First hepatitis B virus full-length genomic study among blood donors from Argentina: unexpected mutations in the circulating subgenotypes' proteins

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Abstract Hepatitis B virus (HBV) is a worldwide public health concern. The circulation of strains carrying mutations in the viral proteins implies both clinical and therapeutics challenges. HBV complete genomes (HBV-CGs) were reported from injecting drug users and HBV chronically infected patients from Argentina—including Amerindians—although no studies were conducted in blood donors. Here, we described HBV-CG sequences from the latter population. Some of the HBV sequences classified as B2 and C2 subgenotypes clustering together with Asian isolates, while others, such as D3, F1b, and F4, were homologous to European and Latin America sequences. New substitutions for all analyzed open reading frames and changes in the HBsAg hydrophobicity profiles were detected. Several HBV-CG subgenotypes are described for the first time in this population. Mutations observed in X, PreS, and P proteins have been associated with advanced liver disease, hepatocellular carcinoma, and/or natural resistance to nucleos(t)ide antiviral treatment. It deserves to be highlighted that these substitutions were detected in a population without epidemiological risk factors for viral infection, and most importantly, without any previous antiviral treatment (natural resistance). Regarding the remaining mutations, further research is warranted in order to determine their clinical and therapeutics relevance.

Keywords Hepatitis B virus · Blood donors · Complete genomes · Subgenotypes · Mutants

M. M. Biglione and V. L. Mathet have contributed equally to this work.

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Introduction

Hepatitis B virus is a worldwide public health concern. Taking into account that 240 million people are chronic carriers. HBV promotes liver inflammation, frequently associated with cirrhosis and/or hepatocellular carcinoma (HCC) [1, 2]. HBV genome length is approximately 3.2 kb, with four overlapping open reading frames (ORFs) (PreS/S, PreCore/Core [pC/C], X, and polymerase [P]) [1]. Two main reasons account for the high level of HBV genetic diversity. First, the virally encoded reverse transcriptase lacks proof-reading ability, and second, the HBV genome undergoes frequent recombination [3]. The presence of HBV strains with mutations in the viral proteins has

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important clinical and therapeutics implications [4]. HBV has been divided into eight genotypes (A–H) and two tentative genotypes (I, J), and some of them also classified into subgenotypes and clades with global distribution. The different genotypes are defined by >8 % and subgenotypes by >4 % divergence over the entire genome [5]. The A genotype is observed in Northwest Europe and in North America. The B and C genotypes are circulating in Southeast Asia, China, and Japan; the D genotype is globally distributed, being predominant in India and the Mediterranean region; the E genotype is mainly circulating in West Africa; and the F genotype can be detected in different populations from American continent [5, 6]. The G genotype was first discovered in France and the United States, and it has recently been reported in Belgium. The H genotype has been described in America and Japan [7].

HBV-CGs were reported for the genotypes A, D, E, F, and H in injecting drug users and HBV chronically infected patients—including Amerindians—from Argentina but until now they have not been reported from blood donors, with the exception of the C2 subgenotype described by our group in a previous study [8–12]. Taking into account such epidemiological background, the main aim of this study was to analyze the genetic diversity of HBV-CG circulating among blood donors from the Central region (Favaloro Foundation [FF] in the city of Buenos Aires) and from the Northeastern of Argentina (Central Blood Bank of Misiones Province [CM]).

Results

A total of 6 HBV-CG sequences were in agreement with the partial ones from those previously obtained at S/P and/or pC/C regions [12]. Two further previously published sequences from Argentina were also included in the phylogenetic analysis [12]. Six HBV-CGs from Central Argentina were ascribed as follows: FF3 (B2 subgenotype) exhibited the highest identity with Chinese and Vietnamese sequence; FF6,FF11, and FF7 classified as C2 together with Chinese and Japanese sequences; whereas FF2 classified as a Chilean strain (F1b subgenotype) and FF5 classified among F4 Argentinean strains. On the other hand, both sequences (CM1 and CM4) from Northeast Argentina the highest identity with Belgian D3 subgenotype sequences (Fig. 1). New substitutions were detected from all analyzed ORFs. Furthermore non-synonymous mutations—previously reported by our group—were also observed (Tables 1, 2) [12].

For the X protein, the triple mutation—I127/N/S/T, K130M, and V131I—was documented in both D3 subgenotype sequences. Moreover, in FF6 and FF7 sequences, the K130I mutation was detected, and in the FF2, the K130M/V131I double mutation was recorded.

In ORF C, the non-previously reported V149I and S180P changes were detected in CM1 and CM4 sequences (D3 subgenotype), being located inside at T cell epitope.

