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Hepatitis Delta virus genotype 8 infection in Northeast Brazil: Inheritance from African slaves?

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ABSTRACT

Hepatitis Delta virus (HDV) is endemic worldwide, but its prevalence varies in different geographical areas. While in the Brazilian Amazon, HDV is known to be endemic and to represent a significant public health problem, few studies have assessed its prevalence in other regions in the country. This study evaluated the seroprevalence of HDV among HBsAg chronic carriers from Maranhão state, a region located in the Northeast of Brazil. Among 133 patients, 5 had anti-HD, of whom 3 had HDV RNA. HDV genotypes were characterized by Bayesian phylogenetic analysis of nucleotide sequences from the HDAg coding region. HDV-3 was identified in one patient who lives in Maranhão, but was born in Amazonas state (Western Amazon basin). Phylogenetic analysis shows that this HDV-3 sequence grouped with other HDV-3 sequences isolated in this state, which suggests that the patient probably contracted HDV infection there. Surprisingly, the other two patients were infected with HDV-8, an African genotype. These patients were born and have always lived in Urbano Santos, a rural county of Maranhão state, moreover they had never been to Africa and denied any contact with people from that continent. This is the first description of the HDV-8 in non-native African populations. This genotype may have been introduced to Brazil through the slaves brought to the country from the West Africa regions during the 16–18th centuries. Our results indicate that the need of clinical and epidemiological studies to investigate the presence of this infection in other areas in Brazil.

1. Introduction

Hepatitis Delta virus (HDV) is a highly pathogenic virus that causes acute and chronic liver diseases. HDV is found only in individuals positive for the hepatitis B virus (HBV) surface antigen (HBsAg) because the virus is defective and needs a helper function provided by HBsAg that is used as its own envelope protein for particle assembly and viral transmission across the human host (Wedemeyer and Manns, 2010).

The prevalence of HDV infection varies according to the geographical area. Around 15 million HDV carriers are estimated worldwide (Rizzetto, 2009). In Brazil, HDV is endemic in some areas of the Amazon region where it is associated with severe forms of the disease (Bensabath and Dias, 1983; Bensabath et al., 1987; Gomes-Gouvea et al., 2008, 2009). In the Amazon region, anti-HD antibodies can be found in up to 34% of HBsAg carriers (Fonseca et al., 1988). Except for the studies done in this endemic region, studies on HDV prevalence in Brazil are scarce (Ferraz et al., 1985; Oliveira et al., 1999; Strauss et al., 1987).

HDV genomes isolated from around the world show 39% divergence over the entire RNA genome. This HDV diversity is currently classified into eight genotypes named HDV-1 to HDV-8. Distinct clinical courses are associated with different HDV genotypes: HDV-1 has been associated with a broad spectrum of pathogenicity, HDV-2 and HDV-4 cause milder forms of liver disease, and HDV-3 has been associated with outbreaks of fulminant hepatitis. The pathogenicities of genotypes 5–8 are not well known.

HDV-1 is geographically widespread (Shakil et al., 1997), but all other genotypes are closely associated with specific geographic areas. HDV-2 and HDV-4 are found in East Asia (Ivaniushina et al., 2001; Lee et al., 1996; Sakugawa et al., 1999); HDV-3 has been isolated in the northern area of South America only (Amazon Basin of Brazil, Peru, Colombia, and Venezuela) (Casey, 1996; Casey et al.,

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1993; Gomes-Gouvea et al., 2008; Quintero et al., 2001); and HDV-5 to HDV-8 have been identified in individuals from Africa (Le Gal et al., 2006; Makuwa et al., 2008, 2009; Radjef et al., 2004).

In this study we describe the presence of HDV infection in chronic HBsAg carriers from a region in Brazil other than the HDV endemic area and report for the first time the infection with HDV genotype 8 in non-native African populations.

2. Materials and methods

2.1. Patients

A total of 133 patients with chronic hepatitis B virus infection (HBsAg positive for at least 6 months) and followed at the Center for Liver Studies, University Hospital of the Federal University of Maranhão, Brazil, between January 2008 and February 2010 were enrolled in this study. Blood samples were collected and the sera separated and stored at −70 °C.

Patients' demographic and clinical data were retrieved from their medical files. All patients were from Maranhão, a state in the Northeast of Brazil. Most of them (69.2%) were from São Luís, the state capital, and the remainder were from different regions in the state (Fig. 1).

The status of chronic hepatitis B was defined according to the following criteria:

(1) *Inactive carrier*: positive HBsAg, negative HBeAg, positive anti-HBe, normal alanine aminotransferase (ALT) levels, and HBV DNA below 2000 UI/mL.
(2) *Chronic hepatitis B*: positive HBsAg, positive HBeAg, high ALT levels or positive HBsAg, negative HBeAg, positive anti-HBe, and HBV DNA higher than 2000 UI/mL.
(3) *Hepatic cirrhosis*: positive HBsAg with positive or negative HBeAg regardless of the viral load, signs or symptoms of advanced hepatic disease (ascite and/or presence of esophageal varices), or histological diagnosis of hepatic cirrhosis.
(4) *Immunotolerance*: positive HBsAg and HBeAg with normal ALT levels.

