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Molecular epidemiology of Hepatitis B virus in Córdoba, Argentina

Fernando Gallego[a,*], María Belén Pisano[a], Carolina Torres[b], Luciana Caeiro[a], Maribel Martínez Wassaf[c], Marcos Balangero[a], Rodolfo Campos[b], Viviana Ré[a]

[a] Instituto de Virología "Dr. J. M. Vanella", Facultad de Ciencias Médicas, Universidad Nacional de Córdoba, Enfermera Gordillo Gómez s/n, Ciudad Universitaria, CP 5016 Córdoba, Argentina
[b] Cátedra de Virología, Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires, Junín 956, 4° piso, C1113AAD Ciudad Autónoma de Buenos Aires, Argentina
[c] Laboratorio de Análisis Clínicos Especializados-LACE, Vélez Sársfield 528, CP 5000 Córdoba, Argentina

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ABSTRACT

Background: The analysis of the genomes of hepatitis B virus (HBV) identifies phylogenetic variants called genotypes, which may lead to distinct biological and clinical behaviors.
Objectives: The aim of this study was to describe the current molecular epidemiology and genetic diversity of HBV in Córdoba, Argentina.
Study design: A total of 52 HBV samples, 40 from HBV mono-infected and 12 from human immunodeficiency virus (HIV)-HBV co-infected patients, were sequenced in the S gene and in the basal core promoter-precore (BCP-pC) region.
Results: Presence of subgenotypes F1b (35%) and F4 (17.5%), subgenotype A2 (37.5%), C (5.0%) (subgenotype could not be defined) and D (5.0%) (subgenotype D2, and the other could not be defined) were observed among mono-infected patients. The co-infected individuals displayed a different genotype distribution: sub-genotype A2 was the most common (75.0%), followed by subgenotype F1b (25.0%).
Conclusions: These results showed two epidemiologic scenarios: the mono-infected population may represent the ethnic composition of the current human population of Córdoba, where the Amerindian (genotype F) and European origins (subgenotype A2) account for the 90% of the samples; for the co-infected patients, the high prevalence of subgenotype A2 resemble previous analyses from Buenos Aires. In addition, mutations in hepatitis B surface antigen (HBsAg), polymerase and BCP-pC regions were identified, mainly in chronic or co-infected patients.

1. Background

It is estimated that more than 2 billion people have been infected with hepatitis B virus (HBV) [1], and 350 million individuals have been diagnosed with chronic HBV infection worldwide [2,3].

Based on a genetic diversity of 8% in the entire genomic sequence, the HBV has been classified at least into nine genotypes (A-I) which have a worldwide geographical distribution [4,5]. These distribution of HBV genotypes (gt) and subtypes (sgt) are markedly heterogeneous throughout the world, even among nearby geographical regions [6]. Genotype A is more frequently found in Europe, North America, and Sub-Saharan Africa [7]; gts B and C are confined to Asia and Oceania [7–10]. Genotype D is the most widely distributed and has been found universally in southern Europe, North Africa, India, China, and West and South Africa, and intravenous drug users in all continents [7,11]. Genotype E is found mainly in West and South Africa; gt F is restricted to Central and South America, while gt G to the United States and France [6,12]. Genotype H has been found in North and Central America [12,13], and gt I has been isolated in Vietnam and Laos [14]. Studies in Argentina from the Metropolitan region (Buenos Aires) have demonstrated that gts A, D and F are the most prevalent on similar proportions (approximately 30%) [15–17], but sgt A2 is the most prevalent in HIV-HBV co-infected patients [18,19]. On the other hand, in Northern regions of the country (provinces of Salta, Jujuy, Formosa and Chaco) sgts F1b and F4 represent more than 90% of

Abbreviations: HBV, hepatitis B virus; HIV, human immunodeficiency virus; BCP-pC, basal core promoter-precore; HBsAg, hepatitis B surface antigen; gt, genotype; sgt, subgenotype; HBc, hepatitis B core protein; IgM, immunoglobulin M; HBeAg, hepatitis B "e" antigen; DNA, deoxyribonucleic acid; PCR, polymerase chain reaction; ORF, open reading frame; MHR, major hydrophilic region.