Within the ORF S, three sequences from C2 (FF6 and FF11) and D3 (CM1) subgenotype exhibited mutations affecting the start codon of the PreS2 domains (M1I in C2 sequences and M1 deletion in D3 sequences). Other changes for PreS1 and S are listed in Table 1.

In FF6 and FF11, hydrophobicity changes were documented for I1M in PreS2 and M198I, W199L, Y200F in ORF S. In FF7, the I45T and T49I mutants within the PreS2 generate slight changes in their hydrophobicity profile. Moreover, the larger number of mutations described in CM1 and CM4 isolates change the hydrophobicity pattern over the entire ORF S, while for isolate FF2, the S96P mutation in the PreS1 region does not affect such parameter. On the other hand, the C220S mutation recorded in FF5 sequence alters this profile (supplementary figure). Regarding the ORF P, several coding changes were documented in the terminal protein, spacer, rt, and RNAse H domains (Table 2). For rt domain, the mutations rtS317A and rtN337H were observed in the FF7 sequence (C2 subgenotype). The changes rtI16T, rtY54H, rtV266I, and rtQ267R were present in CM1 sequences, while rtN238H, rtV266R, and rtQ267H were identified in CM4 sequences (both D3 subgenotype). The mutations rtM344A were documented in the FF2 sequence (F1b subgenotype).

Discussion

The global human migrations affect the pattern of genotype distribution, introducing genotypes differing from those circulating in the original inhabitants [6]. This is the reason why genotypes B, C, and D are frequently observed in Argentina, together with the autoctonal F the most prevalent one [12, 13]. B2 and C2 subgenotype sequences clustered together with those from China, Japan, and Vietnam, possibly due to recent migrations from these countries to Argentina [14]. Moreover, the presence of D3 subgenotype in the Northeastern region of Argentina could be explained mainly by the contact with immigrants from the *post*-Columbian era, coming from different parts of Europe [15]. Therefore, CM1 and CM4 samples classified together with Belgian sequences, suggesting European immigrants as the original source of this virus strain. Genotype F can be detected in different populations from the America continent, and furthermore, in agreement with that FF2 sequence (F1b subgenotype) classified together with sequences from Chile as a putative consequence of ancient communities harboring HBV F genotype and/or to the recent historical migrations to and from this neighboring country [6, 16].

