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Short Communication

Hepatitis E virus in swine and effluent samples from slaughterhouses in Brazil

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ABSTRACT

Hepatitis E is an infectious disease which virus (HEV) is highly disseminated in swine herd populations. Sporadic acute human hepatitis E cases have been associated to genotype 3 and 4 strains of HEV also reported in swine populations of endemic and non-endemic areas. With the aim to evaluate the incidence of animals with current infection of HEV, 115 bile samples were collected from three slaughterhouses under inspection by Animal Sanitary Protection Agency of Rio de Janeiro, Brazil. In parallel, effluent samples were collected from six sewage pipe exit sites of two slaughterhouses. HEV RNA was detected in 11 out of 115 (9.6%) bile samples collected and three waste samples from one slaughterhouse. Viral loads observed for bile samples varied from 10^1–10^5 genome copies/mL and for effluent samples mean load was 10^2 genome copies/mL. Sequencing and phylogenetic analysis classified samples within genotype 3 subtype 3b closely related to the sample obtained from the first reported autochthonous human case and samples from swine of commercial herds in Brazil. Our data demonstrates that although most animals achieve slaughter age (around 20 weeks old) already immune to HEV, a significant number of animals are with current infection at commercial age. Further studies should be addressed to consider risk analysis and possible evaluation of inspection regulations considering food safety measures regarding hepatitis E zoonotic aspect in Brazil.

1. Introduction

Hepatitis E is a viral disease with clinical and morphological features of acute hepatitis. In many developing countries it represents an important public health concern, where the primary route of transmission is the fecal–oral through contaminated food and water supplies associated to epidemic outbreaks (Emerson and Purcell, 2003). Hepatitis E particles are small (27–34 nm diameter) and non-enveloped classified as Hepevirus genus within the *Hepeviridae* family (Emerson et al., 2004). Besides the four major genotypes already established (G1–G4), recently, two additional HEV genotypes have been proposed after its characterization from rabbits and rodents (G5–G6) (Johne et al., 2009; Zhao et al., 2009). Each HEV genotype may be classified into subtypes being the genotype 3 genetically more variable (Lu et al., 2006). Genotype 1 is associated to outbreaks occurring in Asia and Africa, whereas genotype 2 is restricted to an outbreak that occurred in Mexico and sporadic cases in Nigeria (Emerson and Purcell, 2003). Genotypes 3 and 4 have been described in sporadic acute cases from non-endemic areas and in

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some animal species. After the characterization of the first swine strain of HEV (Meng et al., 1997), strains have been described from pigs worldwide, mostly belonging to genotypes 3 and 4. Sporadic human strains from non-endemic hepatitis E areas have been demonstrated to have a close phylogenetic relationship to autochthonous swine strains, demonstrating that swine might play a role as reservoir of the infection and that zoonotic transmission of HEV is relevant in industrialized countries (Clemente-Casares et al., 2003). A study demonstrated that even being less stable than hepatitis A virus (HAV), infectious HEV particles could likely survive the internal temperatures of rare-cooked meat (Emerson et al., 2005). Some evidences reported acute cases in consumers of undercooked contaminated meat and organs from *Sika* deer, pigs and wild boars. It has been demonstrated that HEV strains sequences obtained from subjects that consumed undercooked meat were identical to the strains detected in the meat samples obtained from leftovers (Tei et al., 2003). Additionally, occupational or accidental exposure should also be considered since several studies reported that employees in close contact with swine, such as veterinarians, farmers and slaughterhouse workers, present higher seroprevalence rates compared to normal populations (Withers et al., 2002). HEV isolates were also detected in sewage samples of livestock origin which may also represent a source of exposure to infection (Pina et al., 2000; Clemente-Casares et al., 2003). Inspection measures applied to products of animal origin should be capable to prevent potential dissemination of microbiological pathogens and potential infection acquired through direct consumption. Nevertheless, if considered viruses, such as HEV, which are associated to subclinical infections in swine, additional control and prevention strategies should be considered since inspection criteria are mainly based on clinical aspects. In this study, we evaluated the presence of HEV in pigs from three public slaughterhouses submitted to official inspection by the Animal Sanitary Protection Agency in Rio de Janeiro State (ASPA). In parallel, raw effluent samples were collected from two slaughterhouses. Detection and quantification by real-time PCR followed by RT-PCR for genetic characterization of detected strains were performed. Sequences obtained in this study were compared with human and swine prototypes of endemic and non-endemic areas.

