



Communication

[Comunicação]

High genetic diversity of hepatitis E virus in swine in São Paulo State, Brazil

[Diversidade genética do vírus da hepatite E em suínos no estado de São Paulo, Brasil]

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Hepatitis E virus (HEV), a member of the *Orthohepevirus A* species, can infect mammals and humans and may be classified into genotypes and subtypes (Smith *et al.*, 2020). In South America, genotype 1 (HEV-1) is restricted to humans, and transmission occurs via the fecal-oral route. Zoonotic genotype 3 (HEV-3) is related to humans, pigs, wild boar, sewage, rivers, drinking water plants, and slaughterhouse effluent (Pisano *et al.*, 2018).

Both serological and molecular detection of HEV-3 infection in domestic pigs have been demonstrated in several Brazilian regions and South America, and the subtypes are 3a-f and h (Campos *et al.*, 2018; Pisano *et al.*, 2018; Silva *et al.*, 2018; Oliveira-Filho *et al.*, 2019), but there are only two records of HEV-3 in pigs in São Paulo state (Paiva *et al.*, 2007; Bertolini, 2017).

Therefore, due to scarce information concerning this viral infection, the aim of this work was to assess the occurrence of HEV in samples from a biological bank of swine feces from the state of São Paulo.

Fecal samples from 89 swine collected between 2008 and 2009 from the metropolitan region of Campinas were analyzed using nested reverse transcriptase-polymerase chain reaction (RT-nPCR) and Sanger sequencing. This study was carried out following the recommendations in the

Brazilian National Guidelines on Animal Experimentation. The protocol was approved by the Universidade Santo Amaro Ethics Committee on Animal Use (32/2013).

RNA extraction was conducted using a 10% aqueous diethyl pyrocarbonate (DEPC) fecal suspension and TRIzol Reagent (Ambion™ Carlsbad, USA) according to the manufacturer's instructions. Complementary DNA (cDNA) was synthesized using random primers (Invitrogen™, Carlsbad, USA) and MML-V Reverse Transcriptase (Invitrogen™, Carlsbad, USA) following the manufacturer's instructions.

ORF-1 nested PCR (Wang *et al.*, 1999) amplification was carried out with the primers Cons-ORF1-s1 and ConsORF1-a1 (external) and ConsORF1-s2 and ConsORF1-a2. The first amplification was carried out in a final volume of 20 µL using 2.0µL of cDNA, 8 pmol of each primer, 1x Promega GoTaq® Green Master Mix, and DEPC water q.s.p.. The second amplification was performed using 1µL of the amplicon and the same reagents and concentrations. Both PCRs were carried out under identical conditions: (1) initial denaturation at 94°C for 4 min; (2) 40 cycles of amplification (94°C/15 s denaturation, 50°C/15 s annealing, and 72 °C/45 s extension); and (3) a final extension at 72 °C for 5 min. Nested ORF-2 PCR (Huang *et al.*, 2002) included the primers 3156NF and 3157NR (external) and 3158NF and 3159NR (internal).

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The first amplification was carried out in a final volume of 25 µL, comprising 2.5 µL of cDNA 1 X Promega Go Taq® Green Master Mix (Promega, Madison, USA), 10 pmol of each primer and DEPC water q.s.p. Conditions for the first amplification were initial denaturation at 94°C for 4 min, followed by 40 cycles (94°C/15 s denaturation, 52°C/30 s annealing, and 72°C/1 min extension) and a final extension at 72°C for 5 min. The second amplification was performed using 1 µL of the amplicon and the same reagents, concentrations, and amplification conditions from the first amplification, except for the extension time (72 °C/30 s). The ORF-1 (287 bp) and ORF-2 (348 bp) fragments were run on a 1.5% agarose gel, stained with SYBR Safe® (Thermo Fisher Scientific, Carlsbad, USA), and visualized under UV light.

PCR products were purified with a QIAquick PCR purification kit (Qiagen Inc. Valencia, CA), and nucleotide sequencing of PCR products was performed in an ABI 3500 (Applied Biosystems®, Carlsbad, USA) automatic sequencer using BigDye 3.1 (Applied Biosystems®, Carlsbad, USA). Electropherogram quality analysis and consensus sequence analysis were performed using Phred and CAP3 software (<http://asparagin.cenargen.embrapa.br/phph/>). Similarity searches were conducted using BLASTn (<http://www.ncbi.nlm.nih.gov/BLAST/>).

