Arch Virol
DOI 10.1007/s00705-015-2477-0

ORIGINAL ARTICLE

Genetic variability of hepatitis B virus in Uruguay: D/F, A/F genotype recombinants

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Received: 3 December 2014 / Accepted: 29 May 2015

Abstract Hepatitis B virus (HBV) infection is a serious global health problem. Approximately 2 billion people worldwide have been infected, and approximately 350 million individuals currently suffer from HBV-induced chronic liver infection, which causes 600,000 deaths annually from chronic hepatitis, cirrhosis and hepatocellular carcinoma. HBV is classified in eight genotypes (A-H), and two more have been proposed (I-J). In this paper, complete genome sequences of nine Uruguayan HBV are reported. Five samples belong to genotype F1b and one to genotype A2. Three HBV recombinants were detected: A1/F1b, A2/F1b and D3/F1b. The following mutations were detected: a G1896A substitution, a 33-nucleotide deletion from position 2896 to 2928 in the Pre-S1 region involving Pre-S1 residues 3-13, a 33-nt deletion in the Pre-S1 region involving nt 2913-2945 and Pre-S1 residues 9-19. More F genotypes strains than expected were detected in this study, supporting the hypothesis that there are more people of indigenous origin than declared in our population. Also, one third of the samples analyzed were recombinants. This cannot be explained by the low HBV prevalence in Uruguay, but a high HBV infection rate in drug addicts and dialysis patients could act in favor of multiple-genotype HBV infections that could lead to recombination.

Electronic supplementary material The online version of this article (doi:10.1007/s00705-015-2477-0) contains supplementary material, which is available to authorized users.

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Introduction

Hepatitis B virus (HBV) belongs to the family *Hepadnaviridae* and is characterized by a partially double-stranded 3-kb DNA genome and an RNA intermediate for genome replication. This RNA intermediate contributes to the genetic diversity of this virus because the host RNA polymerase II, which is responsible for the production of all viral transcripts, lacks proofreading activity. The HBV genome contains four open reading frames that are partially overlapping and correspond to the polymerase, surface antigen, core (nucleocapside), e antigen (HBeAg) and X proteins.

Hepatitis B virus is responsible for an infection that causes acute and chronic disease. It is transmitted through contact with blood or other body fluids of an infected person. As a consequence of hepatitis B infection, 780,000 people die every year in the world [1]. In Latin America, prevalence varies depending on the region; according to the Pan American Health Organization (PAHO), the Amazon basin and Peru are considered to have high prevalence (equal to or more than 8 % HBV surface antigen [HBsAg] prevalence), Brazil and Venezuela show intermediate HBsAg prevalence, and Uruguay, Argentina, Chile, Paraguay and Bolivia show low prevalence of less than 2 %. In Uruguay, a high prevalence of HBV infection has been

Published online: 23 June 2015

found in drug addicts, multitransfused patients (including hemophiliacs), and dialysis patients [2, 3].

HBV has a high degree of genetic variation [4, 5] and has been classified into eight groups (genotypes A-H) deduced from complete genome sequence comparisons [3–9]. More recently, two additional genotypes (I and J) have been proposed [10, 11]. HBV genotypes have been further separated into several subgenotypes that differ by 4 to 7.5 % in their whole nucleotide sequence [12, 13].

HBV genotypes have a distinct geographical distribution [7, 13]. Genotype A is divided into several subgenotypes with distinct geographic origin: A1, Afro-Asian; A2 European; and A3-A7, African [14–19]. Genotype B is found mainly in Asia [12], while genotype C is found in Asia and in Australian aborigines [12, 20–22]. Genotype D is widespread but predominates in the Mediterranean region [20] and the Middle East, and at least six subtypes have been reported (D1–D5 and D7) [23]. Genotype E is associated with West Africa [24]. Genotype G has been isolated in different countries, including Germany, France, Japan and the USA. Genotype H is associated with Mexico and Central America [25]. For HBV genotypes E, G and H, no subtypes have been proposed yet.

