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ORIGINAL ARTICLE

Hepatitis B virus and hepatitis D virus in blood donors from Argentina: circulation of HBsAg and reverse transcriptase mutants

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Abstract In Argentina, current procedures to ensure the safety of the blood supply for transfusion include the serologic detection of specific blood-borne infections. The aim of this study was to evaluate the prevalence and the genetic diversity of hepatitis B virus (HBV) and hepatitis D virus (HDV) in blood donor populations from two distantly located Argentine regions. Data from 56,983 blood donations from the Favaloro Foundation, in the city of Buenos Aires (Central Region), and the Central Blood Bank of Misiones Province (Northeast Region) were analyzed. Samples that were reactive for HBsAg were analyzed for HBV-DNA characterization and HDV serological and molecular analysis. The HBV prevalence was 0.12 % for HBsAg and 1.68 % for anti-HBc antibodies in Buenos Aires, and 0.73 % and 8.55 %, respectively, in Misiones. Seventy-seven HBsAg-reactive samples were analyzed by polymerase chain reaction for HBV-DNA. Subgenotypes A2, B2, C2, F1b and F4 (Buenos Aires) and F1b and D3 (Misiones) were detected. Several mutations within the major hydrophilic region of HBsAg, the reverse transcriptase, the basal core promoter, and the precore/core were detected. HDV genotype 1 was identified in Buenos Aires. This study confirms the circulation of several HBV subgenotypes, as well as known and newly identified variants, and the presence of HDV1 in this population. A thorough investigation has to be carried out to evaluate the clinical importance of some of the documented mutations as well as those detected in the HDV1 case.

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Introduction

Hepatitis B virus (HBV) causes liver inflammation, frequently associated with cirrhosis and/or hepatocellular carcinoma (HCC) [1]. Hepatitis D virus (HDV) requires the helper function of HBV for its assembly and transmission [2]. At least nine major HBV genotypes (A-H, J), including multiple subgenotypes, and eight HDV genotypes (HDV 1-8) have been defined [3–5].

HBV infection is globally distributed, with high (>8 %), intermediate (between 2 and 8 %) and low (<2 %) endemic areas, while HDV is highly endemic in Mediterranean countries, the Middle East, Central Africa, and the northern areas of South America [6, 7]. Argentina shows a low prevalence of HBsAg, which ranges from 0.2 % to 0.3 % in blood donors (BDs) [8–10]. Genotypes A-F and H have been reported in different populations from Argentina, with

the F genotype being the most common [11–16]. In addition, HDV infections were also observed in the 1990s, with a prevalence ranging from 0.7 % to 5.7 %, depending on the study group [17, 18]. Contrary to the recommended recruitment model proposed by the World Health Organization, the blood supply in Argentina is based mainly on repository or replacement donations. Current procedures to ensure the safety of blood transfusions include reviewing the records of BDs and the serological detection of specific blood-borne infections [19]. At present, only the detection of HBsAg and anti-HBc antibody markers for HBV is mandatory in Argentina.

The main objective of this study was to estimate the prevalence of HBV and HDV infections and to analyze the genetic diversity of the viruses detected in voluntary BDs from two geographically distant Argentine regions (approximately 1,600 km distance).

Materials and methods

Plasma samples

A retrospective analysis of serial cross-sectional data collected at two blood banks from the Central (Favaloro Foundation [FF], in the city of Buenos Aires) and Northeastern (Central Blood Bank of Misiones Province [CM]) regions of Argentina was carried out. Data from consecutive blood donations were obtained from a total of 56,983 donors: 50,445 from Favaloro Foundation (2003-2008) and 6,538 from Central Blood Bank of Misiones Province (2009). In both blood banks, serological tests were performed by enzyme immunoassays for HBsAg (Murex and Axsym, Abbott Diagnostics, USA) and total IgG anti-HBc (Hepanostika, Biomerieux, The Netherlands).