The resulting sequences were genotyped by phylogenetic reconstruction using reference sequences (Silva *et al.*, 2018; Smith *et al.*, 2020) and GenBank sequences. GenBank accession numbers, genotypes, and countries of origin are related in the phylogenetic tree. Phylogenetic trees were generated by the neighbor-joining method with the Kimura 2-parameter method (ORF-1) or the maximum composite likelihood method (ORF-2) for evolutionary distances, complete deletion and 1,000 bootstrap replicates with Mega 7.0.21 (Kumar *et al.*, 2016). Avian HEV was used as an outgroup. The sequence identity matrix was generated with BioEdit software version 7.0.8.0 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>).

Seven out of 89 stool samples were positive for the ORF-1 region, three collected in 2008 and four in 2009. Of the seven, three showed positive

amplification for ORF-2, which suggests differences in sensitivity between protocols or gene diversity (Silva *et al.*, 2018). A possible explanation is that there are differences in the anchoring of the primers due to possible mutations in the Brazilian sequences, which is reflected in the lower proportion of detection.

Phylogenetic analysis of both partial ORF-1 and ORF-2 sequences (fig. 1 and 2) showed that all seven samples belonged to HEV-3 zoonotic genotypes (Pisano *et al.*, 2018; Oliveira-Filho *et al.*, 2019). The finding of HEV-3 in the metropolitan region of Campinas increased the viral dispersion previously described in the southeastern region (Paiva *et al.*, 2007; Bertolini 2017) and may explain the presence of HEV seropositivity in human populations in 2000 (Gonçalves *et al.*, 2000). The sequences were deposited in GenBank under accession numbers MF981263-MF981272.

Figure 1 shows the genetic diversity of HEV samples from the state of São Paulo. The presence of subgenotypes 3b (MF981263, MF981264, and MF981265), 3h (MF981266 and MF981267), and 3j (MF981268) can be observed by phylogenetic partial ORF-1 analysis.

The sequences MF981266 and MF981267 obtained in this study clustered with Brazilian sequences (KU888662-KU888663) from pork bioproducts at Rio Grande do Sul (Heldt *et al.*, 2016), with 98.3% to 98.9% nucleotide identity, and clustered with subtype HEV-3h, showing 87% nucleotide identity with the reference subtype JQ013794 (Smith *et al.*, 2020) from humans. This clade forms a sister group with swine isolates from Rio Grande do Sul that have similarities with human samples from Uruguay. This subtype was first reported in backyard pigs in Rio Grande do Sul (Silva *et al.*, 2018). It is noteworthy that both MF981266 and MF981267 were identified in 2008, suggesting the presence of this genotype for at least one decade in the state of São Paulo.

The sequence MF981268 clustered with 90.8% similarity with the Arkell HEV strain swine HEV-3j. The subtype HEV-3j has not previously been in Brazil/South America (Pisano *et al.*, 2018).

High genetic diversity...

