Hepatitis B virus genotypes: a retrospective survey in Southwestern France, 1999-2004

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Introduction

Chronic hepatitis B virus (HBV) infection remains a major public health problem worldwide with approximately 300 million chronic carriers and the development of severe complications such as liver cirrhosis and hepatocellular carcinoma [1]. The course of HBV infection depends on several factors modifying the immune response, 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. Indeed, 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, seven major viral genotypes, designated A to G, have been identified and correspond to viral isolates sharing more than 80% homology in their nucleotide sequences [4-7]. HBV genotypes have distinct geographical distributions. Genotype A is found in North America [4] and Northern Europe, as well as in parts of Africa [8, 9],
while genotypes B and C are the most common in Southeast Asia [4]. Genotype D is found universally [8]. Genotype E is predominantly found in Africa [8], and F clusters in Central and South America [10]. Genotype G has been reported in the United States and France [7].

Accumulating evidence suggests that HBV genotypes have an impact on the pattern of mutations in the pre-core (PC) and core (CP) regions, the natural course of chronic HBV infection [11-15], or the severity of underlying liver disease [16, 17]. Genotypes B and C have been studied most extensively due to their co-circulation in Asia, allowing differences in ethnic or racial background of the patients. These studies have shown that genotype B, compared to genotype C, is associated with a higher rate of severe liver disease and is more prevalent among HBsAg-positive patients than genotype C. Similarly, compared to genotype D, genotype A is more prevalent in HBsAg-positive than in anti-HBe-positive antibody patients. Several studies indicate that HBV genotypes may be associated with differences in treatment response [18-20]. Genotype C is associated with a lower response rate to alpha interferon therapy compared to genotype B. In Caucasian patients, a better response to peginterferon alpha-2b and lamivudine was found when patients were infected with genotype A vs genotype D. Investigating the clinical significance of HBV genotypes is becoming increasingly relevant. The identification of HBV genotypes could be useful to monitor HBV infection and related diseases and may also help in implementing appropriate therapeutic regimens.

To date, there are little data on the prevalence and clinical significance of HBV genotypes in France. One previous study suggested that HBV genotypes A and D were predominant in this country and that genotype A was associated with HBeAg positivity [19]. However, genotype D was associated with HBsAg negativity [21]. Another study by Ganne-Carrié et al. concluded that HBV genotypes A, B, C, D, and E circulated in the Seine-Saint-Denis District, close to Paris, reflecting the multiple geographical origins of patients [22].

The aim of this study was to determine the prevalence and the distribution of HBV genotypes among a consecutive sample of patients with HBV infection with or without HBV coinfection in Bordeaux hospital (France) that caters to patients from various parts of Southwestern France. Additional objectives were to determine whether there was an association of HBV genotypes with patients' demographics, clinical status, and PC and CP variants.

### Materials and methods

#### Patients

Patients with HBV infection and referred to our virological laboratory (Pellegrin hospital, Bordeaux, France) from different clinical departments between November 1999 and May 2004 were retrospectively and systematically included in the survey. The inclusion criteria were serologic evidence of chronic HBV infection (HBsAg positivity >6 months), HBV DNA positivity with concurrent HBeAg, and anti-HBe tests. Patients with HBV positivity or HBV treatment were also included.

#### Clinical findings

Information relating to the patients' demographics, clinical and virological status and hepatitis disorders (clinical, biological, and histological) were recorded anonymously and retrospectively from the patients' medical files. Whenever a trusted patient had a liver biopsy, it was performed before treatment. In HBV infected patients, epidemiological, clinical, biological, and therapeutic data were retrieved from the ANRS CO3 Aquitaine Cohort database of the Groupe d’Epidémiologie Clinique du Sida en Aquitaine (GECSA) [23]. Epidemiological characteristics included sex, age, and ethnicity. Coinfection with the immunodeficiency virus (HIV), the hepatitis C virus (HCV), and the delta virus (HDV) were also recorded. The presumed source of HBV infection was determined by inquiring about the patients' sexual behaviour (intravenous drug abuse with or without other modes of transmission), history of intramuscular drug use at least once, blood transfusion, vertical transmission (HBsAg-positive mother), intravenous drug use (endoscopic or coelioscopy examination, history of surgery, acupuncture, hemodialysis).

### Detection of virological markers in serum

The study population was tested routinely for HBsAg, HBcAg, and anti-HBe antibody (Dade Behring Laboratories, Marburg, Germany), HIV antibodies (Amplicor HIV-1 Monitor, Roche Diagnostics, Branchburg, NJ), HCV antibodies (Ortho Clinical Diagnostics, Raritan, NJ), and anti-delta antibodies (DiaSorin, Saluggia, Italy).

