Characterization of the Genotypic Profile of Hepatitis Delta Virus: Isolation of HDV Genotype-1 in the Western Amazon Region of Brazil

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Key Words

Hepatitis delta virus · Genotype-1 · Sequencing · Phylogenetic analysis · Clinical analysis

Abstract

The hepatitis delta virus (HDV) is a hepatotropic subvirus that is dependent on the hepatitis B virus (HBV) and supplies the viral envelope containing the surface antigen of hepatitis B. Viral genetic diversity is related to the geographical origin of the isolates, and there are at least eight genotypes that are referred to as HDV-1 through HDV-8. HDV-3 is responsible for epidemics of severe and fulminant hepatitis, which are common in northeastern South America. HDV-3 is prevalent in the Brazilian Amazon and is associated with the increased aggressiveness of HDV infections. Although isolated, the characteristics of the clinical presentation of HDV-1 in the Amazon region have not yet been clearly reported. **Objective:** This study aims to assess the genotypic and clinical characteristics of individuals with the HDV-1 genotype in the western Amazon region. **Methods:** The HDV was genotyped by nested PCR-RFLP and sequencing from serum samples of 56 patients with HBV/HDV infection. The genotypes were correlated with the clinical characteristics presented by patients with HBV/HDV infection. **Results:** A prevalence of 92.3% for the HDV-3 genotype (n = 48) and 7.6% (n = 4) for the HDV-1 genotype was

observed. **Conclusion:** To date, this is the most extensive clinical study of HDV-1 genotype infections in the nonindigenous population of Western Amazonia.

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Introduction

The hepatitis delta virus (HDV) is a hepatotropic subvirus that is dependent on the hepatitis B virus (HBV) and supplies the viral envelope containing the surface antigen of hepatitis B (HB_sAg) [1, 2]. The HDV virion has a ribonucleoprotein that is composed of a circular ssRNA of approximately 1.7 kb encoding a single viral protein, the delta antigen (HDAg). The HDAg is a phosphoprotein that is found in 2 forms: a short version referred to as HDAg-S (24 kDa, 195 amino acids) that plays an important role in replication [3] and a long version referred to as HDAg-L (27 kDa, 214 amino acids) that promotes the packaging of HDV RNA [4–9].

The HDV genome is highly variable [10], with a variation of up to 16% within the same genotype and 20–40% between different genotypes [11–15]. This heterogeneity is located within the C-terminal region of the virus assembly signal in HDAg-L. However, the sequence of the ribozyme and the RNA-binding domain are highly con-

served [6, 14–16]. Viral genetic diversity is related to the geographical origin of the isolates, and there are at least eight genotypes which are classified as HDV-1 through HDV-8 [11–15, 17, 18].

There are 400 million chronic carriers of HBV worldwide, and over 20 million of these carriers show serological evidence of exposure to HDV [15, 19]. Regions with high levels of endemicity include Central and North Africa, the Amazon Basin, Eastern Europe, the Mediterranean, the Middle East, and parts of Asia [20]. HDV-1 is ubiquitous [21] and has been isolated in the USA, Europe, the Middle East, Russia, Africa, Asia, and Brazil [6, 7, 12, 18, 21–24]. HDV-2, formerly referred to as genotype-IIa, is found in Japan, Taiwan and Russia [11, 13, 25]. HDV-3 has been isolated in the Amazon region of Peru, Colombia, Ecuador, Venezuela, and Brazil [12, 23, 26, 27]. HDV-4, formerly referred to as genotype-IIb, is found in Taiwan and Japan [13, 25, 28]. HDV-5, HDV-6, HDV-7, and HDV-8 genotypes are found in Africa [17, 18]. Recently, HDV-8 was isolated from 2 native individuals in the countryside of the state of Maranhão (Brazil) [29]. HDV-3 is responsible for epidemics of severe and fulminant hepatitis, which are common in northeastern South America [12]. HDV-3 is prevalent in the Brazilian Amazon and is associated with an increased aggressiveness of HDV infection [12, 23, 24, 26, 27].