This study was approved by the local institution ethics committee and all patients provided informed consent.

2.2. Serological tests

All serum samples were tested for hepatitis B serological markers (HBsAg, total anti-HBc, anti-HBs, HBeAg, anti-HBe) and HDV antibody (anti-HD) using enzyme-linked immunosorbent assay (ELISA) kits (DiaSorin, Italy).

2.3. HDV RNA and HBV DNA amplification and sequencing

Viral nucleic acids were detected by PCR in all samples that were both positive for HBsAg and anti-HD. For this, HBV DNA and HDV RNA were initially extracted using QIAamp DNA mini kit and QIAamp Viral RNA viral mini kit (Qiagen, Germany), respectively, according to the manufacturer's instructions.

A fragment of the delta antigen genomic region (403 nucleotides) was amplified by nested polymerase chain reaction (PCR), as previously described (Gomes-Gouvea et al., 2008). HBV DNA detection was performed by a quantitative in-house real-time PCR assay (sensitivity = 50 UI/mL) (Sitnik et al., 2010). HBV genotypes were characterized by amplifying a nested PCR 1306 nucleotide fragment partially comprising HBsAg and the DNA polymerase coding regions (S/POL) using primers PS3132F/2920R and PS3201F/P1285R. To avoid false-positive results, the precautions and procedures suggested by Kwok and Higuchi (1989) were strictly followed.

Amplified PCR products were purified using ChargeSwitch® PCR Clean-Up Kit (Life Technologies, USA). The sequencing reactions were carried out from both strands using inner primers and fluorescence-labeled dideoxynucleotide chain terminators using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). The sequences were determined in an automated DNA sequencer (ABI Prism 3100 Genetic Analyser, Applied Biosystems, USA). The quality of each electropherogram was evaluated using the Phred-Phrap software and consensus sequences were obtained by alignment of both sequenced strands (sense and antisense) using CAP3 software available at the web page *Electropherogram quality analysis Phred* (http://asparagin.cenargen.embrapa.br/phph/).

Sequences were submitted to EMBL/GenBank/DDBJ under accession numbers JF298898–JF298900 (HDV sequences), and JF298901–JF298903 (HBV sequences).

2.4. Phylogenetic analyses

Genotyping classification for HBV and HDV was performed on the basis of phylogenetic reconstructions using representative reference sequences from different HDV genotypes ($n = 105$) and HBV subgenotypes ($n = 281$) obtained from the GenBank public database (http://www.ncbi.nlm.nih.gov/) (data available upon request). Sequences were aligned using CLUSTAL_X and edited using BioEdit software. Bayesian phylogenetic analyses were conducted using the Markov Chain Monte Carlo (MCMC) simulation implemented in BEAST v.1.6.1 (Drummond and Rambaut, 2007). HDV and HBV datasets were analyzed under relaxed uncorrelated lognormal molecular clock using the model of nucleotide substitution (GTR + G + I) obtained previously by Modeltest v3.7 (Posada and Crandall, 1998) and 10 million generations were sufficient to obtain the convergence of parameters. The maximum clade credibility (MCC) tree was obtained from summarizing the 10,000 substitution trees and then 10% of burn-in was removed using Tree Annotator v.1.6.1 (Drummond and Rambaut, 2007).

3. Results

Altogether, 133 samples from chronic HBsAg carriers were screened for the presence of antibodies against HDAg by serology. Among these patients, 69 (51.9%) were women and 64 (48.1%) were men, their mean age was 40.4 years (ranging from 13 to 84 years) and in terms of race (based on skin color), 66.2% were brown (*pardo*), 18.8% were black, and 15% were white. Sixty-nine (52%) patients were inactive carriers, 39 (29.5%) had chronic hepatitis, 15 (11%) had cirrhosis, and 10 (7.5%) were in the immunotolerance phase.

Out of 133 samples, 5 (3.8%) were positive for anti-HD and HDV RNA was detected in 3 (60%) of these anti-HD positive samples. All anti-HD positive patients showed detectable HBV viral load. HBV-DNA levels, HBeAg and anti-HBe status, HBV and HDV genotypes, and clinical/epidemiological data of anti-HD positive patients are summarized in Table 1.

A phylogenetic tree constructed with sequences of the partial delta antigen genomic region of HDV RNA is shown in Fig. 2A. The phylogeny showed that from the three sequences characterized in this study, one clustered with HDV-3 and two with African HDV-8. The HDV-3 sequence obtained herein closely grouped with other HDV-3 sequences isolated in the Amazonas state (Western Amazon Basin).

The two HDV-8 sequences from Maranhão state in Brazil showed 78.8–87.7% (mean 83.5%) similarity with the other ten HDV-8 African sequences previously described and 89.4% with