HBV-DNA amplification

Seventy-seven reactive samples for HBsAg were analyzed for HBV-DNA, which was extracted from 200 µl of the sample using a QIAmp DNA Mini Kit (QIAGEN AG, Basel, Switzerland). A polymerase chain reaction (PCR) for the S/Pol overlapping genomic region (S/P, nt 256-796) [20] and a nested-PCR (nPCR) for the preCore-Core region (preC/C, nt 1736-2471) [21] were performed. An amplification protocol for the full-length genotype C HBV-DNA was adapted from one described elsewhere [22]. Appropriate precautions were strictly followed to avoid cross-contamination [23]. Each HBV DNA amplified product was validated, since negative controls were added from the extraction step rendering the expected negative results. All positive PCR results were confirmed from the DNA extraction step in a second independent experiment. The potential Taq-dependent DNA misincorporation rate was investigated by bi-directionally sequencing PCR products derived from a GBV-C/HGV clone as previously described [24].

HBV serological markers

HBV-DNA-positive samples were tested for the detection of HBeAg (AxSYM, Abbott Diagnostics, USA), anti-HBe antibodies (AxSYM, Abbott Diagnostics, USA), and anti-HBs antibodies (AxSYM, Abbott Diagnostics, USA).

HDV serology and HDV-RNA amplification

The 77 HBV-reactive samples were tested in duplicate by ELISA for total anti-HDV antibodies (Murex, Abbott Diagnostics, USA). Concomitantly, RNA extraction was performed using 200 µl of plasma and TRIzol Reagent (Life Technologies, USA). The cDNA was prepared by reverse transcription (RT) with random hexamers and AMV reverse transcriptase (Promega, USA). Amplification of a partial region coding for HDAg was performed by nested PCR, in which one of the primers had been described elsewhere [26] and the remaining one was designed by the authors [27]. The first round produced a fragment of 415 bp (853-1267) [25], and the second round spanned a total of 353 bp (889-1241),

RFLP and cloning

To solve discrepancies in the assignment of S/P and preC/C genotypes, two different analyses were performed for some strains: a) RFLP of S amplicons according to a reference protocol [20] and b) cloning of the PCR products corresponding to different regions of the HBV genome (GR1: nt 1736-2471 and GR2: nt 1824-2654) into the pGEM-T Easy Vector system (Promega, Madison, WI) according to the manufacturer's instructions.

Sequencing and phylogenetic analysis

PCR products and plasmid DNA were sequenced directly in both directions with the corresponding amplification primers using a BigDye Terminator Cycle Sequencing Ready Reaction Kit on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Sequences were aligned using CLUSTALX v1.83 software [28]. Phylogenetic trees were obtained using the neighbor-joining algorithm with the Kimura two-parameter model of molecular evolution in MEGA v 4.0 [29]. The HBV genotype/subgenotype classification was assigned according to Shi et al. [30].

Table 1 HBV serological markers and genotypes from 19 HBV-DNA-positive samples from blood banks in Buenos Aires (FF) and Misiones Province (CM)

Serological markers								S/P regions		pC/C region	
Sample	Age	Sex	HBsAg	anti-HBc	anti-HBs	HBeAg	anti-HBe	PCR	Genotype	PCR	Genotype
FF1	27	M	R	R	NR	NR	IS	Negative	NA	Positive	D3
FF2	25	M	R	R	NR	IS	IS	Positive	F1b	Positive	F1b
FF3	26	M	R	R	NR	R	NR	Positive	B2	Positive	B2
FF4	28	M	R	R	NR	NR	NR	Positive	A2	Positive	A2
FF5	27	M	R	R	NR	IS	IS	Positive	F4	Positive	F4
FF6	31	M	R	R	NR	NR	R	Positive	C2	Positive	A2
FF7*	49	M	R	R	NR	R	NR	Positive	C2	Positive	C2
FF8	40	M	R	R	NR	NR	NR	Positive	C2	Negative	NA
FF9	31	M	R	NR	NR	NR	NR	Positive	C2	Negative	NA
FF10	41	F	R	R	NR	NR	R	Positive	C2	Negative	IS
FF11*	55	F	R	NR	NR	NR	NR	Positive	C2	Positive	C2
FF12	51	F	R	R	NR	NR	R	Positive	C2	Negative	NA
FF13	18	M	R	NR	NR	NR	NR	Positive	C2	Negative	NA
CM1	55	M	R	R	NR	NR	R	Positive	D3	Positive	D3
CM2	28	M	R	NR	NR	R	NR	Positive	F1b	Negative	NA
CM3	40	F	R	R	NR	NR	R	Positive	F1b	Negative	NA
CM4	46	M	R	R	NR	NR	R	Positive	D3	Positive	D3
CM5	32	M	R	R	NR	IS	IS	Negative	NA	Positive	D3
CM6	21	M	R	R	NR	IS	IS	Negative	NA	Positive	D3

