Hepatitis B Virus Genotypes and Precore and Core Mutants in Brazilian Patients

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A method for genotyping hepatitis B virus by partial HBsAg gene sequencing with primers common to all known genotypes was developed. Mutations related to anti-HBs resistance are also detected with this method. Samples from 103 Brazilian patients were analyzed. Precore and core region of these viruses were also sequenced in 101 patients. Genotypes A, B, C, D, and F were found with frequencies of 49.5, 2.9, 13.6, 24.3, and 9.7%, respectively. Genotypes B and C were found only in Asian patients, whereas genotypes A, D, and F were more common in patients without an Asian background. Precore mutants were found in 32 (31.7%) of 101 patients, with a higher frequency in those infected with genotype D (22 of 25 [88.0%]). Analysis of nucleotide 1858 showed presence of thymine in all patients with genotypes B, C, and D and in a few patients with genotypes A (10.0%) and F (30.0%), who showed more frequently the presence of cytosine. This nucleotide was closely related to the presence of precore mutants. Mutations in the basal core promoter were found in 64 of 101 (63.4%) samples. These mutations were more frequent in patients infected with genotype F (90.0%) and less frequent in patients infected with genotype B (33.3%). Deletions in this region were found in two genotype C-infected patients.

HBV vaccination, anti-HBV hyperimmunoglobulin (HBIg), and anti-HBs monoclonal antibody may induce resistant mutations, as previously shown by other researchers (5–7, 11, 40–42, 46, 53). These mutations are especially common in regions where HBV is endemic and widespread anti-HBV vaccination have already been established and in patients undergoing liver transplantation due to hepatitis B who are using specific prophylaxis with HBIg or anti-HBs monoclonal antibodies.

Precore mutants were first described in Italy (4) related to cases showing negative HBcAg but with detectable HBV-DNA, HBsAg, anti-HBe, and anti-HBc. These strains have mutations in the nt 1896 (G→A) and 1899 (C→A) in the precore region, leading to a stop codon that prevents HBcAg synthesis. Precore region also contains the ε encapsidation signal, a secondary structure maintained by hydrogen bonds among bases in the same strain. Precore mutations are especially rare in genotypes that usually contain C at position 1858, since this nucleotide pairs with the nt 1896 in the secondary structure of ε encapsidation sign (23). Precore mutants have been associated with more severe hepatitis B evolution. This effect was related to the absence of the immune response modulator role from HBcAg and to alterations in the binding of the ε signal, leading to more efficient viral replication (50), but the overall pathogenic meaning of these mutations remains unclear (15, 28, 39). Since HBV genotypes are not uniformly distributed around the world, precore mutants also have a particular distribution, being more frequent in geographic regions such as Asia and the Mediterranean basin, where genotypes B, C, or D are predominant, and rare in North America and Europe, where genotype A is commonly found (24, 45). In South America, the frequency of this mutation is poorly studied, but Castro et al. (9) found a low frequency of precore
TABLE 1. Partial S-gene sequences utilized in the alignment carried out to distinguish different HBV genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Sequence (GenBank accession no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A.........</td>
<td>X75666, X75669, X65258, M54898, J02205, X51970, and M74498</td>
</tr>
<tr>
<td>B.........</td>
<td>X75660, D23677, D23678, D00329, D00330, D00331, M54923, S74815, and S74867</td>
</tr>
<tr>
<td>C.........</td>
<td>X75667, X75665, X75792, X75565, X01587, D23680, D23682, D23683, D16665, S62754, S75184, S81945, S81946, and U19777</td>
</tr>
<tr>
<td>D.........</td>
<td>X75668, X75662, M32138, X77309, X65259, X59795, X68292, X77308, J02202, V01460, X02496, and X77310</td>
</tr>
<tr>
<td>E.........</td>
<td>X75657, X75654, and L24071</td>
</tr>
<tr>
<td>F.........</td>
<td>X75638, X75661, and S69798</td>
</tr>
<tr>
<td>G.........</td>
<td>AF369553 and AF160501</td>
</tr>
<tr>
<td>H.........</td>
<td>U91819, U91827, and AY900460</td>
</tr>
</tbody>
</table>

mutations (7 of 29 [24%]) in anti-HBe-positive Brazilian carriers.

HBsAg, anti-HBe, and HBV-DNA positive serological profile can also be related to mutations affecting the basal core promoter (BCP), especially A1762T and G1764A. These mutations have been associated with active hepatitis, severe disease after liver transplantation, development of hepatocellular carcinoma, and fulminant hepatitis (3, 51).

