

Short
CommunicationHepatitis B virus and hepatitis delta virus genotypes
in outbreaks of fulminant hepatitis (Labrea black
fever) in the western Brazilian Amazon region

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The genotypes of hepatitis B (HBV) and delta (HDV) viruses circulating among fulminant hepatitis cases from the western Amazon Basin of Brazil were characterized in this study. HBV and HDV isolates were obtained from liver samples from 14 patients who developed fulminant hepatitis and died during 1978–1989. HBV DNA and HDV RNA were detected in all samples. Phylogenetic analyses of HDV sequences showed that they all clustered with previously characterized sequences of HDV genotype 3 (HDV-3). HBV genotypes F, A and D were found in 50.0, 28.6 and 21.4% of cases, respectively. These results confirm the predominance of HDV-3 in South America and its association with the severe form of hepatitis, and the finding of the co-infection of HDV-3 with different genotypes of HBV suggests that the association between HDV-3 and HBV-F is not necessarily causally related to a more severe clinical course of infection.

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The Brazilian western Amazon Basin is considered a highly endemic area for hepatitis B (HBV) and delta (HDV) viruses (Bensabath & Dias, 1983; Bensabath *et al.*, 1987; Braga *et al.*, 2001; Fonseca *et al.*, 1988; Viana *et al.*, 2005). Severe hepatitis cases with peculiar clinical and histopathological features have been reported in this region. Cases of fulminant hepatitis with similar features have been described in other countries in northern South America (Colombia, Venezuela, Peru and Ecuador) and in the Central African Republic. This severe form of liver disease has been identified as HBV and HDV super- or co-infection (Bensabath *et al.*, 1987; Buitrago *et al.*, 1986; Casey, 1996; Casey *et al.*, 1996; Hadler *et al.*, 1984; Lesbordes *et al.*, 1987; Ljunggren *et al.*, 1985; Manock *et al.*, 2000; Popper *et al.*, 1983; Sjogren & Colichon, 1991; Torres & Mondolfi, 1991).

The GenBank/EMBL/DDBJ accession numbers for the sequences reported in this paper are FJ010634–FJ010647 (HDV) and FJ010648–FJ010661 (HBV).

A supplementary table and two supplementary figures are available with the online version of this paper.

The disease associated with HDV infection is typically more severe than that due to HBV infection alone, but its clinical spectrum ranges from asymptomatic carriage of the virus to very severe disease. A factor that may influence the course of disease is the genetical heterogeneity of HDV prevalent in different geographical areas (Casey, 1996; Rizzetto & Durazzo, 1991; Smedile *et al.*, 1982). Only HDV genotype 3 (HDV-3) was identified in cases of fulminant hepatitis from different countries of South America and has been associated with the co-infecting HBV genotype F (HBV-F), which is also indigenous to South America, suggesting that HDV-3 alone or in combination with HBV-F could be an important determinant of the particularly severe form of HDV-related disease in this region (Casey, 1996; Nakano *et al.*, 2001a, b).

Bensabath *et al.* (1987) carried out a 5 year protocol during 1979–1984 in the Brazilian western Amazon Basin to study the epidemiology of HDV infection and its role in the aetiology of fulminant hepatitis. In that study, the authors observed a high endemicity of HBV and HDV infection and confirmed the presence of HDV as a major factor for

fulminant hepatitis development in this area (Bensabath *et al.*, 1987). Nevertheless, as HDV infection could not be demonstrated in all cases of hepatitis fulminant, its exact role in these cases remains unclear. In the present study, we investigated the presence of HDV by molecular techniques and characterized the circulating HBV and HDV genotypes among fulminant hepatitis cases from the Brazilian western Amazon Basin detected during 1978–1989.

Liver samples from 14 patients who developed fulminant hepatitis and died were collected in three contiguous municipalities on the Purus River in the western Amazon Basin of Brazil (Boca do Acre and Pauini, in Amazonas state, and Sena Madureira, in Acre state) (Fig. 1). All patients were positive for HBsAg; two were also positive for anti-HBc IgM, a serological pattern consistent with acute HBV infection. Only eight (57%) patients had evidence of HDV infection in serum (HDAg and anti-HD) and/or liver tissue (HDAg) as shown by serological and immunoperoxidase assays, respectively. Anti-HAV IgM and anti-HCV were negative in all samples. The serological patterns found in these cases suggested HDV superinfection of HBV chronic carriers in 12 cases and HDV/HBV co-infection in two cases. Supplementary Table S1 (available in JGV Online) shows the demographic, clinical and epidemiological aspects of the studied cases. This study received institutional review board approval.

