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ORIGINAL ARTICLE

Serological and molecular markers of hepatitis E virus infection in HIV-infected patients in Brazil

A. C. Ferreira[1] · Michele Soares Gomes-Gouvêa[1] · G. Lisboa-Neto[2] · M. C. J. Mendes-Correa[2] · C. M. Picone[3] · N. A. Salles[4] · A. Mendrone-Junior[4] · F. J. Carrilho[1] · J. R. R. Pinho[1,5]

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Abstract In Brazil, the circulation of hepatitis E virus (HEV) has been demonstrated in distinct groups of individuals and some animals, but its prevalence among individuals with human immunodeficiency virus (HIV) infection is unknown. This study aimed to assess the frequency of serological and molecular HEV markers in individuals infected with HIV from São Paulo, Brazil. Serum and plasma samples of 354 HIV-infected patients collected between 2007 and 2013 were included. All samples were tested for anti-HEV IgG and IgM antibodies and HEV RNA. Anti-HEV IgG and IgM antibodies were detected in 10.7% (38/354) and 1.4% (5/354) of the samples, respectively. Both antibodies were detected simultaneously in only two samples. HEV RNA was not detected in any sample. There was no significant correlation of anti-HEV serological status (positivity to anti-HEV IgG and/or IgM) with sex, age, $CD4^+$ T cell count, HIV viral load, antiretroviral therapy, liver enzyme levels, or coinfection with hepatitis B virus and/or hepatitis C virus. Our study provides serological evidence of past and recent HEV infections in HIV-infected patients from São Paulo, Brazil. However, the occurrence of ongoing HEV infection appears be a rare event in this population.

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✉ Michele Soares Gomes-Gouvêa
gomesmic@yahoo.com.br

[1] Laboratorio de Gastroenterologia e Hepatologia Tropical LIM/07, Instituto de Medicina Tropical da USP, Departamento de Gastroenterologia, Hospital das Clinicas HCFMUSP, Faculdade de Medicina, Universidade de Sao Paulo, Av. Dr. Enéas Carvalho de Aguiar, 500, Sao Paulo, SP 05403-000, Brazil

[2] Laboratorio de Virologia LIM/52, Instituto de Medicina Tropical da USP, Departamento de Doenças Infecciosas e Parasitarias, Hospital das Clinicas HCFMUSP, Faculdade de Medicina, Universidade de Sao Paulo, Sao Paulo, SP, Brazil

[3] Departamento de Doenças Infecciosas e Parasitarias, Hospital das Clinicas HCFMUSP, Faculdade de Medicina, Universidade de Sao Paulo, Sao Paulo, SP, Brazil

[4] Fundação Pró-Sangue Hemocentro de São Paulo, São Paulo, Brazil

[5] Hospital Israelita Albert Einstein, São Paulo, SP, Brazil

Introduction

Hepatitis E virus (HEV), initially identified in 1983, is a single-stranded, non-enveloped RNA virus that belongs to the genus *Orthohepevirus* of the family *Hepeviridae*. HEV can be classified into seven genotypes (1–7); genotypes 1–4 infect humans, while genotypes 3 and 4 are also found in other mammals, particularly pigs [56]. The main route of HEV transmission in highly endemic regions is fecal-oral. In industrialized countries, zoonotic transmission through the consumption of raw or undercooked meat of HEV-infected animals accounts for most HEV transmissions [2, 32].

Until recently, HEV was thought to cause only acute and self-limiting disease; however, since mid-2008, several cases of chronic hepatitis due to HEV infection have been observed in immunocompromised patients, such as human immunodeficiency virus (HIV)-infected subjects, organ transplant recipients, and patients with hematological disorders receiving chemotherapy [8, 22, 30, 36, 58]. Liver cirrhosis has also been described as an outcome of chronic HEV infection in these patients [19, 22, 48, 55].

In general, HEV genotype 3 has been identified in these cases, but two cases involving different genotypes were recently reported: genotype 4 was described in a child with acute lymphoblastic leukemia [18], and genotype 7 was found in a liver-transplant recipient from the United Arab Emirates [38].

The epidemiological mechanisms involved in coinfection of HEV and HIV are poorly understood. Additionally, the clinical implications of HEV infection in HIV-infected patients require further analysis to better understand the outcome of this coinfection [13].

Studies of HEV prevalence among HIV-infected individuals carried out in different countries have reported rates of anti-HEV IgG ranging from 1% to 45% [13]. Some studies revealed a greater frequency of this serological marker among HIV patients than that in other populations, such as blood donors, but the studies results have been controversial. The large discrepancies found in HEV seroprevalence are associated with the studied region, habits of the local population, and different sensitivities and specificities of the serological kits used [13, 16, 49, 52, 54].

