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Short Communication

Serological and molecular retrospective analysis of hepatitis E suspected cases from the Eastern Brazilian Amazon 1993-2014

***Alex Junior Souza de Souza*[1],[2], *Andreza Pinheiro Malheiros*[2], *Vânia Pinto Sarmento*[2], *Fabricio de Souza Resende*[2], *Max Moreira Alves*[2], *Heloisa Marceliano Nunes*[2], *Manoel do Carmo Pereira Soares*[2] *and Lilian Rose Marques de Sá*[1]**

[1]. Departamento de Patologia, Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, SP, Brasil.
[2]. Seção de Hepatologia, Instituto Evandro Chagas, Belém, PA, Brasil.

Abstract

Introduction: We evaluated the anti-hepatitis E virus (HEV) antibody prevalence and HEV-RNA in archived serum samples of non-A–C hepatitis, or suspected cases of HEV infection from the Eastern Brazilian Amazon from 1993 to 2014. **Methods**: Serum samples (n = 318) were tested using ELISA and immunoblotting, and screened for HEV-RNA by RT-qPCR. **Results**: Anti-HEV IgM and IgG were detected in 3.4% (11/318) and 5.9% (19/318) of the samples, respectively. All samples were HEV-RNA negative. **Conclusions**: HEV was detected at a low prevalence. Broader serological and molecular evaluation of HEV infection in the Amazon region should be carried out.

Keywords: Latin America. HEV. Serology. Molecular biology. Acute hepatitis.

Hepatitis E virus (HEV) is a fecal-oral transmissible agent currently classified in the *Hepeviridae* family, *Orthohepevirus* genus, and *Orthohepevirus A*, *B*, *C*, and *D* species[1]. *Orthohepevirus A* is subdivided into seven genotypes with four (numbered 1–4) main genotypes that infect humans[1].

Acute HEV is clinically indistinguishable from other acute viral hepatitis but is not routinely tested in cases of acute hepatitis. Inconsistencies in the sensitivity and specificity of commercial diagnostic tests may have miscalculated the HEV prevalence and its influence on public health in several regions worldwide, including Latin America[2,3].

Only five human cases of HEV genotype 3 infections confirmed by HEV-RNA detection have been reported in Brazil; all being from Southeastern Brazil[4-6]. Previous serological surveys suggested that the Brazilian Amazon region has a low seroprevalence of HEV. For example, anti-HEV IgG antibodies were detected in only 4% of 349 asymptomatic residents in riverine communities in the Western Brazilian Amazon[7] and in 4.5% of 2–9-year-old children from the Amazon basin[8].

A cross-sectional serological study showed a prevalence of 12.9% of anti-HEV IgM antibodies and 16.3% of anti-HEV IgG antibodies in asymptomatic rural communities of the Western Brazilian Amazon[9]. The variations in the seroprevalence of anti-HEV among different geographical regions of the Amazon basin indicated that HEV might be associated with different epidemiological characteristics[9,10].

Pigs are the main source of HEV genotypes 3 and 4 that infect humans[2], and despite the detection of HEV genotype 3 in swine from the Eastern Brazilian Amazon[11], no cases of human HEV infection have been confirmed by HEV-RNA detection in this region. There are also no published data on human HEV infection assessed using serological methods combined with molecular techniques in patients from the Amazon with clinical and/or laboratory suspicion of hepatitis E. In short, the epidemiology of HEV infection remains unknown as a cause of acute and chronic liver diseases in the Brazilian Amazon.

Corresponding Author: Dr. Alex Junior Souza de Souza.
e-mail: souzajralex@gmail.com
Orcid: 0000-0001-6201-2777
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The present study aimed to determine the seroprevalence of anti-HEV IgM and IgG antibodies and to detect HEV-RNA in archived serum samples of suspected cases of hepatitis E in the Eastern Brazilian Amazon from 1993 to 2014.

Serum samples (n = 318) collected between October 1993 and August 2014 at the Hepatology Section of the Evandro Chagas Institute (IEC/SVS/MS), a regional viral hepatitis reference laboratory located in the city of Belém, in the state of Pará in Northern Brazil, were studied. The storage at −20 °C and use of the samples for this study were approved by the Ethics Committee for Human Research of the Evandro Chagas Institute (report number 280.087).

The serological and molecular results for hepatitis viruses A (HAV), B (HBV), C (HCV), and Delta (HDV) in the patients were obtained from the existing database. We evaluated serum samples from (1) patients with a previous positive or inconclusive serological results for HEV infection (n = 158); (2) patients with clinical and laboratory findings of acute hepatitis of undetermined etiology and/or negativity for HAV, HBV, HCV, and HDV infection (n = 114); and (3) patients with a specific demand for hepatitis E virus infection tests (n = 46).

