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ORIGINAL ARTICLE

Molecular epidemiology of hepatitis B virus in an isolated Afro-Brazilian community

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Abstract This study was conducted in an Afro-Brazilian, slave-descendant community with high (42.4%) hepatitis B virus (HBV) prevalence. Twenty (8.4%) out of the 239 subjects under study were HBsAg-positive, and HBV-DNA was detected in 59 (25%) individuals. A high rate (18.3%) of occult infection was therefore observed that was associated to low HBV loads (mean, 1.8×10^4 copies/ml) and to a specific amino acid substitution (C100Y) in the small surface antigen. Genotyping of 50 isolates showed that 43 (86%) were of subgenotype A1, one (2%) from subgenotype A2, and five (10%) from subgenotype D. Mixed genotypes A1 and E were observed in one (2%) sample. The genetic distance ($0.8 \pm 0.3\%$) among the HBV/A1 isolates from the community was smaller than the intragroup divergence among A1 isolates from Brazil as a whole, but it was similar to that found between A2 isolates from different countries, suggesting that HBV/A1 was introduced in the community through different sources. The substitution W501R (polymerase), previously reported only in Gambia, was observed in 46% of the HBV/A1 isolates. The precore/core promoter region of HBsAg-positive isolates showed several substitutions that could explain the anti-HBe phenotype found in 18 of 20 (90%) of the HBsAg-positive subjects.

Introduction

Hepatitis B virus (HBV) is still a major cause of liver disease worldwide despite the introduction in the last decades of vaccination programs. It is estimated that 2 billion people have been infected with HBV and that more than 350 million are chronic carriers of the virus. HBV is an etiological agent of acute self-limited hepatitis B, asymptomatic HBV status, fulminant hepatitis and chronic hepatitis that can progress to liver cirrhosis and hepatocellular carcinoma (HCC) [22].

Based on sequence divergence of 8% or more in the entire genome, HBV isolates are classified into eight genotypes, designated A–H, with a distinct geographical distribution [31, 32]. Genotype A circulates in Europe, India, Africa, and North and South America [9, 32]. This genotype is now divided into three subgenotypes: one of African origin that was originally designated subgroup A′ [5] and renamed subgenotype Aa [39] or subtype A1 [17]. The subgenotype of European origin has been designated Ae [39] or A2 [17]. A third subgenotype, A3,

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers EF547828 to EF547858 and FJ174794 to FJ174800.

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has been identified in Cameroon [21]. Isolates belonging to genotypes B and C have been observed in Southeast Asia and the Far East. Genotype D is widespread, with a high prevalence in the Mediterranean area and in the Middle East region. Genotype G is infrequent and has mainly been found in Europe, Mexico, and the USA [32], while genotype E is native to West Africa [19, 28], and genotypes F and H are considered indigenous to Latin America [9]. Genotypes A, D and F are the most prevalent in Brazil [4, 24, 26, 38]. Recently, a high proportion of genotype A, subtype A1, was observed among HBV Brazilian isolates [4, 24].

Occult HBV infection is defined as the presence of HBV-DNA in blood and/or liver of patients in the absence of detectable hepatitis B surface antigen (HBsAg). Prevalence data of occult infection reported in different studies are difficult to compare due to important differences in the composition of the studied populations and the level of sensitivity of the HBV-DNA detection assay. However, high rates of occult hepatitis B have been observed in endemic areas for HBV infection [12, 43]. Occult HBV infection rates varying from 5 to 55% among subjects with chronic hepatitis, with or without HCC, have been observed [3, 6]. It has also been reported in populations without symptomatic liver disease, such as blood donors (BD), individuals with normal liver tests or general populations (rates from 0 to 17%) [6, 25].

Mutations in HBV nucleotide sequence may occur at any stage of infection, allowing the emergence of variant viruses, including mutations in the precore, basal core promoter (BCP), pre-S/S and polymerase regions [8]. The precore and BCP regions play an important role in HBV replication and HBeAg production. Thus, mutations in these regions have been detected in many HBeAg-negative patients with persistent viremia and active liver disease [2, 10].