Genotype F is the most divergent of the HBV groups and has been found mainly throughout the American continent. It is subdivided into four subgenotypes (F1, F2, F3 and F4] [26]. Subtype F1 includes two clusters: F1a is related mostly to strains from Central America, and F1b comprises strains from southern South America and Alaskan natives; F3 is related to HBV strains from northern South America, and F4 includes strains from Bolivia and Argentina [25–30].

In the Americas, as in the rest of the world, the genotype distribution reflects the patterns of migration. There is a high prevalence of genotypes A and D as a reflection of the migratory movements that occurred during the colonial period, especially subtype A1, related to the slave trade [31, 32], and A2 and several subtypes of genotype D, associated with European settlers. Strains of genotypes B and C have been isolated in Peru and Panama, as well as in Argentina and Brazil [33–35]. Genotypes A, H and G have been detected in Mexico, while genotypes A and H have been found in Nicaragua. Genotypes A and D have been found in Venezuela, Uruguay and Costa Rica [27, 36, 37].

Nevertheless, the most prevalent genotype of Central and South America is genotype F [38]. It is highly prevalent in the Amerindian population [37], and this fact supports the theory that genotype F is indigenous to the native population of the New World and precedes the European discovery of the Americas five centuries ago [39]. Genotype F subgenotypes also have a distinct geographic distribution. Subgenotype F1 has been found in Alaska, El Salvador, Guatemala, Costa Rica, and Nicaragua [25, 29, 40], whereas the F1b genotype has been reported in Peru, Chile and Argentina [27, 30, 34, 40–43]. Subtype 2 is the most common in Venezuela and Brazil [31, 32, 35, 37–44], particularly F2a. Subgenotype F3 has been described in Venezuela, Colombia, and Panama [45, 46]. In Venezuela, genotypes F1a, F3 and F4 have been found in Amerindians [28, 37].

HBV diversity is associated with the clinical outcome of the infected patients. Types C and D may be related to poor clinical outcomes [47]. Some mutations are also linked to severe chronic hepatitis, hepatocellular carcinoma, fulminant hepatitis and resistance to treatment [13, 48–50].

Despite the large amount of information available about HBV in South America, only limited information exists about Uruguayan HBV strains [36], based on partial sequences. This is the first study designed to determine the genotypes and genetic variability of HBV in Uruguay using complete HBV genome sequences.

Materials and methods

Patients

Nine complete HBV genomes, isolated from serum samples (collected between 2007 and 2013) obtained from six asymptomatic blood donors and three symptomatic patients (HBsAg and Anti-HBc positives) were characterized. The study protocol was approved by the Bioethical Committee of Centro Hospitalario Pereira Rossell.

DNA extraction

DNA extraction from serum samples was performed using a High Pure PCR Template Preparation Kit (Roche) following manufacturer's instructions.

PCR

Five different sets of PCR primers were used in order to amplify the whole genome of each HBV strain (see Table A, Supplementary Materials). Overlapping regions of the HBV genome were amplified in duplicate to evaluate sequence discrepancies and detect possible errors in the sequences introduced by the polymerase. PCR amplification was done as follows: for a 50-µl final volume, 5 µl of 10X PCR buffer, 1 µl of 10 pM dNTPs, 1.5 µl of 50 mM $MgCl_2$, 1 µl of each primer (10 nM), 0.3 µl of HotStart Polymerase (QIAGEN catalog no. 203203), 35.2 µl of distilled water and 5 µl of extracted DNA were added to a 200-µl PCR tube. The PCR programme used was 1 cycle of 95 °C for 15 minutes, 42 cycles of 95 °C for 40 seconds, 50 °C for 45 seconds, and 72 °C for 60 seconds, followed by 1 cycle of 72 °C for 10 minutes.

