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Hepatitis E Virus Genotype 1 and Hepatitis A Virus Dual Infection in Pediatric Patients with a Low Socioeconomic Status from Mexico

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Abstract
Objective: We aimed to detect and characterize hepatitis E virus (HEV) RNA in sera samples from a pediatric population infected with the hepatitis A virus (HAV) exhibiting acute hepatitis and to correlate the infection status with the clinical outcome. ***Methods:*** Seventy-five ELISA-positive samples from children containing anti-HAV and anti-HEV IgM were used to amplify and characterize partial regions within HEV ORF2. A statistical comparison of clinical data between HEV IgM-positive/HEV RNA-positive patients and HEV IgM-positive/HEV RNA-negative patients was performed. ***Results:*** Thirteen out of 75 IgM-positive samples provided amplification of discrete regions of the HEV genome. Nested RT-PCR-based detection and subsequent sequencing of 5 samples confirmed the identity of HEV genotype 1 (G1), which had not been previously reported in Mexico. Though not significant, a trend towards exacerbated clinical manifestations was found in HEV RNA-positive patients relative to HEV RNA-negative patients. ***Conclusions:*** An elevated rate of G1 RNA was detected. Hepatitis E seems to be a neglected disease in Mexico and epidemic strains of HEV are likely to play a role as causative agents of acute hepatitis in highly exposed children. Although HAV is endemic in Mexico, an HEV-RNA detection rate of 17% in co-infected samples shows the need for screening for HEV as a part of future vaccination strategies.

Introduction

Hepatitis E virus (HEV) is a leading causative agent of acute hepatitis globally, with a high incidence in developing countries [1]. The pediatric population is not typically considered an at-risk population despite the suboptimal sanitation conditions and the low income that prevails in these countries. Altogether, these factors may promote infection in this population.

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HEV is mainly transmitted via the fecal-oral route, and it is unique among hepatitis viruses because it is zoonotic and infects animal reservoirs (mainly pigs and wild boars) [1, 2]. Infection results in an enteric self-limiting hepatitis, but it could also potentially lead to fulminant liver failure in pregnancy and chronic hepatitis in immunocompromised patients [2]. Most studies on HEV have been historically conducted in adults, although studies in HEV-infected patients support the notion that children with altered transaminases should be tested for HEV [3, 4]. However, the information about clinical manifestations in pediatric acutely HEV-infected patients is still limited.

HEV is a small non-enveloped particle with a size of 27–32 nm with a single-stranded positive-sense RNA genome of 7.2 kb that encodes 3 overlapping open reading frames (ORF), i.e., ORF1, ORF2, and ORF3 [1]. HEV belongs to the *Orthohepevirus A* species (Hepeviridae family), and sequences infecting humans are classified into 5 main genotypes (G). G1 and G2 have exclusively been found in humans and are common in developing countries, where they are responsible for sporadic infections and large outbreaks in endemic areas. G3 and G4 are zoonotic viruses and have been detected in humans and animals such as pigs, deer, and boars. G3 circulates in industrialized and in developing non-endemic countries and G4 has been described in Asia and a few European countries [5]. On the other hand, G7 is zoonotic, it has been detected in humans and camels, and it is widely distributed in several African and Asian countries [6]. To date, the limited sequences of G5, G6, and G8 detected in other mammals have not been associated with infection in humans.

In Latin America, several countries, including Uruguay, Cuba, and Mexico, have reported the circulation of HEV [1, 2]. Many of these countries have also reported molecular studies on HEV of G1 and G3 exclusively detected in adult patients. However, data regarding the clinical features and molecular epidemiology of HEV in pediatric populations is scarce. In Mexico, detection of HEV has been reported in adults and swine [2, 7]. The epidemic strains from G2 were first described in 1992 in Mexico, and no additional detection of this genotype in the country has been reported since then [2]. In addition, G3 has been also reported from animal reservoirs in the country [8], and in a recent study we found a high frequency of anti-hepatitis A virus (HAV) and anti-HEV IgM antibodies in pediatric patients with acute hepatitis [9]. Thus, hepatitis E could represent an insufficiently attended hepatic infection in Mexico that may cause up to 12% of acute viral hepatitis cases, with no etiological agent detected, in which pediatric populations living in poor and non-sanitary conditions may be an at-risk group. This study has 2 goals. First, to detect and genetically characterize HEV-RNA in serum samples from low-income pediatric patients with serologic markers for HEV and HAV clinically diagnosed with acute hepatitis and, second, to analyze and compare clinical features of HEV infection among viremic and non-viremic patients in order to investigate to what extent hepatitis E might influence the clinical outcome resulting from HAV infection, which is the main cause of liver disease in children in Mexico.