In a previous study with 12 semi-isolated communities in Central Brazil, almost exclusively composed of descendants of African slaves, a mean overall HBV infection prevalence of 19.8% was found. Large variations in infection rates were observed from one community to another. The highest prevalences were detected in the Furnas dos Dionísios (FD) community, with 42.4% and 7.4% for hepatitis B core antibody (anti-HBc) and HBsAg, respectively [27]. In the present study, this community was chosen in order to investigate the prevalence of occult HBV infection, the HBV genotype distribution, and the occurrence of intrafamilial transmission. In addition, genetic variability of precore, BCP, and small S regions, as well as of the overlapping parts of the polymerase gene, was examined.

Materials and methods

Study population

This study was conducted in Furnas dos Dionisios (FD), a semi-isolated Afro-Brazilian community in the State of Mato Grosso do Sul (MS), Central Brazil. The FD community consists of about 80 families. Two hundred thirty-nine (88%) of the 271 individuals previously examined for HBV epidemiological status [27] were included in this study. Sera from the 32 remaining subjects were not available for molecular studies. The age range of the individuals was 2–87 years (mean, 25.7 ± 18.4 years). One hundred twenty-one (50.6%) individuals were male and 118 (49.4%) were female. This study was approved by the Human Ethics Committee of the Federal University of Mato Grosso do Sul, and informed consent was obtained from all subjects.

HBV serological tests

Detection of HBsAg, anti-HBc and anti-HBs were performed using enzyme-linked immunosorbent assay (ELISA) (Hepanostika Uni-form Organon Teknika B. V., Boxtel, Holland). HBsAg-positive samples were tested for the presence of anti-HBc IgM, HBeAg and anti-HBe (Hepanostika Uni-form Organon Teknika B. V.).

PCR amplification and genotyping

Viral DNA was extracted from all 239 serum samples as previously reported [29] and subjected to a semi-nested PCR performed with Taq polymerase (Invitrogen, San Diego, CA) to amplify the almost complete pre-S/S region. This assay uses, in the first round, sense primer PS1 and a mixture of antisense primers S2 and S22, which makes possible the amplification of all HBV genotypes. The second round of amplification was performed with 1 µl of the first-round PCR product, using primers PS1 and SR (Table 1). This two-round PCR assay, resulting in amplicons shorter than those described in our original genotyping study [4], was recently standardized to increase sensitivity (to three DNA molecules per assay) without a loss of specificity [27]. Isolates were genotyped by using a restriction fragment length polymorphism (RFLP) analysis of PCR products. For this, PCR products were digested separately with *Bam*HI, *Eco*RI and *Stu*I restriction endonucleases [4, 27].

Nucleotide sequencing

HBV-DNA-positive samples were subjected to amplification of the entire S region using a semi-nested PCR assay.

Table 1 Primers used for PCR amplification and sequencing

Primer	nt position*	Sequence
PS1 (sense)	nt 2,826–2,845	5′-CCA TAT TCT TGG GAA CAA GA-3′
S2 (anti-sense)	nt 841–819	5′-GGG TTT AAA TGT ATA CCC AAA GA-3′
S22 (anti-sense)	nt 841–819	5′-GTA TTT AAA TGG ATA CCC ACA GA-3′
SR (anti-sense)	nt 704–685	5′-CGA ACC ACT GAA CAA ATG GC-3′
S1 (sense)	nt 124–143	5′-CTT CTC GAG GAC TGG GGA CC-3′
S4 (sense)	nt 416–436	5′-TGC TGC TAT GCC TCA TCT TCT-3′
S7 (anti-sense)	nt 676–656	5′-TGA GCC AGG AGA AAC GGG CT-3′
S14 (sense)	nt 184–203	5′-GGA CCC CTG CTC GTG TTA CA-3′
S17 (anti-sense)	nt 273–249	5′-GAG AGA AGT CCA CCM CGA GTC TAG-3′
C1 (sense)	nt 1,935–1,958	5′-CTG TGG AGT TAC TCT CGT TTT TGC-3′
C2 (anti-sense)	nt 2,458–2,432	5′-CTA ACA TTG AGA TTC CCG AGA TTG AGA-3′
C3 (anti-sense)	nt 2,056–2,075	5′-TTG CCT GAG TGC AGT ATG GT-3′
X4 (sense)	nt 1,644–1,663	5′-AAG GTC TTA CAT AAG AGG AC-3′

