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Phylogenetic analysis and genotype distribution of Hepatitis B Virus (HBV) in Roraima, Brazil

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ABSTRACT

Hepatitis B virus (HBV) infection is a serious global health problem. HBV has a high viral genetic diversity, with 10 genotypes recognized. In Brazil, the Roraima State is the third in the Northern region regarding the number of hepatitis B cases. On the other hand, few data on HBV genotyping and phylogenetic analysis are available. The purpose of this study is to characterize the HBV genotypes circulating in Roraima State. Of the 113 chronic hepatitis B patients enrolled in this study, 40 were HBV-DNA positive. A fragment of 280 bp (S gene) was amplified by PCR and submitted to nucleotide sequencing. A dataset containing the viral sequences obtained in this study, plus 130 obtained from GenBank was used for genotyping by phylogenetic analysis. The HBV subgenotype distribution found was A1 (62.5%), A2 (7.5%), D2, D3, D4 (2.5%), F2a (12.5%), and F3 (10%). We characterized the genotypes and subgenotypes of HBV circulating among patients in the State of Roraima. In addition, our study shows for the first time the HBV/F3 genotype circulating in Brazil. In conclusion, our findings showed a high diversity of HBV genotypes in Roraima, which is also found in other Brazilian geographical regions.

KEYWORDS: Hepatitis B. Genotypes. Roraima State. Phylogeny.

INTRODUCTION

Hepatitis B virus (HBV) evolution is driven by a high mutation rate during replication, estimated to be 1.4-3.2 x 10^{-5} nucleotide substitutions per site per year, which is more than 10-fold higher than other DNA viruses[1]. This leads to a high degree of genetic heterogeneity, resulting in ten genotypes (A to J) which have distinct geographical distribution. HBV genotypes are characterized by a difference in the nucleotide sequences of 8% or more, whereas differences between 4 to 8% are classified as subgenotypes[2]. Indeed, most genotypes are now divided into subgenotypes with distinct virological and epidemiological properties. HBV genotypes, subgenotypes and mutations in specific regions of the HBV genome play an important role in the rates of HBeAg and HBcAg seroconversion, viremia levels, immune escape, emergence of mutants, pathogenesis of liver fibrosis, disease progression, response and resistance to antiviral therapy, in addition to being predictive of clinical outcomes[3].

Genotype A is mainly found in the North America and Africa. Genotypes B and C are prevalent in Southeast Asia and the Far East. Genotype D is found worldwide, mainly in the Mediterranean and Central Asia. Genotypes E and F are prevalent

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in West Africa and among the indigenous peoples of the Americas, respectively. Genotype G has been reported in the United States, France, Colombia and Brazil. Genotype H has been shown to be prevalent in Central and North America. In addition, two new genotypes, I and J, have been recently reported in Vietnam, Laos, and Japan[4-7]. In Latin America, the presence of genotypes F and H is related to indigenous origins, and genotypes A and D result from the mixture between European and African populations[8].

Overall, genotype A is the most prevalent in Brazil, followed by genotypes D and F. However, genotypes B and C have also been detected in some individuals[9]. Even though Brazil is as a country with intermediate hepatitis B endemicity, there is a large heterogeneity between Brazilian regions. Some studies show that across South and Southeast of Brazil, less than 2% of the population is infected with HBV. Towards the North of the country the incidence increases, and thus the Amazon basin presents a high prevalence of HBV infection[10].

Epidemiological data[11] shows that, among the different etiological agents of viral hepatitis, hepatitis B is the second most prevalent in Roraima. According to the National Immunization Program[12], even though Roraima is considered an endemic region and the HBV vaccine is available for all age groups, coverage did not reach the goal stipulated by the Brazilian Ministry of Health in the following age groups: less than 1 year with 88.87%, 3 years 94.34%, 15 to 19 years 88.92%, 20 to 24 years 62.69% and 25 to 29 years 61.74%.

Valaydon *et al.*[13] associated mutations in gene S with resistance to vaccine; however, the correlation between HBV genotypes, possible mutants and escape from vaccination is still unknown. Studies suggest that the infection with HBV in vaccinated children was related to a wild type of the genotype E[14] or to the genotypes B and C[15].