PCR-RFLP (PCR-restriction fragment length polymorphism), which is a variation of conventional PCR in which amplicons are treated with restriction enzymes, is frequently used for HDV genotyping [13, 30–34]. RFLP analysis is typically performed using electrophoresis with agarose or polyacrylamide [35]. HDV genotyping can also be performed using sequencing of the amplicons. The sequences generated are then compared with known sequences of HDV deposited in databases in order to determine the evolutionary lineage of the viral isolates by using phylogenetic analysis and to identify the genotypes of the isolates [6, 17, 18, 21, 26, 27, 36, 37].

There is a potential relationship between the HBV and HDV genotypes and the clinical outcome of patients. HDV-3 infection is associated with severe clinical features of HDV [12, 38]. The production of viral particles in HDV-3 infections is approximately 20-fold greater than that with HDV-1. The level of editing is approximately 2-fold less for HDV-3 compared with HDV-1, and lower levels of editing are consistent with a more aggressive infection [12]. It is thought that infection with HDV-3 and HBV genotype-F could result in an aggressive clinical course [39]. Similarly, this relationship could extend to the response to treatment, as with other types of hepatitis.

Materials and Methods

Clinical Specimens

Serum samples from 54 patients with HBV/HDV infection (with detectable HDV RNA and HB_sAg) [40] were obtained from the Research Center for Tropical Medicine of Rondônia – CEPEM. A control group of 30 donors who were negative for HIV-1, HIV-2, HB_sAg, anti-HBc, and anti-HCV (hepatitis C virus) was used. Serum samples from 5 individuals with chronic HCV mono-infection and 5 individuals with chronic HBV mono-infection were also tested. This study was approved by the research ethics committee of CEPEM (approval No. 33/10 CEP/CEPEM; registration No. 110/2010).

Clinical Analysis of Patients

Clinical and epidemiological data were obtained from the medical records of outpatients that were compiled by specialists. All information was stored in a database that was developed solely for this research. The results of laboratory tests were extracted from medical records and stored similarly. The following test results were used: hematological tests (hematocrit level, leukocyte count, platelet count, and determination of prothrombin time), biochemical tests (levels of aspartate transaminase, alanine transaminase and unconjugated and conjugated bilirubin, as well as levels of total protein, albumin, alkaline phosphatase, and γ -glutamyl transferase), imaging (ultrasound of the abdomen), examination of liver biopsy (for patients with indication), and serological markers for HBV, HCV and HDV, all following international standards.

HDV RNA Extraction

HDV RNA extraction was performed using QIAamp Viral RNA Mini Kit (Qiagen, Germany) with 200 μ l of serum, according to the manufacturer's instructions. The precipitated RNA was resuspended in 50 μ l of elution buffer. To avoid false-positive results, strict procedures that are proposed for nucleic acid amplification diagnostics were followed [41].

Reverse Transcription

The HDV RNA was denatured at 95°C for 5 min and converted to cDNA by using 15 μ l of extracted RNA, 200 U of M-MLV (Moloney murine leukemia virus) enzyme (Sigma Aldrich, Saint Louis, Mo., USA), 1 μ l of 10 $\times$ M-MLV reverse transcriptase buffer, 1 μ l of random primer, and 1 μ l of 10 mM dNTP Mix. The temperature conditions for cDNA synthesis were as follows: 70°C for 10 min, 37°C for 50 min and 94°C for 10 min.

HDV PCR and Nested PCR

To characterize the genotypes of HDV, PCR was performed using 10 μ l of cDNA in 40 μ l of PCR Mix with 0.2 mM dNTPs, 1.5 mM MgCl₂, 1 $\times$ enzyme buffer, 2 U of Taq DNA polymerase (Invitrogen, Waltham, MASS., USA), and 25 pmol of 853IU (5'-CGGAT GCCCAGGTCGGACC-3') and 1302OD (5' GGATTCACCGA CAAGGAGAG-3'), which were previously described [37] and are known to amplify a 450-bp fragment of the HDV genome corresponding to a portion of HDAg-L. Nested PCR was performed using 5 μ l of the PCR products generated as described above and 45 μ l of PCR Mix with 25 pmol of HDV-E (5'-GAGATGCCATGC CGACCCGAAGAG-3') and HDV-A (5'-GAAGGAAGGCCCTC GAGAACAGA-3'), which were previously described [12] to amplify a 406-bp fragment. The following cycling conditions were