Materials and Methods

Seventy-five serum samples, obtained from pediatric patients (aged less than 16 years) with acute hepatitis, diagnosed through clinical symptoms, abnormal alanine aminotransferase (ALT), aspartate aminotransferase (AST), and direct bilirubin (D. Bil) values, and positivity for anti-HEV IgM and anti-HAV IgM antibodies [9], were retrospectively selected to look for HEV viremia through RNA detection. Excluded from this study were sera from patients with liver disease who were undergoing treatment with a hepatotoxic drug, those with acute or chronic hepatitis B or C infections, and those diagnosed with autoimmune hepatitis. The samples collected between 2015 and 2016 were obtained from the Centro de Referencia de Hepatitis Virales del Occidente de México, Servicio de Biología Molecular at the Hospital Civil de Guadalajara Fray Antonio Alcalde (HCGFAA), a tertiary health care center located in Western Mexico, which serves patients with limited economic resources and poor sanitary conditions. Clinical and sociodemographic data and risk factors associated with HEV infection were retrospectively analyzed. Serum samples were tested for the presence of the HEV genome. RNA was extracted using a QIAamp® Viral RNA Kit (Qiagen, Germany), and HEV infection was confirmed by a nested reverse transcription PCR (RT nested PCR) amplification of a conserved sequence within the HEV ORF 2/3 overlapping region [10]. Following confirmation, 2 additional RT nested PCR methods were used for amplification targeting regions of 330 [11] and 728 bp [12] within ORF2. Phylogenetic analyses and tree reconstruction were performed with MEGA v6 software.

A comparison of clinical data between HEV IgM-positive/HEV RNA-positive patients relative to HEV IgM-positive/HEV RNA-negative patients was performed. Statistical analyses were done using GraphPad software version 5.01 (GraphPad Software, San Diego, CA, USA). Continuous values were reported as means ± SD. Demographic and clinical data were reported as simple frequencies. Statistical differences were evaluated by applying χ^2 and Mann-Whitney U tests. $p < 0.05$ was considered statistically significant.

This study was approved by the Ethical Committee of Research of the HCGFAA and the Centro Universitario de Ciencias de la Salud, Universidad de Guadalajara.

Table 1. Clinical and demographic characteristics of the patients

Feature	HEV IgM+		*p* value
	HEV RNA (–)	HEV RNA (+)	
Patients, *n* (%)	62 (82.7)	13 (17.3)	–
Age, years	7.5 ± 3.6 (1–16)	7.8 ± 3.1 (1–16)	ns
Female/male ratio (%)	33/29 (53.2–46.8)	6/7 (46.1/53.9)	ns
ALT, IU/L	1,116 ± 840.9 (3–3,303)	1,348 ± 110.6 (75–3,389)	ns
AST, IU/L	812 ± 767.7 (29–3,501)	1,049 ± 977 (58–2,753)	ns
D. Bil, mg/dL	3.8 ± 3.1 (0.1–14.4)	4.5 ± 3.1 (0.08–11.3)	ns
Hepatomegaly, %	93.4	100	ns
Acute liver failure, %	0	0	ns
Nausea, %	89.1	100	ns
Vomiting, %	89.1	75	ns
Fever, %	91.3	100	ns
Jaundice, %	67.3	90	ns

Values are presented as percents or means ± SD (range) unless otherwise stated. ALT, alanine aminotransferase (abnormal values >41 IU/L); AST, aspartate aminotransferase (abnormal values >38 IU/L) D. Bil, direct bilirubin (abnormal values >0.3 mg/dL); HEV, hepatitis E virus; ns, not significant.

Results

Overall, the children included in this study belonged to a socioeconomic context where individuals, including their parents, have basic/null levels of education and a low income (data not shown). Thirteen out of 75 (17.3%) serum samples were positive for the presence of HEV RNA. Both HEV RNA-positive and HEV RNA-negative patients exhibiting IgM anti-HEV antibodies showed abnormal values of AST/ALT enzymes and D. Bil. No significant differences in clinical features (symptoms and severity of disease) were found among viremic and non-viremic patients for HEV. However, hepatomegaly, nausea, fever, and jaundice tended to be more frequent in HEV RNA-positive patients relative to HEV RNA-negative ones. On the other hand, a slight trend towards increased AST, ALT, and D. Bil values was found in HEV RNA-positive patients (Table 1).

In 3 of the 13 samples in which HEV RNA was detected, the 330-bp amplicon within ORF2 could be amplified and sequenced, while the 728-bp region close to the genome 3′ end was amplified and sequenced from 2 other samples (GenBank accession No. MF039701 to MF039705). Phylogenetic analyses showed that the HEV strains identified in this study clustered within G1 with high bootstrap values (Fig. 1a, b). The nucleotide identity between these isolates and other strains from G1 ranged from 97.2 to 99.8%. Furthermore, a very close phylogenetic relationship was observed between the Mexican strains and Indian isolates (AF076239 and AF459438) (98.8–99% nucleotide identity) and between the Mexican strains and Cuban sequences (EU284748 and EU284749) (98.5–99%).