2. Materials and methods

2.1. Slaughterhouses

The study was developed in accordance with the protocol approved by the Institutional Committee for Ethics in the Use of Research Animals (CEUA-Fiocruz: PO 0132/01) and followed the regulations established in the Guide for Care and Use of Laboratory Animals published in 1996 by the US National Institutes of Health National Research Council. Sampling was performed on swine populations from three slaughterhouses located in Itaocara (SITC), Itaperuna (SITP) and Petrópolis (SPET) (North and Hill region of Rio de Janeiro State). Commercial establishments are submitted to controlled inspection by an official agency of Rio de Janeiro State (Serviço de Inspeção Estadual—SIE). Each slaughterhouse has a daily routine demand of animals according to respective capacity. All animals were estimated to have over 5 months of age and provided from different commercial herds of Rio de Janeiro state. During the process of sanitary inspection, staff responsible for each establishment carried out *ante* and *post-mortem* inspection of animals and carcasses. Inspection criteria include clinical observance of animals and histopathological analysis of carcasses for the presence of minor or major lesions associated to parasites. All animals from the three slaughterhouses were classified healthy and approved for slaughter and further commercialization according to inspect evaluation criteria.

2.2. Bile and effluents samples collect

On December 2008, 115 bile samples were collected from three slaughterhouses being 13 from SITC, 76 from SITP and 26 from SPET. Bile samples have been demonstrated to be more sensitive for HEV RNA detection (de Deus et al., 2007; Leblanc et al., 2010). The samples were collected during the evisceration process under sanitary requisites determined by regulations of Animal Sanitary Protection Agency in Rio de Janeiro State (ASPA). Five milliliters of bile were collected by vaccum-punction through the gallbladder wall with sterile syringe and immediately stored into cryogenic tubes in dry ice. At the laboratory, bile samples were stored at −80 °C until analysis. Effluent samples were collected from 6 selected sewage pipe exit sites from slaughterhouses SITC and SITP. Each sample was collected in a sterile container and kept chilled until arrival and process in the laboratory. All effluent samples were composed by raw sewage generated from stocking yards room (SYR) (where the animals remain to recover for a holding period of 24 h between arrival from commercial herds and slaughter), from the stunning and bleeding room (SBR), from the evisceration room (EVR) and from a final waste site (FW). In slaughterhouse SITC, two available sites were selected for collection, one from the stocking yard and another one from the final waste site.

2.3. Concentration of viral particles from effluent samples

The recovery and concentration of viral particles in samples were carried out as described in previous studies (Puig et al., 1994; Pina et al., 2000). Briefly, 42 ml of effluent sample were ultracentrifuged (110,000 × *g* for 1 h at 4 °C). The sediment was eluted with 3.5 ml 0.25 N glycine buffer (pH 9.5) and kept on ice for 30 min. After the addition of 2× phosphate-buffered saline (PBS, pH 7.4), the suspension was centrifuged at 12,000 × *g* for 20 min to separate suspended solids. Particles in the supernatant were pelleted by ultracentrifugation (110,000 × *g* for 1 h at 4 °C), resuspended in 0.1 ml 1× PBS and stored at −70 °C.