The present study aims to characterize different HBV subgenotypes circulating in Roraima State, an important epidemiological issue, especially in a border area. Thus, our data will probably contribute to a better understanding of HBV genotypes and subgenotypes dispersion in the Northernmost region of Brazil.

METHODS

Roraima State is located in the Amazon region (Figure 1). It is the Northernmost and least populated State of Brazil. It is bordered to the East by the State of Para and Guyana, to the South and West by the State of

Figure 1 - Geographic localization of Roraima State, Brazil

Amazonas, and to the North by Venezuela. Roraima has 15 municipalities, including the State capital, Boa Vista, where 63.53% of the State's population is concentrated[16].

In the study period, from 2013 to 2015, the prevalence rate of Hepatitis B was 13.9 per 100,000 habitants in 2013; 16.5 in 2014 and 9.29 in 2015. The number of samples in this study represents 20.3% of the total of 197 chronic Hepatitis B patients registered in SINAN (*Sistema de Informação de Agravos de Notificação*) during this period[12].

This analysis included whole blood samples collected from 113 patients with chronic Hepatitis B, defined as the persistence of the HBsAg for at least 6 months, associated with the presence of total anti-HBc and non-reagent anti-HBc IgM. The number of samples analyzed accounts for 71.97% of the total number of patients on therapy during the entire studied period. Samples were collected at the Central Laboratory of Roraima State (LACEN/RR) between January 2013 and March 2016, during a blood test to assess viral load. HBsAg-positive individuals of both genders aged 18 years or more were enrolled in this study. Subjects signed an informed consent form prior to sample collection. This study was approved by the Research Ethics Committee of the Federal University of Roraima (Protocol Nº 121005) on October 26, 2012.

Genomic DNA of each sample was extracted from 200 µl of whole blood using the QIAamp DNA Blood kit (Qiagen, USA) according to the manufacturer's instructions. HBV DNA amplification was performed by a nested PCR using type-specific primers for the target region. The first-round PCR primers amplify a 447 bp fragment of the S region with primers FHBS1 (position 244 to 267; 5'-GAGTCTAGACTCGTGGTGGACTTC-3'), and RHBS1 (position 688 to 691; 5' GCTAAATKGCACTAG TAAACTGAGCCA-3'), and the second-round primers amplify a 417-bp fragment with primers FHBS2 (position 255 to 278; 5'-CGTGGTGGACTTCTCTCAATTTTC-3') and RHBS2 (position 648 to 671; 5'-GCCARGAGAAAC GGRCTGAGGCCC-3'). PCR amplification was carried out (unpublished data).

The positive samples were further purified with polyethylene glycol (PEG) at a final concentration of 20% according to the protocol used in the genomic platform of the Leonidas and Maria Deane Institute (ILMD), FIOCRUZ unit in Manaus, Amazonas. Samples were sequenced with BigDye® Terminator V3.1 Cycle Sequencing Kit (Applied Biosystem, Thermo Fisher Scientific, USA). The sequencing reaction was analyzed on an ABI 3130 automated sequencer (Applied Biosystems, Thermo Fisher Scientific, USA). The quality of each electropherogram and assembly of the consensus sequence was performed using the Geneious-R6® software. Sequence identity was estimated using the BLASTn tool and compared with other HBV genomic sequences available at public databases. Sequences were genotyped by phylogenetic reconstruction using reference sequences from each HBV genotype obtained from GenBank (130 in total), comprising 280 bp of the partial S gene sequence. Bayesian phylogenetic analysis was conducted using the MCMC method implemented in MrBayes 3.1.2, using the nucleotide substitution model (GTR + G), as previously estimated in jModelTest v2.1.3. Fifteen million generations were enough to reach the convergence. A non-human primate external sequence was used as an external group to root the phylogenetic tree. The maximum clade credibility (MCC) tree was obtained by summarizing 15,000 trees and was then modified using a 10% burn-in. Effective sample sizes >200, for all estimated parameters, were obtained after analysis with Tracer v.1.5. The resulting phylogenetic tree was visualized in FigTree v1.3.1. Sequences were deposited into the GenBank under the following accession numbers: KY780240 - KY780279.

