Prevalence of hepatitis B virus genotypes in chronic carriers in Santiago, Chile

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Abstract The eight genotypes (designated A–H) of hepatitis B virus (HBV) display distinctive geographical distribution worldwide, with genotypes A, D and F frequently detected in South America. To determine the prevalence of HBV genotypes in Santiago, Chile, 131 samples from chronic carriers were used for PCR amplification, and genotyping was performed by RFLP. The results indicated that genotype F was the most prevalent among HBV carries (84% of the cases), whereas genotypes A, B, C and D were found at a prevalence of 3.8, 3.8, 6.1, and 2.3%, respectively. We discuss these data in the complex scenario of HBV epidemiology.

Chronic hepatitis B virus (HBV) infection is currently a major health problem, with approximately 400 million virus carriers worldwide [10]. Based on an inter-group divergence of around 8% in the complete nucleotide sequence or more than 4% at the level of the S gene, HBV has been classified into eight genotypes, designated A–H [1, 2, 14, 21]. Importantly, there is increasing evidence regarding the influence of the different HBV genotypes on the progression of liver disease [15, 22], hepatitis B e-antigen (HBeAg) seroconversion [11] and response to antiviral therapy [3, 6].

The eight HBV genotypes show a distinctive geographical distribution. Genotype A is more prevalent in northwestern Europe, North America, India, and sub-Saharan Africa, although it has also been found in some regions of South America. Genotypes B and C are characteristic within Asia. Genotype D has a worldwide distribution, but it predominates in the Mediterranean region. Genotype E is mainly restricted to Western Africa, while genotypes F and H have been detected in South and Central America, respectively. Finally, genotype G has been identified in France, Germany, Mexico and the United States [4, 7, 10, 20, 25].

Although genotypes A, D and F have usually been detected in South America, these variants show important differences in their prevalence among the countries of this region. Data are available from Argentina, Bolivia, Brazil, Colombia, Peru and Venezuela [5, 9, 13, 18, 24]. To date, only data about the prevalence of HBV infection in the Chilean population, based on the detection of either surface antigen (HBsAg) or antibodies against the viral core protein (anti-HBc), have been reported [8, 17, 23], but an analysis of the molecular epidemiology of HBV in our country has not been published. For these reasons, we undertook this study to determinate, for the first time, the prevalence of HBV genotypes in Santiago, Chile.

The present study involved serum samples collected between November 2000 and March 2008 from 131 patients with chronic HBV infection (76 active chronic infections with HBeAg+/AntiHBe− and 55 inactive chronic infections with HBeAg−/AntiHBe+). Chronic infection was defined based on the detection of HBsAg for at least 6 months. Among these patient samples, we found only four co-infections with human immunodeficiency

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virus and no cases of co-infection with hepatitis C virus. One hundred and one of the patients were male and 30 were female; age ranged from 5 to 85 years (average age of 47 years). The majority of patients were born in Chile, but 13 were immigrants from Asia. The samples were received in the Gastroenterology Section, Clinical Hospital, University of Chile (Santiago, Chile), for routine HBV DNA detection or quantification. Our laboratory is the national reference center for molecular diagnostics of viral hepatitis, processing samples from all medical centers of the country. However, in this study, we only analyzed samples from residents of the Santiago Metropolitan Area.

Viral DNA was isolated from 500 μL of serum using a High Pure System Viral Nucleic Acid kit (Roche Molecular Systems, Branchburg, NJ, USA). Genotyping was carried out by polymerase chain reaction (PCR) and restriction fragment length polymorphism (RFLP) as previously described [26], with some modifications. The strategy described in the current study was applied according to the most prevalent HBV genotype predicted for the South American region. A segment of the S gene was amplified by PCR using primers sense YS1 (nt 203–221, 5′-GCG GGG TTT TTC TTG TTG A) and antisense YS2 (nt 787–767, 5′-GGG ACT CAA GAT GTT GTA CAG) in a reaction volume of 50 μL. PCR was performed after an initial 5-min denaturation step at 94°C, by 40 cycles of amplification including denaturation for 60 s at 94°C, annealing for 40 s at 53°C, and elongation for 60 s at 72°C, and followed by a final extension period of 10 min at 72°C. Each PCR reaction product was visualized on a 2% agarose gel stained with ethidium bromide. The 585-bp PCR products were then subjected to RFLP analysis, using 5 U of the restriction enzymes *Sau*3A I, *Bsr* I or *Hpa* II (New England Biolabs, MA, USA), and incubation according to the manufacturer's instructions.