FF, Favaloro Foundation samples; CM, Central Blood Bank of Misiones samples; M, male; F, female; NR, non-reactive; R, reactive; NA, non-amplified; IS, insufficient sample

* Full length-characterized HBV DNA; D3, "D3 new"; C2, quasi-subgenotype C2 [30]

Analysis of the regulatory regions and ORFs of HBV and HDV

The nucleotide sequences representing the basal core promoter (BCP), the pC, C, and partial S (amino acids [aa] 55-210)/P (aa 64-220) regions for HBV and the HDAg-S (aa 110-195) and HDAg-L (aa 110-214) for HDV were translated into aa sequences according to their corresponding open reading frames (ORFs) and compared with the consensus sequences from the corresponding genotypes using BioEdit software.

Statistical analysis

Statistical tests were performed with the Student *t*-test and the chi-square test (Epidat v3.1 software) (PAHO/WHO, 2006) as appropriate; *p*-values <0.05 were considered significant.

Results

Prevalence of HBV/HDV infection

The seroprevalence was 0.12 % (61/50445; 95 % CI: 0.09-0.152) for HBsAg and 1.68 % (851/50445; 95 % CI: 1.57-1.8) for anti-HBc antibodies in BDs from the Favaloro Foundation and 0.73 % (48/6538; 95 % CI: 0.52-0.95) and 8.55 % (559/6538; 95 % CI: 7.86-9.23) in BDs from the Central Blood Bank of Misiones Province. These last values were significantly higher than those from the Favaloro Foundation ($p<0.0000$ and $p<0.0000$, respectively). Of the total HBsAg-reactive samples, 77 had enough volume for subsequent analysis (47 from Buenos Aires, and the remaining 30 from Misiones). Out of the 47 HBsAg-reactive samples from Buenos Aires, a single one (FF14) was found to be anti-HDV antibody reactive, reaching a final prevalence of 0.0000198 % (1/50,545) and 2.12 % in HBsAg-reactive samples. FF14 was reactive for both anti-HBc and anti-HBe antibodies. All HBsAg-reactive samples from Misiones were non-reactive for anti-HDV antibodies.

Detection of HBV-DNA and HDV-RNA

Out of the 47 samples from the Favaloro Foundation (39 males; mean age = 33.5 ± 11.6 years), 12 allowed the amplification of HBV S/P region; and 8, the preC/C region. Out of 30 samples from Central Blood Bank of Misiones Province (27 males; mean age = 35.6 ± 10.6 years), 6