All samples were screened for anti-HEV IgM and IgG antibodies using enzyme-linked immunosorbent assays (ELISAs) recomWell HEV IgM and recomWell HEV IgG kits (Mikrogen GMBH, Neuried, Germany). Positive samples were submitted to a confirmatory test using a recombinant immunoblot test (RIBT) using RecomLine HEV IgM/IgG (Mikrogen). The commercial kits were used according to the manufacturer's instructions (and contained recombinant antigens of genotypes 1 and 3 of HEV, corresponding to viral variants from endemic and non-endemic regions).

Additionally, 250 µL of each of the 318 samples was subjected to total RNA extraction using the phenol/chloroform/guanidine isothiocyanate method with the Trizol™ LS reagent (Thermo Fisher Scientific, Waltham, MA, USA) following the manufacturer's instructions. The extracted RNA was stored at −70 °C until further use.

HEV-RNA was detected using quantitative real-time reverse transcription polymerase chain reaction (RT-qPCR) using QuantiFast Pathogen RT-PCR + IC kit® (QIAGEN, Valencia, CA, USA) according to the manufacturer's guidelines. The final reaction volume was 25 µL, containing 2 µL of sample RNA, with primers (0.4 µM) and the TaqMan® probe (0.2 µM), as described previously[12].

The RT-qPCR assays were carried out in a Rotor-Gene Q (QIAGEN) thermocycler for 20 min at 50 °C (RT step) and 5 min at 95 °C (PCR activation), followed by 45 cycles of denaturation at 95 °C for 15 seconds and annealing/extension at 60 °C for 30 seconds.

The standard curve used in RT-qPCR assays was developed through the cloning (using TOPO® TA Cloning®, Thermo Fisher Scientific) from a swine HEV-positive stool sample (genotype 3) from the Brazilian Eastern Amazon[11]. The quantification standard curve was constructed using nine ten-fold serial dilutions (10^9–10^0 copies/mL).

The RT-qPCR assays were performed and results were analyzed together with the standard curve in duplicate, for the absolute quantitation of HEV-RNA in the samples, combined with a negative control (NC), positive control (PC, swine genotype 3 HEV), and no template control sample (NTC).

Additionally, the first World Health Organization (WHO) International Standard (IS) for Hepatitis E Virus RNA Nucleic Acid Amplification Techniques (NAT)-based Assay (Paul-Ehrlich Institute) was included as a control in all experiments. This IS contained 250,000 IU/mL of HEV-RNA (genotype 3a) and was subjected to automated RNA extraction in an EZ1 Advanced XL (QIAGEN) in four serial dilutions (1:10, 1:100, 1:1000, and 1:10000).

Among the 318 patients, 51% (162/318) were female, and the average age was 30.5 years (range ± SD; 0–84 ± 20,21). The overall ELISA seroprevalences of anti-HEV IgM and IgG were 5.0% (16/318) and 9.1% (29/318), respectively. Individuals aged between 16 and 30 years old had a higher seroprevalence of anti-HEV IgM antibodies (1.5%; 5/318), and individuals aged between 31 and 45 years had a higher seroprevalence of anti-HEV IgG (3.7%; 12/318).

Based on the RIBT, the prevalence of anti-HEV IgM was 3.4% (11/318), and that for anti-HEV IgG was 5.9% (19/318) (**Table 1**). No samples were positive for both antibody classes.

All samples were negative for HEV-RNA. The detection limit of the standard curve obtained by cloning was 100 copies/mL, while the detection limit using the standard curve obtained from the IS ten-fold serial dilutions was up to the 1:1000 dilution, containing 250 IU/mL. Software analysis of the RT-qPCR assays determined that the coefficients of efficiency and reproducibility were greater than 90%.

We found low seroprevalences for anti-HEV IgM and IgG, which were similar to previous serological surveys in riverine communities[7], children[8], and rural populations[9] from the Amazon. In the present study, the occurrence of HEV infection was investigated in patients with clinical and/or laboratory suspicion of HEV infection in the Brazilian Amazon region.

In contrast to the low seroprevalence of HEV, a higher seroprevalence (>80%) for HAV infection is frequently described in the Brazilian Amazon[7-9]. Total anti-HAV antibodies were detected in six cases that presented anti-HEV IgG and one

TABLE 1: Distribution of the population by sex for anti-HEV IgM and IgG positive cases detected by ELISA and RIBT suspected cases of HEV infection from the Eastern Brazilian Amazon from 1993 to 2014.