*Nucleotides (*nt*) are numbered starting at the cleavage site for restriction enzyme *Eco*RI, located in the pre-S2 region of a full-length HBV genome of 3221 bp

The first round of amplification was carried out with sense primer PS1 and antisense primers S2 and S22. The second round of amplification was performed with sense primer S1 and antisense primers S2 and S22 (Table 1). BCP and precore regions were amplified by semi-nested PCR, using primers X4 and C2 in the first round and X4 and C3 in the second round. Nucleotide sequences of the amplified regions were determined by direct sequencing using a BigDye Terminator kit (Applied Biosystems, Foster City, CA) with specific HBV primers (S1, S2, S22, S4, S7 and C1, Table 1). Sequencing reactions were analyzed on an ABI3730 + automated sequencer (Applied Biosystems). Bioinformatics analysis of the sequences was performed using the University of Wisconsin Genetic Computer Group package. A neighbor-joining phylogenetic tree was drawn and rearranged using the Mega program version 3 [20]. Phylogenetic analysis was performed and genetic distances were determined using 37 HBV isolates from the FD community, 18 HBV/A Brazilian isolates from other places, three of which were from blood donors (BD) living in the city of Campo Grande, located at 45 km from the FD community, 33 HBV/A1 from Africa, and 44 GenBank sequences representative of HBV genotypes from other geographical regions.

Quantification of HBV-DNA

HBV-DNA-positive samples were quantified using the real-time PCR TaqMan technology, according to Pas and Niesters [34], with some modifications. A panel of reference sera was used for quantification by real-time PCR. This panel was tested by comparison to commercially available panels and contained known numbers of HBV-DNA molecules. Amplification assays were performed in a final volume of 25 μL of TaqMan universal Master Mix (Applied BioSystems), containing 2 μL of DNA, 1 μM each of sense S14 and antisense S17 primers (Table 1), and 0.3 μM of probe (5′-FAM-TGTTGACAARAATCCTC ACAATACCRCAGA-TAMRA-3′, nucleotide position 218–247). After initial incubation steps of 2 min at 50°C and 10 min at 95°C, the PCR cycling program consisted of 50 cycles of 15 sec at 95°C and 60 sec at 60°C. Reactions were performed in a 7700 SDS system (Applied BioSystems). The assay has a limit of detection of 10 copies/reaction, i.e., 100 copies/ml of serum.

Avoiding PCR contamination

Extreme care was taken to avoid PCR contamination. Positive and negative controls were included in all runs of DNA extraction and PCR amplification reactions. No more than 10 serum samples were extracted together. In addition, water was subjected to the same steps of extraction and PCR amplification, as a negative control. All samples subjected to PCR-RFLP amplification were tested in two independent assays of DNA extraction and PCR amplification. Only samples that were positive in both independent assays were considered positive for HBV-DNA.

Results and discussion

Hepatitis B serological profile and HBV-DNA results

Table 2 shows that 20 out of 239 (8.4%) individuals were HBsAg-positive. An anti-HBs-positive/anti-HBc-positive/anti-HBc-IgM-negative serological pattern, corresponding to past HBV infection, was observed in 87 (36.4%) subjects. Five (2.1%) individuals were ‘anti-HBc only’. Isolated anti-HBs was detected in 68 (28.4%) subjects, probably as a result of previous vaccination. The pre-S/

Table 2 Serological status and HBV-DNA data of individuals living in the Furnas dos Dionisios community, Central Brazil

