Influence of Hepatitis B Virus (HBV) Genotype on the Clinical Course of Disease in Patients Coinfected with HBV and Hepatitis Delta Virus

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Objective. We evaluated the influence of hepatitis B virus (HBV) genotype on the course of disease in patients coinfected with HBV and hepatitis delta virus (HDV).

Methods. We evaluated HBV genotypes in 190 patients, 140 of whom had chronic HBV monoinfection and 50 of whom had chronic HBV-HDV coinfection. Real-time polymerase chain reactions for the amplification of HBV DNA and HDV RNA were developed, and we compared the patient groups with respect to HBV genotype, viral load, alanine aminotransferase (ALT) and bilirubin levels, and disease severity.

Results. Coinfected patients had higher ALT and bilirubin levels as well as a higher prevalence of liver cirrhosis and liver carcinoma. ALT levels were higher among individuals coinfected with HDV and HBV genotype F than among individuals infected only with HBV genotype F. Among HDV-HBV–coinfected patients, HDV load was lower among those infected with HBV genotype A than among those infected with HBV genotype D or genotype F.

Conclusion. Liver inflammation and HDV load are influenced by HBV genotype in individuals coinfected with HBV and HDV.

Hepatitis B virus (HBV) genotypes A, D, and F have been found in the Amazon basin, and genotype F is the most prevalent among Amerindian populations [1, 2]. Studies have shown that coinfection with HBV F and hepatitis delta virus (HDV) genotype III has played a role in increasing the severity of the clinical manifestations of hepatitis found in this region [3, 4]. However, few studies have attempted to clarify the role of HBV genotypes in the clinical evolution of HBV-HDV coinfection [5]. In the present study, we analyzed and compared patients with HBV monoinfection and patients with HBV-HDV coinfection, evaluating viral genotypes, viral load, serologic results, and clinical characteristics.

Methods. From February 2003 through November 2006, we studied 190 patients with chronic HBV infection. We excluded patients who were coinfected with hepatitis C virus, human T cell lymphotropic virus, or human immunodeficiency virus. We also excluded patients who were receiving treatment with interferon or antiviral medication, patients with alcoholism, and patients who were users of illegal drugs. Participants were from the city of Manaus in the State of Amazonas, in northern Brazil. This study received institutional review board approval, and all participants provided written informed consent.

Of 190 participants, 140 (104 men and 36 women) had HBV monoinfection and 50 (32 men and 18 women) had HBV-HDV coinfection (defined as 2 consecutive serological tests positive for antibodies to HDV and HBV surface antigens). The time since diagnosis was defined as the interval in months between diagnosis of the infection and inclusion in the study. Subjects were divided into 4 diagnosis groups, defined as follows: (1) patients with asymptomatic carriage (AC), who had normal hematological, biochemical, and ultrasonographic parameters; (2) patients with chronic hepatitis with evidence of liver inflammation (CH), who had had elevated alanine aminotransferase (ALT) levels for >6 months, without evidence of liver cirrhosis; (3) patients with liver cirrhosis (LC), who had esophageal varices, splenomegaly, and thrombocytopenia or imaging findings consistent with the same; and (4) patients with hepatocellular carcinoma (HCC), as identified by computed tomography or magnetic resonance imaging.

For HBV genotyping, DNA was amplified with primers to the S gene. Subsequently, internal primers were employed to identify genotypes A–H [6–8]. HBV quantification was performed with real-time quantitative reverse-transcriptase polymerase chain reaction (qRT-PCR). The primers used (pre-S/S region) were 5’-CACA-CCTCCAATCTACCTGACAA3’ and 5’-ATATGATAAACGCGC-CAGACAC-3’, and the probe was 5’-FAM-TCTCTCAAATTTGTCTGGTTATCGCT-3’TAMRA.