Sequencing of PCR products

Sequences were obtained at Macrogen (Korea). The same primers that were used for PCR were used for sequencing with an ABI 3700 Genetic Analyzer (Sanger method). The complete genomes were assembled using DNA Baser Sequence Assembler v3.5.4.2 (Heracle BioSoft SRL Romania, http://www.DnaBaser.com).

Sequence alignment

Reference HBV strains and Uruguayan isolates were aligned using Clustal Omega software [51].

Phylogenetic analysis

Once aligned, the FindModel program (available at http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html) was used to identify the optimal evolutionary model that best fitted our sequence data. Akaike information criteria (AIC) and the log of the likelihood (ln L) revealed that the GTR+Γ model was the best fit to the data (AIC of 39325.98 and ln L of -19653.990584).

For the complete HBV genome sequence, maximum-likelihood phylogenetic trees were constructed using the PhyML program with the GTR+Γ model [52]. As a measure of the robustness of each node, we used an approximate likelihood ratio test (aLRT) [53]. The aLRT value was calculated using a Shimodaira-Hasegawa-like procedure (SH-like) [54, 55].

Nine additional Uruguayan HBV partial genomes have been analyzed for genotype and subgenotype assignation based both on phylogenetic analysis of partial sequences and deduced amino acid sequence comparisons for the S/P genes (nucleotide position 502 to 770). For phylogenetic analysis, sequences obtained in this study and HBV sequences from the GenBank database were aligned using the ClustalX (v2.1) software [56] and edited with the BioEdit (v7.2.0) software [57]. Phylogenetic trees were constructed by the neighbor-joining method performed with Kimura's two-parameter model of molecular evolution as implemented in MEGA v6 software [58]. The robustness of reconstructed phylogenies was evaluated by bootstrap analysis (1000 replicates). In order to confirm the genotype and differentiate among subgenotypes, amino acid and nucleotide signature patterns of each subgenotype were identified. The S/P open reading frames were analyzed with VisSPA v1.6.2 software [59], considering that a candidate signature pattern should be present in more than 90 % of the sequences of the query set and in fewer than 10 % of the samples of the references background set.

Recombination analysis

Recombination events were detected using the Simplot program [60], with a window size of 200 bp and a step size of 20.

Sequences accession numbers

The GenBank accession numbers for the Uruguayan strains reported in these studies are KJ586803 to KJ586811 (see also Table 1).

Results

This is the first study reporting the HBV genotypes and molecular characterization of complete HBV genome sequences in Uruguay. Nine complete Uruguayan HBV genome sequences were determined.

A preliminary analysis of possible recombination sites within Uruguayan HBV genomes showed that three strains (P1, P342 and P3283) showed evidence of having a recombinant origin and were therefore excluded from the phylogenetic analysis (Fig. 1). Subgenotyping of recombinants was also done by phylogenetic analysis (Supplementary Material, Figures B and C). The HBV P342 strain showed D3/F1b genotype recombination with the breakpoint located at positions 2388 and 2867 (corresponding to the amino terminus of the HBV polymerase). This region has only one ORF, and it is the most variable region of the HBV genome. P3283 also showed evidence of recombination between genotypes A2/F1b between nt 227 and 1593 (corresponding to pre-S2 and the carboxy terminus of HBV polymerase, respectively). Due to a recombination point located at the end of the genome, in order to obtain the same information density at this region, for the analysis of isolate HBV P1, a dataset composed of two consecutive concatenated complete genome sequences was used. Based on this analysis, the recombination points of HBV P1 were located between nucleotides 85 and 2390 (recombination points correspond to the pre-S2/polymerase region and the amino terminus of the HBV polymerase region, respectively). Simplot analysis of the concatenated HBV P1 genome is included in the Supplemental Material section.

In order to gain insight into the degree of genetic variability of HBV strains isolated in Uruguay, a phylogenetic analysis was performed. The six non-recombinant full-length genome sequences (P5, P9, P11, P17, P18 and P2729) were aligned with the corresponding sequences from 34 HBV strains isolated elsewhere for which the genotype and subtype had been determined previously. The results are shown in Figure 2.

