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High Prevalence of Hepatitis B Subgenotype D4 in Northeast Brazil: an Ancient Relic from African Continent?

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ABSTRACT

Introduction. Hepatitis B virus (HBV) infection leads to a chronic liver disease that is distributed worldwide. The characterization of HBV into genotypes/subgenotypes is not only a mere procedure for distinguishing different HBV strains around the world because determining their geographic distribution is crucial to understanding their spread across the world. **Material and methods.** We characterized different HBV genotypes and subgenotypes in five municipalities located in northeastern Maranhão, in the Brazilian north Atlantic coast. 92 HBsAg-positive individuals were submitted to PCR (polymerase chain reaction). Fifty samples were sequenced using automated Sanger sequencing and classified by phylogenetic methods. **Results.** Subgenotypes D4 and A1 were found in 42 (84%) and eight (16%) samples, respectively. To our knowledge, this is the first study to describe a high frequency of subgenotype D4 in any population. Subgenotype A1 is frequently found across Brazil, but D4 has been rarely detected and only in a few Brazilian states. This study shows the characterization of HBV subgenotypes from a population based study in the state of Maranhão, particularly in populations that do not have frequent contact with populations from other regions of the world. **Conclusion.** Our findings showed a HBV subgenotype profile that probably reflect the viruses that were brought with the slave trade from Africa to Maranhão. This study also reinforces the need to evaluate the status of HBV dispersion not only in large urban centers, but also in the hinterland, to enable the implementation of effective control and treatment measures.

Key words. HBV. Genotype. Slave trade. Maranhão.

INTRODUCTION

Even though an efficient vaccine to prevent infection from the hepatitis B virus (HBV) is available since the 1980s more than 700,000 people have died worldwide from its complications in 2010.[1,2] HBV is a highly prevalent and silent infection.[3] HBV infection is a public health problem in regions where complete vaccination coverage has been difficult to achieve, particularly in infants, who are more prone to develop the chronic course of the disease.[4,5]

East Asia and Sub-Saharan Africa show high HBV prevalence, whereas Tropical Latin America, Central Latin America, Western Europe, and North America are considered low prevalence areas.[6] According to a national survey, Brazil is considered a low prevalence area,[7] although some areas with higher prevalence have been reported in the Amazon Basin and in the Southeast and South regions.[8]

HBV is a DNA virus classified into eight genotypes (A-H) that differ by 7.5% or more in their genomic sequence and have distinct geographical distributions.[9,10] A putative genotype I has also been described,[11] although some au-

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thors have contested it, based on new recommendations for sorting a new genotype.[12,13] Additionally, a genotype J has also been described in Japan.[14] This tentative genotype shows similarities to gibbon and orangutan viruses and further studies are needed to ratify its classification.[15] HBV genotypes may also be further divided into subgenotypes based on a nucleotide intragenotypic difference between 4 and 7.5%.[9]

The classification of HBV into genotypes/subgenotypes is not only a mere procedure for distinguishing different strains around the world because determining their geographic distribution is crucial to understanding their spread and analyzing evolutionary pathways.[16,17]

The distribution of genotypes around the world is a reflection of human population movements and is related to each ethnical background.[15,18] In Brazil, genotype A is the most frequent genotype found followed by D, and F,[19-26] whereas A1, D3, and F2a seem to be the most prevalent subgenotypes.[19-22,24,27]

The population of Brazil is composed of three main ancestral backgrounds: Europeans, Amerindians, and Africans.[28] The latter group consisted of African slaves brought to work in many regions of the New World. Brazil received the largest numbers of slaves, followed by the Caribbean.[29] Maranhão is a state in the north Atlantic Coast of Brazil that has particular features in the history of African slave trade: later establishment of slavery, isolation from other regions of the country due to the lack of river connection with the continent, and the similarity of slave trading routes with those in North Atlantic regions.[30]

The state of Maranhão is one of the Brazilian states with a higher percentage of Afro-descendants and remained isolated from the rest of the country until the mid-19th century.[30] There are only two previous studies analyzing the distribution of HBV genotypes in the state of Maranhão: one that analyzed patients followed up in the city of São Luís, the state capital,[19] and another that analyzed a quilombo community in northwestern Maranhão.[31] In both studies, HBV genotypes also found in Africa were the most frequent, but genotype D4 found at the São Luís Referral Center for Liver Studies, São Luís, Maranhão, Brazil, has rarely been detected in other Brazilian regions.