Sex	Tested samples (%)	Anti-HEV IgM (%)		Anti-HEV IgG (%)	
		ELISA*	RIBT¥	ELISA	RIBT
Male	156 (49)	8 (2.5)	6 (1.8)	16 (5.0)	10 (3.1)
Female	162 (51)	8 (2.5)	5 (1.5)	13 (4.1)	9 (2.8)
Total	318 (100)	16 (5.0)	11 (3.4)	29 (9.1)	19 (5.9)

***ELISA:** enzyme-linked immunosorbent assay; **¥RIBT:** recombinant immunoblot test.

case that was positive for anti-HEV IgM, suggesting serological evidence of previous co-exposure to HAV and HEV, which is similar to findings from studies in other Latin American countries[3]. Co-infection with HAV and HEV suggested that the routes of exposure to both viruses might be similar in the Amazon. This result also indicated that HEV co-infection in acute HAV cases might occur; therefore, its epidemiological significance requires further investigation.

Echevarría et al.[3] indicated that HEV isolates have not yet been detected and characterized among Amerindian and isolated rural communities, although there is serological evidence of HEV transmission in these populations. Our results confirmed three anti-HEV-positive cases among Amerindians of the Parakanã ethnicity from an isolated rural community in the Pará state, suggesting recent and past exposure to HEV. This finding may be associated with the consumption of game or pork meat and/or exposure to possible unknown wildlife HEV reservoirs[2,3], and requires further investigation.

The use of the confirmatory RIBT test after ELISA for anti-HEV IgM and IgG positive has been observed to reduce the numbers of false positive cases. Five cases were determined as positive for anti-HEV IgM by ELISA but were not confirmed by RIBT. The reduction in the number of anti-HEV IgM positive cases after the use of confirmatory RIBT can be up to 50% in anti-HEV IgM cases[13], which highlights the importance of complementary tests to confirm a diagnosis of acute hepatitis E made using serological tests.

Additionally, we consider that because the commercial kits available for serological diagnosis of HEV have important differences in their sensitivity and specificity, seroprevalence results obtained only using screening tools should be interpreted carefully [13].

HEV-RNA was not detected, even among the 11 acute phase samples confirmed by RIBT. This result may be related to the absence of HEV-RNA, low viral load, and/or HEV-RNA degradation in the samples caused by: 1) The short viremia period of HEV infection, which in acute cases occurs predominantly in the pre-icteric stage and disappears with the onset of symptoms[2]; 2) the long period of serum storage (up to 21 years)[14]; and 3) the previous exposure of patients to low HEV viral loads resulting in subclinical infections and seroconversion[2].

Our results indicated that HEV has a low circulation rate even between suspected cases in the Eastern Amazon. Among three other studies conducted in Brazil that characterized HEV-RNA sequences and indicated a low molecular prevalence, only one positive case was reported in 64 patients with acute non-A, non-B, non-C hepatitis from the state of Rio de Janeiro[4], one case among 2,271 tested samples of patients from a laboratory in the state of São Paulo[6,15], and three positive samples among 96 tested samples from renal transplant recipients from the state of São Paulo[5]. The authors were unable to determine whether HEV infection was the primary or secondary cause of acute liver failure (ALF) but a recent study has indicated that HEV is not a common cause of ALF in North America[14].

Non-detection of HEV-RNA was not related to technical failure, because we used strict efficiency controls in the RT-qPCR assays, which amplified both the standard curves obtained by cloning and the WHO IS effectively and reproducibly. The detection sensitivity of the RT-qPCR assay and quantification of the tenfold dilutions of WHO IS were similar with other in-house and commercial tests used for the molecular detection of HEV in human samples. Hence, the RT-qPCR method used in the present study was demonstrated as suitable for use.

The low prevalence of HEV infection among patients with clinical and/or laboratory suspicion of hepatitis E corroborates with previous data suggesting low levels of HEV circulation in the Brazilian Amazon. However, a diagnosis of HEV infection must be considered in the differential diagnosis of acute and chronic viral hepatic diseases, especially in cases that the most frequent causes have been excluded. Broader serological surveys covering healthy individuals and patients with acute and chronic liver disease should be developed to better characterize the epidemiology and the impact of on public health in the Amazon region and Latin America.

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Conflict of Interest: The authors declare that there is no conflict of interest.

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