially those that work on pig farms, because the existence of a risk to public health cannot be excluded.

In conclusion, the present study demonstrated the presence of HEV infection in animals at slaughter age, suggesting that pigs in the Eastern Brazilian Amazon are exposed to HEV during different stages of their life cycle. The epidemiological HEV dynamics in pig farms at this region deserves additional studies. Herein, we reported the first identification and genotyping of HEV in Eastern Brazilian Amazon. The HEV subtypes circulating in pigs in the Pará state were found to be related to human and animal samples from other non-endemic regions of the world, suggesting that these isolates had zoonotic potential. The present investigation also detected the simultaneous presence of HEV subtypes 3c and 3f in some pigs, which suggests that mixed infection by multiple and/or recombinant viral strains occurs in these animals. Further studies should be conducted to clarify the epidemiological significance of HEV circulating in the state of Pará and its impact on public health in the Amazon region and in other Brazilian regions.

Conflict of interest statement

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

Acknowledgments

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