RESULTS

In this study, the HBV DNA was found in 40 patients (35.40%). Gender, age, demographical, virology information, genotype and subgenotype of these patients are presented in Table 1.

The age average of the patients was 39.6 ± 13.7 SD years, with 21 women and 19 men ($X^2 = 0.22$, $p = 0.6394$). Of the total number of individuals analyzed in the study, 37 declared their ethnicity with a majority of 59.4% declaring to be of mixed ethnicity (mixture of white and black) ($X^2 = 31.75$, $p < 0.0001$).

Of the 33 patients who declared their place of birth, 25.8% reported having been born in Boa Vista, capital of the State of Roraima ($X^2 = 9.645$, $p = 0.0218$). Only one individual claimed to have been born in Guyana, but he has lived in Brazil for more than 20 years. Most patients, 67.5%, lived in the capital Boa Vista ($X^2 = 4.90$, $p = 0.0269$). All the individuals presented detectable viral load, with 50% of them with viral load above 2,000 IU/mL.

When we analyzed the genotypes (Figure 2), we found a higher frequency of genotype A, corresponding to 70% of the analyzed samples ($X^2 = 25.55$, $p < 0.0001$). For the subgenotypes, A1 was the most frequent one ($X^2 = 71.30$, $p < 0.0001$), representing 60% of the samples, followed by subgenotypes A2 = 10%, D2 = 2.5%, D3 = 2.5 %, D4 = 2.5%, F2a = 12.5%, F3 = 10%.

A1 genotype was more prevalent among the group with mixed ethnicity ($X^2 = 9,364$, $p = 0.0093$); genotype A2 and F2a were equivalent in those with mixed ethnicity and Caucasians; D1 and D4 were reported only in those

Table 1 – Characteristics of the 40 chronic hepatitis B patients included in this study

Parameters		P- value*
Patients	40	
Age (average±SD)	39,62(±13,74)	
Gender [n(%)]		
Male	19(47,5)	NS[a]
Female	21(52,5)	
Race [n(%)]		
White	7(18,9)	
Mixed ethnicity	22(59,4)	< 0.0001[a]
Black	4(10,8)	
Indigenous	4(10,8)	
Not informed	3	
Residence place[n(%)]		
Boa Vista (capital)	26(67,5)	= 0.0269[a]
Another Roraima's municipality	14(32,5)	
Birth place [n(%)]		
Boa Vista (capital)	8(25,8)	
Another Roraima's municipality	10(29)	= 0.0218[a]
Another Brazil's state	15(41,9)	
Viral load (UI/mL) [n(%)]		
> 2000	20 (50)	NS[a]
< 2000	20 (50)	
Genotypes [n(%)]		
A	28(70)	< 0.0001[a]
D	3(7,5)	
F	9(22,5)	
Subgenotypes [n(%)]		
A1	24(60)	< 0.0001[a]
A2	4(10)	
D2	1(2,5)	
D3	1(2,5)	
D4	1(2,5)	
F2a	5(12,5)	
F3	4(10)	

a: Teste X^2; p>0,05; NS- not significant; SD – standard deviation

with mixed ethnicity , D2 only in blacks and F3 only in the indigenous group.

Phylogenetic tree reconstructions (Figure 2) showed two distinct clades inside the subgenotype A1. One clade is predominantly "African" and includes isolates from South, East and Central Africa, and the other is an "Asian-American" clade with isolates from Asia, Somalia, Caribbean islands and a restricted number of strains from South America[17]. Phylogenetic analysis of the subgenotype A1 indicated a posterior probability value of 1, splitting the "African" and "Asian-American" clades. In the "African" clade, strains were grouped into clusters previously isolated in Brazil, Cameroon, Gambia and Nigeria. The "Asian-American" clade clustered a larger number of analyzed strains, together with isolates from Brazil, Rwanda, Kenya, Colombia, Belgium and Martinique.