2.4. RNA extraction, cDNA synthesis, PCR and quantitative PCR for bile and effluents final eluates

Total RNA was extracted from 140 μl of bile samples diluted 1/10 in RNAse free water using QiaAmp viral RNA

kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Effluent samples and the suspension used in seeding experiment were extracted by the glass powder method (Boom et al., 1990). RNA reverse transcription (RT) was conducted using a Superscript III (Invitrogen, Carlsbad, CA, USA) at 50 °C and submitted to both quantitative (qPCR) and qualitative (RT-PCR) assays. For effluent samples, reverse transcription was performed with concentrated and diluted RNA (1/10 in RNase free water) to avoid inhibition in PCR reaction. Quantitative PCR was performed according to a modified 2-step protocol described by Jothikumar et al. (2006) since cDNA syntesis was performed separately. All samples were tested in duplicate. A plasmid clone from a Brazilian swine HEV strain previously characterized was constructed with TOPO® TA cloning® kit (Invitrogen, Carlsbad, CA, USA) and the primers described (Jothikumar et al., 2006; dos Santos et al., 2009). Plasmid DNA was purified using the QIAprep spin miniprep kit (QIAgen, CA, USA) and quantified with the Nanodrop ND-1000 instrument according to manufacturer's instructions (Wilmington, DE). Standard curves were generated using 10^0 to 10^9 copies of plasmid DNA. The equivalent genome titers of HEV were determined based on the standard curve.

The qualitative PCR reaction was conducted in a Termocycler (TC313, Techne, Cambridge, UK). Protocols, previously described, were applied to amplify partial regions of ORF1 and ORF2 of HEV genome corresponding to methyltransferase and capsid genes, respectively (Erker et al., 1999; Wang et al., 1999). Primers sequences for ORF1 amplification were: CTGGCATYACTACTGCYATTGAGC (external sense); CCATCRARRCAGTAAGTGCGGTC (external anti-sense); CTGCCYTKGCGAATGCTGTGG (internal sense); GGCAGWRTACCARCGCTGAACATC (internal anti-sense). Primers sequences for ORF2 amplification were: GACAGAATTRATTTCGTCGGCTGG (sense); CTTGTTCRTGYTGGTTRTCATAATC (anti-sense). ORF1 is a conserved region of the genome, therefore a more sensitive region for genome detection. Final fragments expected for ORF1 and ORF2 were 287 and 197 base pairs, respectively. At each stage of the reaction a negative control (DEPC water) was included to further minimize the possibility of cross-contamination. Strict anti-contamination procedures, including the use of separate rooms, safety hoods, frequent discharging of gloves and accurate cleaning of surfaces and materials were implemented. Amplified products were visualized in a 2% agarose gel stained with ethidium bromide.

2.5. Sequencing and phylogenetic analysis

Fragments obtained from partial ORF1 and ORF2 genome region were subsequently amplified and submitted to direct sequencing by the dideoxynucleotide chain termination method, using the ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Kit® and the ABI Prism 3730 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA) at PDTIS sequencing platform at Oswaldo Cruz Institute (IOC) (Otto et al., 2008). Sequences from human and swine HEV strains were collected from public databases, and a phylogenetic tree was reconstructed by the neighbor-joining method, with Kimura two-parameter as the model of nucleotide substitution, using the MEGA v. 4.0 software package. Bootstrap analysis with 2000 pseudoreplicate data set was used for statistics. The Genbank accession numbers of the sequences reported in this paper are from HM154537–HM154547.

3. Results

An initial screening with real-time PCR showed that 11 out of 115 (9.6%) bile samples and 3 out of 6 effluent samples were positive for HEV genome. It was not observed significant difference in the detection of diluted and non-diluted RNA of effluent samples for both real-time PCR and RT-PCR. Among bile samples, 9 were from SPET and 2 were from SITP slaughterhouses. Viral loads observed for bile samples varied from 10^1 to 10^5 genome copies/mL and for waste water samples was 10^2 genome copies/mL. HEV RNA was detected in effluent samples collected from SYR, EVR and FW of SITP slaughterhouse. To further characterize the samples respective genotype, RT-PCR was performed and sequences were obtained from seven SPET bile samples (Bile 01, Bile 05, Bile 16, Bile 21, Bile 23, Bile 24, Bile 26), two SITP bile samples (Bile 51 and Bile 62) and one effluent sample from SITP ('effluent' from SYR site), which were positive either for ORF1 or ORF2 protocol and compared with existing human and swine origin of endemic and non-endemic areas available in Genebank. Results from the phylogenetic analysis are shown in Fig. 1 for ORF1 partial region and Fig. 2 for ORF2 partial region. For both analysis, the samples from bile and effluents clustered within genotype 3 and were closely related to a human isolate from Japan (subtype 3b) and, particularly, to two swine strains previously characterized in Brazil and to the first human autochthonous hepatitis E case reported in Brazil (dos Santos et al., 2009; Lopes dos Santos et al., 2010).