Serological status	HBV-DNA positivity ($n = 239$)	HBV load (copies/ml)		
		($n = 48$)	Range	Mean
HBsAg/anti-HBc	19/20 (95%)	17	2.7×10^3–1.3×10^8	1.8×10^7
Anti-HBc only	2/5 (40%)	2	6.9×10^3–6.7×10^4	3.7×10^4
Anti-HBs/anti-HBc	24/87 (27.5%)	16	2.2×10^2–3.9×10^4	1.4×10^4
Anti-HBs only	8/68 (11.8%)	8	2.3×10^2–8.1×10^4	4.9×10^4
No serological marker	6/59 (10.2%)	2	2.8×10^4–3.4×10^4	3.2×10^4

partial S region was successfully amplified in 59 (24.7%) samples. HBV-DNA was detected in 19 of 20 (95%) HBsAg-positive- and 40 of 219 HBsAg-negative samples, showing an overall occult infection rate of 18.3%. Such a high rate of occult HBV infection (18.3%) in an Afro-Brazilian slave-descendant community where high prevalence rates of anti-HBc (42.4%) and HBsAg (7.4%) had been detected previously [27] was in accordance with some studies which reported that occult HBV infection is frequent in regions where HBV is endemic [3, 6, 25, 43, 45]. Six HBsAg-negative samples, which gave PCR-positive, although not reproducible, results were considered HBV-DNA negative. The presence of HBV-DNA was detected in samples showing an HBsAg-negative pattern, varying from 40% (2/5 subjects) among individuals with anti-HBc alone to 10.2% (6/59) among subjects without any HBV serological marker (Table 2). HBV-DNA levels were measured by real-time PCR in 48 of 59 HBV-DNA-positive samples. HBV-DNA loads were significantly higher ($p = 0.041$) in the HBsAg-positive patients (mean, 1.9×10^7 copies/ml) than among those with occult infection (1.8×10^4 copies/ml). In agreement with previous reports [11, 16, 23, 30, 44], the most probable reason for occult infection is the occurrence of low HBV loads.

Molecular characterization and evolutionary analysis of S and P regions

Fifty out of 59 (85%) HBV-DNA-positive samples were successfully genotyped by PCR-RFLP. Forty-four (88%) of them belonged to genotype A, 43 of which were from subgenotype A1 and one was from subgenotype A2. Subgenotype A1 has been shown to be common in both Brazilian [4, 24, 38] and African [5, 17–19, 33] populations and has also been detected at a high frequency in Afro-Venezuelan groups [35]. These results suggested an African origin for the HBV isolates circulating in the Afro-Brazilian community under study, probably as a result of the slave trade that took place during three centuries (1,551–1,857). Five (10%) isolates belonged to genotype D, which is common in Brazil [24, 38]. The presence of few genotype D isolates in the FD community may be explained by a recent entry of young black people who moved from an urban zone to the community. A mixture of genotypes A1 and E was observed in one sample (2%). Nucleotide sequencing of the small S gene was successfully performed in 37 of the 50 isolates. Phylogenetic analysis (Fig. 1) of these 37 isolates confirmed RFLP patterns, with 34 isolates belonging to subgenotype A1. One isolate (FD151) belonged to subgenotype A2 and another one (FD09) clustered with genotype D sequences. The sequence from isolate FD123 that displayed mixed A + E genotypes by PCR-RFLP analysis clustered with genotype E sequences, indicating that the major HBV population belonged to genotype E. Interestingly, the HBV genotype E carrier was born in Angola (he came to the FD community in 2000). This genotype circulates in western, sub-Saharan Africa but has never been found in people born in Brazil. The absence of genotype E is compatible with the hypothesis of Mulders et al. [28], who suggested the emergence of genotype E as a human pathogen in the mid-to-late nineteenth century, at a time when the slave trade was already over.

The possibility of intrafamilial transmission was investigated among members of the FD community. Family was defined as father, mother and offspring. Five sequences were derived from family F1, eight from F2, five from F3, seven from F4, two from F5, two from F6, and two from F7. The remaining six sequences, namely FD191, FD123, FD09, FD174, FD97 and FD210, were the unique representatives of families F8 to F13, respectively. The phylogenetic tree (Fig. 1) shows that some HBV sequences, derived from members of the same family, were located in distinct groups. Inversely, two identical sequences (FD95-F1 and FD219-F3) were derived from members of different families. In areas that are highly endemic for HBV, different modes of horizontal transmission may occur as a consequence of close and frequent contacts between family members, especially in populations with low socioeconomic level and poor hygiene conditions [13, 40, 42]. In this study, no correlation could be established between families and HBV nucleotide sequences. This may be due to a high rate of inter-family/intra-community transmission.