HEV infection was described recently in Brazil in renal and liver transplant recipients, demonstrating its circulation among immunocompromised individuals patients [26, 45]. However, among HIV carriers, the epidemiology of HEV is unknown, and therefore, this study aimed to assess the frequency of serological and molecular HEV markers in an HIV-infected population from São Paulo, in the Southeast region of Brazil.

Materials and methods

Study population

In this study, we analyzed serum and plasma samples from 354 HIV carriers who regularly attended the AIDS Outpatient Clinic of the Clinics Hospital, University of São Paulo School of Medicine and Infectology Institute Emílio Ribas, São Paulo, Brazil.

Demographic and clinical data [age, sex, alanine and aspartate aminotransferase (ALT/AST) levels, $CD4^+$ T cell count, HIV viral load, and antiretroviral treatment] were retrieved from the patients' medical records.

The status of Hepatitis B virus (HBV) and Hepatitis C virus (HCV) coinfections was also obtained from these records. When this information was unknown, the samples were tested for HBV (HBsAg, anti-HBs, and anti-HBc) and HCV (anti-HCV) serological markers by enzyme-linked immunosorbent assay (ELISA; MONOLISA HCV Ag–Ab, Bio-Rad, Hercules, CA, USA; ARCHITECT Anti-HBc II, Anti-HBs and HBsAg Qualitative II, Abbott, Wiesbaden, Germany).

The ethics committees of the involved institutions approved this study. All participants gave written informed consent prior to enrollment in the study.

Laboratory procedures

To detect IgM and IgG anti-HEV antibodies in serum samples, commercial ELISA kits (RecomWell HEV, Mikrogen GmbH, Neuried, Germany) were used. Immunoblot analysis using RecomLine Kits (Mikrogen GmbH) was performed to confirm the ELISA results. Samples showing anti-HEV IgG and/or anti-HEV IgM borderline or isolated anti-HEV IgM positivity in ELISA were tested with RecomLineHEV IgG and IgM. All assays were performed according to the manufacturer's guidelines.

The occurrence of ongoing HEV infection was investigated in all patients by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Plasma samples (200 µL) were subjected to RNA extraction using a QIAmp® MinElute® Virus Spin Kit (QIAGEN, Hilden Germany) according to the manufacturer's instructions. Viral RNA was eluted in 60 µL of elution buffer, and 5 µL was used for amplification by one-step real-time PCR using the kit QuantiFast Pathogen RT-PCR + IC (QIAGEN) and primers and a TaqMan probe described previously [28] that target the highly conserved ORF3 region.

The 95% detection limit was calculated by probit analysis using 12 replicates of serial dilutions of the WHO international standard (6329/10, Paul Ehrlich Institute, Germany) to give 25,000, 2,500, 250, 200, 150, 100, 50, 25 and 2.5 HEV RNA international units (IU)/mL. The limit of detection (LOD) predicted for this PCR was 240 IU/mL (95% confidence interval: 173–513).

Patient samples were tested in triplicate with negative controls included in each run in addition to serial dilutions of the HEV reference standard.

Statistical analysis

Categorical data were described as absolute and relative frequencies. Continuous data are shown as the median and interquartile range. Pearson chi-square test or Fisher's test was performed to compare proportions. Continuous variables were evaluated using the nonparametric Kruskal-Wallis or Mann-Whitney test as appropriate. Two-tailed p-values were calculated and considered statistically significant if $p < 0.05$. Analyses were conducted using SPSS software, version 15.0 (SPSS, Inc., Chicago, IL, USA).

Results

A total of 354 HIV-infected patients were included in the study, with 93 (26.3%) monoinfected and 261 (73.7%) coinfected: 40.1% HIV/HBV (n = 142: 69 HBsAg-positive, 63 anti-HBc/anti-HBs-positive, and 10 with isolated anti-HBc), 19.5% HIV/HCV (n = 69), and 14.1% with triple infection HIV/HBV/HCV (n = 50: 17 HBsAg-positive, 17 anti-HBc/anti-HBs-positive and 16 with isolated anti-HBc).

The patients were divided into groups according to monoinfection or coinfection status to analyze demographic, clinical, and laboratory data (Table 1).