Recently, several methods based on sequencing have been described that allow determination of viral features related to prognosis and therapeutic response, such as genotype, precore and BCP mutants, and drug resistance.

We describe here a new set of primers for determining HBV genotypes with the same reagents by nested PCR and automated DNA sequencing. We also verified the frequency of HBV genotypes and precore and BCP mutations in Brazilian patients.

MATERIALS AND METHODS

Patients. We studied 103 samples from HBV chronic carriers (determined by HBsAg) from different Brazilian regions that were sent to Laboratório Bioquímico Jardim Paulista, São Paulo, Brazil, from 1998 to 2001. Serum samples were stored at −20°C and thawed immediately before use. The Ethical Committee of the University of São Paulo School of Medicine approved this protocol (research protocol 145/00).

Detection of HBV-DNA by PCR. Extraction and amplification of HBV-DNA were carried out by nested PCR as described by Kaneko et al. (16). All reagents utilized were obtained from Life Technologies do Brazil, São Paulo, Brazil.

Analysis of HBV sequences from different genotypes for primer design. For HBV genotyping, we developed a system amplifying a 417-bp fragment covering a region of S gene with marked heterogeneity among genotypes, flanked by a conserved region to allow equal amplification of them. In this way, an alignment was made with S gene sequences from the different genotypes already described.

Samples were treated as described above and subjected to two rounds of amplification with sequentially outer (HBFS1 and RHBS1) and inner (HBFS2 and RHBS2) primers. The amplification conditions for the two rounds of the nested PCR were as follows: initial denaturation at 94°C for 20 s, followed by 30 cycles of 94°C for 20 s, 56°C for 20 s, and 72°C for 30 s, with a final extension step at 72°C for 1 min in a PTC-200 Thermocycler (MJ Research, Watertown, Mass.).

Detection of BCP and precore mutants. For the detection of BCP and precore mutants, HBV-DNA-positive samples were amplified by using the primers described by Takahashi et al. (51).

Sequencing reaction. PCR products were subjected to cycle sequencing reactions as described previously (48) by using the second-round primers and the ABI Prism BigDye terminator cycle sequencing ready reaction kit (Applied Biosystems). After purification, samples were denatured and loaded in a 5% Long Ranger M urea gel (Long Ranger gel solution; FMC) in an automated ABI Prism 377 DNA sequencer (Applied Biosystems).

Sequence analysis. Genotyping, BCP, and precore mutant analysis were carried out by sequence comparison with other known sequences from different HBV genotypes described previously and were aligned as described above. For this purpose, EditSeq and MegAlign programs from the DNAStar package (LaserGene, Inc.) were used.

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Phylogenetic and molecular evolutionary analyses were conducted by using MEGA version 2.1 (21). Sequences were aligned by using CLUSTAL X version 1.81 (54) and then analyzed by using the neighbor-joining method by a distance matrix calculated according to the Kimura two-parameter model (19). Woolly monkey hepatitis B virus (GenBank accession number AF046996) was utilized as an outgroup. A bootstrap test of phylogeny was performed with 1,000 replicates, and values ≥ 70 are indicated on the branches.

**Statistical analysis.** For statistical analysis, we used the EpiInfo program (version 6.04; Centers for Disease Control and Prevention, Atlanta, Ga., and World Health Organization, Geneva, Switzerland).

**GenBank accession numbers.** Sequences from the S and preC/C genes from Brazilian cases were deposited in the GenBank database under accession numbers AY323306 to AY32408 and AY329494 to AY329594, respectively.

## RESULTS

**HBsAg gene partial amplification analysis.** As shown in the phylogenetic analysis (Fig. 2), genotypes A, B, C, D, and F were found among our samples. The frequency of genotypes in the 103 patients is shown in Fig. 3. The following subtypes were found: adw, ayw, adr, and adw4. Genotypes A and B had only subtype adw, and genotype F had only adw4. Genotype C showed subtype adr more frequently, with one patient infected with subtype adw. Genotype D samples were all subtype ayw except for one (adw). In two Asian patients, mutations in the epitope “a” region were found. One genotype B sample had a T126S mutation, previously associated HBIG resistance, and the other one (genotype C) had a T118A mutation, which was associated with vaccine resistance.