4. Discussion

Several studies have demonstrated the enzoonotic aspect of HEV dissemination amongst swine in commercial herds. At the age of slaughter, around 20 weeks old, most animals (>70%) are already immune to HEV with no history of clinical signs or abnormalities associated to hepatitis E virus infection (Chang et al., 2009). In Brazil, this pattern was also shown in commercial herds that presented high prevalence of anti-HEV (Vitral et al., 2005; dos Santos et al., 2009). Although most animals are already immune to HEV by slaughter age, some studies have demonstrated a significant percentage of animals either with viremia or spreading HEV in feces. HEV RNA was detected in 11.8% of sera samples and in 41.2% of stool samples in naturally infected animals from a prospective study developed in Canada (Leblanc et al., 2007). In China and in Italy, 78.4% and 7.3% of sera and stool samples, respectively, collected from slaughtered animals were positive for HEV RNA (Li et al., 2009; Di Martino et al., 2010). Studies have demonstrated the presence of HEV in 0.83%, 6.5% and 11% of liver samples from retail stores in India, the Netherlands and USA (Bouwknegt et al., 2007; Feagins et al., 2007; Kulkarni and Arankalle, 2008). It has

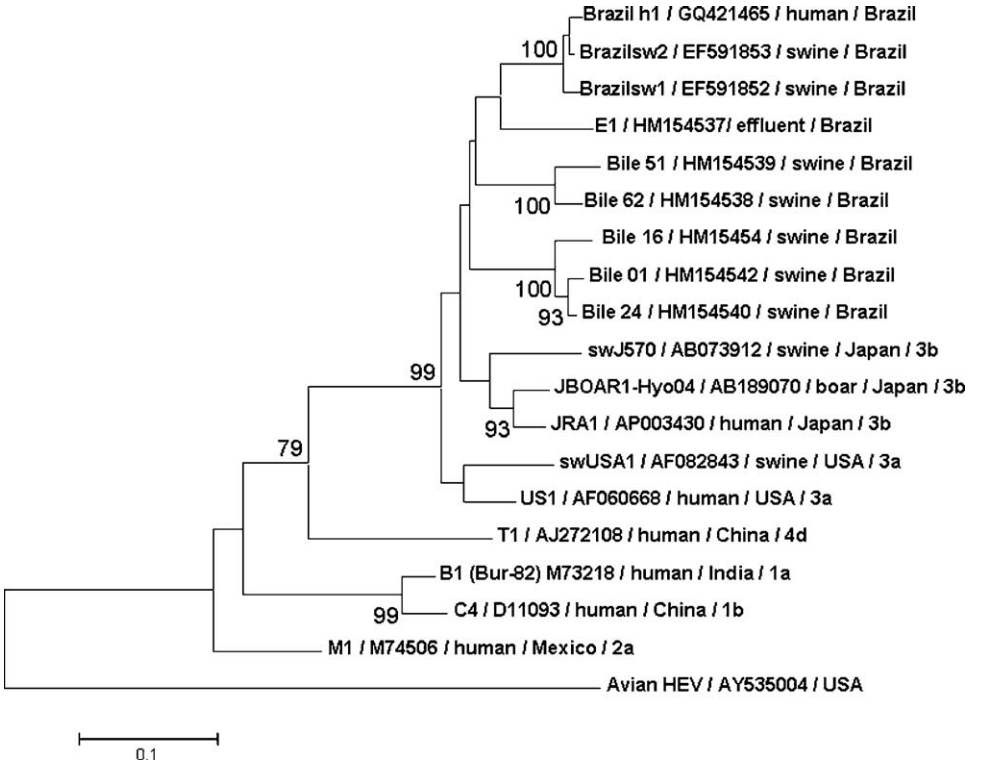

Fig. 1. Phylogenetic tree reconstructed by the neighbor-joining method with common 242-nt ORF1 sequences from 19 HEV isolates, including two porcine isolates from Brazil (EF591852-3) and the human isolate described (GQ421465) Each viral strain is identified by the Genebank accession number, species origin or type of sample, the name of the country of origin, and respective genotype and subtype. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 2000 replicates (bar: 0.05 substitutions per site). Genotypes groups are indicated by major branches. Avian HEV is the outgroup.