* Corresponding author. Tel.: +54 351 4334022.
E-mail addresses: fergallego88@gmail.com, ferel22@gmail.com (F. Gallego), mbelenpisano@gmail.com (M.B. Pisano), carolina_torr@yahoo.com.ar (C. Torres), lucianacaeiro@hotmail.com (L. Caeiro), mari_mw@hotmail.com (M. Martínez Wassaf), marcosbalangero@yahoo.com.ar (M. Balangero), rcampos@ffyb.uba.ar (R. Campos), vivianare@fcm.unc.edu.ar (V. Ré).

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the founded HBV [16,20], while in Misiones province there is a higher prevalence of gt D (58%) [21]. A recent study in Mar del Plata city (province of Buenos Aires) described high prevalence of gt F (69%), and the presence of gt G in low proportion (6.9%) [22]. These information shows that regional differences of gt distribution are present in Argentina, perhaps as a consequence of each particular conformation of human population and immigration waves [16,17]. At the moment, no information about the distribution of HBV subtypes in central region of Argentina is available.

Determining the HBV gt, sgt and isolate has been helpful for understanding the evolution and the epidemiology of the virus. Several clinical and epidemiological observations suggest that genetic differences in viral gts may underlie differences in biological and clinical behaviors [5]. In addition, recently released clinical practice guidelines and consensus conference statements point to the importance of HBV genotyping in therapeutic algorithms for the treatment of chronic hepatitis B [23]. To perform effective public-health surveillance for new variants, modes of transmission, and further vaccine and treatment development efforts, detailed information about sequence variation of gt prevalence is needed.

Several mutations have been described in different regions of the HBV genome associated with varied forms of disease progression and response to therapy. There are evidences that amino acid substitutions within the hepatitis B surface antigen (HBsAg) [24], the HBV polymerase domains [25,26] and the precore (pC) or basal core promoter (BCP) region [27–29] are of clinical importance. The epidemiology of these HBV mutants has been widely studied principally in countries where HBV is endemic. In Argentina, previous studies have shown that the prevalence of HBsAg variants and BCP-pC mutations is significant among population [17,30]. However, this situation is still unknown in the central region of the country.

2. Objectives

The aim of this study was to molecularly characterize HBV isolates from central Argentina (Córdoba province), in order to determine the gt, sgt and the presence of mutations of potential clinical relevance.

3. Study design

3.1. Samples

This was a retrospective study (from January 2009 to December 2011) of 52 adult, unrelated individuals with HBV infection, determined by the presence of surface antigen (HBsAg), inhabitants of Córdoba City, and other small cities and towns from Córdoba province.

3.1.1. Mono-infected individuals

Forty samples belonged to mono-infected patients: 24 males and 16 females (median age 40 years old, range 22–64 years). Twenty-three subjects were anti-HBc IgM positive (+) (IMMULITE 2000 System, SIEMENS, Los Angeles, USA), and 15 were negative (−) to its detection. For samples CbaArg38 and CbaArg50 this data was not available (Table 1). In 18 samples HBeAg was detected (ELISA HBeAg/Ab kit, KHB4IW RADIM, Roma, Italy).

3.1.2. HBV–HIV co-infected individuals

Twelve individuals were co-infected with HIV; they were all males (median age 38 years old, range 25–73 years). Only one sample was reactive to anti-HBc IgM detection, and for specimen CbaArg53 this data was not available (Table 1). HBeAg was detected in 8 samples.

3.2. HBV DNA extraction, amplification and sequencing

The viral DNA was extracted from serum samples using QIAmp DNA Mini Kit (Qiagen GmbH, Hilden, Germany).

Two HBV genomic regions were amplified by nested PCRs, corresponding to the S gene and the BCP-pC gene as described by Pezzano et al. [17]. The resulting amplicons were purified using QIAquick Gel Extraction Kit (Qiagen, Valencia, CA, USA) and submitted to direct nucleotide sequencing reaction in both directions (Macrogen, Inc. Seoul, Korea).

3.3. Phylogenetic analysis and HBV genotyping

Genotype and subgenotype assignation was based on a combined phylogenetic analysis of the S gene and BCP-pC regions using reference sequences ($n = 39$) obtained from GenBank database. The reference dataset was selected according Torres et al. [31].