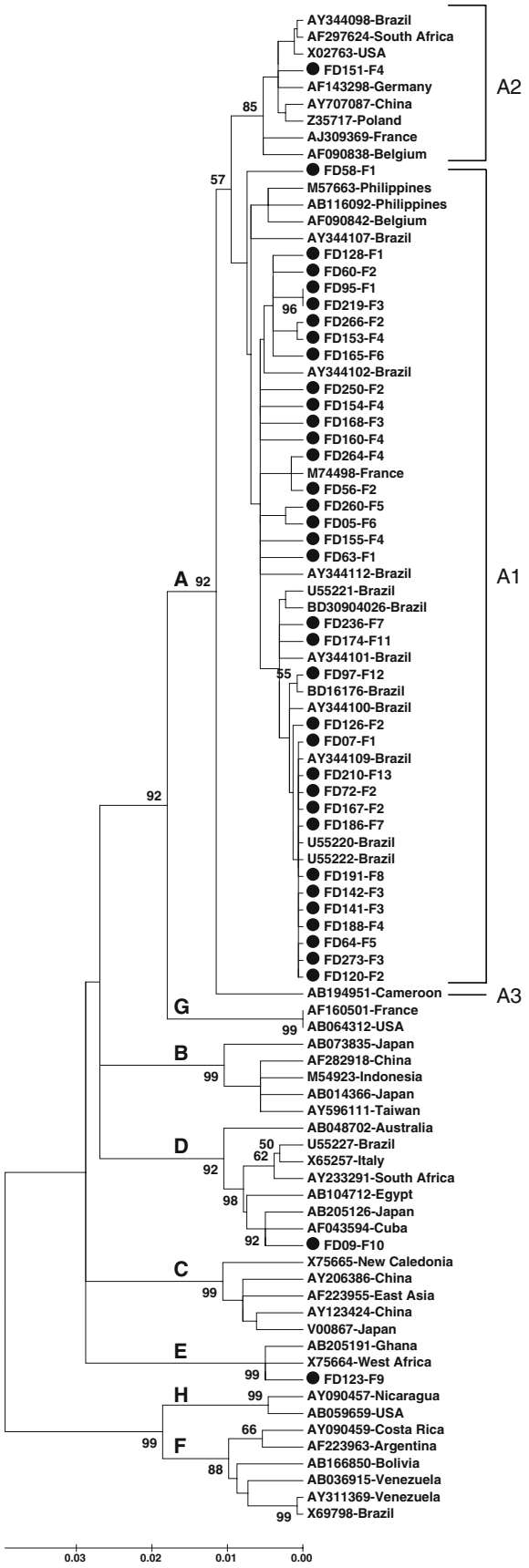

Fig. 1 Phylogenetic analysis based on nucleotide sequencing of the small S region. Sequences whose names begin with FD (*filled circle*, isolated community) and BD (blood donors) are from this work. F1 to F13 refer to the 13 families of the FD community under study. Genotypes are indicated at the branches of the tree

To further explore the phylogenetic relatedness and genetic diversity of FD sequences, molecular genetic distances were calculated among 100 HBV sequences divided into four groups. Nucleotide sequence divergences in the small S region within (1) 18 HBV/A1 Brazilian sequences from different geographical regions, (2) 33 HBV/A1 sequences from Africa, (3) 28 HBV/A1-FD sequences, and (4) 21 HBV/A2 isolates from different continents were $1.3 \pm 0.4\%$, $1.5 \pm 0.3\%$, $0.8 \pm 0.2\%$ and $0.9 \pm 0.4\%$, respectively. The sequence variation ($0.8 \pm 0.3\%$) within the group of HBV/A1-FD isolates was smaller than intragroup divergence found between HBV/A1 isolates circulating in Brazil ($1.3 \pm 0.4\%$) but similar to sequence variation between HBV/A2 isolates from different countries ($0.9 \pm 0.4\%$). Hannoun et al. [15] have speculated that the A2 subgenotype has evolved from a subgroup of A1 isolates exported from southern Africa to Europe about 500 years ago or later. In a similar way, one could argue that the HBV/A1-FD isolates were derived from a subset of A1 African isolates and may have been evolving in relative isolation.