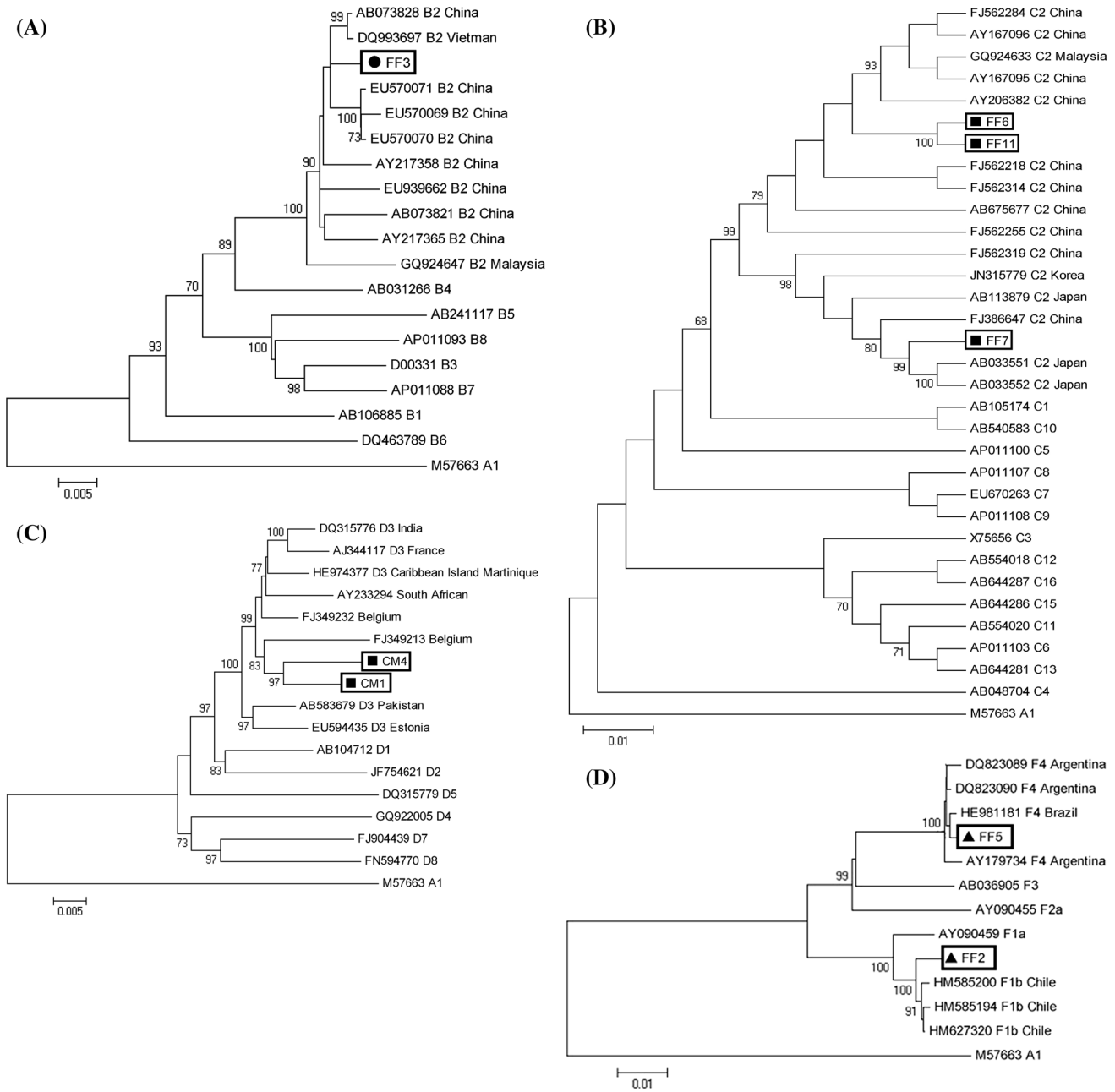

Fig. 1 Phylogenetic relationships of HBV-CG sequences shown in this study were compared to representative sequences belonging to all reportedly known genotypes and subgenotypes using neighbor-joining. Bootstrap statistical analysis was performed using 1,000 bootstrap iterations. *Bars* indicate the number of substitutions *per* site. **a** this *tree* represents the genotype B sequences; **b** genotype C; **c** genotype D; **d** genotype F. All sequences deposited in the GenBank are named with the corresponding accession number. The GenBank accession numbers of those sequences reported in this study are JX079936, JX079937, KF485389-90, KM359440-2, and KM3866 76 sequences mentioned by adding the underlined initials FF (Favaloro Foundation, Buenos Aires city) and CM (Central Blood Bank of Misiones)

Regarding the X protein, it is considered as a promiscuous transcriptional transactivator. Different authors have documented that K130M and V131I mutants exhibited higher activity, which implies a higher risk of HCC development. Moreover, the presence of the triple mutant is another crucial factor [17–19]. Even though these mutations are frequently documented in patients with advanced liver disease (cirrhosis and/or HCC), these strains were also isolated in asymptomatic blood donors recruited in blood banks, where they undergo a complete background questionnaire [20, 21].

A high mutation rate has been previously documented in specific epitopes of the Core protein for T and B lymphocytes in blood donors [22]. In this context, all

Table 1 Mutations in ORF S corresponding to 8 Argentine blood donors HBV complete genome

Samples	Genotype	ORF S		
		PreS1 (1–108/119)	PreS2 (1–55)	S[a]
FF2	F1b	S96P	–	–
FF3	B2	I84L, A91V	–	–
FF5	F4	–	–	C220S
FF6	C2	N39H, H51Q	M1I	*M198I, W199L, Y200F*
FF7	C2	I45T, T49I		
FF11	C2	H51Q	M1I	*M198I, W199L, Y200F*
CM1	D3	D16E, T40N, S98T, N103D	M1 deletion, N4T, T6S, F22L P41H, I49T, P52L	F8L, *C107G, L186S, S204R, Y206C*
CM4	D3	T40N, S90T, N103D	P41H	L42P

FF Favaloro Foundation, Buenos Aires city, *CM* central blood bank of misiones

[a] Positions 1–48 and 210–226 are here in analyzed, the remaining sequence was previously reported by Delfino et al., 2014 (in *Italic*)

Table 2 Mutations in ORF P corresponding to 8 Argentine blood donors HBV complete genome

Samples	Genotype	ORF P domains			
		TP (1–183)	S (184–348)	Rt [349(rt1)–692(rt344)][a]	RH (693–845)
FF2	F1b	–	M125I, S259, F276S	rtM344A	–
FF3	B2	R183G	P215S	–	–
FF5	F4	–	D81N	*rtQ149K*	–
FF6	C2	Q177H	K219T, L232 M, S292A, A301T	*rtY124H, rtV207I*	–
FF7	C2	–	L205F, C213S, V214I	rtS317A, rtN337H	L694I
FF11	C2	Q177H	L232M, S292A, T301A	*rtY124H, rtV207I*	R713Q
CM1	D3	K118N, D178E	P197T, Q220H, A273T, K283R, L294H, F310S	rtI16T, rtY54H, *rtL115W, rtA194V, rtS213T*, rtV266I, rtQ267R	A698T, I699 M
CM4	D3	D178E	Q220H, A234S, V270D, A273T, K283R, A285S, L294H, S300F	*rtS213T, rtQ215H;* rtN238H, rtV266R, rtQ267H	A698T, S787Y