**Genotypes, age, gender, and ethnicity.** The gender, age mean, and ethnicity ranges according to genotypes can be seen in Table 2. Most of the patients studied had a Western ethnicity (n = 83, 80.6%). Genotype A was found mainly in such patients (94.1%), and genotypes D and F were exclusive to them. The only three Asian patients infected with genotype A belonged to the same family (father, mother, and son). Among the 20 (19.4%) Asian patients, 17 (85.0%) had genotypes B or C. None of non-Asian patients had genotype B or C. Statistical analysis showed that infection with genotypes B and C is significantly more frequent among Asian patients in our population (P = 0.01 and P < 0.0001, respectively).

**Genotypes, precore and BCP mutants, and nt 1858 analysis.** In 101 patients, we also analyzed the BCP and precore region of HBV. In the other two patients, it was not possible to amplify this region. The frequency of mutants in these regions among the different genotypes, as well as the results of the nt 1858 analysis, are shown in Table 2. Precore mutants were more frequent in patients infected with genotype D virus (P < 0.0001).

Analysis of nt 1858 showed the presence of thymine in all patients with genotypes B, C, and D and in a few patients with genotypes A (5 of 50 [10.0%]) and F (3 of 10 [30.0%]). This nucleotide was closely related to the presence of precore mutants (P < 0.0001) since, of 49 isolates with nt 1851, 32 (65.3%) also had the stop codon mutation at nt 1896, whereas none of the isolates with a cytosine at nt 1858 showed this mutation. All patients with genotypes A and F that had a mutation in the precore region also had a thymine at position 1858.

BCP mutants were less frequent among genotype B-infected patients (33.3%) and more frequent among genotype F-infected patients (90.0%), but no statistical significant difference was found. Interestingly, in two patients infected with HBV genotype C, we found deletions in the BCP encompassing the studied mutation. These deletions extended from nt 1760 to 1770 in one patient and from nt 1760 to 1769 in the other.

## DISCUSSION

HBV genotyping has been extensively analyzed recently. Better responses to treatment have been reported for genotypes A and B compared to genotypes D and C, respectively (44, 47, 56). On the other hand, evolution to chronic hepatitis or to more severe diseases such as hepatocellular carcinoma has been related to genotypes A and C, respectively (12, 30, 43, 47, 56).
higher frequency of progression to cirrhosis and hepatocellular carcinoma (17). In China, Ding et al. (12) showed that genotype C infections were more severe than genotype B infections. Similar results have also been found in other studies in China and Japan, and it has been hypothesized that the higher frequency of BCP mutants in genotype C could be associated with the higher pathogenicity of this genotype (18, 44). Studies addressing the evolution of hepatitis B and the viral genotypes should also be carried out among Brazilian patients.

Genotypes E, G, and H were not found in our study population. Genotype E has only been found in some regions of Africa (31), and only one study outside Africa found a Haitian child infected with this genotype in Belgium (26). Genotype G has been found in ca. 10% of studied cases from France and United States (49). Genotype H seems to be a divergent clade from genotype F and was described after our primer selection and sample processing, but a later analysis with three sequences from GenBank (U91819, U91827, and AY090460) showed that this method is also able to distinguish this new genotype.

Although there are many African descendants in Brazil due to slaves being brought from Africa from the 16th to the 19th centuries, we did not find genotype E in our sample. There were few patients of known African descent in the studied population. Other studies in Brazilian states with a higher percentage of African descendants, such as Bahia, should be carried out to better evaluate this issue.

Different subtypes were also found in our samples. As expected, it would not be possible to classify the genotypes only based on that.

Mutations related to epitope a recognition were found in only two patients. Mutation T126S has already been described and associated with vaccination resistance (40, 55). Mutation T118A, on the other hand, is outside the main region of epitope a and was related to HB Ig resistance (11). Unfortunately, we were not able to obtain more information about the progress of the disease in these two patients, but it is noteworthy that both had genotypes common to Asiatic regions (43). The finding of these mutations among our cases highlights the need for a continued surveillance to address the appearance of such cases and to better analyze their real meaning.