This study characterized two different HBV subgenotypes found in five municipalities located in northeastern Maranhão.

MATERIAL AND METHODS

Sample selection and ethical approval

A total of 3,860 samples were screened for HBsAg serological marker using commercial kits according to the manufacturer's instructions (DiaSorin, Italy) in the five municipalities located in northeastern Maranhão along the Brazilian north Atlantic Coast (Figure 1) from 2012 to 2014. The representative sample number for the study region was calculated by considering an HBsAg prevalence of 0.5% in Brazil and the total number of individuals in each municipality. The collection procedure was per-

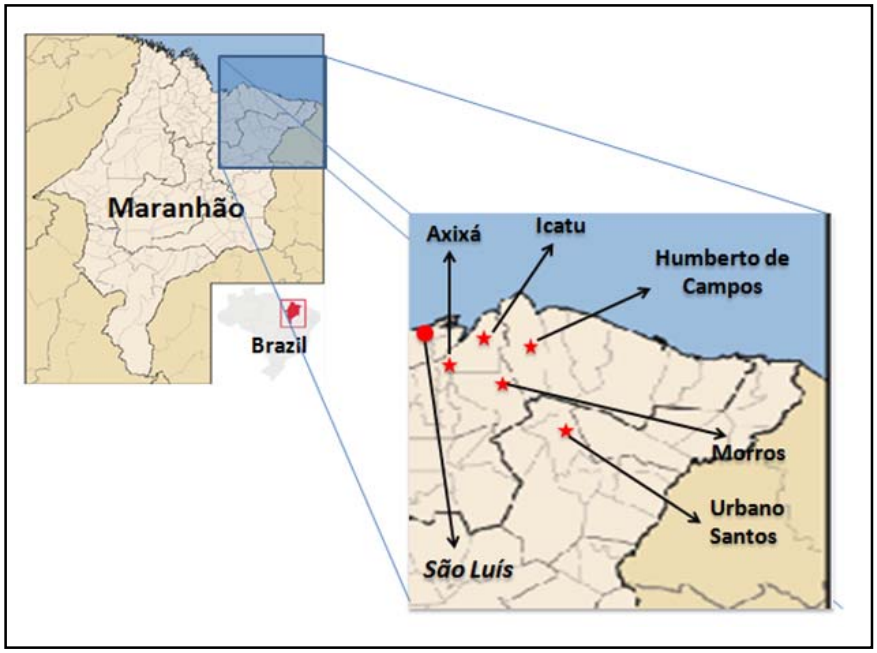

Figure 1. *Geographic location of the five municipalities sampled in northeastern Maranhão, Brazil. Each star represents one municipality. The red circle indicates de state capital, São Luis.*
Source. Modified from: Wikimedia Commons -https://commons.wikimedia.org/wiki/File:Maranhao_MesoMicroMunicip.svg#/media/File:Maranhao_MesoMicroMunicip.svg

formed by two-stage cluster sampling: first by choosing sectors according to population proportion and second by selecting the squares in these sectors by random sampling.

The study was approved by the research ethics committee at University Hospital, Federal University of Maranhão (HUUFMA), São Luís, Maranhão, Brazil, and written consent was obtained from all individuals that agreed to participate in the study.