Table 3 shows the amino acid changes observed in the S protein in comparison with a consensus of 33 African HBV/A1 (HBV/A1-AF) sequences. HBV/A1-FD sequences were also compared to a consensus (HBV/A1-BR) obtained by alignment of the 18 sequences from HBV/A1 isolates of different geographical Brazilian regions. FD isolates sharing the same S protein sequence were grouped. All positions in which amino acid variations were observed in more than one FD sequence are shown in Table 3. Most amino acid substitutions found in the S protein were linked to genotypes. All HBV/A-FD sequences displayed Lys^{122} residues (Table 3) as well as Pro^{127}, Phe^{134} Lys^{160} (not shown), allowing the classification of these samples as serotype *adw2*. The deduced serotypes of isolates FD123 (genotype E) and FD09 (genotype D) were *ayw4* and *ayw3,* respectively. Some variations specific for HBV/A1-FD isolates were observed when compared with amino acid residues of the consensus HBV/A1-AF and with residues typical of HBV/A1-BR. Except for one residue (aa 194), the consensus sequences from African and Brazilian sequences were identical. At position 194, most of the HBV/A1-FD isolates displayed the same amino acid, Ala^{194}, as in the HBV/A1 Brazilian consensus sequence, whereas African sequences showed Val^{194}. Fifteen FD isolates displayed the same amino acid sequence as the HBV/A1-BR consensus. The most prevalent substitution

Table 3 Amino acid variability of S protein in FD sequences

Isolate	Genotype	S protein (position)																			
		3	8	19	20	45	66	68	100	114	119	122	124	131	143	159	161	168	194	207	208
HBV/A1-AF	A1	N	F	F	F	S	P	I	Y	T	G	K	C	N	T	A	Y	V	V	N	I
HBV/A1-BR	A1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–
Group 1	A1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–
FD154*	A1	–	–	S	–	–	–	–	–	–	–	–	–	–	–		–	–	A	S	–
FD 160*	A1	R	–	S	L	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–
FD126	A1	–	–	–	L	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–
FD165	A1	–	–	–	L	–	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–
FD168	A1	D	–	Y	W	–	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–
Group 2	A1	–	–	–	–	–	–	–	C	–	R	–	–	–	–	–	–	–	A	–	–
Group3	A1	–	–	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–
FD56*	A1	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–
FD05	A1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FD236	A1	–	–	–	–	–	A	–	–	–	–	–	–	–	–	–	–	–	A	–	–
FD210	A1	S	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	–
FD174	A1	–	–	–	–	–	A	–	–	–	–	–	S	–	–	–	–	–	A	–	–
FD60	A1	–	L	–	–	–	–	–	C	–	–	–	S	–	–	–	–	–	A	–	–
FD260*	A1	–	L	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
FD128	A1	–	L	–	–	–	–	–	C	–	–	–	–	–	–	–	–	–	A	–	–
FD58*	A1	–	L	–	–	–	–	T	–	–	–	–	–	–	–	–	–	–	A	–	T
FD63*	A1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	F	–	A	–	–
AY707087	A2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	S	–
FD151	A2	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	S	–
AB205126	D	–	–	–	–	T	–	T	–	S	–	R	–	T	S	G	F	A	–	S	–
FD09	D	–	–	–	–	T	–	T	–	S	–	R	–	T	S	G	F	A	–	S	–
AB205191	E	–	–	–	–	A	–	–	–	S	–	R	–	T	S	G	F	A	–	–	–
FD123*	E	S	–	–	–	A	–	–	–	S	–	R	–	T	S	G	F	A	–	–	T

