Distribution of Hepatitis B Virus Genotypes in Blood Donors and Chronically Infected Patients in a Tertiary Care Hospital in Southern India

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Hepatitis B virus (HBV) genotypes differ in their potential for causing disease. Consecutive patients with chronic HBV infection (CHBV) \( (n = 122) \) and blood donors \( (n = 67) \) positive for hepatitis B surface antigen and HBV DNA were genotyped using polymerase chain reaction–restriction fragment–length polymorphism. The ratio of male to female subjects was significantly higher in the blood donor group than in the group of patients with CHBV \( (P = .0004) \). Among patients with CHBV, genotype D was detected in 57.3%, genotype A was detected in 18%, and genotype C was detected in 11.5%. Only genotypes D and A were detected in blood donors. The difference between the detection rate of genotype C in patients with CHBV and in blood donors was significant \( (11.5\% \text{ vs. } 0\%; \ P = .009) \). Patients with CHBV who had genotype C had higher alanine transaminase (ALT) levels than those who had genotype A \( (P = .044) \) or genotype D \( (P = .014) \). Detection of genotype C in patients with CHBV and the association of genotype C with higher ALT levels may predict that this genotype has a greater potential for causing disease than other genotypes.

Chronic hepatitis B virus (HBV) infection (CHBV) remains a major health problem worldwide, with >300 million chronic carriers [1]. The course of HBV infection depends on several factors that can influence the immune system, including age at infection and host genetic factors [2], and it probably also depends on the genetic variability of the virus, which influences the expression of viral antigens. HBV replicates via a reverse-transcription step and is estimated to have a high mutation rate [3]. On the basis of a comparison of complete genomic sequences, HBV has been classified into 7 genotypes, designated A to G [4–7]. Recently, an additional genotype, genotype H, has been reported from Central America [8]. HBV genotypes appear to show a geographical pattern in their distribution. Genotype A is found in North America [4] and northern Europe, as well as in some parts of Africa [9, 10]. Genotypes B and C are common in southeast Asia [4]. Genotype D is found universally [9]. Genotype E has been reported from western and southern Africa [9]. Genotype F has been detected in South America and Central America [11]. Genotype G has been reported in France and North America [6].

Differences in pathogenicity among HBV genotypes remained unknown until recently. Studies from southeast Asia have shown that HBV genotype C is associated with severe liver disease [12] and with lower rates of response to IFN-α, compared with rates of response associated with genotype B [13]. In Taiwanese patients, genotype B has been shown to be associated with the development of hepatocellular carcinoma at a younger age [14]. Differences among HBV genotypes in the rate of seroconversion to hepatitis B e antibody (anti-HBe)
have been demonstrated in patients from Hong Kong [15]. In studies from Europe, differences among HBV genotypes in the selection of basal core promoter and/or precore mutations [16] and disease progression have been demonstrated [17]. The clinical significance of HBV genotypes is becoming increasingly relevant. The identification of HBV genotypes may help in the early identification of potential sequelae after chronic HBV infection and may also help in implementation of appropriate therapeutic regimens.

Apart from nucleotide sequencing, various techniques used for HBV genotyping include PCR–restriction fragment–length polymorphism (PCR-RFLP) [18, 19], PCR with type-specific primers [20], DNA hybridization assay [16], and serological assay [21]. Genotypes A and D have been recently reported in India that caters to patients from various parts of the Indian subcontinent. Additional objectives were to determine whether there was an association of HBV genotypes with sex, age, or subcontinent. Additional objectives were to determine whether there was an association of HBV genotypes with sex, age, or alanine transaminase (ALT) levels. The presence of IgM anti-HBc was investigated as a marker to HBV core antigen (anti-HBc) was investigated as a potential marker of active liver disease [24].