DISCUSSION

This study reports the geographic distribution of HBV genotypes in the Brazilian State of Roraima. Molecular characterization of HBV isolates is invaluable in establishing HBV evolutionary origins and dispersion patterns[18]. Molecular evolutionary studies to characterize HBV in Roraima have an even more vital role, once this State shares international land borders with Venezuela and British Guyana, countries where hepatitis B is highly endemic. The distribution of HBV genotypes varies across different geographical regions, some are distributed worldwide, whereas others are more geographically confined[19].

The results of genotyping are in agreement with previous records found in Brazil, showing that genotype A prevails (28/40 - 70%), followed by genotypes F (9/40 - 22.5%) and D (3/40 - 7.5%); (X^2 = 25.55, p < 0.0001)[20-23]. Recently, a study involving samples of HBV representing the five Brazilian regions showed that the genotypes A, D and F are the main genotypes found in the country[24]. The distribution of genotypes in the Brazilian population is correlated with its heterogeneous ethnic origin[25], which is an admixture of European, Native American and African ancestors[6].

Genotype A is subdivided into subgenotypes A1 to A7. Among them, A1 is highly prevalent in sub-Saharan Africa and Northern Europe; subgenotype A2 and A3 are prevalent in Western Africa; A4 and A5 were found in sub-Saharan Africa (Mali, Gambia, Nigeria); A5 was found in people of African descent in Haiti[26,27].

In Northern Brazil, genotype A,mainly the subgenotype A1, is predominant and was probably introduced in Brazil during the slave trade[28,29]. A study carried out in the Western Brazilian Amazon at Labrea municipality, Amazonas State, identified the presence of genotypes A (60%), D (35%) and F (5%)[22]. Another study on HBsAg-positive blood performed in Manaus, Amazon Region, Northern Brazil, showed a high prevalence of genotype A, as well as the predominance of HBV genotypes A and F[20].