Sequences were aligned with ClustalX (v1.83) [32] and edited with Bioedit (v7.1.3.0) [33]. Phylogenetic analyses were performed using the maximum likelihood method with PhyML v3.1 [34] under the appropriate model of nucleotide substitution selected by jModeltest v2.1 [35], according to the Akaike Information Criterion. The robustness of the phylogenetic grouping was evaluated with bootstrap analysis with 1000 replicates.

3.4. Analyses of mutations in ORF-S, ORF-P and BCP-pC genetic regions

Nucleotide and amino-acid sequences were aligned and compared with prototype strains of each sgt using program MEGA (v4.0) and Mutation Reporter Tool [36]. Amplicons corresponding to the S region were translated into amino-acid sequences according to the open reading frames of the S (ORF-S) and P (ORF-P) genes.

3.5. Nucleotide sequence accession number

Nucleotide sequences analyzed in this work were deposited at GenBank under accession numbers KC680722 to KC680773 for the S gene and KC999418 to KC999469 for the BCP-pC genomic region.

3.6. Statistical analysis

Statistical analyses were conducted using InfoStat program, version 2011 [37]. For qualitative variables the data were stratified into binary outcomes, and measures of association were tested. The strength of the relationship was estimated by using Odds Ratio (CI: 95%).

4. Results

4.1. Phylogenetic analysis

Based on the phylogenetic analysis, the 40 sequences obtained from mono-infected patients grouped as following: 14 belonged to sgt F1b (35%), 7 to sgt F4 (17.5%), 15 to sgt A2 (37.5%), 2 to gt C (5%), and 2 to gt D (5%). Samples assigned to gt C could not be subgenotyped ($n = 2$); for gt D, one sample grouped within sgt D2, and the other could not be defined. Among HIV/HBV co-infected patients, sgt A2 was the most prevalent (75%), followed by sgt F1b (25%) (Fig. 1).

Statistical analyses showed association between HIV/HBV co-infected patients and sgt A2 of HBV (p value = 0.0266; OR = 4.80; CI = 1.21–19.09). However, there was no association between determined gt with HBeAg status, gender or age.

A further discrimination in relation to the course of infection revealed that among the HBV mono-infected individuals with

Table 1
Main characteristics of the studied samples and mutations observed in each one.

	Genotype (*n*)	Sample	Anti-HBc IgM	HBeAg	Genotype/Subgenotype	S (MHR)	P (rt)	pC-BCP		
								1762	1764	1896
HBV mono-infected (*N* = 40)	A (15)	CbaArg05	+	+	A2	No	No	No	No	No
		CbaArg09	+	+	A2	No	rtL180M, rtM204V	No	No	No
		CbaArg10	+	+	A2	No	rtL180M, rtM204V	No	No	No
		CbaArg15	+	+	A2	No	No	No	No	No
		CbaArg34	+	–	A2	No	No	No	No	No
		CbaArg36	+	+	A2	No	rtQ125K	No	No	No
		CbaArg37	+	–	A2	No	rtQ125K	No	No	No
		CbaArg41	+	–	A2	No	No	No	No	No
		CbaArg45	+	+	A2	No	No	No	No	No
		CbaArg29	–	–	A2	No	No	No	No	No
		CbaArg32	–	–	A2	No	rtI53V, rtN76D, rtW153R	A1762T	G1764A	No
		CbaArg43	–	–	A2	I110M, M133T, F134L	rtI53S, rtS119A, rtS143T	A1762T	G1764A	No
		CbaArg49	–	+	A2	No	No	No	No	No
		CbaArg52	–	+	A2	No	No	No	No	No
		CbaArg38	ND[a]	–	A2	I110L, deletion nt335-43, T116N, G119E, P120T, C121G, K122Q, C124R, P127L, M133T	rtN118A, deletion nt360-68, rtN124K, rtT128N, rtM129R, rtQ130P, rtL132P, rtW153R	A1762T	G1764A	No
	C (2)	CbaArg31	+	–	C	No	rtP109S, rtH126Y	No	No	No
		CbaArg12	–	–	C	No	No	No	No	No
	D (2)	CbaArg06	–	–	D2	No	rtY54N, rtM129L	A1762T	G1764A	No
		CbaArg39	–	–	D	No	No	No	No	No
	F (21)	CbaArg02	+	+	F1b	No	No	No	No	No
		CbaArg03	+	–	F1b	No	No	No	No	No
		CbaArg08	+	+	F1b	No	No	No	No	No
		CbaArg14	+	–	F1b	No	No	No	No	No
		CbaArg17	+	+	F1b	No	No	No	No	No
		CbaArg18	+	+	F1b	No	No	No	No	No
		CbaArg22	+	+	F1b	No	No	No	No	No
		CbaArg26	+	+	F1b	No	rtL199V	No	No	No
		CbaArg46	+	–	F1b	No	No	A1762T	G1764A	G1896A
		CbaArg48	+	–	F1b	No	No	No	No	No
		CbaArg21	–	+	F1b	No	No	No	No	No
		CbaArg27	–	–	F1b	No	rtS40A, rtN134D, rtQ149K	A1762T	G1764A	G1896A
		CbaArg33	–	–	F1b	No	rtS40A	A1762T	G1764A	G1896A
		CbaArg50	ND[a]	+	F1b	No	No	No	No	No
		CbaArg28	+	+	F4	No	No	No	No	No
		CbaArg44	+	–	F4	No	No	No	No	No
		CbaArg47	+	–	F4	No	No	No	No	No
		CbaArg04	–	–	F4	No	rtY122N, rtQ149K, rtL151F	A1762T	G1764A	G1896A
		CbaArg16	–	–	F4	I110L, V177A	rtN118T, rtS119A, rtY122H, rtN134D, rtQ149K, rtL151F	No	G1764A	G1896A
		CbaArg25	–	–	F4	I110L	rtN118T, rtY122H, rtN134D	A1762T	No	G1896A
		CbaArg42	–	+	F4	I110L, P120Q, K160R	rtN76D, rtN118T, rtY122H, rtN134D, rtS137T, rtQ149K	No	No	G1896A