Table 1. Genotypic profile generated by digestion with SmaI and XhoI to obtain a 406-bp fragment that corresponds to HDAg-L

	Length of enzyme restriction fragments	
	SmaI, bp	XhoI, bp
HDV-1	225+181	388+18
HDV-3	300+106	24+382
HDV-8	225+181	315+91

used: 2 min at 94°C, 36 cycles of 30 s at 94°C, 50 s at 55°C, and 45 s at 72°C, with a final stage of extension of 5 min at 72°C. Results of the amplification were analyzed using electrophoresis with 1.5% agarose gel.

Restriction Fragment Length Polymorphism

The RFLP technique was used for genotyping [31]. Briefly, 15 µl of the amplicons was digested overnight at 30°C using a digestion mix containing 10 U of SmaI (Invitrogen) and 1× enzyme buffer to a final volume of 20 µl. The amplicons were then digested overnight at 37°C using a digestion mix containing 10 U of XhoI (Invitrogen) and 1× enzyme buffer to a final volume of 20 µl. The resulting restriction fragments were analyzed using electrophoresis with 3% agarose gel (table 1).

Sequencing and Phylogenetic Analysis

A total of 12 samples that were identified using nested PCR-RFLP as HDV-3 and 4 samples that were identified as HDV-1 were selected for sequencing. These samples were sequenced at the Laboratory Center for Genomics, São Paulo, Brazil to confirm the results obtained using nested PCR-RFLP. Phylogenetic analyses were performed using the Molecular Evolutionary Genetics Analysis software, MEGA5 [42]. The evaluation, processing, editing, and production of consensus sequences were performed following the Phred-Phrap-Consed method [43, 44], using CodonCode Corporation 5.0 software (CodonCode Corporation, Centerville, Mass., USA). The consensus sequences were aligned using Clustal X [45, 46] with 241 HDV sequences deposited in GenBank (NCBI). To elucidate the evolutionary lineage of the isolated HDV sequences, the maximum likelihood method based on the Hasegawa-Kishino-Yano replacement model [47] was used to generate a phylogenetic tree. The bootstrap consensus tree was inferred from 1,000 replications [48, 49].

Results

Viral Genotyping

Nested PCR-RFLP

Of the 54 samples analyzed using PCR-RFLP, 52 samples from patients with HBV/HDV infection were determined to be positive for HDV RNA, and 2 samples were determined to be negative. All samples from the control group were determined to be negative. Of the 52 positive

samples, a prevalence of 92.3% for the HDV-3 genotype (n = 48) and 7.6% for the HDV-1 genotype (n = 4) was observed.

Phylogenetic Analysis

The results of a phylogenetic analysis of 16 sequenced samples confirmed the genotypic profiles obtained using PCR-RFLP (fig. 1).

Clinical Description

Analysis of patient records suggested that 4 patients were members of the HDV-1 genotype group and were positive for HB_eAg, anti-HDV and HDV RNA. All patients were HB_eAg negative and anti-HBe positive. The group consisted of 3 women and 1 man, with an average age of approximately 41 years. None of the patients was indigenous. In detail, 1 patient had a history of transfusion, and 2 had previously undergone surgeries; these patients were symptomatic, and 2 had splenomegaly. The mean hemoglobin rate was 13.3 g/dl and the mean platelet rate was 51.806 per mm³ of blood; 3 patients had a platelet count below 50,000 per mm³ of blood. The average transaminases values were 52 and 53 U/l for aspartate transaminase and alanine transaminase, respectively. A total of 3 patients exhibited symptoms of chronic liver disease, as determined using an abdominal ultrasound, and they had esophageal varices detected during an endoscopy. Using the METAVIR system, the liver biopsy for 1 patient exhibited a fibrosis value of 2 and an activity value of 1.