Variations and identity (–) are indicated in comparison with consensus HBV/A1-AF, representing African A1 isolates. HBV/A1-BR represents the Brazilian A1 consensus. Sequences AY707087, AB205126 and AB205191 are representative of genotypes/subgenotypes A2, D and E, respectively.*HBsAg-positive samples. Group 1 includes isolates FD72*, FD97, FD64*, FD273, FD141, FD155*, FD191, FD188, FD186, FD167*, FD142*, FD120*, FD07, FD250*, and FD264. Group 2 includes isolates FD219 and FD95*. Group 3 includes isolates FD153 and FD 266

among HBV/A1-FD isolates was Y100C, observed in 8 of 37 isolates. Interestingly, all samples displaying Y100C were from cases of HBV occult infection. This mutation has been previously found in HBsAg-negative and anti-HBc-positive BD from Venezuela [14]. Further studies of in vitro HBsAg expression of HBV isolates with the Y100C substitution should be conducted to verify the association between this mutation and the HBsAg-negative phenotype.

As shown in Table 4, the deduced amino acid sequences of polymerase (aa 359–569) in FD isolates demonstrated the presence of mutations in several positions. Except for two positions (401 and 457; not shown), the consensus sequences from the African and Brazilian sequences were identical. In these positions, all but one HBV/A1-FD isolate displayed the same amino acids (Ile^{401} and Ser^{457}) as in the HBV/A1-BR consensus. The most frequent specific mutation of the Afro-descendant community was W501R, found in 17 HBV/A1-FD isolates. This substitution in the viral polymerase was previously identified only in Gambia [15]. Interestingly, it is known that a number of slaves who came to Brazil were from Gambia [36]. This suggested that substitution W501R may be used as a marker of HBV isolates originating in Gambia.

Precore/core mutations linked to HBeAg phenotype

Several studies have emphasized the potential importance of BCP and precore mutations in viral persistence during residual infection. The most common naturally occurring variations include the precore stop codon mutation G1896A, which abolishes HBeAg production [7, 10], and

Table 4 Amino acid variability of partial HBV polymerase in FD sequences

Isolate	Genotype	Polymerase (position)																	
		361	364	368	376	377	401	421	422	451	470	472	475	477	487	499	501	505	561
HBV/A1-AF	A1	H	I	P	F	L	L	L	S	I	H	N	G	L	Q	Y	W	L	S
HBV/A1-BR	A1	–	–	–	–	–	I	–	–	–	–	–	–	–	–	–	–	–	–
Group 4	A1	–	–	–	–	–	I	–	–	–	–	–	–	–	–	–	–	–	–
FD 174	A1	–	–	–	–	–	I	V	C	–	–	–	–	–	–	–	–	–	–
FD 236	A1	–	–	–	–	–	I	V	C	–	–	–	–	M	–	–	–	–	–
FD210	A1	–	–	A	–	–	I	–	–	–	–	–	–	–	–	–	–	–	–
FD154*	A1	–	–	–	–	F	I	–	–	–	–	–	–	–	–	–	R	–	T
FD273	A1	–	–	–	–	F	I	–	–	–	–	–	–	–	–	–	–	–	–
FD168	A1	–	–	–	L	G	I	–	–	–	–	–	–	–	–	–	R	–	–
FD160*	A1	–	–	–	S	–	I	–	–	–	–	–	–	–	–	–	–	–	–
Group 5	A1	–	–	–	S	–	I	–	–	–	–	–	–	–	–	–	–	–	–
FD 165	A1	–	–	–	S	–	I	–	–	–	–	–	–	–	–	–	R	–	–
Group 6	A1	Y	–	–	–	–	I	–	–	–	–	–	E	–	–	–	R	M	–
Group 7	A1	–	–	–	–	–	I	–	–	–	–	–	–	–	–	–	R	–	–
FD260	A1	–	T	–	–	–	I	–	–	–	–	–	–	–	–	–	R	–	–
FD58*	A1	–	T	–	–	–	I	–	–	–	N	–	–	–	–	–	R	–	–
FD56*	A1	–	–	–	–	–	S	–	–	–	–	–	–	–	–	–	R	–	–
FD60	A1	D	T	–	–	–	I	–	–	–	–	–	–	–	–	–	R	–	–
FD128	A1	–	T	–	–	–	I	–	–	–	–	–	–	–	–	–	R	–	–
AY707087	A2	–	–	–	–	–	I	–	–	V	–	–	–	M	–	F	–	–	–
FD151	A2	R	–	–	–	–	V	–	–	–	N	–	–	M	–	–	–	–	–
AB205126	D	–	–	–	–	–	D	–	–	V	F	H	–	M	N	F	R	–	–
FD09	D	–	–	–	–	–	N	–	–	V	F	H	–	M	N	F	R	–	–
AB205191	E	–	–	–	–	–	–	–	–	V	I	H	–	–	N	F	R	–	–
FD123*	E	–	–	–	–	–	S	–	–	V	I	H	–	–	N	F	R	–	T