SUBJECTS, MATERIALS, AND METHODS

Subjects. We performed a cross sectional study of 122 consecutive patients with CHBV who were HBV DNA positive and who were referred to the clinical virology department of our hospital (Christian Medical College, Vellore, India) from the gastroenterology department during the period of September 2000 through November 2001. All patients were known to have been positive for hepatitis B surface antigen (HBsAg) for >6 months. Also included in the study were 102 healthy blood donors (volunteer and/or replacement donors) who were found to be positive for HBsAg by the John Scudder Memorial Blood Bank (Christian Medical College) during the period of March 2001 through November 2001. Most blood donors were family members or friends of the patient and were recruited by the patient to replace blood used during the course of treatment. Additionally, a blood bank officer screened blood donors for authenticity before blood donation (to eliminate professional members or friends of the patient and were recruited by the

other concomitant causes of liver disease and those coinfe

other concomitant causes of liver disease and those coinfected with hepatitis C virus (HCV), hepatitis D virus, or HIV were excluded from the study. Individuals receiving drugs active against HBV were also excluded.

HBV DNA was detected in plasma samples by a nested PCR, which was performed as described elsewhere [27]. Among samples obtained from the 102 HBsAg-positive blood donors, HBV DNA was detectable in 67 (66%). Those blood donors whose test results were negative for HBV DNA were excluded from further analysis. Thus, a total of 189 samples positive for HBV DNA were further studied.

The study population included 160 male subjects (85%) and 29 female subjects (15%), aged 5–71 years (mean ± SD, 35.18 ± 11.98). The study population was stratified into 2 groups: group 1 consisted of 122 patients with CHBV who were positive for HBV DNA, and group 2 consisted of 67 healthy blood donors who were positive for HBV DNA.

Serological markers. The study population was tested for HBsAg (Axsym HBsAg [V2], Abbott Laboratories; Hepanostika HBsAg Uni-Form II, bioMérieux), hepatitis B e antigen (HBeAg) (HBe 2, Abbott Laboratories; DiaSorin), anti-HBe 2 (Abbott Laboratories), anti-HBc IgM (DiaSorin), HCV antibody (UBI HCV EIA 3.0, Axsym HCV, version 3.0, Abbott Laboratories) and anti-δ antibody (Anti-delta EIA, Abbott Laboratories; DiaSorin). ALT levels were estimated for all samples in the Department of Clinical Biochemistry using a fully automated analyzer (Hitachi 912). We had earlier established an ALT level of 64 U/L as the upper limit of normal (ULN) in our population [26]. This was based on testing of samples obtained from 134 blood donors who were negative for markers of HBV, HCV, hepatitis G virus, and TT virus. In this study, an ALT level of 64 U/L was considered the ULN.

Nested PCR for HBV DNA detection (PCR I). Plasma from anticoagulated blood was aliquoted and stored at −60°C until further testing. DNA was extracted from 200 μL of plasma using QIAamp DNA blood mini kit (Qiagen). A 10-μL sample of the DNA extract was used in a previously standardized nested PCR [27], using primers targeting the core gene of the HBV genome.

Genotyping of HBV (PCR II). HBV genotypes were determined by using PCR-RFLP, as described elsewhere [18]. Briefly, 10 μL of DNA extract was amplified in a 50-μL reaction volume containing 1.5 mmol/L MgCl2, 200 μmol/L of each dNTP, 1U of Taq polymerase (Genecraft), and 12.5 pmol of each primer P7 and P8. Amplification was performed under the following conditions: initial denaturation at 94°C for 2 min, 45 cycles of amplification at 94°C for 45 s, at 53°C for 1 min, and at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Samples that were not amplifiable by the nonnested PCR were subjected to a heminested PCR that we standardized. Briefly, 5 μL of DNA extract was amplified in a 50-μL reaction...
volume containing 1.5 mmol/L MgCl₂, 200 μmol/L of each dNTP, 1 U of *Taq* polymerase, and 7.5 pmol of each primer P1 [20] and P8. The thermal cycling profile used was as follows: initial denaturation at 94°C for 3 min, 35 cycles of denaturation at 94°C for 45 s, annealing at 55°C for 45 s, and extension at 72°C for 2 min, followed by a final extension at 72°C for 10 min. Two microlitres of the first round PCR product were further amplified with primers P7 and P8 for 35 cycles using the same protocol described above for this set of primers.