HBV–HIV co-infected ($N = 12$)	A (9)	CbaArg23	+	+	A2	Y161F	rtY126H	No	No	No	No
		CbaArg01	–	+	A2	No	No	No	No	No	No
		CbaArg07	–	+	A2	No	rtY126H	No	No	No	No
		CbaArg13	–	–	A2	No	No	No	No	No	No
		CbaArg19	–	–	A2	No	No	No	No	No	No
		CbaArg20	–	+	A2	No	rtL180M, rtM204V	No	No	No	No
		CbaArg24	–	+	A2	No	rtL164M, rtL180M, rtM204V	No	No	No	No
		CbaArg30	–	+	A2	No	rtI103V, rtL180M, rtM204V	No	No	No	No
		CbaArg53	ND[a]	+	A2	No	No	No	No	No	No
	F (3)	CbaArg11	–	–	F1b	No	No	No	No	No	No
		CbaArg35	–	–	F1b	No	No	No	No	No	No
		CbaArg40	–	+	F1b	M103I, K122R, L127P	rtV112L, rtL180M, rtM204V	No	No	No	No

[a] ND, no data.

positive anti-HBc IgM, gt F was the most prevalent (56.5%), comprising sgt F1b (76.9%) and sgt F4 (23.1%), followed by sgt A2 (39.1%) and gt C (4.3%). In addition, in patients with negative anti-HBc IgM, gt F was present in 46.7%, followed by sgt A2 (33.3%), gt D (13.3%) and gt C (6.7%).

On the other hand, among HBV–HIV co-infected individuals, only one sample was reactive to anti-HBc IgM detection, which corresponded to sgt A2. All the rest of the samples resulted negative for anti-HBc IgM, and belonged to sgt A2 (70%) and sgt F1b (30%).

4.2. Detection of mutants

Mutations in three different genomic regions (ORF-S, ORF-P and BCP-pC) were analyzed (Table 1).

Among HBV mono-infected individuals, mutations in the pC-BCP region associated with lower levels of HBeAg were almost exclusively found in patients with anti-HBc IgM (–), some of whom also presented amino-acid substitutions associated with resistance to antiviral therapy (ORF-P) (3/15) and/or associated with failure in diagnostic assays (4/15), vaccine or immunoglobulin therapy escape (MHR in ORF-S).