Most of the patients were male (71.5%), and males were more frequently coinfected with HBV or HBV/HCV than coinfected with HCV or monoinfected with HIV ($p < 0.001$). The median age of the enrolled subjects was 48 years (range: 19–76 years). The median $CD4^+$ T cell count was 530.5 cells/$mm^{3,}$ and 275 individuals (89.3%) had values above 200 cells/mm^3. Only 43 of 261 patients (16.5%) had detectable HIV RNA in the serum. Two hundred fifty-eight subjects (97%) were on antiretroviral therapy. There was no significant difference in the distribution of any variables between the four mono/coinfection groups (Table 1).

The median ALT and AST serum levels were 28 IU/mL (range: 8–249 IU/mL) and 27 IU/mL (range: 11–180 IU/mL), respectively. ALT and AST levels were higher in HIV/HCV- and HIV/HBV/HCV- coinfected patients ($p < 0.001$) (Table 1).

Anti-HEV IgG antibodies were detected in 38 patients (10.7%), and IgM antibodies were identified in 5 patients (1.4%), both antibodies were detected simultaneously in only two samples. HEV RNA was not detected in any sample. According to different groups of patients (HIV monoinfected or coinfected with HBV and/or HCV), the prevalence of anti-HEV IgG was 6.4% (6/93) in HIV monoinfected subjects, 10.1% (7/69) in HIV/HCV-coinfected subjects, 14.1% (20/142) in HIV/HBV-coinfected subjects, and 10% (5/50) in HIV/HBV/HCV-coinfected subjects. There was no difference in the frequency of isolated IgG anti-HEV between these four groups ($p = 0.321$). Concurrent positivity for anti-HEV IgG and IgM was only observed among HIV/HBV/HCV-coinfected patients, and isolated anti-HEV IgM was identified in two monoinfected patients and one HIV/HCV-coinfected patient.

No significant differences were observed when sex, age, $CD4^+$ T cell count, HIV viral load, antiretroviral therapy, and ALT/AST levels were evaluated with respect to presence or absence of the HEV serological markers anti-HEV IgG and/or IgM (Table 2).

Discussion

HEV infection in immunocompromised individuals has been implicated with chronic liver disease and several studies of HEV prevalence in HIV-infected patients have been conducted in different regions of the world [31, 33, 34, 55]. However, no studies have examined HEV infection among HIV-infected population in Brazil, where data regarding HEV seroprevalence remain limited. In the present study, we evaluated the prevalence of HEV infection (past and

Table 1 Demographic, clinical and laboratory data of HIV-infected patients according to monoinfection and coinfection status

Features	HIV monoinfected	HIV/HCV coinfected	HIV/HBV coinfected	HIV/HBV/HCV coinfected	Total	*p*-value[&]
Male gender (n, %)	48/93 (51.6)	39/69 (56.5)	127/142 (89.4)	39/50 (78)	253/354 (71.5)	<0.001[#]
Age (median, IQR)	48 (41-56)	46 (43-53)	47.5 (42-55)	47.5 (44-53)	48 (42-55)	0.878[$]
Age ≥ 40 years (n, %)	77/93 (82.8)	25/27 (92.6)	107/124 (86.3)	32/34 (94.1)	241/278 (86.7)	0.298[#]
CD4+ cel/mm^3 (median, IQR)	584 (359.2-772.7)	514 (383.5-706)	524 (340-754)	506 (306-872)	530.5 (348.2-760.5)	0.853[$]
CD4+ ≥ 200 cel/mm^3 (n, %)	84/92 (91.3)	54/61 (88.5)	102/115 (88.7)	35/40 (87.5)	275/308 (89.3)	0.896[#]
HIV viral load undetectable (<50 copies/ml) (n, %)	71/92 (77.2)	58/61 (91.8)	58/70 (82.9)	33/38 (86.8)	218/261 (83.5)	0.109[#]
HIV antiretroviral therapy (n, %)	68/68 (100)	57/59 (96.6)	100/104 (96.2)	33/35 (94.3)	258/266 (97)	0.352[#]
AST IU/L (median, IQR)	22 (17-26)	45 (29-74)	25 (19-34)	37 (26.5-60.5)	27 (20-39)	<0.001[$]
AST (>1.5x ULN) (n, %)	1/92 (1.1)	22/63 (34.9)	7/123 (5.7)	13/45 (28.9)	43/323 (13.3)	<0.001[#]
ALT IU/L (median, IQR)	23.5 (14-28.7)	50 (29-79)	27 (18-44)[c]	38 (28.5-59.5)	28 (19-49)	<0.001[$]
ALT (>1.5x ULN) (n, %)	2/92 (2.2)	25/63 (39.7)	13/123 (10.6)	12/45 (26.7)	52/323 (16.1)	<0.001[#]

Continuous data presented in median and interquartile range (IQR)