After amplification by nonnests or heminested PCR, 10 μL of the 541 bp PCR product were digested with 5 U of *Hinf* (New England BioLabs) and 5 U of *Tsp 509I* (New England BioLabs) for 3 h at 37°C and 65°C, respectively. Electrophoresis was carried out in a 3% agarose gel containing 0.5 μg/mL of ethidium bromide. The PhiX 174 DNA digested with *HaeIII* (Roche Diagnostics) was used as the molecular weight marker. Amplified products were viewed on a UV transilluminator (Mighty Bright, Hoefer Scientific Instruments) and the genotypes were assigned on the basis of the digestion pattern, as recommended elsewhere [18]. This technique was validated using representative genotypes from the Health Protection Agency (Colindale, London; courtesy of Dr. C. G. Teo).

**HBV DNA sequencing.** To confirm PCR-RFLP findings, nucleotide sequencing was performed following amplification using primers P7 and P8. Ten representative samples were sequenced, including the first 2 samples that were typed as genotype A, the first 2 samples that were typed as genotype C, and the first 6 samples that were typed as genotype D using PCR-RFLP. The amplified products were subjected to cycle sequencing with ABI Prism Big Dye Terminator Cycle Sequencing Ready Reaction Mix (Applied Biosystems). Sequencing was carried out using ABI Prism 310 genetic analyzer (PE Applied Biosystems).

**Statistical analysis.** Data were analyzed by Fisher’s exact test, χ² test with Yates’ correction, Student’s *t* test, and Mann-Whitney *U* test, as appropriate. The NCSS/PASS 2000 Dawson edition, EpiInfo software, version 6.04b (Centers for Disease Control and Prevention), and Stata software, version 8.0 (Stata), were the statistical packages used. Results were considered statistically significant at *P* < .05.

**RESULTS**

The demographic profile, HBcAg status, and ALT levels of the study population are shown in table 1. The ratio of male subjects to female subjects was significantly higher in group 2 than in group 1 (*P* = .0004). There was no statistically significant difference between the 2 study groups in the proportion of subjects with HBcAg-positive status. Multivariate analysis showed that the mean ALT levels differed significantly between the 2 groups (*P* = .014), despite differences in the age, sex, and geographical origin of subjects. ALT levels greater than ULN were found in a significantly higher proportion of subjects in group 1 (40.1%) than in group 2 (17.9%) (*P* = .001). The distribution of HBV genotypes, ALT levels, and anti-HBc IgM-positive status across genotypes is shown in table 2. In both of the study groups, genotype D was the most frequently detected genotype, and genotype A was the second most frequently detected genotype. Genotype C was found in 14 (11.5%) of the subjects in group 1 and was found in none of the subjects in group 2 (*P* = .009).

Among the subjects studied, 111 were from southern India, 77 were from eastern India, and 1 was from northern India. The geographical distribution of HBV genotypes in the study population is shown in table 3. Genotype C was detected in a significantly higher proportion of individuals from east India (16.8%), compared with those from southern India (0.9%) (*P* < .0001). Genotype D was more frequently detected in sub-

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**Table 1.** Demographic profile, hepatitis B e antigen (HBcAg) status, and alanine transaminase (ALT) levels of patients with chronic hepatitis B virus (HBV) infection (group 1) and healthy blood donors who were positive for HBV DNA (group 2).

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1 (n = 122)</th>
<th>Group 2 (n = 67)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, mean years ± SD</td>
<td>37.37 ± 13.03a</td>
<td>31.19 ± 8.65b</td>
</tr>
<tr>
<td>Ratio of male to female subjects</td>
<td>95.27</td>
<td>65.2</td>
</tr>
<tr>
<td>HBcAg positive, no. (%) of subjects</td>
<td>17 (13.9)</td>
<td>12 (17.9)</td>
</tr>
<tr>
<td>HBcAg negative, no. (%) of subjects</td>
<td>105 (86)</td>
<td>55 (82)</td>
</tr>
<tr>
<td>ALT level, U/L</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>78.47 ± 79.13b</td>
<td>45.01 ± 39.64</td>
</tr>
<tr>
<td>Median (range)</td>
<td>54 (19–482)</td>
<td>42 (5–196)</td>
</tr>
<tr>
<td>ALT level greater than ULN, no. (%) of subjects</td>
<td>49 (40.1)c</td>
<td>12 (17.9)</td>
</tr>
</tbody>
</table>

**NOTE.** ULN, upper limit of normal.

* a *P* < .001.
* b ULN was defined as 64 U/L.
* c *P* = .001.