HBV DNA amplification and sequencing

HBsAg positive samples were analyzed by nested polymerase chain reaction (nested PCR) to detect HBV DNA. HBV DNA was extracted using the QIAamp DNA Blood Mini kit (Qiagen®, Hilden, Germany) following the manufacturer's instructions. Fragments of 1,306 base pairs (bp) comprising the whole HBsAg and part of the DNA polymerase genes were amplified by nested PCR. Primers' sequences, PCR conditions, and sequencing procedures are described elsewhere.[19,27] All procedures were strictly done following measures for avoiding contamination as well as negative and positive controls were used in PCR assays.[32]

Viral load

PCR negative samples were analyzed for detection of viral load by real time PCR. We used the TaqMan® system (Life Technologies, Carlsbad, CA, USA) and probes, primers, and thermocycling conditions used are described elsewhere.[33] The amplification was performed using a 7500 Fast Real-Time PCR system (Applied Biosystems, Foster City, USA).

HBV genotyping and subgenotyping

Chromatograms were evaluated using Phred-Phrap software[34,35] and a quality score of 20 was used to select good quality readings. The consensus sequence of each sample was obtained using CAP3 software available at the Electropherogram quality analysis webpage (http://asparagin.cenargen.embrapa.br/phph/). Sequences were aligned using BioEdit (v. 7.0.8) software and edited using CLUSTAL W software.[36] HBV genotypes and subgenotypes were classified by phylogenetic reconstructions with published reference sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/).

Phylogenetic trees were constructed using the Bayesian Markov Chain Monte Carlo (MCMC) method implemented in the BEAST package v.1.6.1[37] under a relaxed molecular clock using GTR + G + I as nucleotide substitution model; MCMC was run for 20 million generations and trees were sampled every 2000 generations. Maximum clade credibility tree was summarized using TreeAnnotator v.1.6.1 and the tree was visualized in FigTree v1.4.2. software (available at: http://tree.bio.ed.ac.uk/software/figtree).

Serotypes were sorted according to the position of amino acids in the S gene by visual analysis.[10]

All sequences were submitted to GenBank under accessions numbers KX302085 - KX302134.

Statistical analysis

The Student's t-test was used to determine the difference in mean age between subgenotypes; the Fisher's exact test was used to compare the other variables between subgenotypes. Differences were considered significant at $p < 0.05$. All analyses were performed using STATA software version 11.0.

RESULTS

Demographic characteristics of HBV-DNA positive individuals

Of the 3,860 samples, 92 were positive for HBsAg serological marker. Fifty-one (55.4%) of the 92 HBsAg-positive individuals had detectable HBV DNA by nested PCR. Most HBV-DNA positive individuals were men (56.9%; 29/51) and their mean age was 44.3 (SD = 21.1) years. In addition, most HBV-positive samples were from Humberto de Campos (43.1%; 22/51). We also found that most HBV-positive individuals were from the rural zone (70.6%; 36/51), had a family income of < 1 minimum wage (64.7%; 33/51), and declared themselves as being Afro-American or mestizo/mullato (76.5%; 39/51).

Viral load

Forty-one of the 92 positive HBsAg samples were negative for HBV-DNA. These negative samples were submitted to viral load detection which was performed in 39 of the 41 samples because two serum samples were unusable. In total, 36 of the 39 samples analyzed had undetectable HBV viral load confirming our findings by nested PCR.

Genotype/subgenotype and serotype distribution

All 51 positive samples had their partial HBV sequence determined. One sample was excluded from the final analysis because the sequence did not achieve a good quality

Table 1. Demographic characteristics of HBV-carriers and subgenotypes found in this study.