Table 1 Accession numbers of complete genome sequences of Uruguayan HBV strains studied in this paper. Asymptomatic patients were blood donors. Symptomatic patients were detected as HBV infected for the first time. None of the patients were drug addicts or undergoing dialysis at the time of sample collection

Isolate	Accession number	Genotype	Clinical assessment	Serological assessment	Age	Sex	Origin
P1	KJ586810	A1/F1b recombinant	Symptomatic	Chronic infection	19	Female	Pando
P5	KJ586808	F-1b	Symptomatic	Acute infection	32	Female	Montevideo
P9	KJ586807	F-1b	Symptomatic	Acute infection	43	Male	Montevideo
P11	KJ586804	F-1b	Symptomatic	Chronic infection	28	Female	Montevideo
P17	KJ586805	F-1b	Asymptomatic	Acute infection	31	Male	Montevideo
P18	KJ586809	A2	Asymptomatic	Acute infection	39	Male	Montevideo
P342	KJ586811	D3/F-1b recombinant	Symptomatic	No data	43	Male	Montevideo
P2729	KJ586806	F-1b	Asymptomatic	Acute infection	40	Male	Montevideo
P3283	KJ586803	A2/F-1b recombinant	Asymptomatic	Acute infection	56	Male	Montevideo

All strains formed clusters, supported by high aLRT values, with references sequences. Based on these data, Uruguayan strains P2729, P17, P11, P9 and P5 were assigned to genotype F1b, and strain P18 to genotype A2. F1b strains clustered together with samples from Argentina, Chile and Venezuela in a single group in the phylogenetic tree. HBV F1b strains from Peru and Japan were located in a separate clade.

In order to assess the genetic diversity of HBV in Uruguay, the genotypes of eighteen partial S/P gene sequences (nt 502-770) were determined by phylogenetic analysis (Supplementary Material, Figure D). The overall genotype distribution was as follows: 5 genotype A (29.4 %), 4 genotype D (23.6 %) and 8 genotype F (47 %). The genotype of P3283 was not determined, and subsequently, this sample was analyzed in depth for evidence of recombination.

The amino acid signature patterns in the S and P ORFs corroborated the results of the phylogenetic analysis and allowed subgenotype assignment. On this basis, samples P1, P10 and P13 were classified as A1 due to the presence of rtL129 in the POL region. P12 and P18 were assigned as subgenotype A2 based on the presence of rtV163. The four samples belonging to genotype D showed the following characteristic pattern: nt A555 and amino acid rtY135 (P ORF), R122/T127/Y134 in the HBsAg protein. Moreover, the samples P342, P15 and P108 contained M125 in HBsAg, which has been described as a polymorphic site in subgenotype D3, but it was not found in the other subgroups of genotype D isolates [61]. The genotype F isolates showed the characteristic pattern (nt T562 and amino acid rtL151) and accordingly were classified as F1b.

Also, the best three matches for each complete Uruguayan HBV genome sequence described in this paper were identified using the available complete HBV genome sequences in the GenBank database. The best matches were mainly from the region. The best matches were as follows: P1, KJ854700 (Brazil); KJ854694 (Brazil), and KJ854690 (Brazil); P5, KJ843200 (Argentina), JN688691 (Argentina), and HM585197 (Chile); P9, KJ843200 (Argentina), JN688691 (Argentina), and HM585197 (Chile); P11, KJ843190 (Argentina), KJ843197 (Argentina), and KJ843174 (Argentina); P17, KJ843200 (Argentina), KJ843197 (Argentina), and JN688691 (Argentina); P18, KJ843218 (Argentina), KJ843217 (Argentina), and KJ843186 (Argentina); P342, FJ349209 (Belgium), KP322602 (China), and KP090177 (Brazil); P2729, KJ843200 (Argentina), KJ843197 (Argentina), and DQ823094 (Argentina); P3283, KJ843200 (Argentina), KJ843197 (Argentina) and HE981182 (Argentina).