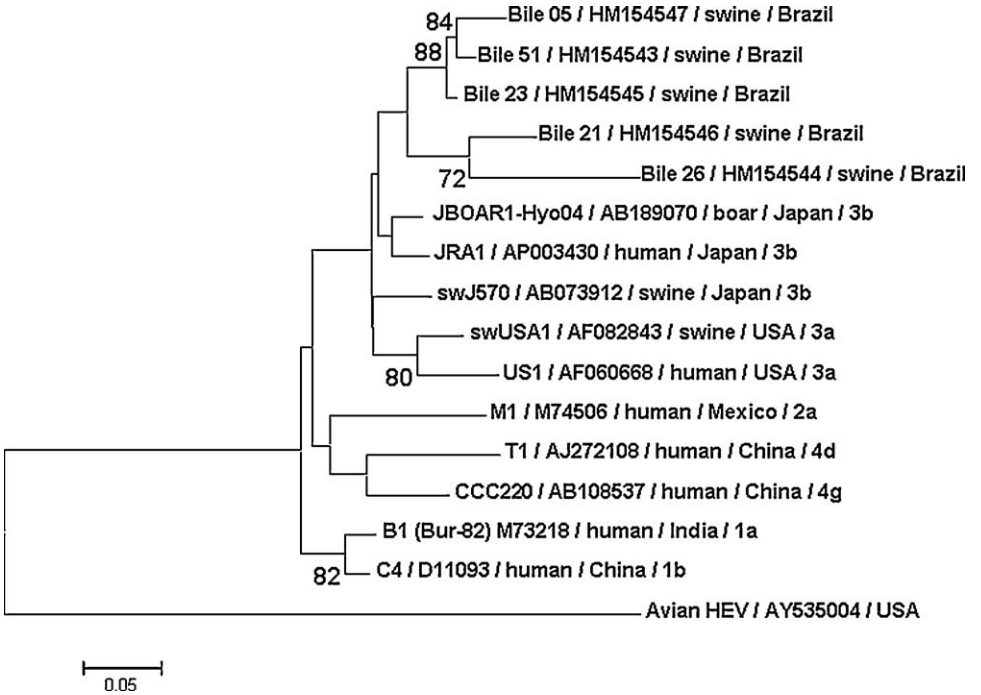

Fig. 2. Phylogenetic tree reconstructed by the neighbor-joining method with common 144-nt ORF2 sequences from 16 HEV isolates. Each viral strain is identified by the Genebank accession number, species origin, the name of the country of origin, and respective genotype and subtype. Bootstrap values are indicated for the major nodes as a percentage of the data obtained from 2000 replicates (bar: 0.05 substitutions per site). Avian HEV is the outgroup.

been demonstrated that some animals may be infected by different strains of HEV during their productive lives and the highest sensitivity of viral partial genome detection was achieved with samples not accessible in live animals, such as liver, mesenteric lymph node and bile (de Deus et al., 2007). Brazil represents the fourth major producer and exporter of pork meat worldwide. Government agencies of inspection are designated for the maintenance of the sanity status of farrow-to-finish farms addressed to achieve the commercial scale of national and export production. Nevertheless, inspection criteria are based on visual analysis of animals and carcasses, thus molecular techniques are not applied for microbiological control purposes. This fact raises an important aspect to be re-evaluated considering that animals infected by HEV presents no clinical or histopathological signs. In the present study, 9.6% of bile samples obtained from animals of three slaughterhouses evaluated were positive for the presence of HEV with viral loads ranging from 10^1 to 10^5 genome copies/mL. Our results demonstrate that even submitted to regulations of control and inspection of products of animal origin, this is an issue to be considered and discussed once a proportion of pigs at the commercial age of slaughter are with current infection of HEV and have no clinical signs or macroscopically evidences of acute hepatitis. In Europe, a study suggested that from the eight zoonotic pathogens investigated, including HEV, only two (Shiga-Toxin producing *Escherichia coli* and Bovines Johne's disease), were likely to be effectively controlled by interventions at farm level (Adam and Brulisauer, 2010). Except for some viruses included in inspection regulations and guidelines, other recognized zoonotic viruses are still not included as a potential risk of infection. It is also relevant to consider that, in Brazil there is also a significant number of family-scale farms in which the practice of slaughtering is not submitted to inspection. Reuter and colleagues described the first autochthonous hepatitis E human case as potentially related to the consumption of pork sausage prepared from house-slaughtered meat (Reuter et al., 2006). Considering the practical aspect of inspection in the production line, fast, reliable and economically affordable diagnostic tests should be considered for zoonotic viruses screening.