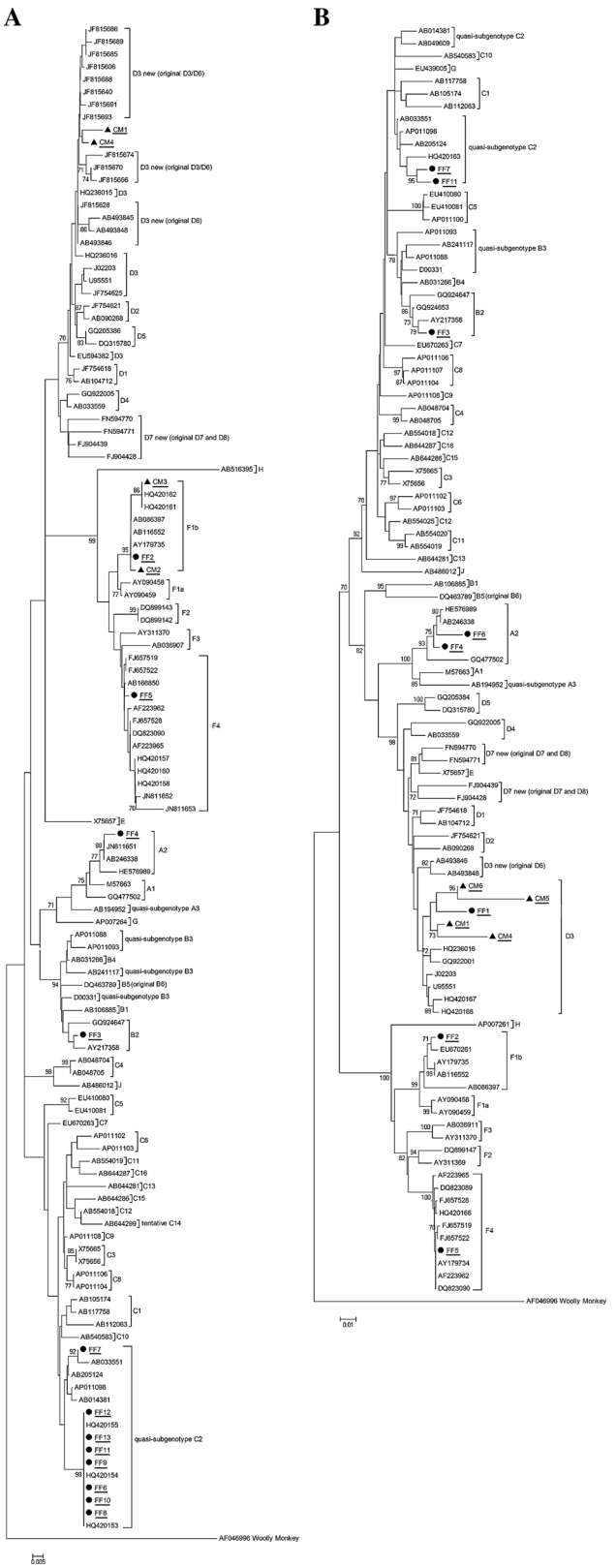


Fig. 1 Phylogenetic relationships of 31 HBV sequences from this study compared to representative sequences belonging to all known genotypes and subgenotypes, determined by neighbor joining. Bootstrap statistical analysis was performed using 1000 datasets, and the numbers at the nodes indicate the bootstrap values. **A.** Phylogenetic tree based on a portion of the S region (nt 369-769) of the HBV genome. **B.** Phylogenetic tree based on the the pC/C region (nt 1784-2391). All sequences obtained from GenBank are identified by accession number. The GenBank accession numbers of the sequences reported in this study are JX079922-JX079947. These sequences are indicated by the underlined initials FF (Favaloro Foundation, Buenos Aires) and CM (Central Blood Bank of Misiones). The scale bar indicates the number of nucleotide substitutions per site. The nomenclature of subgenotypes was proposed by Shi et al. [30]

were positive for HBV-DNA (Table 1). FF14 was found to be HDV-RNA positive, although HBV-DNA could not be detected at either the S/P or preC/C region.

HBV and HDV phylogenetic analysis

The S/P region sequences from Buenos Aires were classified as subgenotype A2, B2, quasi-subgenotype C2, F1b and F4. Two of the four HBV sequences from Misiones were assigned to subgenotype F1b, while the remaining two clustered with "new D3" according to the nomenclature proposed by Shi et al. [30] (Fig. 1A). The preC/C region sequences (FF1, CM5, CM6 samples)–from which the S/P region could not be amplified–were classified as subgenotype "new D3" (Fig. 1B). Seven out of eight HBV C2 sequences clustered together, with a bootstrap value of 98 % (FF6, FF8-FF13). The complete genomes from one of these sequences (FF11) and from another C2 sequence (FF7; at a separate position in the phylogenetic tree) were analyzed. These two sequences were classified as quasi-subgenotype C2 (Fig. 2), and the identity matrix revealed 98 % identity between FF11 and C2 reference sequences obtained from GenBank.

The preC/C region of FF6 was assigned to the C2 quasi-subgenotype, and the S/P region was assigned to the A2 subgenotype (Table 1). To solve this discrepancy in the S/P and preC/C genotypes, two different analyses were performed. RFLP of the S region demonstrated the presence of only the C2 genotype, while phylogenetic analysis of the clones documented the presence of HBV A2 (n = 2) and C2 (n = 2) subgenotypes.

The FF14 HDV sequence was classified as genotype 1, clustering together with sequences from Asia and Argentine Amerindians from Misiones (Fig. 3).