### Determination of HBV genotypes and detection of PC stop codon (G1896A) and CP variants (A1762T, G1764A) and A1762T

HBV DNA was extracted from 200 µl of serum using QIAamp DNA mini-kit (Qiagen, Hilden, Germany). HBV genotyping was performed by sequencing a part of the polymerase region. Two rounds of polymerase chain reaction amplification were used for polymerase sequence studies [24]. The first round used primers CHBV-3 (5’-CCTGCTGGTGACGCGTGGGAC-3’) and HBV17 (5’-CGTCCCGCGNAGGATCCAGTT-3’), nested polymerase chain reaction using primers VT301 (5’-CTGGGC- CWMAXATTGGCAGTCCCT-3’) and VT102 (3’-GCAAANCCACAAAGGA- CAAAAT-3’) yielded a 721-bp DNA fragment encoding part (nt 1214-1934) of the polymerase region. Amplification of the precore sequence was performed by nested polymerase chain reaction as previously described [25]. The following primers were used for the amplification of the precore region (nt 1,814 to 1,900) and the core promoter (nt 1,742 to 1,849) of the HBV genome. The external primers were 5’-CAAAAGGAGGACCTCTGGACT-3’ (sense, nt 1,651 to 1,672), and 5’-GGCGGAGGAGTCTCTGGACG-3’ (antisense, nt 2,394 to 2,369), and primers for nested PCR were 5’-CAACGACCGACCTTG-3’ (sense, nt 1,679 to 1,698) and 5’-AACG- TACGGGACCTGCGGAGA-3’ (antisense, nt 1,783 to 2,009).

The amplified PCR products were purified by the QIA quick PCR purification kit (Qiagen) and then used for direct sequencing using internal antisense primer. Sequencing of the purified products was conducted on a CEQ automatic sequencer (Beckman Coulter, Fullerton, CA) with the CEQ DTCS Quick Start kit (Beckman Coulter). Analysis of each sequence was conducted using Seq Analysis and Seq Investigator software (Beckman Coulter).

For genotype characterization, all sequences were compared to at least 24 Genbank sequences representative of all known HBV genotypes.

### Statistical analysis

Quantitative variables are described by their median and range and categorical variables by percentage. Comparisons between patients were performed using Kruskall Wallis non-parametric test for quantitative variables and Fisher’s exact test for categorical variables. Multiple logistic regression with forward stepwise analyses were used to determine the independent factors associated with HBV genotypes A and those associated with HBV genotypes D. The variables related to genotype A and genotype D with P<0.10 in univariate analysis were entered into the logistic model. Results were considered statistically significant at P<0.05. Data were analyzed using SAS version 8.2 software package (SAS Institute Inc., Cary, NC, USA).

### Results

At total, 194 patients (152 males and 42 females) were identified and enrolled. Their median age was 45 years (range 7-76.9 years). The characteristics of the patients are listed in table I. Of the 146 patients with a documented ethnic background, 104 (71.2%) were Caucasians. HIV coinfection was present in 57/159 (36%) patients screened. Eighty-two patients (42.3%) were currently receiving anti-HBV treatment (interferon: 9 patients, pegylated interferon: 3, nucleoside-nucleotide analogues: 70).
There was a strong association between HIV status and HBV genotypes (P<0.001). HIV coinfection was common in patients with genotypes A and D (45.3% and 34.2%, respectively) and absent in patients with genotypes B and C (table II).

The proportion of patients with increased ALT over the upper limit of normal was not significantly different between HBV genotypes (P=0.4). No association was found between liver activity and HBV genotypes (P=0.47). On the other hand, there was an association between liver fibrosis and HBV genotypes (P=0.01). The prevalence of severe liver fibrosis (F3 or F4 METAVIR score) was higher in HBV genotypes A and E-infected patients (table II).

**HBV genotypes, HBeAg status and PC/CP variants**

Genotypes A and C were associated with a higher prevalence of HBeAg (53% and 55%, respectively) (table II). Among the 188 patients documented, the overall prevalence of PC variant was 35%. PC variant (G1896A) was more common in patients with genotypes E (86.7%), B (61.5%), C (60%), and D (54%), and rare in patients with genotype A (7.3%) (P<0.001). Among the 186 patients documented, the overall prevalence of CP variants (A1762T and/or G1764A) was 43%. CP variant was most common among patients with genotype C (77.8%), followed by genotype A (46.9%), E (46.7%), and D (33.3%), and less common in patients with genotype B (15.4%) (P<0.05). PC (54% vs 8%, P=0.001) as well as CP variants (53% vs 29%, P=0.001) were more often found in HBeAg-negative than HBeAg-positive patients.