The results of the serological tests were confirmed by using bioMérieux kits for HBV and HDV serological markers. HBV DNA and HDV RNA were extracted from frozen liver samples stored at -70°C with QIAamp DNA and RNeasy Mini kits (Qiagen), respectively, according to the manu-



Fig. 1. Geographical locations of the Boca do Acre, Pauini and Sena Madureira municipalities in the western Amazon Basin of Brazil.

facturer's instructions. HDV RNA was amplified as described previously (Gomes-Gouvêa *et al.*, 2008) and HBV DNA was amplified by using a set of primers that amplify a 734 bp fragment partially covering the overlapping DNA polymerase- and surface antigen-encoding genes. Primers used for HBV amplification were FHBS1 (Sitnik *et al.*, 2004) and RADE1M (5'-TGCRTCA-GCAAACACTTGGC-3'; nt 1175–1194) for the first round, and FHBS2 (Sitnik *et al.*, 2004) and RADE2M (5'-TGRCANACYTTCCARTCAATNGG-3'; nt 992–970) for the second round. The nucleotide position was based on the HBV-A sequence (GenBank accession no. M57663). The sequences of primers RADE1M and RADE2M were designed for this study. The PCR was carried out for 35 cycles of [94 °C for 30 s, 56 °C (first round) or 50 °C (second round) for 30 s, 72 °C for 1 min] after a step of 1 min at 94 °C. The amplified products were separated in a 2% agarose gel stained with ethidium bromide to confirm the expected length of the amplicon. Strict procedures for nucleic acid amplification diagnostic techniques were followed to avoid false-positive results (Kwok & Higuchi, 1989). Moreover, positive and negative controls were included in each set of reactions.

HBV DNA and HDV RNA were positive in all analysed samples. The nested PCR products were purified by using a ChargeSwitch PCR Clean-Up kit (Invitrogen). Cycle-sequencing reactions of purified PCR products were performed by using a BigDye Terminator kit v3.1 (Applied Biosystems) and the same primers as were utilized in the second-round PCR for HDV and the primers FHBS2 (sense), RHBS2 (antisense), 5'LAM5 (sense) (Da Silva *et al.*, 2001; Sitnik *et al.*, 2004) and RADE2M (antisense) for HBV, according to the manufacturer's instructions. Each amplicon was analysed in both sense and antisense directions. The sequences were read in an automated ABI Prism 377 DNA Sequencer (Applied Biosystems).

Sequences obtained were aligned by using CLUSTAL_X (Thompson *et al.*, 1997) with previously reported sequences retrieved from GenBank, resulting in alignments of 627 nt for HBV and 319 nt for HDV. Phylogenetic analyses for genotyping were estimated by using distance and maximum-likelihood (ML) approaches implemented in the PAUP* v4.0b package (Swofford, 2002). The evolutionary model of DNA substitution and parameters used (HKY+I+G for HDV and GTR+I+G for HBV) were estimated by MODELTEST v3.06 (Posada & Crandall, 1998).

Phylogenetic analyses of HDV sequences from fulminant hepatitis cases showed that all of them clustered with previously characterized sequences of HDV-3 from South America (Fig. 2). Considering all sequenced isolates from the Brazilian Amazon (western and eastern), we observed that they did not group in one specific, closely related cluster. In the tree obtained by using the neighbour-joining method, three main clusters are clearly observed: clusters