Analysis of the HBV/HDV regulatory regions and ORFs

Six out of 12 preC/C sequences showed mutations in the BCP (A1762T and/or G1764A). Other mutations were detected in T cell core epitopes (Table 2). Several sequences showed different mutations within the major hydrophilic region (MHR) within the S protein. Regarding

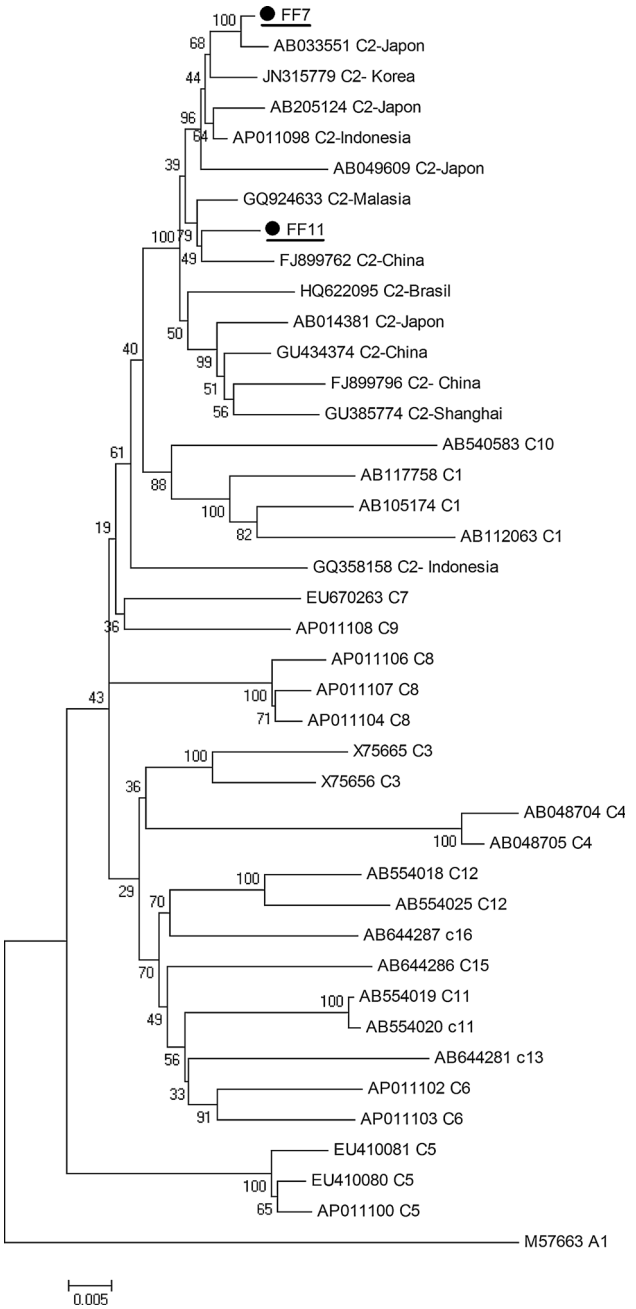


Fig. 2 Phylogenetic relationships of full-length C2 HBV genome sequences from this study (FF7 and FF11; Favaloro Foundation, Buenos Aires city) compared to representative sequences belonging to subgenotypes C1-C13, C15 and C16 using neighbor joining. Bootstrap statistical analysis was performed using 1000 datasets, and the numbers at the nodes indicate the bootstrap values. All sequences obtained from GenBank are identified by accession number. The GenBank accession numbers of the sequences reported in this study are KF485389 and KF485390. The scale bar indicates the number of nucleotide substitutions per site

the RT domain (P protein), several mutations were observed in 7 of the 16 sequences; moreover, the rtY124H and rtV207I mutations were identified in the above-mentioned seven C2 sequences (Table 3).