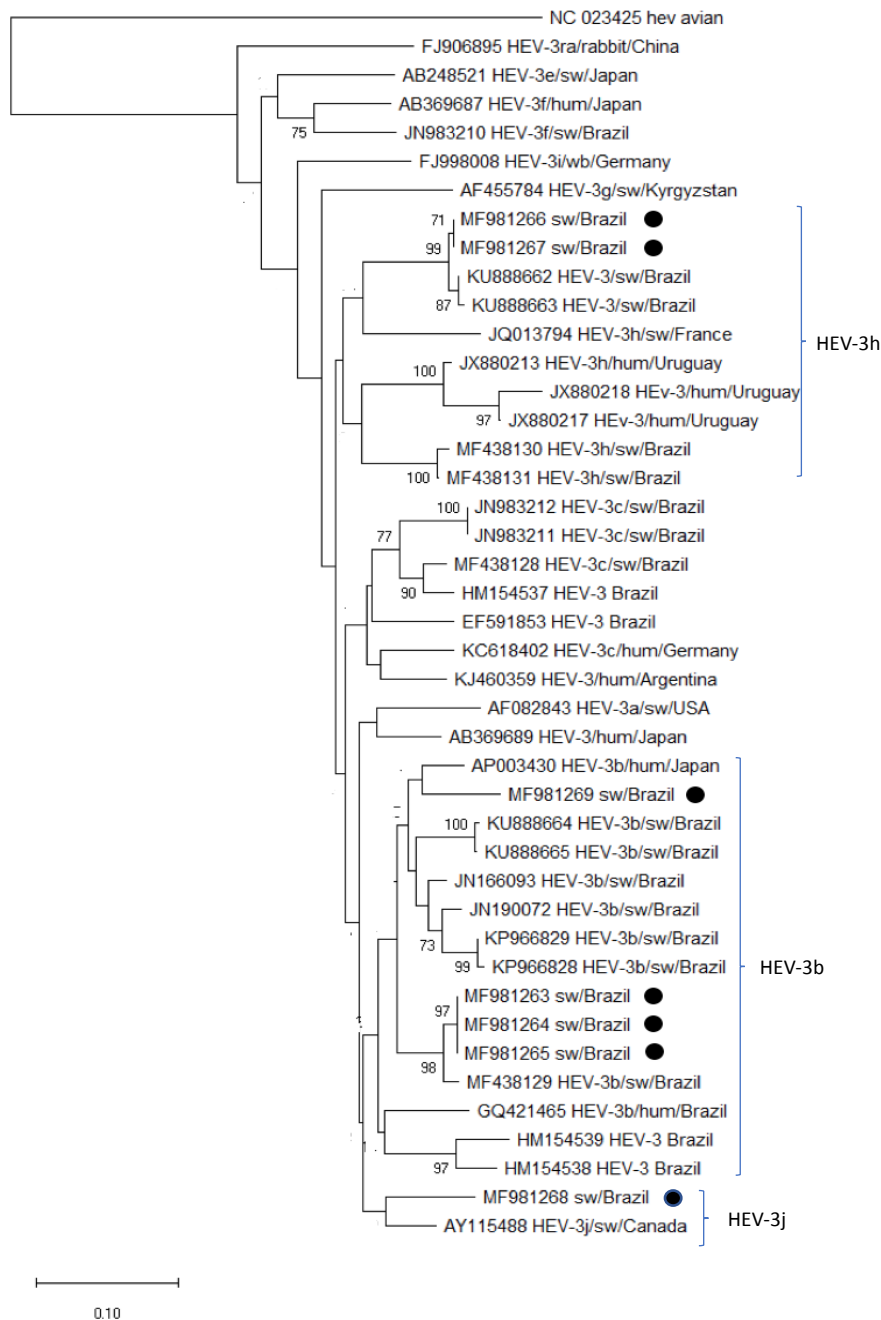


Figure 1. The tree was inferred using the Neighbor-Joining method using the Maximum Composite Likelihood parameter model. It was constructed from 260 nucleotide sequences of a partial ORF- 1. The analysis involved 43 nucleotide sequences. Phylogenetic analyses were conducted in MEGA 7.0.21 with 1,000 bootstrap replicates. Bootstrap values of >70 were shown. Avian HEV was used as an outgroup. The sequences from this study are indicated by a black dot.

The sequence MF981269 clustered with the reference isolate JRA1 (AP003430) for subtype 3b, with 91.9% nucleotide similarity. The MF981263-MF129865 sequences clustered

with the swHEVBR_108-02 isolate (HEV-3b) identified from the swine serum of Rio Grande do Sul (MF438129), with a nucleotide identity of 97.8%. This classification obtained by the

analysis of ORF-1 was supported by the analysis of ORF-2 (MF981270-MF981272) (Fig. 2). These sequences formed a sister group with Brazilian sequences obtained from human patients. The genetic analysis corroborates the study in Rio Grande do Sul (Silva *et al.*, 2018) and reinforces the zoonotic potential of HEV-3b.

The other single sequence (EF491206) from São Paulo (Paiva *et al.*, 2007) clustered with newly described subtype HEV-3i (FJ998008) reference sequences (Smith *et al.*, 2020), which have not been previously described in Brazil.