Discussion

Over the last few decades, the epidemiological scenarios of HEV and HAV have been changing, and accurate epidemiological data regarding the prevalence of anti-HEV and anti-HAV antibodies and infection/co-infection rates are still scant [13]. HEV is not commonly tested as a causative agent of hepatic disease during childhood, and hepatic sickness is mainly associated with HAV infection. Diagnosis of HEV is clinically difficult since hepatitis E is often indistinguishable from the liver disease caused by HAV. In addition, the epidemiological features and dynamics of HEV-HAV dual infection in pediatric patients is poorly understood, and the lack of systematic screening for HEV infection markers in this group probably leads to underestimation of the co-infection rates [14]. In adult populations, by contrast, co-infection of HEV and HAV seems to be a more common event and many data from outbreaks or cross-sectional studies have been reported from developing regions [15–17].

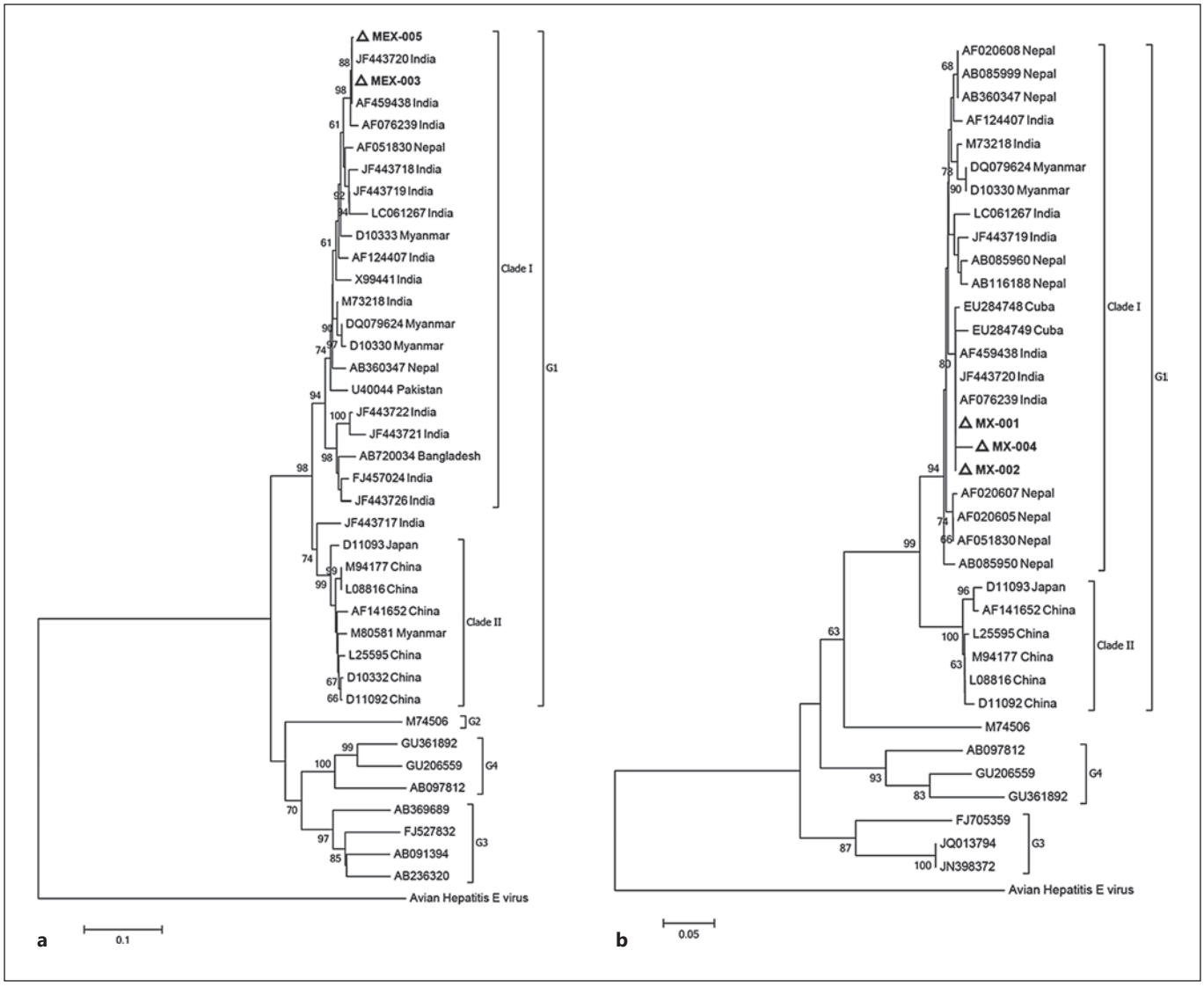

Fig. 1. Phylogenetic trees constructed based on the partial 297-nt region within ORF2 (nucleotides 6007–6303 in the reference strain SAR-55) containing sequences identified as MX-003 and MX-005 (**a**), and the 645-nucleotide sequence of the ORF2 3 end (nucleotides 6468–7113 in the reference strain SAR-55) containing sequences identified as MX-001, MX-002 and MX-004 (**b**). Trees were generated using the neighbor-joining algorithm with Tamura-Nei as the best substitution model as tested by the ModelTest v3.7 tool. The robustness of the trees was determined by bootstrap for 1,000 replicates. Only values ≥60% are shown. Mexican sequences are highlighted in bold and with open triangles.

This study, performed with a relatively small number of tested individuals, showed nonsignificant differences in clinical features among HEV RNA-positive and HEV RNA-negative patients. This remarkable observation suggests that, as in adults, infection of HEV in children might also be neglected, since it could appear completely masked by an HAV-associated liver disease. HAV is not included in the vaccination schedule in Mexico, where the infection is endemic, being the main cause of acute hepatitis in children [18]. However, the results presented here show that sporadic cases of acute hepatitis E are not rare. In this sense, the ESPGHAN Hepatology Committee recently recommended repeated testing for HEV in immunosuppressed children with increased aminotransferases (or in children with persistently-increased liver enzymes) since they may require therapeutic intervention

[4]. The elevated rate of viremic patients infected with HEV detected in this study supports the notion that HEV-RNA should be screened for in the differential diagnosis of pediatric patients with abnormal transaminases and clinical manifestations of liver disease. On the other hand, besides the nonsignificant differences among both groups of patients included in this study, a trend towards pronounced clinical disease (increased values of transaminases and D. Bil and increased frequencies of hepatomegaly, nausea, fever, and jaundice) was found in viremic patients infected with HEV. As previously reported, we found 4 samples to be HEV-positive and HAV-negative. Those samples were included in this study; however, no sequences were obtained. In addition, no significant differences in clinical or demographic characteristics were found in these patients relative to HAV mono-infected patients, supporting the notion of HEV infection as a neglected disease.