Variations and identity (–) are indicated in comparison with consensus HBV/A1-AF, representing African A1 isolates. HBV/A1-BR represents the Brazilian A1 consensus. Sequences AY707087, AB205126 and AB205191 are representative of genotypes/subgenotypes A2, D and E, respectively. *HBsAg-positive samples. Group 4 includes isolates FD72*, FD97*, FD64*, FD141, FD191, FD188, FD167*, FD142*, FD120*, and FD07. Group 5 includes isolates FD165 and FD126. Group 6 includes isolates FD219 and FD95. Group 7 includes isolates FD05, FD153, FD155*, FD250*, FD264*, FD266 and FD63*

the double mutation A1762T-G1764A in the BCP, which down-regulates the expression of HBeAg [37, 39]. In this study, most (18/20) of the HBsAg-positive patients were anti-HBeAg positive. The age of these 20 HBsAg-positive individuals varied from 8 to 52 years (mean, 21 years). To identify mutations in the BCP and precore gene regions that may explain the predominance of the anti-HBe phenotype, nucleotide sequence analysis was carried out. This was performed for 10/20 HBV/A1-FD HBsAg-positive samples (two HBeAg-positive and eight anti-HBe-positive). All isolates were from subgenotype A1. Independent of their HBeAg status, all samples showed C at nt 1,858 and, as expected, G at nt 1,896, which precludes the occurrence of the classical precore stop codon, HBeAg-negative mutation [10]. A high rate (60%, 6/10) of the double substitution A1762T-G1764A, located in the BCP region, was found. This mutation, which has been associated with the HBeAg suppressive phenotype, is also prevalent in isolates from HBV/A1 carriers [1, 7, 15, 41]. The G1862T substitution (in the bulge of the encapsidation signal) was found in all 10 of the HBV/A1-FD isolates that were sequenced. This mutation is commonly detected in HBV/A1 isolates and affects virus replication and production of HBeAg [15, 39, 41]. The G1888A substitution, which introduces an extra precore start codon, was found in 7 of 10 samples. All 10 HBV/A1-FD isolates showed the double G1809T-C1812T mutation, which is only found in subgenotype A1 isolates. This double mutation alters the Kozak sequence preceding the precore start codon at nt 1,814 and may impair HBeAg expression as a result of suboptimal translation initiation [17]. The triple mutations G1809T-C1810T-C1812T and G1809T-A1811C-C1812T were observed in two samples (FD56 and FD58). These changes can severely impair the expression of HBeAg [1,

17, 39]. As in black Southern-African carriers infected with subgenotype A1 [18], a high proportion of anti-HBe was observed. This association seems to be characteristic of subgenotype A1. However, in a previous study conducted with subgenotype A1 isolates from 14 HBeAg-positive children (age 7–29; median, 13 years), mutations G1809T-C1812T, G1862T and G1888A were frequently detected [15]. It was suggested that these changes may produce stable variants that, for some reason, have become prevalent in subgenotype A1, even in HBeAg-positive subjects. However, in the present study, as in other ones, those mutations were mainly detected in anti-HBe-positive individuals. The difference in HBeAg status between these populations may be related to the age of the subjects. Indeed, the median age (21 years) of the subjects studied here was higher than that in the study by Hannoun et al. [15]. One hypothesis is that the occurrence of such mutations, related to the anti-HBe phenotype, may be cumulative, increasing gradually with age. However, the low number of HBeAg-positive isolates observed here makes this conclusion uncertain. Further investigations in populations infected with subgenotype A1 and their different HBeAg status should be conducted to verify this possibility.

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