Precore mutants had a low frequency in our population (31.7%). Such mutations were found more frequently in patients infected with genotype D, with intermediate frequency in pa-

![FIG. 3. HBV genotype distribution in Brazilian patients (n = 103).](Image)

TABLE 2. HBV genotypes and their relationships to patients and viral features

<table>
<thead>
<tr>
<th>HBV genotype</th>
<th>Patient feature</th>
<th>BCP and precore region mutations (no. [%])</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. male (%)</td>
<td>Mean age (yr) (SD)</td>
</tr>
<tr>
<td>A</td>
<td>35 (68.6)</td>
<td>40.7 (11.6)</td>
</tr>
<tr>
<td>B</td>
<td>3 (100)</td>
<td>49.0 (9.87)</td>
</tr>
<tr>
<td>C</td>
<td>9 (64.3)</td>
<td>33.5 (16.5)</td>
</tr>
<tr>
<td>D</td>
<td>19 (76.0)</td>
<td>51.1 (12.9)</td>
</tr>
<tr>
<td>F</td>
<td>10 (100)</td>
<td>33.0 (7.12)</td>
</tr>
<tr>
<td>Total</td>
<td>76 (73.8)</td>
<td>42.0 (13.9)</td>
</tr>
</tbody>
</table>

a *P < 0.0001; †P = 0.01. WT, wild type.
b Includes two cases with a deletion in the BCP region.
c Includes two cases with an adenine at nt 1858.
patients infected with genotypes B and C, and at a very low frequency in genotype A- and F-infected patients. The occurrence of this mutation is dependent upon the nucleotide (cytosine or thymine) at position 1858, which forms a base pair with nt 1896 in the pregenomic RNA loop at the ε encapsidation sign. A thymine at position 1858 is particularly common in genotype D viruses. The presence of a cytosine at position 1858 precludes the G-to-A mutation at nt 1896, since this would destabilize the stem-loop structure of the RNA encapsidation signal (27). Interactions of this encapsidation signal with the viral DNA polymerase is an essential step in the viral replication cycle, and it has been hypothesized that the increased strength of the guanine-to-cytosine base pairing found in precore mutants would implicate in a stronger ε encapsidation sign, allowing a more efficient replication of these strains. Genotype A usually shows a cytosine at this position (23), whereas genotype F may present thymine or cytosine (1). Our results from nt 1858 analysis corroborate with these data, since we also have found the presence of cytosine only in genotypes A and F, which showed a low frequency of precore mutants.

The frequency of this mutation varies widely around the world. Castro et al. (9) studied Brazilian patients and found the precore stop codon mutation in only 24% of them—a result similar to ours. These authors also demonstrated the higher frequency of the stop codon mutation at nt 1896 in the isolates that had T1858. On the other hand, the prevalence of this mutation in China was 86% (10). This discrepancy may reflect differences in genotype distributions in each studied population, since genotype A and D viruses are common in Brazilian populations, whereas in China genotypes B and C are more common. Furthermore, in our population, the frequencies of precore mutants were low even in genotype B- and C-infected patients (33.3 and 23.1%, respectively).

The BCP mutation was found in 63.4% of our patients and was present in all genotypes. BCP mutants frequency ranged from 33.3% in genotype B to 90.0% in genotype F. Changes at positions 1762 and 1764 have been described to be especially common in patients with C1858 (24), but we did not find any correlation between them. Among our patients, the frequency of BCP mutants was high in genotypes A and F patients from whom C1858 is especially common, but genotype C- and D-infected patients, in whom T1858 is common, have similar frequencies. On the other hand, other authors have reported that the presence of these mutants is not related to the presence of precore mutants (9). This point should be further analyzed in our population.

Two genotype C-infected patients in the present study had a deletion in this region. Other authors have reported a number of point mutations, as well as deletions and insertions of nucleotides in the precore region leading to initiation failure or premature termination. The positions of these mutations varied and were normally restricted from 8 to 21 nt, flanking AT-rich regions and overlapping transcription factor binding sites (10, 20, 34). An 8-bp deletion from nt 1768 to 1775 was also found in precore promoter region before exacerbation, but in a later sample this deletion was no longer found (34). The deletions reported here are different from the ones described previously, but they were always in the same region.

In conclusion, we have developed a method for HBV genotyping that can amplify isolates from all of the HBV genotypes found in Brazil. Furthermore, we have characterized this approach the HBV genotypes in our population and their relationship to precore and BCP mutants. We have shown that genotypes E, G, and H were not found in Brazil until now. Genotype A was the most common in our study population. Genotypes A, D, and F are found in non-Asian subjects, whereas genotypes B and C are found among Asian patients. Precore mutants are more common among genotype D-positive patients, whereas BCP mutants were present in all of the genotypes, with a higher frequency among genotype F-infected patients.

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