Categorical data expressed in absolute and relative frequency

ULN upper limit of normality

[&]Comparison between HIV-monoinfected and HIV/HCV-, HIV/HBV- and HIV/HBV/HCV-coinfected subjects

[$]Kruskal-Wallis test

[#]Pearson chi-square test

Table 2 Demographic, clinical and laboratory data of HIV-infected patients according to presence or absence of HEV serological markers

Features	Without markers	Positive serology*	*p*-value
Male gender (n, %)	225/313 (71.9)	28/41 (68.3)	0.632[#]
Age (median, IQR)	48 (42-54)	49 (44-58)	0.113[$]
Age ≥ 40 years (n, %)	212/246 (86.2)	29/32 (90.6)	0.781[#]
CD4+ cel/mm^3 (median, IQR)	534 (346.7-762.5)	516.5 (348.2-768.2)	0.476[$]
CD4+ ≥ 200 cel/mm^3 (n, %)	246/274 (89.8)	29/34 (85.3)	0.386[#]
HIV viral load undetectable (<50 copies/ml) (n, %)	191/232 (82.3)	27/29 (93.1)	0.187[#]
HIV antiretroviral therapy (n, %)	230/238 (96.6)	28/28 (100)	1.000[#]
AST IU/L (median, IQR)	26 (19-39)	29 (21.2-48.5)	0.271[$]
AST (>1.5x ULN) (n, %)	36/287 (12.5)	7/36 (19.4)	0.294[#]
ALT IU/L (median, IQR)	28 (19-49)	29.5 (19.2-49)	0.923[$]
ALT (>1.5x ULN) (n, %)	47/287 (16.4)	5/36 (13.9)	0.702[#]

Continuous data presented in median and interquartile range (IQR)

Categorical data expressed in absolute and relative frequency

ULN upper limit of normality

*Anti-HEV IgG and/or anti-HEV IgM

[$]Mann-Whitney test

[#]Pearson chi-square test or Fisher test

ongoing) in a large group of HIV-infected patients from São Paulo city (southeast region of the country) and found that 10.7% (38/354) of these individuals had serological evidence of infection (anti-HEV IgG) and 1.4% (5/354) showed detectable anti-HEV IgM (two with concomitant anti-HEV IgG). In contrast, no evidence of ongoing HEV infection was found among these individuals, as HEV RNA was not detected using nucleic acid amplification techniques.

The prevalence of anti-HEV IgG varies according to geographic region and the population being studied. Several studies also have detected differences in HEV prevalence in a specific group or region depending on the serological (ELISA) kits employed, showing great variability in their accuracy [35, 61].

Some studies have demonstrated that HIV-infected populations have a higher seroprevalence of anti-HEV IgG than that in the general population [3, 15]. However, this information is controversial. A recent meta-analysis based on European data from 2003 to 2015 found no significant difference in anti-HEV IgG frequency in the general population, blood donors, patients with liver disease, transplant recipients, and individuals with HIV [23].

In Brazil, the prevalence of HEV varies, and previous studies have shown that this variability depends on population/geographic features. Comparing data published before 2006, when the unique serological assay used in the studies was from Abbott Laboratories, the prevalence of anti-HEV IgG among blood donors from different regions of Brazil varied from 2% to 4.3% [4, 20, 44, 59]. However, the prevalence was much higher in different groups of individuals or patients, reaching 38% of the patients with acute hepatitis A [1, 20, 39, 44, 53, 57, 59]. Recently, new studies of HEV prevalence employing more-accurate serological kits (Wantai, Beijing, China) found a higher prevalence (~10%) among blood donors than previously observed (2–4.3%), suggesting that anti-HEV IgG prevalence in Brazil is underestimated [46, 47]. Therefore, it is difficult to assess whether the frequency of anti-HEV IgG among HIV-infected individuals in Brazil is higher than in other populations without risk factors related to HIV infection. To evaluate HEV prevalence in Brazil, it is necessary to perform studies in distinct regions of the country and include other groups from the same region using the same methods and employing more-accurate serological kits.

Moreover, the age of individuals included in the study should be considered, since several studies reported an increase in anti-HEV IgG prevalence with age [9, 13, 23]. Comparing our results with those of other studies in Brazil that used the same serological kit (Mikrogen), we observed that the prevalence of anti-HEV IgG among HIV individuals was higher (10.7%) than those described in two different populations (recyclable-waste pickers and patients with acute non-A, non-B, and non-C hepatitis) evaluated in Goiânia (5.1 and 5.7%) [17, 40]. This result can be related to the age of HIV individuals included in our study, who had a mean of age 48.4 ± 9.2 years, and most (86.7%) were older than 40 years, whereas most individuals evaluated in previous studies in Goiânia were younger and less than 40 years old.