Variable	Subgenotype (%) A1	D4	Total	P
N	8 (16)	42 (84)	50	
Male	5 (17.86)	23 (82.14)	28 (56)	1
Female	3 (13.64)	19 (86.36)	22 (44)	
Mean age (± SD)	41.5 (±18.7)	45.3 (±21.8)	44.7 (±21.2)	0.647
Origin				
Axixá	1 (50)	1 (50)	2 (4)	**0.015**
Morros	2 (16.7)	10 (83.3)	12 (24)	
Icatu	4 (50)	4 (50)	8 (16)	
Humberto de Campos	1 (4.55)	21 (95.4)	22 (44)	
Urbano Santos	0	6 (100)	6 (12)	
Sector				
Urban	3 (21.4)	12 (78.6)	15 (30)	0.683
Rural	5 (13.9)	30 (86.1)	35 (70)	
Ethnic group*				
Afro-American	3 (25)	9 (75)	12 (24)	0.32
Caucasian	0	5 (100)	5 (10)	
Native American	2 (40)	3 (60)	5 (10)	
Mestizo/Mulatto	3 (11.5)	23 (88.5)	26 (52)	
Other/unknown	0	2 (100)	2 (4)	
Education level				
No education	0	12 (100)	12 (24)	0.336
Incomplete Elementary education	5 (23.81)	16 (76.2)	21 (42)	
Complete Elementary education	0	4 (100)	4 (8)	
Incomplete High school education	1 (20)	4 (80)	5 (10)	
Complete High school education	2 (28.6)	5 (71.4)	7 (14)	
Higher education	0	0	-	
Not applied	0	1 (100)	1 (2)	
Family Income				
< 1 mw**	2 (6.3)	30 (93.7)	32 (64)	
1-3 mw	6 (33.3)	12 (66.7)	18 (36)	**0.019**

** Self-declaration. ** Minimum wage.*

index, but the remaining 50 samples had their HBV genotype and subgenotype determined (Table 1).

Genotypes A and D were only found in our sample and genotype D was the most frequent (84%; 42/50). The phylogeny showed that all genotype A sequences were subgenotype A1 (adw2) and all genotype D individuals were subgenotype D4 (ayw2). Additionally, subgenotype D4 clustered in a monophyletic group together with other sequences from Maranhão, whereas subgenotype A1 samples grouped with different HBV/A1 branches, except with the one containing sequences from Africa only (Figure 2).

HBV subgenotypes and sociodemographic characteristics

We found no significant difference in most sociodemographic characteristics between subgenotypes A1 and D4, except for municipality and family income (Table 1). There were more subgenotype D4 individuals in Morros, Humberto de Campos, and Urbano Santos than in Axixá and Icatu (P = 0.015). Additionally, the frequency of subgenotype D4 was higher in families with income < 1 minimum wage (P = 0.019).

DISCUSSION

Up to the present time, this is the third study to determine HBV genotype/subgenotype distribution in the state of Maranhão, in the north Atlantic coast of Brazil[19,31] but it is the first to obtain HBsAg-positive samples from representative number of individuals of a regional population-based survey. The study region is located in northeastern Maranhão and consists of five municipalities with low socio-economical index.[38]

Most infected individuals lived with less than one minimum wage for the whole family and the level of education was often low. Thus, because of poverty and low educational status, these individuals are often unaware about means of transmission and prevention of diseases such as hepatitis, and thus may be more susceptible to in-

Figure 2A. *Phylogenetic analysis of HBV subgenotype isolates from different regions of Maranhão state, Brazil, detailing subgenotypes A1* ***(A)*** *and D4* ***(B)****. Analyses were performed by Bayesian Inference using the Bayesian Markov chain Monte Carlo (MCMC) method. Sequences identified in this study are highlighted (#) and reference sequences from GenBank are indicated by their corresponding genotype/subgenotype accession number and origin. The posterior probability values are shown for key nodes.*