For the HBV internal control, a partial sequence of the HBV genome was internally modified. After amplification and clon-
copies/mL), which were amplified by use of real-time qRT-PCR. The sensitivity and linearity of the HBV and HDV quantification assays were analyzed with DNA and RNA obtained from plasmids, and the levels of HBV DNA were standardized, in international units per milliliter, by use of the OptiQuant HBV DNA Quantification Panel.

qRT-PCR was used for quantitative detection of HDV RNA. The target region corresponded to nt 942–1032 [9], and the probe was 5′-FAM-CACCCTGGGACCCAGTAATACCGG-3′-TAMRA. For the HDV internal control, a partial HDV sequence was internally modified and amplified by use of qRT-PCR to produce synthetic DNA, and the product was subcloned. For detection of the internal control, the probe was 5′-HEX-GCGATGGTGACTTTGTGGATAGTATCCTCA-3′-TAMRA. For the positive control for HDV, a region corresponding to nt 883–1265 was amplified, subcloned, and purified.

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Plasmids containing the internal control and the positive control were reverse transcribed, and the final concentration was determined using spectrophotometry to calculate the number of copies. We prepared serial dilutions in triplicate (from 10¹ to 10⁶ copies/mL), which were amplified by use of real-time qRT-PCR to establish the calibration curve. Figure 1 shows the linear dynamic range of the real-time qRT-PCR assay designed to amplify HBV DNA and HDV RNA.

Results. The sensitivity and linearity of the HBV and HDV quantification assays were analyzed with DNA and RNA obtained from plasmids, and the levels of HBV DNA were standardized in accordance with World Health Organization guidelines [10]. The correlation coefficients for RNA and DNA, respectively, were, −0.98 and −0.99, whereas the respective efficiencies were 95% and 90%. The HBV DNA detection limit was from 5 × 10⁴ to 5 × 10⁷ IU/mL, compared with 1 × 10² copies/mL to 1 × 10⁶ copies/mL for HDV RNA. Independent experiments were performed, with 4 replicates of 2 dilutions from each standard curve. For HBV, we found the variation coefficient to be 1.4% in concentrations of 1 × 10⁴ IU/mL and 26.0% in concentrations of 1 × 10⁵ IU/mL. For HDV, the precision obtained in 4 replicates was 1.6% in concentrations of 1 × 10⁶ copies/mL and 28.4% in concentrations of 1 × 10⁷ copies/mL. In analysis of 100 samples, no false-positive results were observed in either assay (100% specificity).

In HBV-HDV–coinfected patients, the mean (±SD) interval between diagnosis and inclusion in the study was 15.8 ± 23.2 months, compared with 18.3 ± 30.8 months for HBV monoinfected patients. Because there were no significant differences between the coinfected and monoinfected patients with respect to age, sex, or mean time since diagnosis, we opted for unpaired analysis of the data (table 1).

The majority of HBV-HDV–coinfected patients had a history of hepatitis, jaundice, and household contact with individuals who had hepatitis. Mean levels of ALT and total bilirubin were higher among HBV-HDV–coinfected patients than among HBV monoinfected patients; the former group also presented more advanced stages of liver disease. Among HBV-HDV–coinfected patients, the mean ALT levels were 15.7 U/L for patients in the AC group, 93.1 U/L for those in the CH group, 59.7 U/L for those in the LC group, and 109.3 U/L for those in the HCC group. Among patients with monoinfection, the mean ALT levels were 24.5 U/L for patients in the AC group, 84.4 U/L for those in the CH group, and 138.1 U/L for those in the LC group. The mean ALT level for patients coinfected with HDV and HBV F was 82.7 U/L, compared with 32.6 U/L for patients monoinfected with HBV F (P < .05). In contrast, no differences were detected between ALT levels in monoinfected or coinfected patients who were infected with HBV D or HBV A. In the LC group, we also found a significant difference between coinfected and monoinfected patients with respect to mean (± standard deviation) age, which was 38.4 years (±10.7 years) for HBV-HDV–coinfected patients and 44.8 years (±11.2 years) for HBV-monoinfected patients, indicating earlier progression to severe disease in the former group (P < .05).