Some risk factors and clinical data have been associated with HEV infection among HIV patients, but the results of different studies are conflicting [13]. We also found

no significant association between the presence of HEV serological markers and age, sex, $CD4^+$ T cell count, HIV treatment, HIV viral load, ALT/AST levels, and HBV and HCV coinfections.

We detected anti-HEV IgM (confirmed by immunoblot) in 1.4% (5/354) of HIV-infected subjects (two were also anti-HEV IgG positive), reflecting acute or recent infection. HEV RNA, which was expected to be detectable in acute infections, was not found. In acute HEV infection, viremia is transient and disappears from the blood within 3 weeks, just before the antibody titers reach a peak. However, virus spread through feces may persist for an additional two weeks, making stool samples a better option for diagnosing acute infection [10, 24]. It is possible that HEV RNA can be detected in stool samples of these patients, but feces were not collected.

The frequency of HEV RNA in HIV-infected patients without serological markers of HEV infection is unclear because most studies of this population did not evaluate these factors. Most studies investigated viremia only in cases with serological evidence of HEV infection (mainly in cases with detectable anti-HEV IgM). However, viremia in the absence of anti-HEV antibodies has been found in some cases [12, 29, 34, 37]; therefore, we also investigated HEV RNA in samples from all individuals included in the present study. Among HIV-infected patients without HEV serological markers, we detected no HEV RNA, suggesting that in Brazil, active infection by HEV is uncommon in this population. Indeed, a low frequency or the absence of HIV cases with detectable HEV RNA without anti-HEV IgG and/or IgM is commonly observed in other countries: in England, none of the 138 HIV-infected individuals evaluated had detectable HEV RNA [33]; in the United States, a study of HIV-infected individuals with acute increase in ALT levels detected HEV RNA in only one case [6]; in Iran, among a group of 100 HIV-infected individuals, HEV RNA was not detected [50]. Another study of 204 HIV-infected patients from Argentina found a positive PCR result for HEV in one individual [12]. Among a French group of 55 HIV patients with low CD4 count (<200 cells/mm^3) and elevated ALT levels, HEV RNA was not detected [41]. Additionally, no HEV viral load was detectable in a group of 86 HIV immunocompromised patients from Spain [51]. In a large cohort study consisting of 1544 HIV-positive adults from Ghana and Cameroon, HEV RNA was not detected [16]. Another large study analyzed HEV viremia by high-throughput nucleic acid testing of 2919 plasma samples collected from HIV-infected women and men from the United States and found only three positive cases [37].

In conclusion, our study demonstrates that, among HIV-infected individuals from São Paulo, Brazil, (1) serological markers of HEV infection are frequently found, but it remains unclear whether this frequency is higher than that in other populations without HIV; (2) no significant differences were observed between the presence of HEV serological markers and age, sex, $CD4^+$ T cell count, HIV treatment and viral load, ALT/AST levels, and HBV and HCV coinfections; (3) detection of anti-HEV IgM with undetectable HEV viral load suggests the occurrence of recent infection and therefore circulation of HEV among this population; and (4) the occurrence of ongoing HEV infection appears be a rare event among HIV-infected individuals.

In Brazil, several studies observed circulation of HEV by HEV RNA detection among pigs from different regions of the country [7, 11, 14, 21, 43, 60]. More recently, HEV contamination was detected in pork products (pâté and blood sausage) sold to consumers in the southern region of Brazil [25]. Therefore, although our results demonstrate that, in Brazil, ongoing infection by HEV is uncommon among HIV individuals, some recommendations should be made considering the evidence of HEV genotype 3 circulation in our country: (1) Frequent investigation of HEV infection markers (anti-HEV IgM/IgG and HEV RNA) should be carried out, particularly in HIV-infected patients with low $CD4^+$ T cell count, because this is reported to be a risk factor associated with chronic HEV infection [5, 8, 27, 29, 34, 37, 42, 55]. (2) HEV infection should be considered in cases of HIV-infected patients with an unexplained increase in ALT levels, as persistence of HEV infection was observed in an HIV patient with restored $CD4^+$ T cell count [27].

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Compliance with ethical standards

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Conflict of interest João Renato Rebello Pinho is an employee of Albert Einstein Medicina Diagnóstica, São Paulo, Brazil. All other authors who took part in this study declare that they have no conflicts of interest or disclosures with respect to the manuscript.

Ethical approval All procedures performed in this study were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Declaration of Helsinki and its later amendments or comparable ethical standards.

Informed consent All participants provided written informed consent prior to enrollment in the study.

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