In contrast, among HBV–HIV co-infected individuals, a higher proportion of patients with anti-HBc IgM (–) presented amino-acid substitutions associated with resistance to antiviral therapy (4/10) and no mutations in the pC-BCP region were detected.

5. Discussion

The present study reveals the gt distribution of HBV in Córdoba, Argentina, showing the presence of two distinct epidemiological scenarios, according to the study group: HBV mono-infected and HBV–HIV co-infected patients. The 40 samples from mono-infected individuals grouped as: gts F (52.5%), A (37.5%), C (5.0%) and D (5.0%). The gt F distribution at Córdoba province present an intermediate value between those observed for Buenos Aires (around 30%) [17] and for Northwest Provinces (around 90%) [16], which could be associated with the legacy of Amerindian ethnic original composition of the population of Córdoba. Meanwhile, the presence of sgt A2 might be a consequence of the consecutive European migrations that have taken place in our country since the first Spanish settlers arrived [16].

Genotype distribution among HBV–HIV co-infected patients differed significantly from HBV mono-infected individuals. Genotype A (sgt A2) was the most prevalent in this group, in agreement with previous reports of Argentina [19] and other parts of the world [38–40]. While Buenos Aires have shown a gt A prevalence of 83.3% in this population, and gts D and F have also been detected (8.3% and 4.2% respectively) [19], our results yielded prevalences of 75% and 25% for gts A and F respectively. The precise reason why gt A HBV has become largely prevalent among Argentine patients co-infected with HIV is unknown [19]. The difference on the second most prevalent genotype between Metropolitan and Central regions may respond to the slightly dissimilar gt profile of circulation in each area (gt D is more frequent in Buenos Aires than in Córdoba), possibly representing a more extensive migration pattern in the Metropolitan region. It is notorious that detections among gt F co-infected patients all belonged to sgt F1b.

Mutations in different genomic regions may influence important clinical aspects of HBV infection. Analyses of the nucleotide and amino-acid substitutions revealed that most of the changes observed occurred in patients without presence of anti-HBc IgM (presumably with chronic infection) in both, mono and co-infected patients.

Most of the mutations found in the HBsAg were associated with defective virion secretion [41], failure in diagnostic assays [42–44]