**Factors relating to HBV genotypes A or D**

Factors that may be associated with the most prevalent HBV genotypes present in our patient population (genotypes A and D), including sex, age (<40 vs >40 yrs), ethnicity, presumed mode of infection, PC and CP variant, HBeAg status, liver activity (A0-A1 vs A2-A3) and fibrosis (F0-F1 vs F2-F4) were analyzed by multiple logistic regression analyses. According to univariate analysis, six factors were significantly associated with HBV genotype A (P<0.1): age (>40 yrs), sex (male vs female), ethnicity (non-asian ethnicity), presence of PC variant, and the independent association with genotype D. In multivariate analysis, sex, age (>40 yrs), HBeAg positivity, presence of PC variant, METAVIR fibrosis score F2-F4. In multivariate analysis, age (>40 yrs), HBeAg positivity, presence of PC variant, METAVIR fibrosis score F2-F4. In multivariate analysis, age (>40 yrs), HBeAg positivity, presence of PC variant, METAVIR fibrosis score F2-F4. In multivariate analysis, age (>40 yrs), HBeAg positivity, presence of PC variant, METAVIR fibrosis score F2-F4.

**Discussion**

In view of the distinct geographic distribution of HBV genotypes, it is important to survey their epidemiology in a given country or region, since HBV genotypes may have some influence on the course of liver disease. The seven major HBV genotypes (A-G) were represented in a sample recruited in one major French region outside of Paris. This distribution may reflect local differences caused by various ethnicities of inhabitants and their individual lifestyles that may also differ all over France. In our opinion, our study is the first attempt to systematically investigate patients with chronic hepatitis B (CHB) providing HBV DNA positivity. In addition, it is one of the first attempts to

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**Table I.** — General characteristics of the study population (N=194).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Number (%) of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male gender</td>
<td>152 (78)</td>
</tr>
<tr>
<td>Ethnicity (N=146)</td>
<td></td>
</tr>
<tr>
<td>Caucasian</td>
<td>104 (71.2)</td>
</tr>
<tr>
<td>African</td>
<td>23 (15.8)</td>
</tr>
<tr>
<td>Asian</td>
<td>19 (13.0)</td>
</tr>
<tr>
<td>Infection (N=159)</td>
<td></td>
</tr>
<tr>
<td>HIV</td>
<td>57 (34)</td>
</tr>
<tr>
<td>HCV</td>
<td>15 (9)</td>
</tr>
<tr>
<td>HDV</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Presumed source of HBV infection</td>
<td></td>
</tr>
<tr>
<td>Intravenous drug use</td>
<td>16 (8)</td>
</tr>
<tr>
<td>Transfusion</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Sexual</td>
<td>46 (24)</td>
</tr>
<tr>
<td>Vertical</td>
<td>3 (2)</td>
</tr>
<tr>
<td>Iatrogen</td>
<td>12 (6)</td>
</tr>
<tr>
<td>Unknown</td>
<td>116 (59)</td>
</tr>
<tr>
<td>HBV phenotype</td>
<td></td>
</tr>
<tr>
<td>HBeAg positive</td>
<td>82 (42.3)</td>
</tr>
<tr>
<td>Hbs antibody positive</td>
<td>105 (54.1)</td>
</tr>
<tr>
<td>ALT elevated (N=142)</td>
<td>71 (43.8)</td>
</tr>
<tr>
<td>Liver fibrosis (METAVIR scoring system) (N=83)</td>
<td></td>
</tr>
<tr>
<td>F0 or F1</td>
<td>16 (19)</td>
</tr>
<tr>
<td>F2 or F3</td>
<td>46 (54)</td>
</tr>
<tr>
<td>F4</td>
<td>21 (25)</td>
</tr>
</tbody>
</table>

HBeAg: hepatitis B e antigen; Hbs: hepatitis B surface; ALT: alanine transaminase; A: African; C: Caucasian; E: East Asian; F: French; H: Hispanic; PC: precore; CP: core promoter; H0-A3: hepatitis B virus sequence; H0, A1, A2, A3: hepatitis B virus genotype; P: probability; vs: versus; +: positive; –: negative; HBeAg: hepatitis B e antigen; HBe antibody: antibody to HBeAg; ALT: alanine aminotransferase.
investigate genotypes in mono-infected or HIV co-infected patients. Therefore, the findings of the present survey are likely to be representative of the CHB patient population in Southwestern France.

Our results showed a predominance of genotypes A and D in agreement with other investigators [15, 21]. As evidenced by previous studies concerning the geographic distribution of HBV, genotypes A and D were predominantly found in Caucasians.
Les facteurs indépendants associés à la genotypisation HBV ont été analysés par multivariée. Les odds ratios obtenus sont présentés dans le Tableau III.

Tableau III – Indépendants factors associated with genotypes A or D in multivariate analysis.