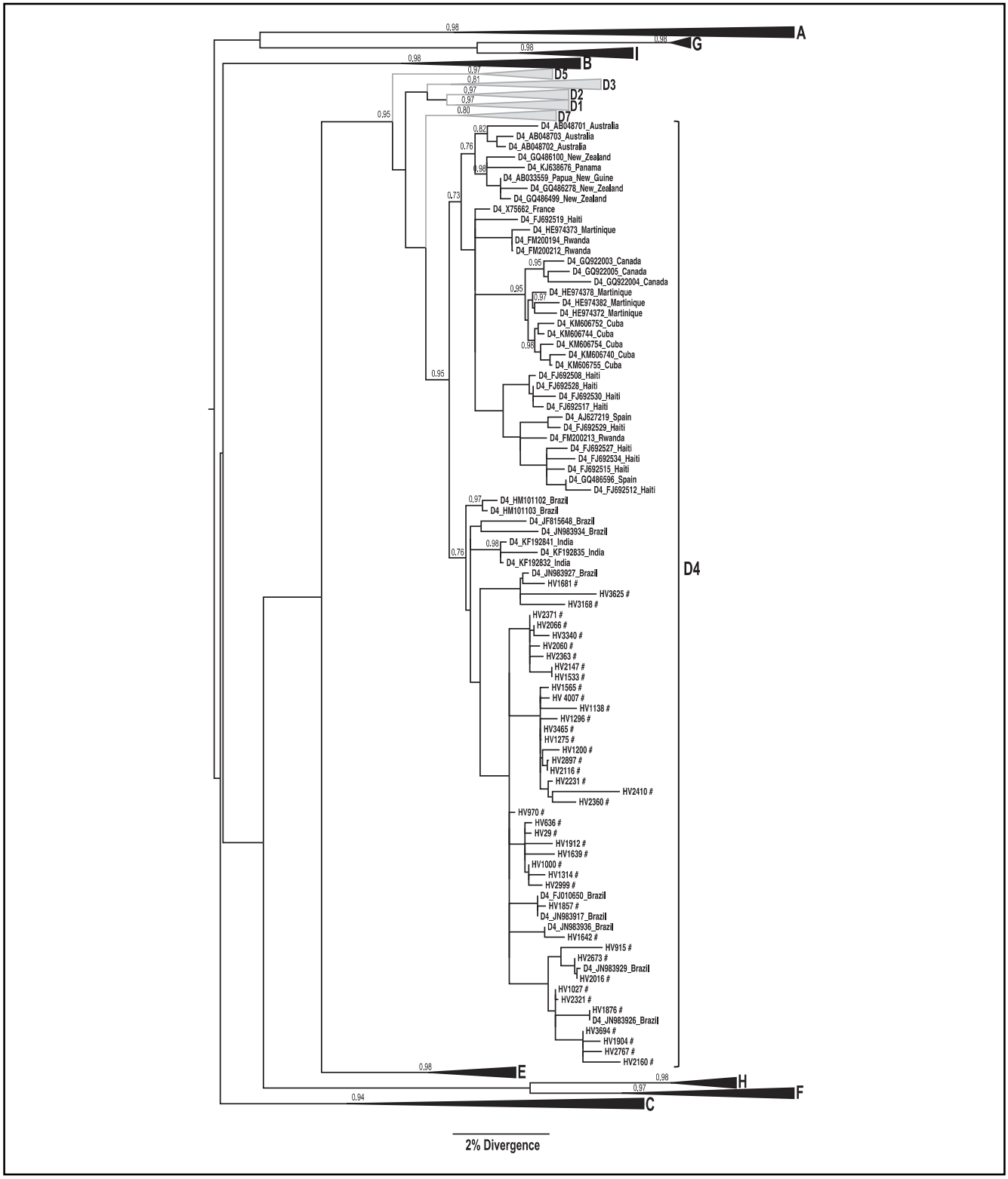

Figure 2B. *Phylogenetic analysis of HBV subgenotype isolates from different regions of Maranhão state, Brazil, detailing subgenotypes A1* ***(A)*** *and D4* ***(B)****. Analyses were performed by Bayesian Inference using the Bayesian Markov chain Monte Carlo (MCMC) method. Sequences identified in this study are highlighted (#) and reference sequences from GenBank are indicated by their corresponding genotype/subgenotype accession number and origin. The posterior probability values are shown for key nodes.*

fection. The delayed improvements in living and hygiene conditions and the difficulties in launching prevention, control, and treatment strategies that are important to control viral hepatitis and other diseases are known and have been described elsewhere.[8]