In order to identify HBV variants with clinical importance, nucleotide and/or amino acid substitutions in the Uruguayan isolates were examined. Consensus reference sequences for each genotype/subgenotype were determined and aligned with nucleotide or deduced amino acid sequences obtained in this study. Each isolate that differed from the consensus sequence was defined as a mutant.

Important mutations involved in HBV pathogenesis were investigated, including mutations related to HCC development (G1896A, G1899A, Pre-S1 deletion, Pre-S2 deletion, A1762T/G1764A double mutation) and "a" determinant mutations (G145R, D144A, P142S, Q129H, I/T126N/A, M133L) [31]. The "a" determinant is an important epitope located at the major hydrophilic region (MHR) of HBsAg (aa 124-127) that is detected by the antibodies of commercial HBV detection kits. Mutations at this site could cause the HBsAg to be undetectable. Also, host antibodies against the "a" determinant neutralize HBV infection and are considered important for clearance of HBV infection.

Strain P342 had a G1896A mutation, which led to an e-negative HBV infection in this patient, and a 33-nucleotide deletion from position 2896 to 2928 in the Pre-S1 region involving Pre-S1 residues 3-13. Pre-S1 deletions

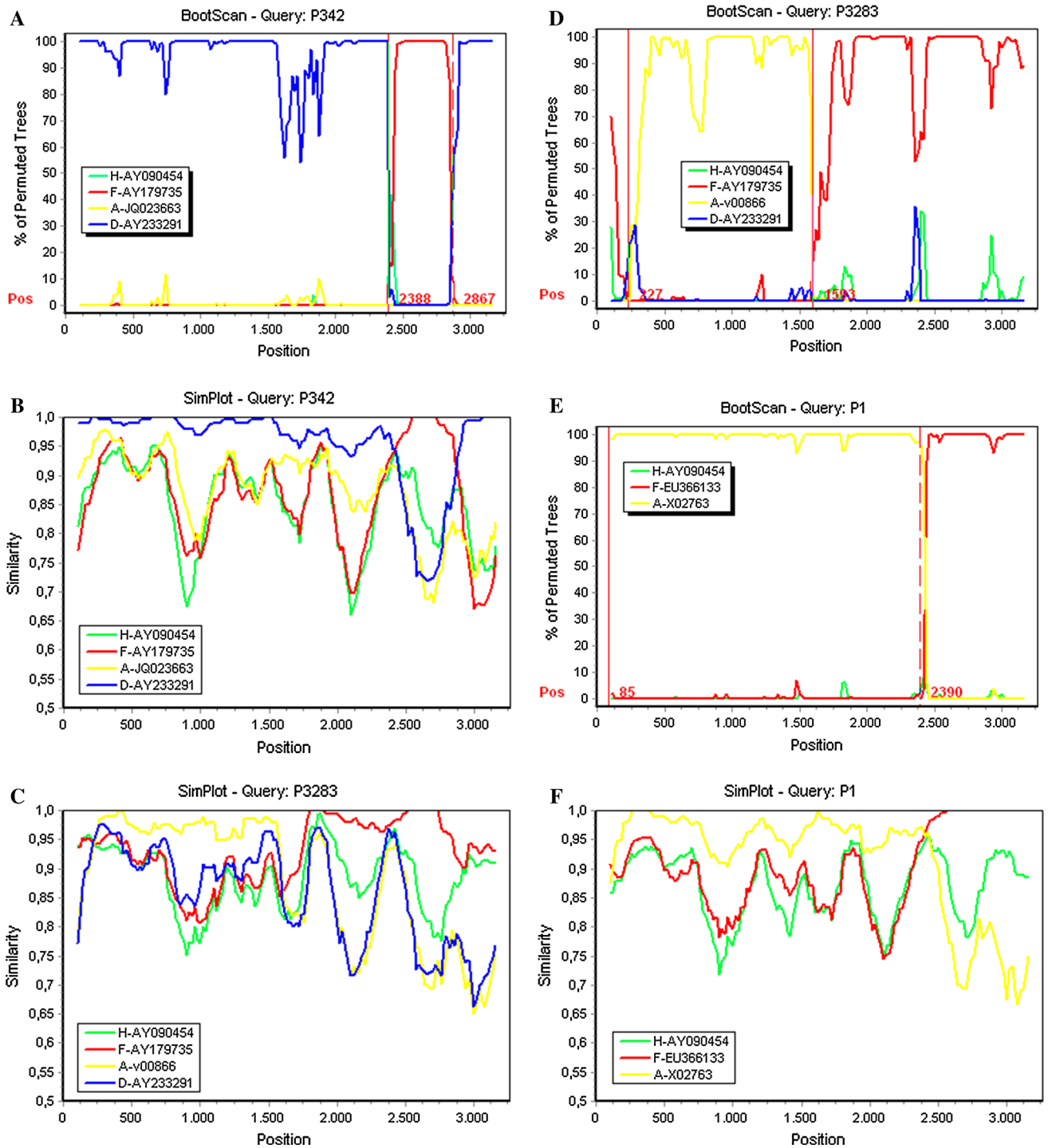

Fig. 1 Simplot analysis for the detection of recombinant HBV sequences. Panels A, C and E show similarity for each position. Panels B, D and F show percentage of permuted trees (BootScan). Reference HBV F, A and D genotypes are colored in *red*, *yellow* and *blue*, respectively. The Uruguayan HBV strain is represented as the horizontal upper line of the rectangle. The Uruguayan HBV strains are identified by a P, followed by a number. The three sequences P342, P3283 and P1 show two breakpoints that switch from genotype D to genotype F in the first case and from genotype A to genotype F in the last two cases. Concatenated P1 strain genome sequence analysis using Simplot is shown in Supplementary material (color figure online)

were also observed in strains P17, P2719 and P3283 corresponding to nucleotides 2913-2945 and Pre-S1 residues 9-19. Epitope changes in the "a" determinant were not observed for these patients, and only one isolate (P342) showed an amino acid change Q101H related to diagnostic failure [62], located outside of the major hydrophilic region