Even though the hepatic disease in the children included in this study could have been mainly caused by HAV, data presented here suggest that HAV/HEV co-infection might result in an exacerbated clinical outcome. In fact, dual infection with HEV and other hepatotropic viruses has been previously studied in pediatric patients through the analysis of serological markers [14]. Results from Zaki et al. [14] suggested an association of co-infections involving HEV with a greater elevation of AST and ALT. However, further research is necessary to shed light on this possibility.

Surprisingly, in our study HEV G1 was the main one identified in viremic children. However, the possibility of co-infection with strains with a minor viral load from other genotypes should not be ruled out. Partial sequencing of the 2 regions within viral ORF2 exhibited a very high percentage of nucleotide identity among the strains. Even though all of the patients had anti-HEV IgM, larger regions within ORF2 could be amplified in a fraction of them. This inconsistency has been previously observed associated with important differences in the performance of serological and PCR assays [19, 20]. In addition, noteworthy differences in the sensitivity and efficiency of detection have been observed among different HEV genome regions [21].

Based on a retrospective analysis of the clinical records, no evidence of time clustering was found, so the possibility that these isolates from Mexico were associated with outbreaks was ruled out. In addition, according to the epidemiological records, no history of traveling outside of Mexico or contact with animal reservoirs was identified (data not shown). The clustering of G1 isolates from Mexico and Cuba and with Indian strains from the Hyderabad and Haryana region is a remarkable finding. A similar high genetic relatedness between Cuban and Indian HEV isolates has been reported [22]. Of note, a limited set of variants from G1 seems to be circulating in the Americas, and all of the reported isolates show a high degree of relatedness [23]. This is the first report of G1 in Mexico, thus making the country unique in terms of circulation of HEV strains belonging to the G1, G2, and G3 genotypes.

In conclusion, the data presented herein suggest that, among pediatric patients, particularly in those with a low socioeconomic status, HEV may be playing a central role in the new epidemiological scenarios of enterically transmitted hepatitis viruses. A high HEV-RNA detection rate, with the predominance of epidemic strains of G1, should be a matter of major concern for public health and mandates HEV screening. Finally, a systematic study and a deeper knowledge of HEV and HEV/HAV-associated clinical-pathologic manifestations will enable clinicians to better handle this underdiagnosed infection.

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Disclosure Statement

The authors declare that they have no conflict of interests.

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