Two samples from each of the genotypes determined by RFLP assays were further analyzed by sequencing using an ABI Prism BigDye Terminator cycle-sequencing ready-reaction mixture (version 3.0, Applied Biosystems) and the YS1 primer. The sequencing reaction products were analyzed on an ABI Prism 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequences obtained were aligned using the EMBOSS Pairwise Aligment Algorithms software at the EMBL-EBI website to compare them with viral reference sequences available through the HBV Regulatory Sequence Database (HBVRegDB) website, for genotypes A (X02763), B (D00329), C (X01587), D (X02496), E (X75657), F (X75658), G (AF160501) and H (AY090454).

Similar to previous reports [26], restriction sites of HBV isolates from different genotypes were verified by multiple alignments using sequences available within public databases. This information was used to design the algorithm depicted in Fig. 1. As anticipated, the use of restriction enzyme *Sau*3A I displayed a specific restriction pattern for HBV genotypes F, C and A (Fig. 2a, lanes 2, 3, 4, respectively). Representative reaction products from unresolved genotypes (B, D, E, G or H) after incubation with this enzyme are shown in Fig. 2a, lane 5. Unresolved samples were subsequently digested with the enzyme *Bsr* I, and two different restriction profiles were obtained (Fig. 2b, lanes 2, 3). One of these patterns was specific for genotype B (lane 3), whereas the other one corresponded to either genotype D or H (lane 2). Using the restriction enzyme *Bsr* I, we did not find any patterns indicative for genotype E or G (see Fig. 1).

PCR products preliminary characterized as being of genotype D or H (three samples) were then identified by digestion with the restriction enzyme *Hpa* II. This enzyme does not digest the PCR fragment from genotype D (data not shown), but it does cut those from genotype H. None of these three samples were digested by *Hpa* II.

In order to verify the genotyping data, amplicons from patient samples were sequenced and then aligned with all of the HBV reference sequences. For genotypes A, B, C, D and F, we obtained 99, 97, 98, 97, and 97% of identity, respectively, which confirmed the results from RFLP

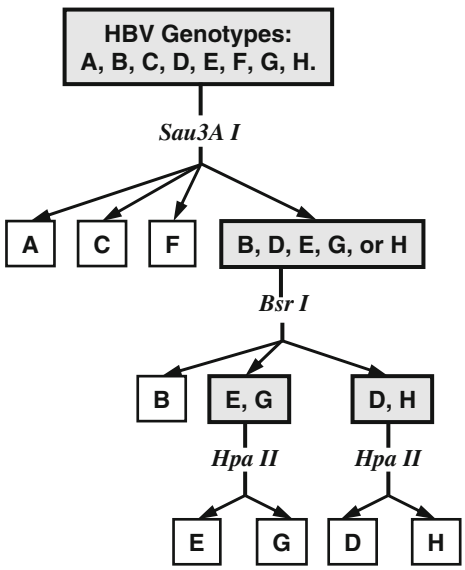

Fig. 1 Flow chart of the strategy for genotyping HBV. PCR products from a fragment of the S gene were subjected to RFLP. Digestion with enzyme *Sau*3A I displays specific patterns for genotypes A (82-, 204-, 299-bp products), C (585-bp) and F (43-, 256-, 286-bp products), whereas treatment with *Bsr* I results in a unique digestion profile (125-, 460-bp products) for genotype B. *Bsr* I digestion of both genotypes E and G results in two fragments (286-, 299-bp products), whereas this restriction site is absent in both genotypes D and H (585-bp product). Finally, unresolved samples are subjected to further digestion with *Hpa* II to differentiate between genotypes E (81-, 504-bp fragments) and G (585-bp product) or between genotypes D (585-bp) and H (90-, 495-bp products). *White boxes* represent assigned sera, *shaded boxes* indicate unresolved samples