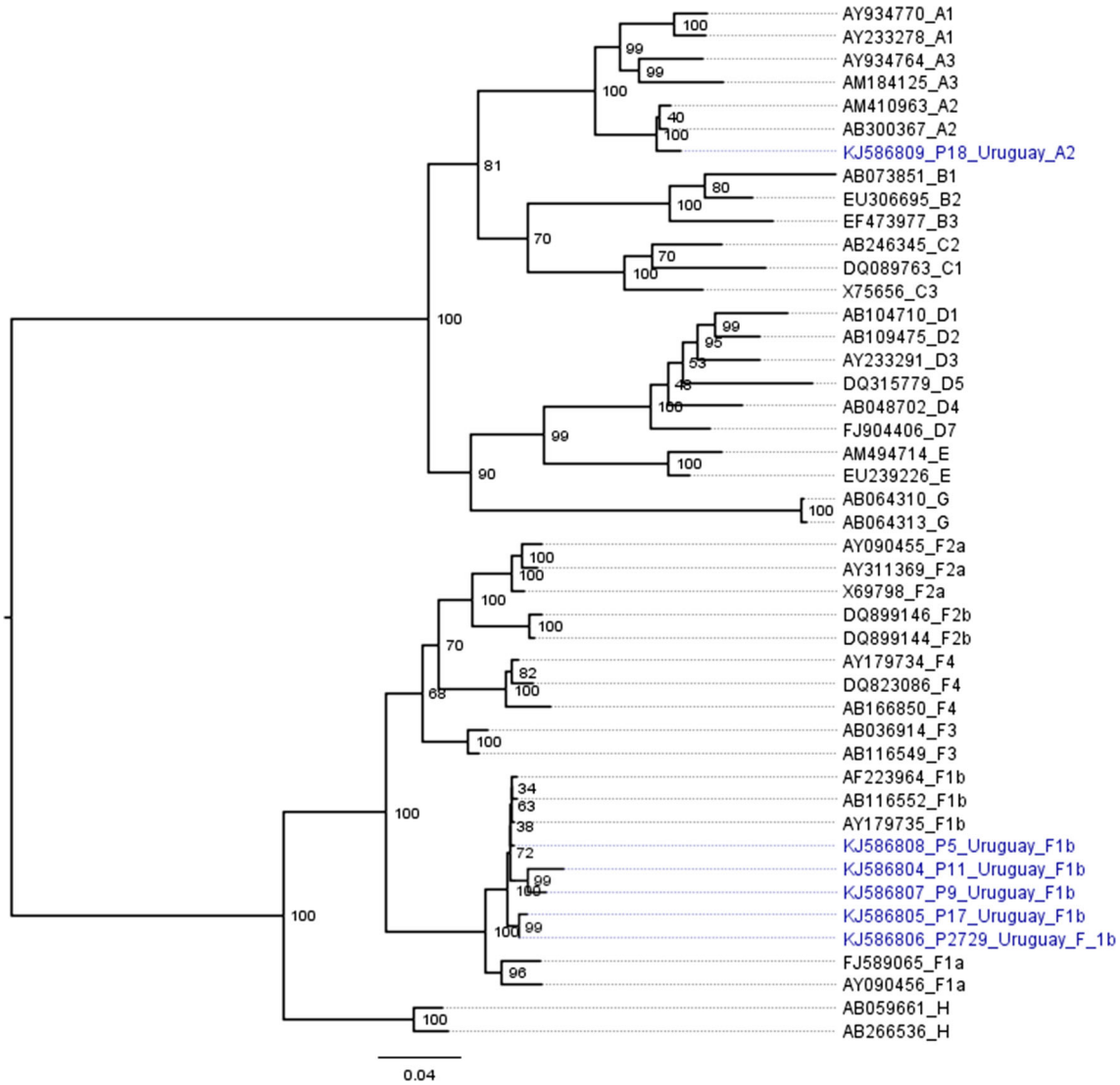

Fig. 2 Maximum-likelihood phylogenetic tree constructed using the PhyML (v3.0) program, based on the complete genome sequences of non-recombinant isolates from this study and a reference sequence of each HBV genotype/subgenotype available in the GenBank database. The tree was rooted using the midpoint method. The numbers at nodes correspond to bootstrap values (1000 replicates). The scale bar indicates the genetic distance. Sequences reported in this work are shown in *blue* (color figure online)

(MHR) of the HBV surface antigen. Substitutions in the reverse transcriptase domain of the viral polymerase were not detected. An HBV A genotype-specific insertion of 6 nt at position 2393 was observed for the novel Uruguayan strains P1 and P18.

Discussion

In spite of the limited number of complete HBV genomes studied and their different origins (asymptomatic blood donors and symptomatic patients), we found a significant proportion of genotype F isolates (5 out of 9, and three recombinants). This was confirmed by analyzing nine additional partial Uruguayan HBV genome sequences (Supplementary Material, Figure D). The following genotypes were identified: 8 genotype F1b (47 %), 3 genotype A3 (17.6 %), 2 genotype A2 (11.7 %) and 4 genotype D (23.5 %). This pattern of HBV genotype prevalence has also been reported in other countries in South and Central America [27, 37, 39, 62, 63]. All genotype F strains were F1b of the subgenotype. The high similarity of Uruguayan genotype F strains (based on complete and partial genome sequences) has also been reported by other authors [62, 63].