Two different HBV genotypes were found in our sample. Genotype D was the most prevalent followed by few genotype A cases. This finding is contrary to the HBV genotype prevalence described for Maranhão and Brazil as a whole, where genotype A is the most prevalente.[19,21-26,31,39] An exception to that pattern is found in southern Brazil, where genotype D is the most frequent genotype,[20,40,41] which is related to the immigration of European people from highly HBV/D prevalent regions.[42]

Only two subgenotypes were identified in our study, A1 and D4 being the latter the most frequent. Subgenotype A1 is the most frequent subgenotype found in cases of genotype A across Brazil[27] and it was also the most frequent subgenotype found in the previous study conducted in São Luís, in Maranhão.[19] In addition to subgenotype A1 that study also identified other four subgenotypes (D2, D3, F2a, and D4). Unlike the current study, subgenotype A1 was the most prevalent in that study, even though subgenotype D4 also had a relatively high frequency (24%; 29/119).[19] The difference between our results and those of Barros, *et al*. (2014) is noteworthy and may be related to the samples analyzed: the population from the latter study consisted of chronic HBV carrier patients treated at a referral center for treatment of liver disease, and even though it covers the entire state of Maranhão, more than 75% of participants were from the metropolitan area of São Luís, the state capital. Thus, subgenotype A1 seems to be more prevalent in the metropolitan region, whereas subgenotype D4 is more frequent in other regions of the state such as the northeast.

Alvarado-Mora, *et al*. (2011) only found subgenotype A1 in four sequenced samples from quilombo Frechal, located in the municipality of Mirinzal, northwestern Maranhão.[31] More studies in other regions of Maranhão should be conducted to analyze a larger number of HBV-positive samples for a better understanding of subgenotype prevalence in this region.

Subgenotype D4 was previously found in the aforementioned study conducted in Maranhão,[19] but it is not frequently found in Brazil. This genotype was initially described in two different studies conducted in the Brazilian Amazon, in the states of Amazonas[21] and Rondônia.[24] Genotype D4 was also found in 5.1% (36/702) of samples collected across Brazil,[27] mostly in Maranhão, but also in other states in the southeast (São Paulo and Minas Gerais) and north (Pará) of Brazil.[43] We hypothesize that internal migrations among different Brazilian states may explain the presence of subgenotype D4 in these regions, particularly in Pará, which has a large border with the state of Maranhão.

The high prevalence of subgenotype D4 in Maranhão supports the hypothesis that this strain was not introduced in Brazil through European immigration because this subgenotype is not frequent in Europe.[42] Zehender, *et al.* (2012) performed for the first time a HBV-D epidemiological reconstruction by phylodynamics and phylogeographic analysis in Europe.[44] Although they did not included D4 sequences in their analysis, a preliminary review of them showed that D4 and D7 subgenotypes are close related. Furthermore, other study also found this relation between these two subgenotypes.[45] HBV-D7 is more often restricted to the Northern Africa[46,47] and may give support to the hypothesis of HBV-D4 distribution from this continente.[19]

In fact, subgenotype D4 has been previously described in many countries worldwide.[10,48-55] HBV D4 has also been found in Caribbean countries of significant African descent such as Haiti, Martinique, and Cuba.[56-58] Interestingly, in Kenya, only subgenotypes A1 (85.7%) and D4 (14.3 %) have been reported,[49] but in proportions that are the opposite of those found in Maranhão. As suggested by Barros, *et al.* (2014), subgenotype D4 may have infected individuals frequently in the past, and during the period of slavery these strains may have reached other continents where African slaves were also traded.[19] For instance, Haiti and Cuba are regions outside Africa with a high frequency of subgenotype D4 cases.[56,58] The Caribbean had intense trading of slaves from Africa, lower only than that in Brazil.[29] Thus, the history of population formation, combined with molecular evidence of virus traits, may support a better understanding of the spread of infections such as HBV.