The proportion of patients who tested positive for hepatitis B e antigen (HBeAg) or for antibodies to HBeAg did not differ significantly between monoinfected and coinfected patients. Among patients positive for HBeAg, the median plasma HBV DNA level was 3.1 log₁₀ IU/mL for HBV-HDV–coinfected patients and 5.5 log₁₀ IU/mL for monoinfected patients (P = .23, by use of the Mann-
Patients had undetectable HBV DNA levels (50 coinfected patients and 52 (37.1%) of 140 monoinfected patients than in HBV-monoinfected patients; 31 (62%) of patients infected with HBV A or HBV D (HBV F were more likely to be coinfected than were individuals groups with respect to HBV genotypes; individuals infected with HBV genotype III. Among patients who tested positive for HDV RNA, the mean ALT level was 70.1 U/L, compared with a mean of 54.3 U/L for patients who tested negative for HDV RNA (P = .37). RNA negativity was more common among patients in the AC group than among patients in the CH group (P < .05). The mean ages of RNA-positive patients and RNA-negative patients were 35.5 and 43.4 years, respectively, which suggests a reduction in serum RNA levels with advancing age (P < .05), a supposition strengthened by the negative correlation found between serum RNA levels and age (r = -0.32; P = .06).

The mean serum RNA level was 5.4 log_{10} copies/mL. In contrast to the pattern observed for monoinfected patients, serum RNA levels were not found to correlate with ALT levels for the HBV-HDV–coinfected patients (r = -0.04; P = .8). In the clinical diagnosis groups, the median RNA levels were as follows: 3.0 log_{10} copies/mL in the AC group, 3.5 log_{10} copies/mL in the CH group, 3.6 log_{10} copies/mL in the LH group, and 4.5 log_{10} copies/mL in the HCC group (P = .86, by use of the Mann-Whitney U test).

Whitney U test). There was a significant difference between the 2 groups with respect to HBV genotypes; individuals infected with HBV F were more likely to be coinfected than were individuals infected with HBV A or HBV D (P < .05).

Serum levels of HBV DNA were lower in HBV-HDV–coinfected patients than in HBV-monoinfected patients; 31 (62%) of 50 coinfected patients and 52 (37.1%) of 140 monoinfected patients had undetectable HBV DNA levels (P < .05). Comparison of the median HBV loads in coinfected and monoinfected patients showed a significant difference between these groups (P < .001, by use of the Mann-Whitney U test).

Among patients coinfected with HBV-HDV, the median HBV load did not differ significantly by diagnosis group. The median levels in the groups were as follows: 10^{5.8} IU/mL in the AC group, 10^{6.1} IU/mL in the CH group, 10^{5.1} IU/mL in the LC group, and 10^{6.2} IU/mL in the HCC group (P = .9). However, among monoinfected patients, there was a gradient increase in the median HBV load according to the clinical diagnosis (from 10^{3.3} IU/mL in the AC group to 10^{3.9} IU/mL in the CH group and to 10^{4.9} IU/mL in the LC group (P = .001, by use of the Mann-Whitney U test). Serum HBV DNA levels were positively correlated with ALT levels for both HBV-HDV–coinfected patients (r = 0.5; P = .02) and HBV-monoinfected patients (r = 0.6; P < .001). There was no correlation between HBV load and age in either group. (r = 0.07 and P = .47 for coinfected patients; r = 0.01 and P = .98 for monoinfected patients).

We identified HDV RNA in samples from 35 (70%) of the 50 HBV-HDV–coinfected patients, all of whom were infected with HBV genotype III. Among patients who tested positive for HDV RNA, the mean ALT level was 70.1 U/L, compared with a mean of 54.3 U/L for patients who tested negative for HDV RNA (P = .37). RNA negativity was more common among patients in the AC group than among patients in the CH group (P < .05). The mean ages of RNA-positive patients and RNA-negative patients were 35.5 and 43.4 years, respectively, which suggests a reduction in serum RNA levels with advancing age (P < .05), a supposition strengthened by the negative correlation found between serum RNA levels and age (r = -0.32; P = .06).