The Uruguayan HBV genotypes identified in this study reflect the three main origins of the Uruguayan population (European, African and Amerindian). However, the

proportion of Amerindian strains (F genotype) was higher than that of the Uruguayan population (9 % mixed Amerindian-European origin, according to the national population census of 2011). This is an interesting finding that supports what Uruguayan geneticists have been claiming: that there are many more people of Amerindian origin than currently recognized [64]. According to Sans *et al.*, Amerindian mitochondrial DNA (mtDNA) has been found in 34 % of the Uruguayan population (average), with the prevalence differing in different zones within the country (62 % in the north and 20 % in the capital Montevideo).

One sample (P18) was found to be genotype A2, which was predictable, since it is present in Europe, India, Africa and the Americas, but mainly in Northern Europe, and most Uruguayan people are of European origin. Another sample (P342) is related to genotype D, which has a Mediterranean origin. A recombinant (P3283) has partial HBV sequences belonging to genotype A2 of European origin. Five samples and three recombinants belong to or have partial sequences of the F1b genotype, respectively, with high similarity to other South American HBV F1b strains from Argentina, Chile and Venezuela. This was expected due to the proximity and close relationship between these nations.

The high percentage of recombinant strains in this study (three out of nine), was unexpected, because the prevalence of HBV infection in Uruguay is low. Two strains of different genotypes infecting the same hepatocyte are needed for recombination to occur. This would be expected in regions where carrier rates for chronic B infection range from 10 to 20 %. However, previous studies in Uruguay have found a high prevalence of HBV serologic markers in the population with risk factors (intravenous drug addicts, chronic dialysis patients) that approaches this rate [2, 3]. Recombination is an important factor contributing to the genetic variability of HBV [65, 66]. HBV recombinants in Latin America have been reported previously [31, 67]. An F3/A1 recombinant was found in an Afro-Colombian population [68], and an F4/D2 recombinant was reported in Argentina [69]. "Hot spots" for HBV genome recombination events have been described within the core region, pre-S1, pre-S2/S, polymerase, and X gene [69]. The recombination points identified for the three Uruguayan strains were located within the HBV genome "hot spots" described previously.

The three putative recombination events did not affect the region between nucleotides 250-1500, corresponding to the surface antigen and carboxy-terminal half of reverse transcriptase and whole RNaseH regions of HBV polymerase, showing its importance in the HBV life cycle. Changes in the HBV genome are in certain genomic regions restricted to the protein functions and are highly conserved. The reverse transcriptase (RT) domain of HBV polymerase and HBsAg overlap at RT aa 8-236, with the HBsAg ORF shifted downstream by 1 nt. This means that the third position of the polymerase codons correspond to the second position of the S codons. Nucleotide substitutions at the polymerase second codon position affects the amino acid in both RT and HBsAg, and this position has the highest degree of conservation in its nucleotide and amino acid sequence, like the RNAseH region of the polymerase or the PreS1 region [68, 69]. This could explain why we did not find recombination events within this conserved region. Other regions show higher genetic variability, like the "spacer" region of the polymerase or the N-terminal end of the X protein [70] and thus can support amino acid changes.

Conclusions

The complete HBV genome sequences from nine samples obtained from natives of Uruguay were analyzed. The high genetic variability of HBV strains and the mixed origin of our population (which is frequently denied) were reflected in the results of this study. Five samples were classified as genotype F1b, one as genotype A2, and three were recombinants (A1/F1b, A2/F1b and D3/F1b). Also, a G1896A mutation in one sample and partial deletion of Pre-S1 were observed in two samples. A higher than expected prevalence of genotype F strains was detected, supporting the hypothesis that there are more people of indigenous origin than declared in our population. Also, one third of the samples analyzed were recombinants. This appears to be inconsistent with the low HBV prevalence in Uruguay and is probably due to a higher HBV infection rate in drug addicts and dialysis patients, which could favor infection with multiple genotypes, and thus recombination.

Conflict of interest The authors declare that there is no conflict of interest regarding the publication of this paper.

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