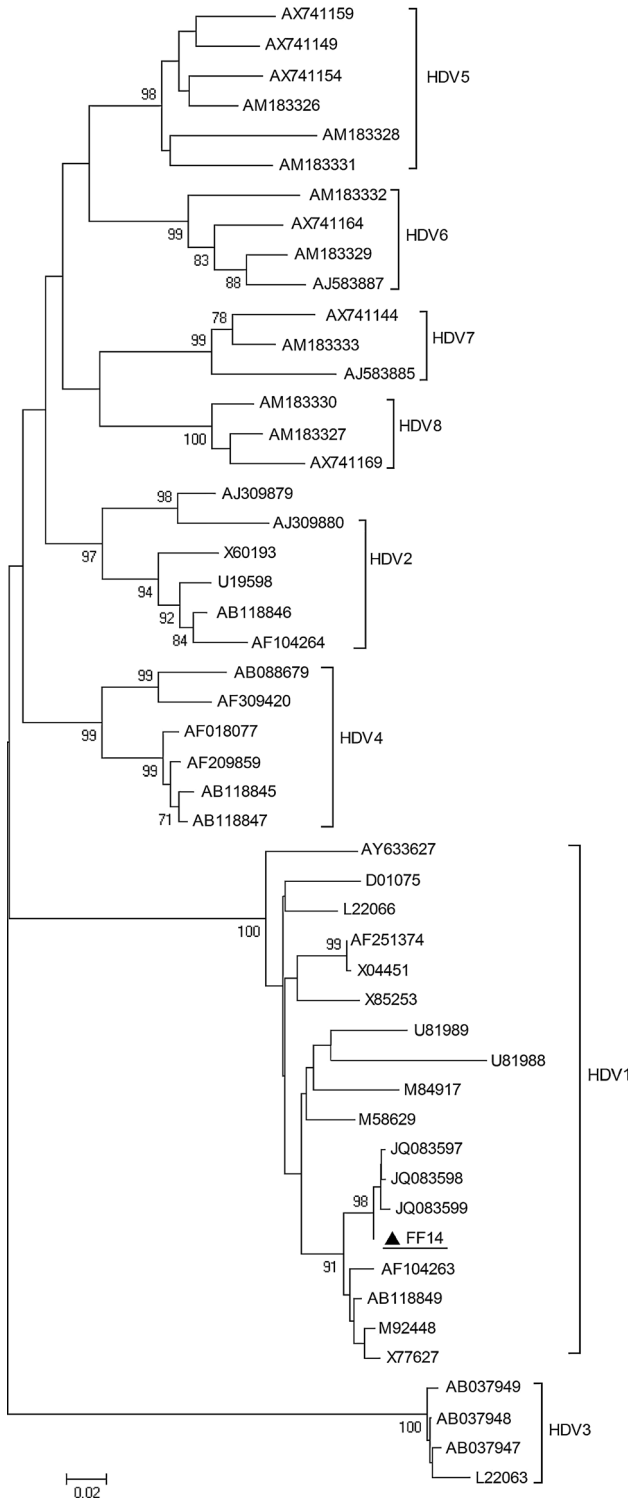
The partial amino acid sequence of HDAg (FF14) exhibited several non-synonymous changes: V149I, G151D, V188L, I198L (within the nuclear export signal), when compared with the HDV1 consensus sequence.

Discussion

Our study confirms a low HBV endemicity in BDs at two blood banks in central and northeastern Argentina and the presence of HDV in one BD in Buenos Aires. Although both HBsAg and anti-HBc antibodies from Buenos Aires donors were found at low prevalence, as reported in 2004 [12], these serological markers were significantly higher in BDs in Misiones. These data are consistent with the previously reported prevalence rates from Amerindians from Misiones [31], highlighting the need to improve health control policies in this region.

Concerning HBV genotypes, this study confirmed the circulation of F1b subgenotype in Misiones, as we have recently reported from Mbyá-guaraní natives living in this province [31]. Also, two sequences (CM1 and CM4) clustered together with D3, D6 and previously proposed "D3/D6 recombinant sequences" [32]. Further analysis of CM1 and CM4 sequences led to their final classification as subgenotype "new D3", in agreement with a very recently proposed nomenclature [30, 33].