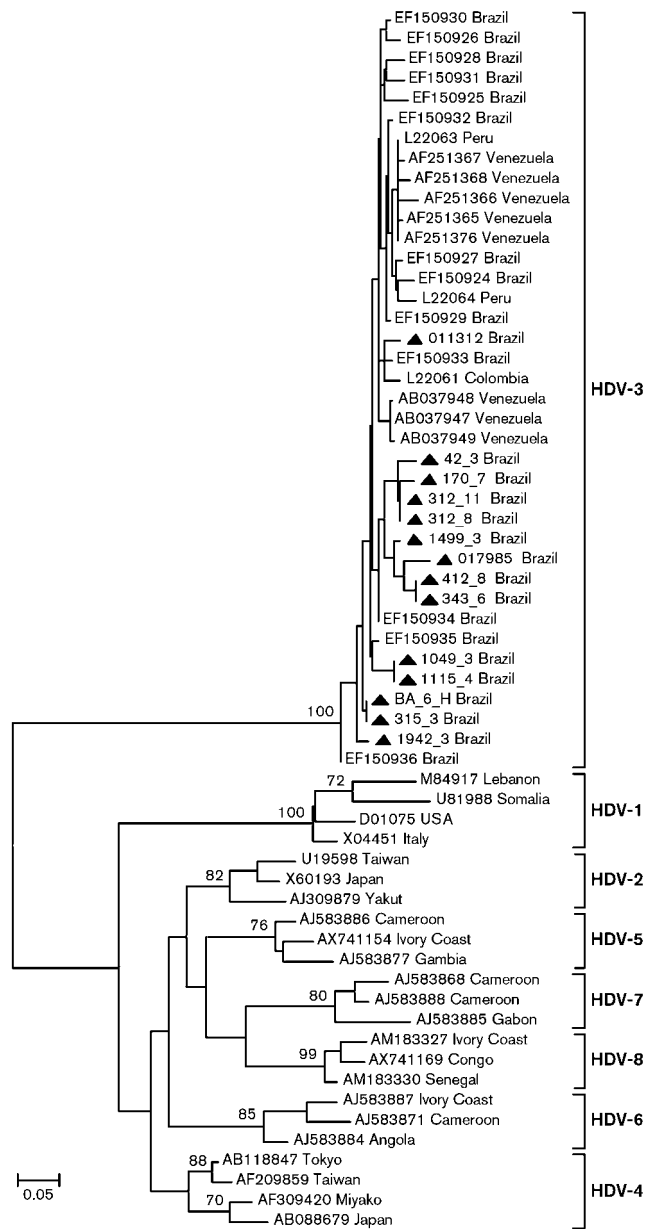


Fig. 2. Unrooted phylogenetic tree constructed by the maximum-likelihood method based on HDV sequences from this study (marked ▲) and from GenBank (denoted by accession no. followed by the country of origin). Percentages at nodes represent bootstrap values obtained with 1000 replications. Only values >70 % are shown. Genotypes are indicated.

with only Brazilian isolates, clusters with western Venezuelan and Colombian isolates and clusters with southern Venezuelan and Peruvian isolates (Supplementary Fig. S1a, b, available in JGV Online).

The phylogenetic trees show that some HDV sequences from patients of the same municipality of the western Amazon were grouped in different clusters. It is also observed in these trees that the fulminant cases occurring

in the same municipality in different years were caused by closely related viruses (Supplementary Fig. S1a, b, available in JGV Online).

The divergence in nucleotide sequence of HDV-3 from fulminant cases characterized in this study ranged from 0 to 7.4 % (4.7 ± 1.6 %, mean \pm SD). Analyses of HDV-3 nucleotide sequences from the Brazilian Amazon (eastern and western) show a divergence of 0–8.4 % (4.4 ± 1.4 %), while between all HDV-3 sequences from other South American countries (Peru, Colombia and Venezuela), this divergence was 0–6.6 % (2.9 ± 1.5 %).

The 19 aa sequences of the C terminus of the large HDAG (the packaging-signal sequence) were not completely conserved between HDV-3 isolates characterized in this study, as only 14 (73.7 %) of the 19 aa were conserved among them (Supplementary Fig. S2, available in JGV Online).

The tree constructed by phylogenetic analysis using the neighbour-joining (Fig. 3) and maximum-likelihood (data not shown) methods showed that HBV genotypes A, D and F were present in acute fulminant hepatitis cases that occurred in the western Brazilian Amazon. Genotype F was found in seven (50.0 %) cases, whereas non-F genotypes were found in the other 50 % of the cases: four (28.6 %) genotype A and three (21.4 %) genotype D. By using this dataset, it was also possible to assign subgenotypes within each genotype characterized in this study; subgenotypes A1, F2a, D3 and D4 were observed in Brazilian strains.