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### Table 1. Comparison of patients coinfected with hepatitis B virus–hepatitis delta virus (HBV-HDV) and patients infected only with HBV.

<table>
<thead>
<tr>
<th>Variable</th>
<th>HBV-HDV–coinfected patients (n = 50)</th>
<th>HBV-monoinfected patients (n = 140)</th>
<th>Statistic</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean ± SD, years</td>
<td>38.1 ± 10.0</td>
<td>37.1 ± 10.3</td>
<td>P = .79</td>
</tr>
<tr>
<td>Alanine aminotransferase level, mean ± SD, U/L</td>
<td>65.4 ± 57.1</td>
<td>49.3 ± 96.8</td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>Total bilirubin level, mean ± SD, mg/dL</td>
<td>1.46 ± 1.0</td>
<td>0.86 ± 1.1</td>
<td>P &lt; .05</td>
</tr>
<tr>
<td>Diagnosis group</td>
<td></td>
<td></td>
<td>P &lt; .001</td>
</tr>
<tr>
<td>Asymptomatic carriage</td>
<td>3 (6.0)</td>
<td>94 (67.1)</td>
<td></td>
</tr>
<tr>
<td>Chronic hepatitis</td>
<td>8 (16.0)</td>
<td>33 (23.6)</td>
<td></td>
</tr>
<tr>
<td>Liver cirrhosis</td>
<td>36 (72.0)</td>
<td>13 (9.3)</td>
<td></td>
</tr>
<tr>
<td>Hepatocellular carcinoma</td>
<td>3 (6.0)</td>
<td>0 (0.0)</td>
<td></td>
</tr>
<tr>
<td>History of hepatitis</td>
<td>31 (62.0)</td>
<td>11 (7.9)</td>
<td>OR, 21.65 (95% CI, 8.4–56.9)</td>
</tr>
<tr>
<td>History of jaundice</td>
<td>41 (82.0)</td>
<td>26 (18.6)</td>
<td>OR, 18.80 (95% CI, 7.6–47.8)</td>
</tr>
<tr>
<td>Household contact with individuals who had hepatitis</td>
<td>34 (68.0)</td>
<td>70 (50.0)</td>
<td>OR, 2.67 (95% CI, 1.28–5.59)</td>
</tr>
<tr>
<td>HBV DNA level, median, IU/mL</td>
<td></td>
<td></td>
<td>P &lt; .05</td>
</tr>
<tr>
<td>&lt;10^{5}</td>
<td>31 (62.0)</td>
<td>52 (37.1)</td>
<td></td>
</tr>
<tr>
<td>10^{5}–10^{6}</td>
<td>17 (34.0)</td>
<td>74 (52.8)</td>
<td></td>
</tr>
<tr>
<td>&gt;10^{6}</td>
<td>2 (4.0)</td>
<td>14 (10.0)</td>
<td></td>
</tr>
<tr>
<td>Positive for antibodies to HBeAg</td>
<td>41 (82.0)</td>
<td>121 (86.4)</td>
<td>P = .59</td>
</tr>
<tr>
<td>Positive for HBeAg</td>
<td>5 (10.0)</td>
<td>15 (10.7)</td>
<td>P = .89</td>
</tr>
<tr>
<td>HBV genotype</td>
<td></td>
<td></td>
<td>P &lt; .05</td>
</tr>
<tr>
<td>A</td>
<td>18 (20.0)</td>
<td>72 (80.0)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>7 (24.0)</td>
<td>22 (76.0)</td>
<td></td>
</tr>
<tr>
<td>F</td>
<td>15 (39.5)</td>
<td>23 (60.5)</td>
<td></td>
</tr>
</tbody>
</table>

**NOTE.** Data are no. (%) of patients unless otherwise indicated. CI, confidence interval; HBeAg, hepatitis B e antigen; OR, odds ratio; SD, standard deviation.

a For comparison of genotype A with genotypes D and F.

b For comparison of genotype A with genotypes D and F.
The median HDV load of coinfected patients varied according to the HBV genotype they were infected with. In patients infected with HBV A, the median load was 1.8 \( \log_{10} \) copies/mL; in patients infected with HBV D, it was 3.9 \( \log_{10} \) copies/mL; and in patients infected with HBV F, it was 3.6 \( \log_{10} \) copies/mL (\( P < .05 \), for comparison of A with D and F).