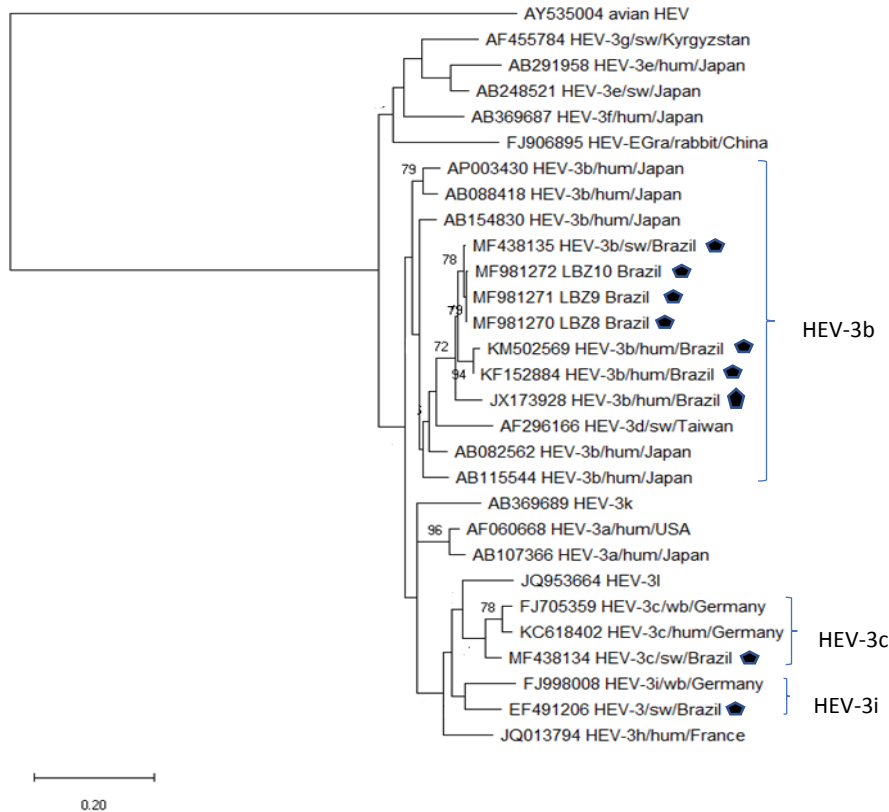


Figure 2. The tree was inferred using the Neighbor-Joining method using the Maximum Composite Likelihood parameter model. It was constructed from 260 nucleotide sequences of a partial ORF- 2. The analysis involved 27 nucleotide sequences. Phylogenetic analyses were conducted in MEGA 7.0.21 with 1,000 bootstrap replicates. Bootstrap values of >70 was shown. Avian HEV was used as an outgroup. The sequences from this study are indicated by a black form.

Considering the genotypic variety found in São Paulo state in samples collected in the late 2000s, molecular epidemiology studies are necessary in humans and animals to understand the dispersion of these genotypes and the current seropositivity found in humans. The present data demonstrated the presence of HEV subtypes not previously

described in Brazil, indicated elevated genetic diversity in the state of São Paulo and reinforced the zoonotic potential of HEV-3.

Keywords: Brazil, domestic pig, food, hepatitis E virus, molecular characterization

RESUMO

A hepatite E é uma zoonose emergente que afeta diversas espécies de mamíferos, inclusive o ser humano. É ocasionada por um vírus da espécie *Orthohepevirus A* que possui diversos genótipos e subgenótipos. No Brasil é descrito o genótipo HEV-3, cujo principal reservatório é o porco doméstico. Testes moleculares e sorológicos demonstram o HEV-3 em diferentes estados, tanto em animais quanto em humanos. No estado de São Paulo, existem diversos estudos sobre a epidemiologia da hepatite E em humanos, mas faltam informações sobre o HEV-3 em suínos. Assim, o objetivo deste trabalho foi verificar a ocorrência de HEV por meio da técnica de RT-PCR e posterior sequenciamento em um banco de amostras de fezes de suínos colhidas entre 2008 e 2009, na região metropolitana de Campinas. Das 89 amostras analisadas, foi possível detectar o HEV-3 em sete e, pela reconstrução filogenética, foram encontrados os subgenótipos HEV-3b, HEV-3h, e HEV-3j. Uma amostra disponível no GenBank, proveniente de São Paulo, que ainda não havia sido subgenotipada, foi agrupada ao HEV-3i. Os subgenótipos HEV-3j e HEV-3i ainda não tinham sido relatados no país. O estudo demonstra uma grande diversidade genética do HEV no estado de São Paulo e reforça o caráter zoonótico da HEV-3.

Palavras-chave: hepatite E, suíno, caracterização molecular, Brasil

CONTRIBUTION

AC and MBH were responsible for supervision. AC, PEB, MBH were responsible for the planning of the experiments, interpretation and discussion of the data and drafted the manuscript. AC, AMVP, CSM, SATM, AOS were responsible for the execution of the experiments and interpretation of the data. AMMGC and FAB were responsible for discussion of the data and manuscript draf. All authors reviewed the results and approved the final version of the manuscript.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are openly available in GenBank at <https://www.ncbi.nlm.nih.gov/genbank/>, reference number MF981263-MF981272.

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