The samples from this study clustered within genotype 3 and subtype 3b of HEV among other Brazilian strains, including the first described autochthonous hepatitis E human case (dos Santos et al., 2009; Lopes dos Santos et al., 2010). Several studies have reported sporadic acute cases, related to animal strains reinforcing the zoonotic transmission of HEV (Rutjes et al., 2009). Direct and indirect sources should be considered to acquire HEV infection including accidental occupational exposure to a final exposure by consumption of pork meat derivative products. Sporadic cases have already been reported with risk factors associated to potential occupational exposure such as a butcher and a slaughterhouse worker (Jary, 2005; Perez-Gracia et al., 2007). In the present study, it was also detected HEV RNA with mean viral load of 10^2 genome copies/mL in effluent samples from one of the slaughterhouses investigated. It could represent a potential exposure and contamination source for the employees, handled carcasses and the environment. Detection of HEV in raw effluents raises the concern of how to deal with manure of swine origin, once it is eventually used as soil fertilizer. It is still yet discussible, the viability of HEV infectious articles in meat and environmental samples. Considering the instability of RNA molecules, it is suggested that the detection in environmental samples refers to complete particles (Carducci et al., 2008). It has yet been demonstrated that the HEV particles remains infective and may resist internal temperatures of rare-cooked meat (Emerson et al., 2005; Feagins et al., 2008). It is therefore suggested that proper cooking can prevent exposure to infection showing that not the frequency but the habit of consuming could represent a risk factor. After slaughter, the carcasses are maintained in chill rooms which could favor the viability of HEV, in case of viremic animals. A study demonstrated the presence of HEV in deer meat consumed by patients that developed hepatitis E (Tei et al., 2003). Extrahepatic sites of replication of HEV, including small intestines and colon, have also been reported which could imply in other exposure possibilities such as the consumption of sausages (Williams et al., 2001). The presence of HEV RNA has already been described in tissues and organs of naturally and infected pigs (Banks et al., 2004; Kasorndorkbua et al., 2004). Bouwknegt et al. (2009) reported the presence of HEV RNA in over 50% muscles samples of animals experimentally infected. The meat parts evaluated in the study are usually designated for commercial purposes. Once, it was detected for a period of four weeks after the onset of faecal shedding the time of infection might be a relevant factor should be considered in food safety standards (Bouwknegt et al., 2009). Recently, Leblanc et al. (2010) determined by quantitative PCR hepatitis E virus loads from 10^3 to 10^7 genome copies in different organs and tissues of animals at slaughter from a experimental herd in Canada (Leblanc et al., 2010).

Hepatitis E virus has been emerging as a public health concern once it has been associated to sporadic acute cases related to the direct or indirect exposure to some animals, their derived products and potentially contaminated water sources. There are no regulations, guidelines or tools that consider the screening for zoonotic agents that are not harmful for the animals but recognizably associated to human diseases such as hepatitis E. The real impact of the presence of HEV in slaughtered animals from inspected or non-inspected establishments should be considered to evaluate the risks involved. So far, except for cooking recommendations, legal measures are not available to prevent the exposure to HEV contaminated meat. Multidisciplinary studies should be addressed to provide possible solutions for food quality control regarding hepatitis E virus zoonotic aspect.

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