Among coinfected patients, those infected with HBV A were more likely to have undetectable RNA levels than those infected with genotypes F or D (\( P < .05 \)). Patients infected with genotype D had higher ALT levels than those infected with genotypes A or F, and the rate of positivity for HBeAg was lower in the group of patients infected with HBV A. In patients positive for HBeAg, the median HDV load was 6.3 \( \log_{10} \) IU/mL, compared with 3.4 \( \log_{10} \) IU/mL in the patients positive for antibodies to HBeAg (\( P < .001 \), by use of the Mann-Whitney \( U \) test). No significant difference was found between the HBV genotypes with respect to patients' median viral load or clinical status.

**Discussion.** In this study, we evaluated HBV-HDV coinfec-
tion in the western Brazilian Amazon region, where HBV infec-
tion affects individuals in childhood and can be aggravated by coinfection with HDV. There is growing interest in the genetic diversity of viral pathogens as well as in the relationship between such diversity and the clinical evolution of disease.

In the present study, the rate of HBeAg positivity among
HBV-monoinfected patients was lowest in those infected with
HBV A. In addition, the mean ALT level was higher in patients
monoinfected with HBV D than in those monoinfected with
HBV A or HBV F. Among the HBV-HDV–coinfected patients, we found that the mean HDV load was lower in those coinfection
with HBV A than in those coinfected with HBV D or HBV F, which suggests that the HDV-HBV combination could promote a differential level of HDV remission. We have confirmed that the inflammatory potential of coinfection is greater than that of
monoinfection, as evidenced by the fact that the mean levels of
ALT and bilirubin were significantly higher in HBV-HDV–
coinfected patients than in patients monoinfected with HBV. In
addition, a history of symptomatic hepatitis and jaundice was
more common among HBV-HDV–coinfected patients than among patients with HBV monoinfection, which corroborates the hypothesis that the combination of the 2 viruses leads to
greater disease severity. Interestingly, although patients mono-
infect ed with HBV F had a lower mean ALT level than patients
monoinfected with HBV D, coinfection with HDV and HBV F
resulted in a mean ALT level that was significantly greater than
the mean ALT level associated with HBV F monoinfection. This
effect was not observed for HBV A or HBV D. Furthermore,
HBV F infection may be particularly harmful, because coinfection
with HDV is more frequent in patients infected with HBV F. Thus, a potentially less severe infection has a higher probability
of becoming a worse disease.

We detected a tendency for the presence of HDV to suppress
HBV replication; the mean viral load for HBV was significantly
lower in coinfected patients. This indicates that, despite the fact
that the damage caused by HBV-HDV coinfection is greater than
is that caused by monoinfection with HBV, it is likely that HDV
plays a greater role in the pathogenic process than does HBV.
The HBV load was found to correlate with the level of disease
among patients with monoinfection but not among patients
with coinfection, thus emphasizing that although the key factor
involved in disease progression among monoinfected patients
is HBV replication, the main factor involved in disease progression
among coinfected individuals is likely to be the HDV infection.
We found that HDV replication is extremely common, occurring
in 35 (70%) of 50 HDV-HBV–coinfected patients. We also
found a gradient increase in the HDV load according to the clinical
stage of liver disease, as has been reported for other viral infections.
In the present study, the limited number of samples evaluated and
the cross-sectional study design prevent us from drawing
definitive conclusions. However, we believe that our findings
illuminate some details related to the molecular aspects of HBV and
HDV infection and add to the body of knowledge regarding the
natural history of these 2 pathogens. We also describe new
molecular research tools that could be applied to diagnostic and
research strategies.

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