Our results confirmed that acute HBV and HDV co-infection and mainly acute HDV superinfection were the primary cause of severe hepatitis in the Brazilian western Amazon Basin, as has been observed in other countries of South America where outbreaks of severe hepatitis also occurred.

All HDV isolates from fulminant hepatitis cases that occurred in the western Amazon were classified as HDV-3. This result is consistent with other studies carried out in Peru, Colombia and Venezuela (Casey, 1996; Casey *et al.*, 1993; Nakano *et al.*, 2001b) and confirms the role of this genotype as the causal agent of outbreaks of fulminant hepatitis in South America.

The results of phylogenetic analysis showed that HDV-3 sequences from Brazil did not group as a specific, closely related cluster, as was observed with isolates from Venezuela (the country with the largest number of sequences characterized after Brazil), where HDV-3 grouped in specific clusters depending on the origin of the samples (northwest or south). These results and the higher nucleotide divergence observed between HDV-3 sequences from the Brazilian Amazon suggest that these isolates have a longer history within this region than HDV-3 characterized in other South American regions. This hypothesis needs to be confirmed by analysis of a larger number of HDV-3 sequences from other regions of South America where few HDV-3 sequences have been

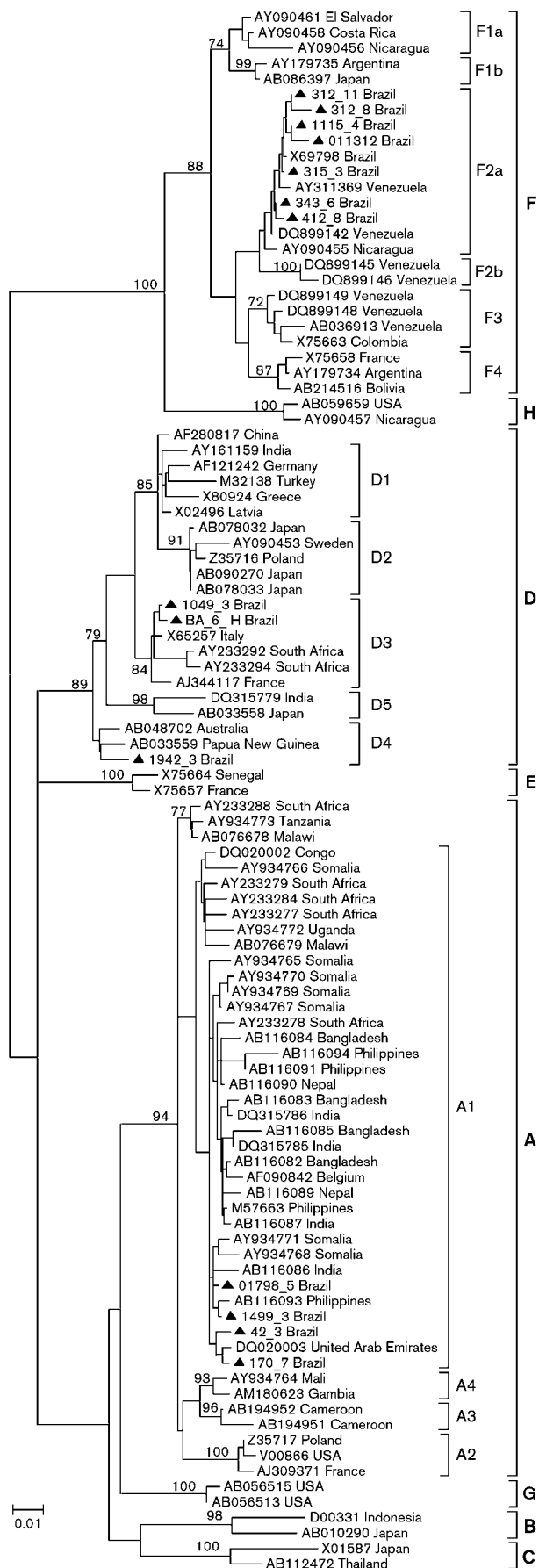


Fig. 3. Unrooted phylogenetic tree constructed by the neighbour-joining method based on HBV sequences from this study (marked ▲) and from GenBank (denoted by accession no. followed by the country of origin). Percentages at nodes represent bootstrap values obtained with 1000 replications. Only values >70% are shown. Genotypes and subgenotypes are indicated.

characterized. More extensive sampling of HDV-3 sequences, mainly full genomes, would be helpful in understanding the evolutionary history of HDV-3 in South America.