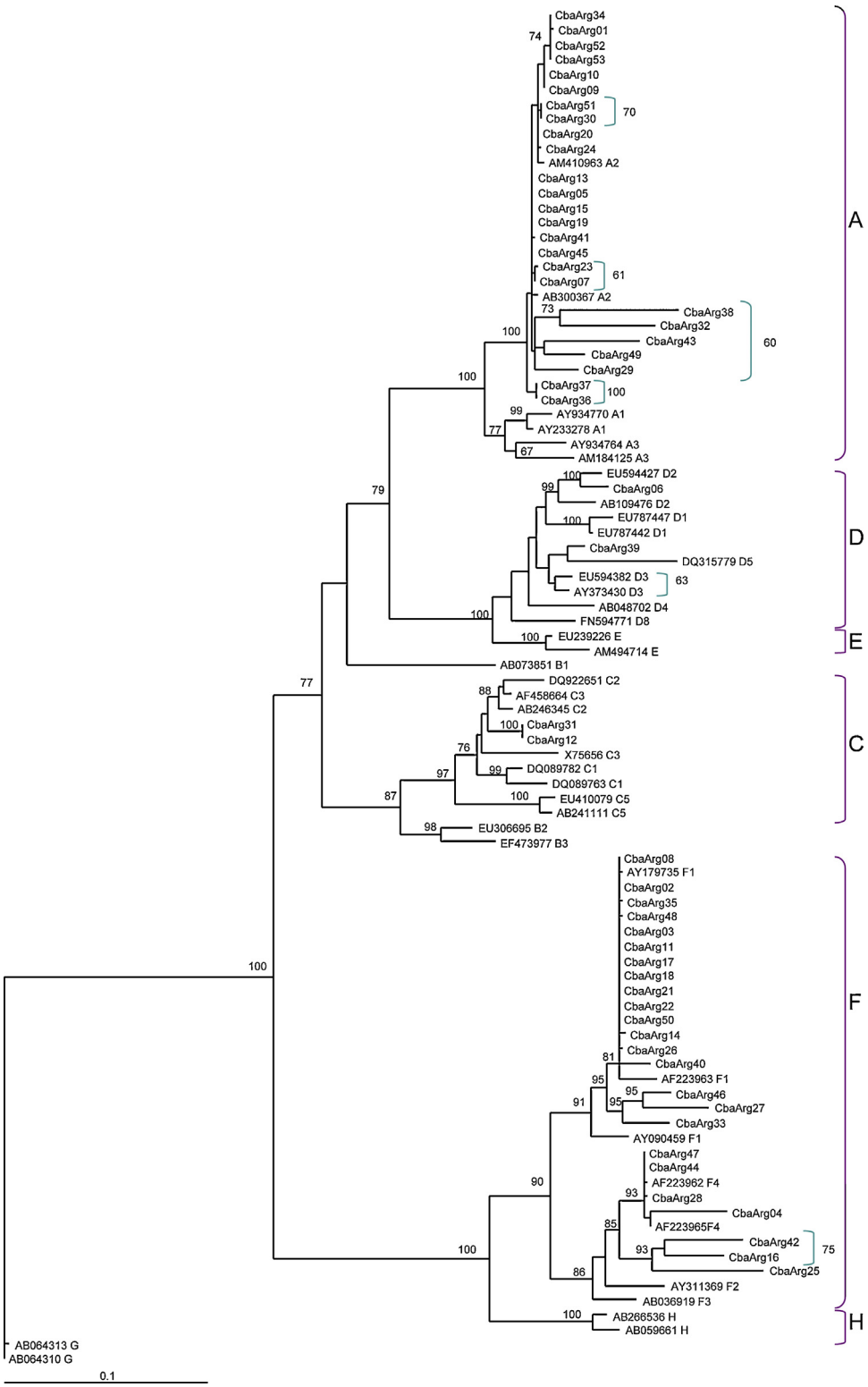

Fig. 1. Maximum likelihood phylogeny of HBV samples of Córdoba, Argentina. Maximum likelihood phylogenetic tree obtained using sequences of 1223 nucleotides, resulting from concatenated sequences of S gene (525 nucleotides) and X gene/BCP-pC regions (698 nucleotides) of 52 HBV samples of central Argentina and reference sequences from each genotype or subgenotype available at GenBank. The numbers next to each node correspond to bootstrap values obtained with 1000 replicates.

and immune escape [45]. As previously reported for other Argentinean isolates [16], none of our samples had failed to be detected by the routine commercial kits utilized, suggesting that the mutations observed have a minimal influence on the performance of diagnostic tests. It is interesting to note that the novel mutation I110L was frequently found in gt F and all belonged to anti-HBc IgM negative patients (Table 1). Further studies are necessary to dilucidate the clinical implications of this mutation.

There are several BCP-pC mutations that lead to a decreased or an abolished expression of HBeAg. G1896A (guanine to adenine substitution on the 1896 position) is the most common -produces a stop codon in the precore open reading frame [46,47], and was

the most frequent for gt F. For gt A this mutation was not present, whereas the most common HBeAg (−) variant was A1762T/G1764A double mutation [27–29], which was also found in one sample of gt D. This agrees in part with findings of the Metropolitan region, where it has been described the same pattern for gts A and F HBeAg mutants [17].

Mutations related to resistance to antiviral therapy or increased clinical severity were identified mainly in samples of anti-HBc IgM negative patients (10/13, 76.9%). The non-availability of clinical information of some of these patients do not allow us to make further speculations.

In conclusion, this work describes, for the first time, gts of HBV that circulate in the central area of Argentina which may represent the current ethnic composition of the region's human population. Mutations were found mainly in anti-HBc IgM negative specimens (probably of chronic patients) or HIV co-infected patients, as was expected. More studies are needed to deepen on the phylogenetic and evolutionary relationships of these strains in the central area of our country.

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Conflict of interests

None declared.

Ethical approval

This work is part of a research project inscribed and approved by the ethics committee of the Health Ministry of the Province of Cordoba (RepisNro002).

Authors' contributions

VR and RC were involved in the study design, the analysis of the data and in the process of writing the manuscript. FG, LC and MMW carried out the experiments. FG, MBP, CT and MB were involved in the analysis of data and in the process of writing the manuscript. CT and RC performed statistical and phylogenetic analyses.

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