FF favaloro foundation, Buenos Aires city, *CM* central blood bank of misiones, *TP* terminal protein, *S* spacer, *rt* reverse transcriptase, *RH* RNAse H

[a] Mutations outside the rt domain (positions 64–220) were described previously by Delfino et al., 2014 (in *talic*)

substitutions detected in the Core protein are relevant due to the fact that in chronically infected HBV patients, a significantly diminished T CD4+ and CD8+ lymphocytes-specific response to viral epitopes has been reported. This event is more evident in the CD8+ population, showing an impaired IFN- γ production in ex vivo studies [23].

Among the mutations affecting the PreS/S region, the most frequently reported are the PreS1 and/or PreS2 deletions, along with the presence of *stop* codons in PreS2 [24]. HBV isolates with these mutations are often naturally selected in HBV carriers, particularly in cases with long-lasting chronic infection. Much evidence indicates that infections with PreS/S HBV variants correlate with the most progressive forms of liver disease and HCC. Moreover, these mutants were associated to A, C, D, or F genotypes [25–27]. All C2 and one D3 subgenotype sequences showed mutations in the start codon of the PreS2. In vitro studies have reported that these substitutions—M1 deletion mainly—produce a significant reduction in the HBsAg secretion, retention of envelope proteins in the endoplasmic reticulum, and decreased virion release efficiency [12, 24]. This impaired secretion could have important consequences in the outcome of screening tests, leading to misdiagnosis in some cases; nevertheless in our samples, they were still detectable.

Several years ago, Mangold et al. determined the significance of cysteine residues in the S protein for proper secretion and/or folding of HBsAg by site-directed

mutagenesis [27]. It is shown that changes in the residues within the HBsAg significantly alter the spatial structure and antigenicity of the protein by disrupting disulfide bonds [28]. In this study, the C200S mutation (FF2) generated a considerable variation on its hydrophobicity profile, despite this HBsAg remains detectable, and therefore, this was not translated into modifications in the antigenicity pattern. Moreover, size and charge changes in such amino acidic position are more likely to be the important structural parameters, since they change less with the C to S change than that does hydrophobicity.

In the P protein, amino acid mutations at positions rtN238T/D have been previously reported as potentially associated with adefovir (ADV) resistance [29]. It is important to point out that this substitution was detected in the CM4 sequence (D3 subgenotype), sample obtained from a blood donors untreated, and therefore, it represents a natural resistance.

It should be considered that all HBV-CG sequences reported herein perhaps represent a small subset of the pool of HBV sequences published from Argentina. However, it should be stressed that this study provides the first HBV-CG of a genotype B isolate.

In summary, several HBV-CG subgenotypes were described for the first time in blood donors. The HBV-CG phylogenetic analysis allowed us to suggest a putative origin of the strains circulating in blood donors from Argentina. All sequences exhibited mutations within the ORFs. The mutations described in X and P proteins have been associated with advanced liver disease and natural resistance, respectively. PreS/S variants are related to more aggressive forms of liver disease and HCC in chronic HBV-infected patients. It deserves to be highlighted that these substitutions were observed in blood donors, usually considered a low risk population. Regarding all these results further research needs to be carried out in order to approach to their biological significance.

Materials and methods

HBV DNA positive serum samples by PCR in blood donors from the Central region (Favaloro Foundation [FF2, FF3, FF5, and FF6 samples ascribed as subgenotype F1b, B2, F4, and C2, respectively] in the city of Buenos Aires) and from the Northeastern of Argentina [Central Blood Bank of Misiones Province (CM1 and CM4 samples classified as subgenotype D3)], that were previously studied, were considered for this work [12]. An amplification protocol adapted for the HBV-CG was carried out [30]. The PCR product was sequenced in both directions using a BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA) and aligned using CLUSTALX v1.83 software [31] with sequences from GenBank. Each alignment was done with 497, 783, 621, and 131 sequences for the genotypes B, C, D, and F, respectively. These sequences constitute the whole dataset of HBV-CG available in the GenBank at the time of submission of this manuscript (November 2014). Phylogenetic trees were obtained using the neighbor-joining algorithm with the Kimura two-parameter model in MEGA v 4.0 [32]. The phylogenetics analysis of FF7 and FF11 sequences included in this study were previously described in a published paper [12]. The nucleotide sequences were translated into amino acid sequences for the corresponding to the ORFs using BioEdit 7.2 software [33]. The hydrophobicity plot for ORF S was performed according to the Kyte & Doolittle method.

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