In the tree topology of HDV sequences, it was observed that isolates from patients of the same municipality of the western Amazon were grouped in different clusters; this pattern may represent the introduction of different virus populations with distinct origins or indicate that they have diverged from a common ancestor and are probably evolving independently. We also observed that one specific strain was involved in fulminant cases in the same municipality in different years, suggesting the continual transmission of this HDV strain. This is likewise suggested by the fact that some cases showed evidence of having been exposed to the same source of infection, as their sequences showed 100% nucleotide similarity and grouped in the same branch in the phylogenetic tree with strong bootstrap support.

Contrary to previous studies involving outbreaks of fulminant hepatitis among Yucpa Indians in Venezuela (Nakano *et al.*, 2001a, b) and military troops in the Amazon Basin region of Peru (Casey *et al.*, 1996), where HDV-3 was always associated with HBV-F, we found HBV-F in 50.0% of the cases, HBV-A in 28.6% and HBV-D in 21.4%, suggesting that the association between HDV-3 and HBV-F is not necessarily causally related to a more severe clinical course of infection. These results confirm that there is not a specific interaction between particular HBV and HDV genotypes and that co-infections reflect the most frequent genotypes found in a particular geographical area. Previous studies have also described co-circulation of HBV-A, -D and -F in the western Brazilian Amazon (Viana *et al.*, 2005; Victoria Fda *et al.*, 2008). Our results also are in agreement with previous *in vitro* studies carried out by Shih *et al.* (2008), who did not observe any influence of the different HBV genotypes on assembly and secretion of different HDV genotypes.

In the western Amazon, HDV infections were described to be associated with different clinical manifestations (Bensabath *et al.*, 1987). This difference in the pattern of evolution may be caused by (i) factors related to the genetic characteristics of HDV or specifically of HDV-3; (ii) characteristics of the HBV helper, mainly the structure and level of HBsAg; or (iii) genetic background of the host or altered immune response due to other chronic infections and other cofactors, such as poor nutrition, alcohol, environmental toxins etc.

Different HDV genotypes may be related to the different outcomes observed between patients from this region. HDV-1 was reported previously in Venezuela (Quintero *et al.*, 2001) and Amazonas state, Brazil (Parana *et al.*, 2006). However, the fact that we previously found HDV-3 associated with chronic infection (Gomes-Gouvêa *et al.*, 2008) and in this study with fulminant infection shows that there may be differences in the pathogenicity of the different HDV-3 isolates. Characterization of the HDV isolates associated with different clinical manifestations that occur in the Brazilian western Amazon may elucidate whether any genomic structure related to virus replication could explain this issue.

We found that the amino acid sequences of the C terminus of the large HDAg were not completely conserved between HDV-3 isolates from fulminant cases characterized in this study. We did not observe a particular pattern in this region associated with fulminant evolution, as some amino acid sequence patterns were similar to those of other HDV-3 isolates from Brazil (eastern Amazon) that are associated with chronic infection (Gomes-Gouvêa *et al.*, 2008). Hsu *et al.* (2002) reported that the differences in amino acid sequence of this region were reflected in variations in packaging efficiencies between HDV-1 and HDV-2 and among isolates of the same genotype. The authors suggested that these differences could at least partially explain the differences observed in disease outcome between patients infected with HDV-1 and those infected with HDV-2. The identification of similar patterns of amino acid sequence at the C-terminus region between HDV-3 isolates associated with chronic and fulminant infection suggests that this factor is not related to disease outcome, at least among these cases. Neither did we observe any relationship between a specific pattern of amino acid sequence at the C-terminus region and a particular genotype of HBV. Further studies are needed to clarify the role of the variation in the C terminus of the large HDAg in HDV-3 packaging efficiencies and in the ability to interact with HBsAg from different HBV genotypes.

In conclusion, these results confirming the predominance of HDV-3 in South America and its association with the severe form of hepatitis, and the finding of co-infections of HDV-3 with different genotypes of HBV, suggest that the association between HDV-3 and HBV-F is not necessarily causally related to a more severe clinical course of infection.

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