In a recent study published by Vasconcelos et al. (2012) [50], we described features of hepatitis delta patients with genotype-3 followed up by our outpatient service. Of the selected patients with regular follow-up, 64.9% were male, with a mean age of 32 years (6–57). Household contact with chronic HDV carriers was significant, as reported in 46.7%. Only 20% of the patients were considered Amerindian. Leukopenia was detected in 36.7%, and alanine transaminase was above the upper normal level in more than half of the patients. Only 9.1% of the coinfecting patients had positive HB_eAg, and 55.8% had liver biopsy, with 16.4% cirrhosis and 20.9% F0 on the METAVIR scale.

Discussion

Performing HDV genotyping using sequencing followed by phylogenetic analysis and/or PCR-RFLP [13, 31, 34] is an established technique. The results of HDV geno-

typing obtained using nested PCR-RFLP were consistent and reliable, as evaluated by sequencing and phylogenetic tree construction. The nested PCR-RFLP was validated by comparing with both sequencing and phylogenetic analyses, which indicates the reliability of this method for determining the genotype of HDV isolates in the Amazon region. These results permit us the autonomy to genotype using sequencing; however, there is no platform for sequencing available in the state.

Here, high endemicity for HDV infection was observed in the study population. This might be related to the high rate of endemicity for HBV in the region [51] and the presence of an indigenous population. Previous studies have indicated a high prevalence of HDV infection in Amerindian people from the Amazon region [23, 24, 38, 52].

A prevalence of 92.3% was observed for the HDV-3 genotype. The results from nested PCR-RFLP analysis were further evaluated using sequencing data from 16 samples (12 HDV-3 and 4 HDV-1 samples). The HDV-3 isolates indeed aligned with global sequences of HDV-3. Similar results were obtained for the HDV-1 isolates.

These results corroborate those of previous studies that have shown a higher prevalence of these genotypes in the Amazon region and have demonstrated that they are commonly associated with severe hepatitis delta outbreaks [12, 23, 24, 27, 38, 51–54]. The HDV-1 genotype characterized in this study has been isolated from non-Amerindian individuals at a frequency of 7.6%. Previous studies have reported a prevalence of 55% for the HDV-1 genotype (22 out of 40 samples). However, only 4 of these patients were of Amerindian ancestry, and they were infected with HDV-3 in 3 cases and with HDV-1 in 1 case. Therefore, a vast majority of patients infected with HDV-1 were of nonindigenous descent [23]. Gomes-Gouvea et al. [27] (2008) examined the genotypes of 15 isolates and found only the HDV-3 genotype.

Although earlier studies established that HDV genotypes influence the natural course of hepatitis delta infection, the most recently identified genotypes (HDV-5 through HDV-8) remain poorly understood [15]. A study in Taiwan [55] suggested that a lower rate of remission and more adverse patient outcomes are associated with

Fig. 1. Molecular phylogenetic analysis using the maximum likelihood method. Taxons marked with a black triangle represent the sequences from this study that were aligned with known sequences from GenBank. Samples DST-0001 through DST-0012 belong to the HDV-3 genotype and samples DST-0013 through DST-0016 belong to the HDV-1 genotype.

the HDV-1 genotype compared with the HDV-2 genotype. Patients with the HDV-4 genotype frequently have mild liver disease [56]. However, a variant of the HDV-4 genotype in Japan is associated with increased progression to cirrhosis compared with that observed with the HDV-4 genotype in Taiwan [57].

Previous studies in patients with hepatitis delta in the state of Rondônia [58] have demonstrated a higher prevalence of the HDV-3 genotype in the Amerindian population, and in this instance no patients were indigenous. This study included young individuals with advanced chronic liver disease, since 3 of the 4 patients already showed signs of portal hypertension and evidence of

esophageal varices. Our results support the notion that HDV represents a public health problem in many countries worldwide, including in the western Amazon region of Brazil where the prevalence of HDV-3 is high. However, we cannot neglect the presence of the HDV-1 genotype, which is generally diagnosed late and determines advanced liver disease in young patients.

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