Regarding Buenos Aires, and in contrast to one published study in which genotype F was reported to be slightly predominant [12], the current survey demonstrates that quasi-subgenotype C2 was the most prevalent in this cohort. However, it should be taken into account that the time period during which the BDs visited the public bank(s) was not mentioned in the previous study, precluding proper temporal comparisons between both reports. A potential bias of the current study with respect to the high C2 prevalence in Buenos Aires might be associated with blood donations from related donors at the blood bank during the same year. These C2 sequences, which were confirmed by analysis of the full-length genome, were clustered with other C2 sequences recently detected in Sagua-Huarpes Argentine Amerindians (Central Western Region) [31], suggesting a recent internal migration event. An alternative hypothesis might involve recent migrations from Asia [34], as well as from other South American countries, since C2 has been recorded in Bolivia in both natives and Japanese immigrants [35]. The remaining HBV genotypes detected were A2, F1b and F4, confirming previous results [12], while a single B2 sequence was detected in BDs for the first time. Moreover, in one of the samples (FF6), the coexistence of variants assigned to C2 and A2 has been confirmed.



◄ **Fig. 3** Phylogenetic relationships of the sole HDV sequence obtained from Argentine blood donors to representative sequences belonging to all known genotypes using neighbor joining. Bootstrap statistical analysis was performed using 1000 datasets, and the numbers at the nodes indicate the bootstrap values. The tree represents the partial HDAg region (nt 912-1230) according to a HDV reference strain (GenBank accession number AB118849). All sequences obtained from GenBank are identified by accession number. The sequence reported in this study is indicated by adding the underlined initials FF14 (Favaloro Foundation, Buenos Aires; GenBank accession number JX011008)

In Argentina, BCP and preC/C mutants have been detected in chronic hepatitis B patients, in HIV-1-coinfected patients, and in BDs [11, 12, 36]. In the present study, mutations in at least one nucleotide of these regions were found in 83.3 % of the samples. The A1762T/G1764A mutation, which was found in 33.3 % of the sequences, could also alter the X protein transactivator activity by causing amino acid changes (L130M and V131I) that are known to be associated with an increased risk of HCC [37]. On the other hand, G1896A preC mutations, which are responsible for the e-minus phenotype, and C1858T (16.66 % of our sequences) are not associated with the risk of HCC, regardless of HBeAg status or HBV genotype [38]. In our study, most of the HBsAg-positive samples proved to be HBeAg non-reactive (Table 1). Such a profile is known to exhibit a higher frequency of mutations at nucleotide positions 1846, 1896 and 1899 in preC/C than is found in HBeAg-positive subjects, as observed herein.

As reported for Amerindians from Misiones with occult HBV infection (OBI), the F179L (F1b) aa replacement [31] was detected in one non-OBI BD (CM3). The D3 sequences exhibited several mutations within the MHR, including the Y100S substitution, which has been reported to be associated with OBI [39]. The remaining D3 sequence exhibited the C107G replacement, which has been shown to be indispensable for efficient secretion. Interestingly, HBsAg was nevertheless detected in these isolates, suggesting that such mutations are not necessarily the sole cause of OBI cases. The M198I, W199L and Y200F mutations, which were present in seven C2 sequences, have been described recently in Sagua-Huarpes Amerindians [31]. Furthermore, the C107G and M198I mutations were shown to disrupt conformational region(s) of the HBsAg protein, affecting its antigenicity [40, 41]. Regarding the RT domain of the P protein, the D3 sequences exhibited rtS213T, rtQ215H and rtA194V mutations (Table 3). In this regard, rtS213T has been reported previously to be associated with resistance to adefovir in treated patients [42, 43]. Moreover, the rtQ215H mutation has been detected at baseline and after 6 months of lamivudine therapy [44], a true pretreatment mutation (also known as natural resistance). With regard to the rtA194V mutation, it is worth mentioning that residue rtA194 is located in a loop at the end of the B domain, which contains an alpha helix that interacts with the nucleic acid template. Since the rtA194T substitution, confers tenofovir resistance [42, 43], may affect polymerisation efficiency by causing allosteric changes that result

Table 2 Mutations in the preC/C genomic and C regions in samples from 12 HBV-DNA-positive blood donors from the city of Buenos Aires and the province of Misiones