<table>
<thead>
<tr>
<th>Factor</th>
<th>Odds ratio</th>
<th>95% confidence interval</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype A</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age &gt; 40 yr</td>
<td>3.97</td>
<td>1.71-9.21</td>
<td>0.001</td>
</tr>
<tr>
<td>HIV positivity</td>
<td>3.48</td>
<td>1.05-11.82</td>
<td>0.03</td>
</tr>
<tr>
<td>Absence of PC mutant</td>
<td>19.23</td>
<td>7.25-50</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Genotype D</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-Asian ethnicity</td>
<td>27</td>
<td>1.65-33</td>
<td>0.04</td>
</tr>
<tr>
<td>Intravenous drug use</td>
<td>6.17</td>
<td>1.55-24.48</td>
<td>0.009</td>
</tr>
<tr>
<td>PC mutant</td>
<td>4.12</td>
<td>1.64-10.35</td>
<td>0.002</td>
</tr>
</tbody>
</table>

HIV = Human immunodeficiency virus; PC = pre-core; CP = core promoter.

La genotypisation HBV est hyperendémique et les génotypes B et C prévalent. Ces études ont montré que le génome C était associé à une hépatite chronique de plus grande gravité [29] et des lésions hépatiques plus agressives [17, 30].

Sur les autres facteurs génétiques, le génome A et C étaient associés à une prévalence plus élevée de HBsAg comparée aux génotypes B et D [12, 31-33]. Des études ont rapporté une corrélation entre le génome HBV et l'absence d'HBeAg. Ces études ont trouvé que la présence d'HBeAg était plus élevée dans les patients avec génomes C et D comparées à ceux avec génome B, suggérant que l'HBeAg était plus élevé dans les patients avec génome B [13, 16].

Les études ont montré que le génome C était associé à une plus grande gravité de l'hépatite chronique [17, 30]. De plus, le génome D était associé à une plus grande gravité de l'hépatite chronique [17, 30].

Nos résultats montrent que la génotypisation HBV a une grande importance sur le pronostic de l'hépatite chronique. La génotypisation HBV est un marqueur fiable de la gravité de l'hépatite chronique et de la réponse au traitement [29].

En conclusion, la génotypisation HBV est un outil important pour la gestion des patients avec hépatite chronique. Les génomes B et C sont associés à une hépatite chronique de plus grande gravité, tandis que le génome D est associé à une hépatite chronique de plus petite gravité. La génotypisation HBV est une étape cruciale dans la prise en charge des patients avec hépatite chronique.
[45-55%] regions. Physicians managing patients with chronic HBV infection must be aware of this condition because the natural course and treatment response are different from that of HBsAg-positive chronic hepatitis [37].

In accordance with previous reports [12, 13, 16, 38], we found that PC variants ([G1896A]) were most common in patients with genotype D and rare in patients with genotype A. In our survey, CP variants ([A1762T, G1764A]) were evenly distributed among HBV genotypes A, C, D, and E and relatively rare among HBV genotype B. The association between HBV genotypes and PC ([G1896A]) variants is related to base pairing in the stem-loop structure of the pregenomic encapsidation sequence [11, 12, 38, 39] but its basis has not been deciphered.

Our study showed that PC variants were predominantly detected in HBsAg-negative patients, whereas CP variants were found in both HBsAg-negative and HBsAg-positive patients, as described by others [40, 41]. A more marked increase in the prevalence of PC versus CP variants in HBsAg-negative patients is attributed to the fact that PC ([G1896A]) variants abolish whereas CP variants ([A1762T, G1764A]) only down-regulate HBsAg production. This study has identified three independent factors associated with genotype A in HBV-infected patients: age >40 yrs, HBV positive status, and absence of PC variant. The proportion of patients with age >40 yrs was significantly higher, a finding consistent with the recent results of a survey performed in France by Ganne-Carrié et al. [22] in which the mean age of patients infected by HBV genotypes B, C, and E, was significantly lower than HBV/A and -D. In keeping with the results from three recent studies [42-44] in which 57%, 92%, and 70% of HIV-HBV coinfected patients were infected by HBV genotype A, this genotype was strongly associated with HBV infection in our study.

In summary, seven HBV genotypes were present, and PC and CP variants could be detected in approximately one-third of patients with chronic HBV infection in Southwestern France. HBV genotypes were related to various ethnicities of inhabitants and influenced the prevalence of serum HBsAg as well as PC and CP variants. Further studies are needed to determine if additional testing for HBV genotype as well as PC and CP variants may help in documenting and predicting clinical prognosis and thus guiding treatment decisions.

REFERENCES

19. Wai CT, Chu CJ, Hassman M, Lok AS. HBV genotype B is associated with better response to interferon therapy in HBsAg (+) chronic hepatitis than genotype C. Hepatology 2002;36:1425-30.