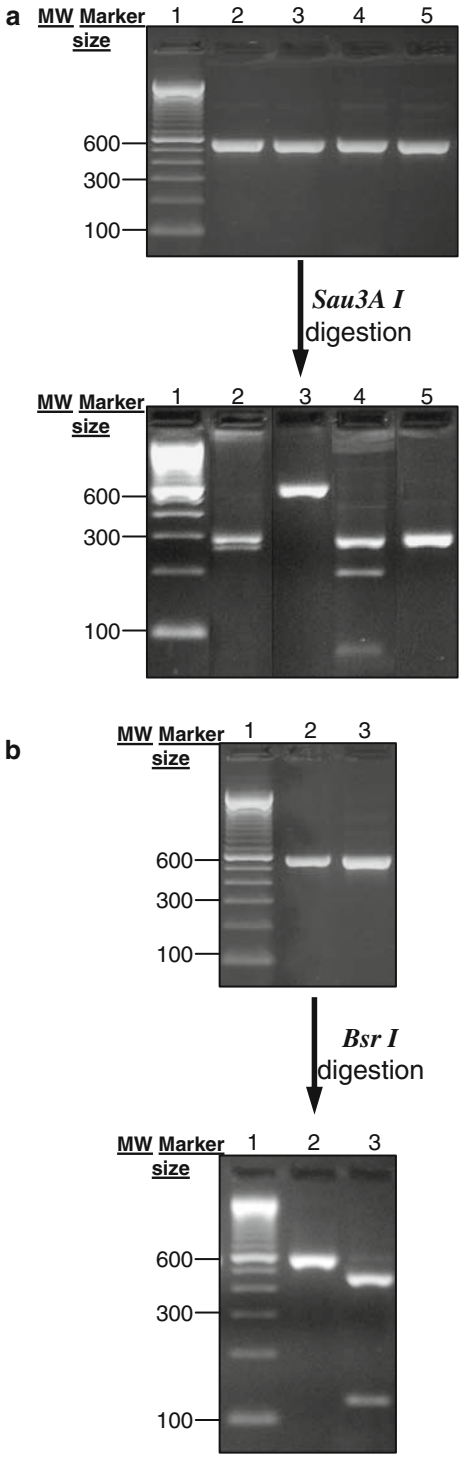

Fig. 2 Representative RFLP profiles of HBV S gene amplicons subjected to digestion with enzymes **a** *Sau*3A I or **b** *Bsr* I. In **a**, *lane 1* 100-bp DNA molecular weight marker, *lane 2* genotype F, *lane 3* genotype C, *lane 4* genotype A and *lane 5* unresolved genotype. In **b** *lane 1* 100-bp DNA molecular weight marker, *lane 2* genotype D or H, and *lane 3* genotype B

Table 1 Distribution of HBV genotypes in Santiago, Chile

HBV genotype	*N* (%)
A	5 (3.8)
B	5 (3.8)
C	8 (6.1)
D	3 (2.3)
F	110 (84)
Total	131 (100)

N (%) number of cases (percentage)

analyses. Alignments with the other HBV genotypes (E, G or H) resulted in less than 95% identity.

Following the current strategy, a summary of the obtained results is presented in Table 1. From 131 samples examined, HBV genotype F was found to be the most prevalent in Santiago, Chile, with 84% of the patients analyzed. The remaining 16% of the sera tested corresponded to carriers of viruses belonging to genotype A, B, C or D. Interestingly, all of the samples with genotypes B and C (Table 1) came from patients of Asian origin, where these two genotypes are very commonly found.

To date, PCR amplification combined with RFLP is a widely used method for HBV genotyping because it is simple and relatively inexpensive. In the current study, we adapted a previously described methodology [26] for identifying, in chronic carriers, the predominant HBV genotypes circulating throughout the Santiago Metropolitan Area in Chile. Currently, there are no publications describing the molecular epidemiology of HBV for our country. Herein, our results have indicated that HBV genotype F is the most prevalent within chronically infected individuals. The high prevalence of this genotype in Chile is consistent to that found in some other countries of this region such as Colombia, Peru, Bolivia and Venezuela [5, 9, 24]. However, the low prevalence of genotypes A and D in our country contrasts with what is found in Argentina and Brazil, where these genotypes are frequently found within hepatitis B carriers [13, 18]. In spite of the fact that HBV genotype F is autochthonous to South America, these marked differences within neighboring countries seem to reflect different rates of foreign immigration between continents [1]. Thus, our study will contribute to understanding the complexity of the molecular epidemiology of HBV within the South American region and in the worldwide context.

On the other hand, our findings should be of interest to clinicians because, in comparative analyses, genotype F has been implicated in a higher rate of death in patients with liver disease, relative to the variants A and D [19]. It has also been significantly associated with the incidence of hepatocellular carcinoma when compared to genotypes A,

B, C or D [12]. Thus, in order to improve the prognosis of hepatitis B patients, it will be essential to continue developing simple methods for the earliest identification of the most aggressive variants within HBV viral carriers.

Given the complex genetic variability of HBV infections, which is increased by phenomena like co-infection with different genotypes or the occurrence of subgenotypes, it is important to point out the limitations of RFLP methodologies. In the first case, to resolve co-infections, different techniques such as line probe assays (INNO-LiPA HBV Genotyping assay, Innogenetics NV., Ghent, Belgium) are more appropriate and commercially available [16]. On the other hand, the study of HBV subgenotypes also requires a different approach [20]. In the particular case of genotype F, which is predominant in some countries of South America, it is known that this genotype is very divergent, where four subgenotypes (named F1–F4) and some clades for certain subgenotypes have been described [4, 24]. For being able to identify these variants, phylogenetic analysis of extensive genome sequences is required. In view of the high prevalence of HBV genotype F within the Chilean population, we are continuing our efforts toward a better characterization of this variant.

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