Samples	PreC/ C genomic region		C region
	BCP (1742-1814)	Precore (1814-1901)	Core*
FF1	A1762T, G1764A	G1816T, A1846T, G1899A	P130V, T147A
FF2	A1762T, G1764A, C1773T	A1846T	-
FF3	-	-	V27I
FF4	C1773T	A1846T, T1850A, T1858C	-
FF5	-	A1846T	-
FF6	A1762T	A1846T, T1850A, T1858C	R82M
FF7	A1762T	-	-
FF11	-	-	-
CM1	T1753C, A1762T, G1764A	A1846T, G1899A	-
CM4	A1762T, G1764A	A1846T, G1896A	S21Q, E64D, M66I, T67N, A69S, V72A, P79Q, A131P
CM5	-	S26G, T67S, E77D,	P79Q, L84Q, A131P
CM6	-	A1846T, G1896A, G1899A	-

FF, Favaloro Foundation, Buenos Aires; CM, Central Blood Bank of Misiones

* Mutations in T cell core epitopes (aa 18-27, 50-85, 120-160)

Table 3 Mutations in S/P proteins in samples from 16 HBV-DNA-positive blood donors from the city of Buenos Aires and the province of Misiones

Sample	Genotype	S/P proteins[a]	
		S	P
FF2	F1b	-	-
FF3	B2	-	-
FF4	A2	**P62L, T63A, A159E**	**rtN71G**
FF5	F4		**rtQ149K**
FF6	C2	M198I, W199L, Y200F	rtY124H, rtV207I
FF7	C2	-	-
FF8	C2	M198I, W199L, Y200F	rtY124H, rtV207I
FF9	C2	M198I, W199L, Y200F	rtY124H, rtV207I
FF10	C2	M198I, W199L, Y200F	rtY124H, rtV207I
FF11	C2	M198I, W199L, Y200F	rtY124H, rtV207I
FF12	C2	M198I, W199L, Y200F	rtY124H, rtV207I
FF13	C2	M198I, W199L, Y200F	rtY124H, rtV207I
CM1	D3/D6	C107G, **L186S, S204R, Y206C**	rtL115W, rtA194V, rtS213T
$CM2^{+}$	F1b	**N204H**	**rtK212T**
CM3	F1b	F179L	**rtI162V, rtI187T**
CM4	D3/D6	Y100S, L109Q, **S204K, S207T**	rtS213T, rtQ215H

Newly described mutations are printed in bold

FF, Favaloro Foundation, Buenos Aires city; CM, Central Blood Bank of Misiones

[a] Partial S (aa 55-210) / P (aa 64-220) proteins

in misalignment between the template and dNTP-binding site, the novel rtA194V replacement deserves to be studied further. It should be stressed that the clinical relevance of these substitutions has not yet been determined. Therefore, since rtA194V, rtS213T and rtQ215H, detected in this study, are not currently considered in genotypic drug resistance algorithms, our data warrant additional studies to analyze their possible associations with antiviral resistance.

On the other hand, the rtY124H and rtV207I mutations were identified in C2 sequences from Buenos Aires and have recently been reported in Sagua-Huarpes Amerindians [31]. The former mutation has been described in untreated Chinese patients, and the latter is associated with resistance to lamivudine [45]. It is worth emphasizing that, in our study, the above-mentioned mutations occurred in untreated (naïve) subjects, as observed previously in randomly selected patients with chronic hepatitis B [46]. These data suggest the circulation of HBV natural resistance variants among BDs. Moreover, other new variants described in Table 3 have not been reported elsewhere.

Concerning HDV, a report published in 1987 showed 1.4 % anti-HDV antibody prevalence among HBsAg-positive Argentine BDs [17], similar to the value reported in this study. One HDV1 sequence (FF14) was detected recently in Amerindians from Misiones [27], confirming the circulation of this genotype in at least two distantly located regions of Argentina. Interestingly, the single HDV case described in this study exhibited several non-synonymous changes when compared with HDV1 reference sequences. With the exception of I198L (involved in the nuclear export signal), there is no published data regarding the functional meaning of the remaining mutations.

In summary, several HBV genotypes were observed in BDs from two Argentine geographic regions. Several sequences exhibited mutations within the BCP, the preC/C region, the HBsAg MHR, and the reverse transcriptase, some of them associated with natural resistance. A thorough investigation has to be carried out to evaluate the clinical impact of some of the documented mutations as well as those detected in the HDV1 case.

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Conflict of interest No conflict of interest to declare.

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