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Table 2. Alanine transaminase (ALT) levels and IgM antibody to hepatitis B virus (HBV) core antigen (anti-HBc IgM) status in patients with chronic HBV infection (group 1) and healthy blood donors who were positive for HBV DNA (group 2), by HBV genotype.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Group 1a (n = 122)</th>
<th>Group 2 (n = 67)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Genotype A</td>
<td>Genotype C</td>
</tr>
<tr>
<td>No. (%) of subjects</td>
<td>22 (18.03)</td>
<td>14 (11.47)</td>
</tr>
<tr>
<td>ALT level, U/L</td>
<td>Mean ± SD</td>
<td>Median (range)</td>
</tr>
<tr>
<td></td>
<td>60.81 ± 25.88d</td>
<td>55 (23–111)</td>
</tr>
<tr>
<td></td>
<td>72.71 ± 69.87f</td>
<td>47 (19–460)</td>
</tr>
<tr>
<td></td>
<td>42.27 ± 21.34</td>
<td>43 (5–83)</td>
</tr>
<tr>
<td>ALT level greater than ULN9, no. (%) of subjects</td>
<td>2 (36.3)</td>
<td>9 (64.2)</td>
</tr>
<tr>
<td>Positive for anti-HBc IgM, no. (%) of subjects</td>
<td>9 (40.9)</td>
<td>7 (50)</td>
</tr>
</tbody>
</table>

NOTE. A total of 12 samples were not amplifiable, and a total of 10 samples were untypeable. ULN, upper limit of normal.

a Mixed infection with genotypes A and D was found in 2 individuals in group 1.

b P = .009 for genotype C, group 1 vs. group 2.
c P = .01 for genotype D, group 1 vs. group 2.
d P = .044 for group 1, genotype A vs. genotype C.
e P = .014 for group 1, genotype C vs. genotype D.
f P = .02 for genotype D, group 1 vs. group 2.
g ULN was defined as 64 U/L.
h ULN was defined as 43 U/L.
i P = .002 for genotype D, group 1 vs. group 2.

The ratio of male subjects to female subjects was significantly higher in blood donors than in patients with CHBV (P = .0004). This may be because of the preponderance of male subjects among blood donors in our hospital. Blood donors were significantly younger than patients with CHBV (P < .001). This finding indicates that frequent monitoring of these blood donors is warranted, as they may subsequently develop HBV-associated chronic liver disease.

Infection with genotype D was detected in a higher proportion of blood donors than of patients with CHBV (76.1% vs. 57.3%; P = .010). In contrast, an earlier study from India reported that genotype D was associated with more-severe liver disease [22]. However, in our study, among subjects infected with genotype D, a higher proportion of patients with CHBV had ALT levels that were greater than the ULN, compared with blood donors (38.5% vs. 13.7%; P < .002). The differences in host genetic make-up, immune response, and modes of transmission among subjects from different parts of the Indian subcontinent may, at least in part, account for the variation in genotype distribution.

ALT levels among patients with CHBV were significantly higher in those infected with genotype C than in those infected with genotype A (P = .004) or genotype D (P = .014). This finding may indicate more-pronounced liver damage in those infected with genotype C. Furthermore, the detection of genotype C in a significant proportion of patients with CHBV and its absence in blood donors (11.5% vs. 0%; P = .009) reflects the greater disease-causing potential of this genotype. However, multivariate analysis showed that the geographic distribution of subjects in the 2 groups differed significantly (P = .001). Therefore, the differences in geographical origin...
Genotype A and D 1 (0.9) 1 (1.3) ...

Nonamplifiable 6 (5.4) 6 (7.8) ...