Previous studies have shown that only two subgenotypes

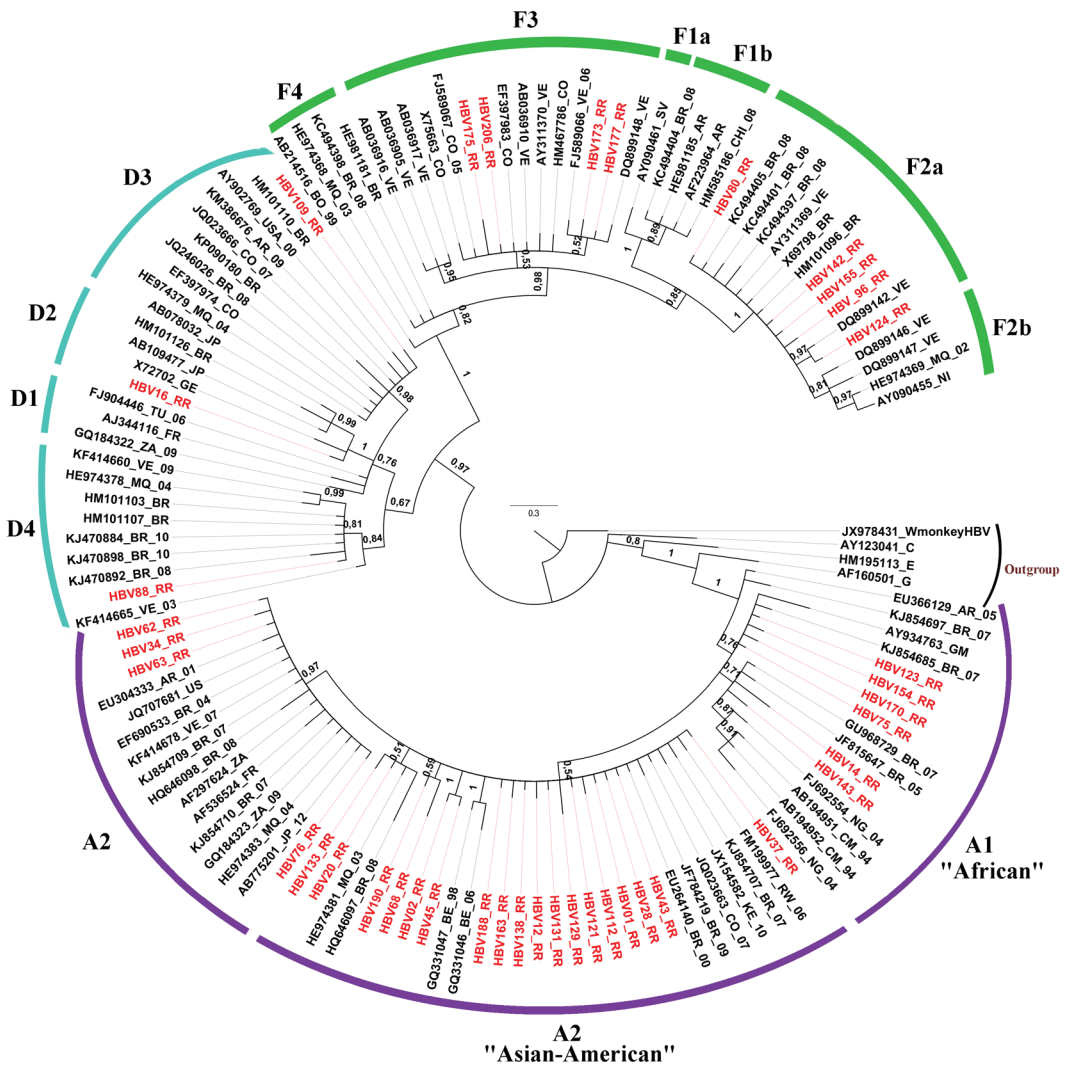

Figure 2 - Phylogenetic analysis of HBV strains isolated in Roraima. The maximum clade credibility (MCC) tree was estimated by Bayesian analysis of 130 sequences according to the S region (280 bp). The posterior probabilities of the key nodes are shown above according to the respective nodes. HBV isolates from Roraima are represented in red and were analyzed together with other worldwide stains.

of genotype A (A1 and A2) have been found in Brazil, where HBV/A1 has been the sole or most prevalent one[18,21,23,30]. Our results corroborated a phylogenetic study with isolates from different regions of Brazil, which showed that two distinct clades have been recognized into subgenotype A1[31]. However, unlike our findings, Brazilian strains grouped into the "Asian-American" clade only.

Similar results of the split between "African" and "Asian-American" clades were also found in a study carried out in Martinique, which identified most of the sequences as belonging to the "Asian-American" clade[17]. The findings in our study are probably due to the presence of a highly mixed-race population in Roraima, which consists of descendants from different geographic regions in Brazil and abroad. Also, the sequences in these two clusters did not form a single clade in the tree and few sequences clustered with other Brazilian sequences described above, indicating these viruses were probably introduced into the State at different moments. These results are similar to those of previous studies carried out in the Brazilian States of Rondonia[18] and Rio de Janeiro[32], which found a large number of HBV isolates belonging to subgenotype A1. Recently, another study performed in Para found the genotypes A and F as the predominant ones in these populations. Genotype A was the most frequentwith 88.46%, and among the subgenotypes, AI represented 78.26%, followed by A2 21.74% and the prevalence of genotype F was 11.54%[33].

In contrast, few sequences in this study were grouped into subgenotype A2, classified as European, which was supposedly introduced in Europe by Portuguese sailors traveling along the coastal waters of South Africa during the 15th, 16th and 17th centuries. Some authors have speculated

that subgenotype A2 has evolved from a subgroup of A1 isolates[34]. This result corroborates previous findings that showed low prevalence or inexistence of genotype A2 in the North and Northeast regions[4,18,20]. This subgenotype is found in Europe, the United States, England, Argentina, Colombia and Spain[19]. Considering the geographic distribution of the two subgenotypes (A1 in Africa and A2 in Europe), the prevalence of A2 in Southern Brazil correlates with the European origin of the population[31].