Subgenotype D4 strains found in Brazil clustered in a separate branch in the phylogenetic tree with other samples described elsewhere (except from three from India), suggesting the introduction of a unique strain in the country. It is noteworthy that only these India sequences appeared to be related to our sequences apart from any other described in the world. These Indian strains are from Tripura State which is the first region in India where subgenotype D4 was found.[53] Although the authors did not found a high prevalence of the HBV-D4 (13/76; 17.1), Portuguese vessels might have brought from India some strains of this infection to Maranhão, mainly when British begun to suppress the Atlantic slave trade and forced the Northern Atlantic traders to change their routes to South-East Africa.[30,53,59,60] Subgenotype A1 strains also clustered in a branch different than the one where most African sequences were grouped and differently than subgenotype D4 strains, A1 strains did not aggregate in a few branches but rather in many branches containing samples from across the world, with only few African samples from So-

malia and South Africa. Thus A1 strains cannot be considered an African clade. In a study that analyzed A1 strains from Brazil,[60] the authors concluded that Brazilian A1 genotypes are not of Central or Western Africa origin, but originated from slaves brought from southeastern Africa during the mid-19th century, because the study's samples clustered into an "Asian-American" clade similarly to the samples in our study. However, there are few available sequences with these genotypes from East African countries, despite the reports of slaves coming to Maranhão from these countries.[61]

The Brazilian population was formed by three ancestral backgrounds in decreasing order of contribution: Europeans, Africans, and Amerindians. Nevertheless, the state of Maranhão is one of the regions in Brazil with the greatest contribution of African ancestry.[28] Our results show that most individuals who were assigned to subgenotypes A1 and D4 declared themselves as being Afro-American (24%) or mestizo/mulatto (52%), reinforcing the association between these two subgenotypes and African ancestry. Moreover, the pattern of slavery establishment in Maranhão was peculiar: due to geographic features (isolation from other regions in the country because of a lack of river connection to the continent) and navigation issues (wind and ocean currents), slave trade routes in Maranhão were more similar to those in Caribbean and North Atlantic countries than those in the rest of Brazil.[30] Thus, we expected to find the same subgenotype in Maranhão and in Caribbean countries, which is not the same one found in Bahia, another Brazilian state with a large number of Afro-descendants.[27] Finally, the recent finding of HDV-8 in Maranhão co-infecting individuals that carried HBV/D4 corroborates the African origin of the latter subgenotype.[62,63]

This study emphasized the presence of HBV genotypes that might be originated from Africa in the state of Maranhão, particularly in populations that do not have frequent contact with populations from other regions of the world. This pattern probably reflects the viruses that were brought with the slave trade from Africa to Maranhão that was interrupted in the middle of the 19th century. Maranhão has a great contribution of African ancestors originated from a particular slave trade route that is similar to the one followed in the Caribbean islands and both areas share a similar pattern of HBV subgenotype distribution.

This study has unexpectedly shown a low prevalence of HBV DNA among the HBsAg individuals. This may be explained by the nature of the sample. The individuals who participated of this study were from an epidemiologic survey rather than individuals searching for medical care. However, the high prevalence of HBV D4 strain may scatter light on the interaction of this virus and the human host, that is, genotype D4 may influence the immunologic state of HBsAg marker.[64] Only with more studies related to this issue with follow up of patients from this population would explain this finding.

This study also reinforces the need to evaluate the status of HBV dispersion not only in large urban centers, but also in the hinterland, to enable the implementation of effective control and treatment measures. Studies involving population samples from wide geographic areas and hard-to-access regions are difficult to perform, mainly due to the high costs and lack of specialized human recourses. Nevertheless, further investigations in isolated areas are needed because a better knowledge of HBV infection across the world is essential for the effective control of this infectious disease.

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