* P < .0001 for genotype C, eastern India vs. southern India.

** P < .0001 for genotype D, southern India vs. eastern India.

between the subjects in the 2 groups could have contributed to the differences in the distribution of genotypes. We are unable to speculate whether infection was acquired differently in the 2 groups, because there were few subjects in either group with documented risk factors. No association was found between the presence of anti-HBc IgM and the infecting genotype.

The geographical variability in the distribution of HBV genotypes has resulted in most studies comparing either genotype B with genotype C or genotype A with genotype D. Studies from southeast Asia comparing genotypes B and C show that genotype C is associated with higher virus loads [28] and more-aggressive liver disease [12, 14]. In India, genotype D is reported to be associated with more-severe liver disease than that associated with genotype A [22]. However, there is a dearth of studies comparing genotype A, C, and D. Therefore, our study population provided us with a unique opportunity to compare these 3 genotypes.

Studies from Taiwan show that genotype C is more refractory to IFN treatment than genotype B [13]. Genotype A is associated with a 20-fold higher risk of lamivudine resistance than that associated with genotype D [29]. These differences among HBV genotypes in response to treatment underscore the need for identification of HBV genotypes. In the near future, different HBV genotypes may warrant implementation of specific treatment regimens.

Genotype D was the most frequently detected genotype in subjects from southern and eastern India. Infection with genotype C was detected in significantly higher proportions of subjects from eastern India than subjects from southern India (16.8% vs. 0.9%; P < .0001). This finding may reflect the geographical proximity of eastern India to southeast Asian countries like Thailand, where genotype C is the predominant genotype [28]. Detection rates of genotype D were higher in subjects from southern India than in those from eastern India (75.6 vs. 46.7%; P < .0001). Furthermore, it may be noted that injection drug abuse is common in eastern parts of the Indian subcontinent [30] and, thus, could often be the mode of transmission of blood borne viruses.

Genotypes could not be assigned for a small proportion (5.2%) of strains. This may be due to the presence of infection with multiple genotypes or with strains that have altered and/or additional recognition sites for the restriction enzymes used in testing. It may be noted that, on studying HBV genotypes from different geographical regions using the same technique used in our study, investigators previously found that most untypeable strains were from southeast Asia [18]. It is possible that the small number of HBV sequences from southeast Asia that were available at that time in the National Center for Biotechnology Information (NCBI; Bethesda, MD) database contributed to a poor representation of the sequence variability in the geographical region, thus leading to the inadequate choice of restriction enzymes. The fact that there were even fewer Indian sequences in the NCBI database may have accounted for the untypeable strains in our study.

However, there were 26 samples (13.7%) that were HBV DNA positive by the nested PCR (PCR I) but were not amplifiable by the PCR used for HBV genotyping (PCR II). These 26 samples were subjected to the heminested PCR that we standardized. Fourteen of these 26 samples were amplified by the heminested PCR, and the genotypes were assigned. Despite amplification by heminested PCR, 12 samples (6.3%) did not yield a PCR product. This phenomenon may be the result of intrinsic differences in the sensitivities of these PCRs or the result of the difference in the region of the HBV genome targeted. PCR I targets the core gene, whereas PCR II targets the surface gene. Earlier published studies [31] have shown that the HBV core gene and surface gene may not always be concomitantly detected.

In conclusion, our results indicate that genotype D and genotype A were the predominant genotypes in this study population. We believe that this study is the first to report the presence of genotype C in India. Genotype C was detected in a significant proportion of patients with CHBV, but it was not detected in blood donors. Furthermore, genotype C was associated with higher ALT levels and was probably associated with greater potential to cause disease. Distribution of HBV genotypes within the Indian subcontinent appears to be markedly different, depending on the geographical origin. Long term follow-up studies are required to understand the clinical, therapeutic, and epidemiological differences among HBV genotypes.

Acknowledgment

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References

15. Chu C-J, Hussein M, Lok ASF. Hepatitis B virus genotype B is associated with earlier HBeAg seroconversion compared with hepatitis B virus genotype C. Gastroenterology 2002; 122:1756–62.