Only three sequences were grouped into genotype D, presenting low prevalence (3/40 - 7.5%). Our study confirms the low prevalence or absence of genotype D found in the North and Northeast regions[20,35]. In contrast, a study carried out among HBV chronically infected patients in the State of Rondonia, Northern Brazil, detected a prevalence of 42.8%[18]. Another recent study, performed in the State of Maranhao, located in the Brazilian Northwest region, showed that genotype D was the most frequent one (84%; 42/50)[36]. Lampe *et al*.[24] showed that among the five Brazilian regions, genotype D is the most prevalent only in the South of Brazil, representing 80% (75/95) of the analyzed samples in that area. Previous studies have indicated that genotype D is more frequent in the Southern Brazilian populations, due to their prevalent European ancestry resulting from the massive migration of 1872-1975. The spread of genotype D strains in the Amazon basin is probably due to the presence of migrants from South and Southeast Brazil, where genotype D is widespread[23,25].

Within genotype D, four subgenotypes can be found: D1 occurs mostly in the Mediterranean and Middle East; D2 has been reported in India, Japan, Europe, and the United States; D3 has been registered in South Africa, Brazil, Rwanda, Costa Rica and the United States and D4 in Australia, South Africa, Somalia, Rwanda and Oceania[19].

The phylogenetic tree showed three D strains subgenotypes: D2, D3 and D4. These subgenotypes were also found in previous studies carried out in the Amazon States of Amazonas[30] and Rondonia[18], and in the Northeast State of Maranhao[21]. The sequence of subgenotype D4, isolated in the State of Roraima, formed a clade more closely related to strains previously isolated in the States of Rondonia[18] and Maranhao[21] and also a sequence similar to a Venezuelan strain[37], an HBV endemic country bordering Roraima, suggesting that these international borders may become the entry of new HBV genotypes into the country.

The sample designated subgenotype D2 formed a closer group with previously isolated sequences in Japan and Brazil. HBV subgenotype D2 was previously described as prevalent in the South and Central-West regions of Brazil[23], and it may have probably been introduced to Roraima by immigrants from those regions. The HBV subgenotype D3 strain was closely related to a Colombian isolate, forming a monophyletic group and exhibiting a genetic distance from other previously described Brazilian sequences. This result suggests that the presence of Colombian immigrants in the State might have introduced a different HBV strain into the country. HBV subgenotype D3 is more frequently found in the North and Central-West regions of Brazil[23]. Due to the small number of representative sequences for each subgenotype of genotype D found in our study, further studies will be required to confirm these observations.

Genotype F is indigenous to the Americas, therefore is the most prevalent HBV genotype among Amerindians of the Amazon basin and Central and South America[18]. In addition, previous studies have indicated that genotype F is indeed the oldest genotype circulating in the Americas[28]. This genotype is highly prevalent in Venezuela[38], a region inhabited by Amerindian populations, supporting the hypothesis that genotype F is inherent in these groups. Genotype F is classified into four subgenotypes (F1-F4) which are further subdivided into different clades. Subgenotype F1, which splits into F1a and F1b, is highly prevalent in Central America, Alaska and Southeast America; F2 (F2a and F2b) is highly prevalent in Venezuela, and is also found in Brazil (clade F2a only); F3 is present in Central America (Panama) and Northern Latin America (Colombia and Venezuela), and is also present in Brazil according to our results and F4 is present in Bolivia and Argentina[39].

Unlike other Latin American countries, where genotype F prevails, Brazil shows a low countrywide prevalence of HBV/F (13%), suggesting that indigenous people are less influential in introducing HBV into the country[9,23]. This genotype has been frequently reported in the Brazilian Amazon, especially among native populations[9,18,20]. Castilho *et al*.[35] reported a high prevalence of genotype F in three riverside communities of Labrea municipality, Amazonas State, Brazil. Another study conducted in the Brazilian Amazon Basin showed the prevalence of genotype F in 50% of the patients who developed fulminant hepatitis B and died[30]. Nevertheless, these results show a low frequency of genotype F (9/40; 22.5%), as observed in most studies conducted across different Brazilian regions, including the Amazon basin[9,18,21,22]. This low frequency in our study can be probably explained by the lower representation of indigenous subjects in our sample, the high miscegenation in Roraima, and the contact between indigenous and non-indigenous people.

Subgenotype F2a was prevalent where the sequences formed a clade with Venezuelan strains and other previously described Brazilian sequences. This result was corroborated by Mello *et al*.[28] who argued that subgenotype F2a is

prevalent countrywide, being found in all geographic regions of Brazil. In addition, HBV/F2a is found almost exclusively in Brazil and Venezuela, with the exception of one HBV/F2a sequence isolated in Nicaragua. By using a phylogeographical approach, the authors also concluded that this subgenotype probably originated in Venezuela and reached Brazil following a North-to-South viral flow. This corroborates that Roraima is a major point of entry for several pathogens into the country. Furthermore, our data are also similar to previous studies carried out in Brazil showing the same phylogenetic pattern for the circulation of subgenotype F2a[18,21,23,28]. In addition, the analyzed strains did not form a homogeneous clade within the F2a subgenotype, suggesting different moments of virus entry into the State. This hypothesis is supported by the intense influx of people on the Brazilian-Venezuelan border, and especially the current increased Venezuelan immigration in Brazil, mainly towards the border State of Roraima, which might probably spread different HBV/F2a strains in the region.

HBV/F3 was the second most frequent subgenotype within genotype F. This work shows for the first time subgenotype F3 isolates found in Brazil. Subgenotype HBV/F3 is highly prevalent in the general population of Venezuela and Colombia[4,40], and has also been found in Panama[41]. In addition, previous studies have identified the HBV/F3 subgenotype circulating among indigenous peoples of Venezuela (Yukpa, Warao, Piaroa, Yanomami)[40]. The four HBV/F3 isolates in our study were detected among members of indigenous people living in the Brazilian-Venezuelan borders (a region comprising part of Roraima and Amazonas). Our phylogenetic analysis showed that those sequences were clustered in different clades. Two of the sequences formed a monophyletic clade with another strain previously isolated from members of the Yanomami in Venezuela (DQ899148)[40], indicating that these strains probably co-circulate in this population, perhaps due to migration between neighboring communities. Other sequences revealing two distinct clusters have a genetic pattern similar to the previously isolated sequences in Colombia and are likely representative of the general population. A possible explanation for this greater genetic similarity is that these strains may have been introduced in Roraima at the Colombia/Amazonas border, thereby reaching the community and spreading throughout this territory. Since the F3 isolates described in our study come from self-identified indigenous individuals, we can infer that different strains of this subgenotype are likely to co-circulate exclusively in these communities. However, due to the few sequences of subgenotype F3 isolated in our study, further analysis is required to support these conclusions.

As our number of samples is low, we cannot perform more correlation studies,however, we believe on the importance of the genotypic characterization and its relationship with ethnicity, since these data will be able to supply us with a better understanding of the association between health data of the general population and their relationship with genotypes and subgenotypes, especially in this area of the country, which is influenced by its geographical location next to the Caribbean and bordering countries.

CONCLUSIONS

In this study, we performed a molecular characterization of HBV strains derived from HBV-DNA-positive patients living in the Brazilian State of Roraima. The frequency and distribution of the genotypes were similar to those found in different geographical regions of Brazil. We have shown that "Asian-American" HBV/A1 is the main circulating subgenotype. HBV subgenotypes A2, D2, D3, D4, F2a were also detected, and F3 was first recorded in Brazil. These findings underscore the importance of continuous molecular epidemiological surveillance at a local level in order to provide a better understanding of the virus behavior patterns and tools to better target this disease.

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AUTHORS' CONTRIBUTIONS

DDS, FG, designed the study, wrote and revised the manuscript. DDS, CRSS, WPLJ and JAB performed the recruitment of the participants and the collection of blood samples, carried out viral RNA extraction and run the molecular biology experiments. DDS, CRSS, WPLJ, IASN and VCS performed the genomic sequencing. DDS, CRSS, WPLJ, IASN and VCS analyzed the genome sequence. DDS, IASN and WPLJ performed the phylogenetic analysis, bioinformatics and the statistical analyses. FGN revised the manuscript and contributed to the literature

review and discussion. All authors contributed to and have approved the final manuscript.

CONFLICT OF INTERESTS

The authors have declared that no competing interests exist.

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