

# Prevalence of Genotypes and Sub-Genotypes of the Hepatitis B Virus in a Population of the Brazilian Amazon Region (Pará State)

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## Abstract

**Objectives:** The present study identified the prevalent hepatitis B virus (HBV) genotypes and subtypes in patients from the Brazilian state of Pará.

**Materials:** A sample of 40 patients infected with HBV was selected from a special facility in Belém between January, 2007, and December, 2008. Clinical, biochemical, serological, virological and pathological variables were used to classify patients as inactive carriers and those with chronic hepatitis, either with or without cirrhosis. Serological tests were performed using enzyme immunoassays and viral DNA sequences and the viral load were assessed using PCR. The HBV genotypes were identified through the sequencing of the S region.

**Results:** All but two of the patients sequenced were genotype A, of which, 92% were A1 and 8% A2. The other two patients were genotype D. The A1 samples were either subtype ayw1 or adw2, while the A2 and D subjects were adw2 and ayw3, respectively. The prevalence of genotype A was 90.5% in group A and 100% in group B.

**Conclusion:** No association was found between genotype and the clinical outcomes of the HBV infection.

**Keywords:** Genotypes; Hepatitis B; Epidemiology; Brazilian Amazon region

## Introduction

The genome of the hepatitis B virus (HBV) is highly variable, allowing the differentiation of eight genotypes, named A to H [1]. All genotypes except for E, G and H are subdivided into several geographically localized subgenotypes [2]. The subgenotypes B1-B7, C1 and C2 predominate in Asia [3], while C3, C4 and D4 are the most prevalent on the Australian continent. Whereas in northern Europe, the subgenotype A2 predominates, D is the most common genotype in southern Europe and North Africa, while subgenotype A1 and genotype E are the most prevalent in central and southern Africa. In the New World, genotype A predominates in North America, while G and F2 are the most common in Central America, and F2 is the most prevalent among the natives of South America [1,2,4,5].

The study of the molecular epidemiology of HBV in Latin America has produced some interesting results. Campos et al. [4] identified subgenotypes F1 and F2 as the region's oldest, which would have arrived during the original migration of the ancestral Amerindians in the Ice Age. According to these authors, genotypes A and D were introduced later, through the immigration of European settlers, while B and C originated from Southeast Asia. In Brazil, genotypes A, D and F are reported most frequently [6,7], although in some isolated communities, genotype F is by far the most common [8,9].

There is evidence that the hepatitis B genotype influences both the clinical course of the disease [10-12] and the therapeutic response in chronic cases [13]. Hepatitis B may also have a variety of manifestations, ranging from acute to chronically asymptomatic, which may lead to cirrhosis and hepatocellular carcinoma [14]. The present study aimed to identify the genotypes, sub-genotypes and subtypes most prevalent in patients with chronic hepatitis B (CHB) in an urban population of the eastern Amazon, in the Brazilian state of Pará, and verify possible correlations between these types and the clinical symptoms.

## Materials and Methods

### Patient samples

A sample of 40 patients infected by HBV were selected from those being treated at a special facility in Belém, capital of the Brazilian state of Pará, between January and December, 2007. Serological analysis of the biomarkers of HBV infection (HBsAg, anti-HBc IgG and IgM, HBeAg, anti-HBe and anti-HBs) was based on immunoenzymatic assays (Organon Teknika<sup>®</sup>, Netherlands<sup>®</sup>; Abbott, USA<sup>®</sup>; Ortho Clinical Diagnostics, Germany). The viral load was quantified by the hybridization method using a commercial AMPLICOR kit (Roche<sup>®</sup>), with a detection limit of 60 IU/mL.

According to the clinical and laboratorial criteria the subjects were divided into two groups: A (inactive carriers) and B (chronic hepatitis with or without cirrhosis). Patients were classified as having inactive hepatitis when they showed persistently normal levels of transaminases (ALT and AST, for a minimum of one year) associated to negative HBeAg, positive anti-HBe, HBV-DNA lower than 2,000 IU/mL and/or the absence of hepatic lesions (METAVIR classification A<1 and F<1). Chronic hepatitis patients were defined as those with clinical and biochemical laboratory alterations, associated to positive

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or negative HBeAg, HBV-DNA higher than 2,000 IU/mL and hepatic lesions indicating fibrosis (METAVIR classification >2-<4). Hepatic cirrhosis was considered with the signs of hepatic failure and portal hypertension associated to the histopathology examination indicative of fibrosis higher than 4 (according to the METAVIR classification).

All patients were evaluated clinically and subjected to biochemical tests, ultrasound, endoscopy and liver biopsy, depending on blood coagulation levels. Patients also infected with HCV, HDV, and HIV-1 were excluded from the analysis.

### Ethics

The proposal for this study was submitted to the Research Ethics Commission of the Santa Casa de Misericórdia Hospital and was approved under protocol 055/2006.

### Statistical analysis

The prevalence of the HBV infection according to the demographic characteristics, as well as, the correlation between genotypes of HBV with the clinical diagnosis the chronic was assessed using the Chi-square ( $\chi^2$ ), Anova and Hottelrig tests. Statistical analyses were conducted in the BioEstat 5.0v package [15].

### Molecular analysis

The DNA was extracted from whole blood samples according to the manufacturer's instructions (Puregene, Genra Systems, Inc., USA).

A total of 1,200 bps of the target DNA (Pre-S1, Pre-S2 and S) was amplified in two steps. In the first step, the primers PS1-5' CATATTCTTGGGAACAAGA 3' and S2-5' GGGTTTAAATGTATACCCAAAGA 3' were used [16]. In the second step, a semi-nested PCR was used to amplify two internal fragments of 600 bps, using the primers PS1-5' CATATTCTTGGGAACAAGA 3' and PS2-5' GGTCCCAGTCCTCGAGGAG-3' (Pre-S1, Pre-S2 and S), and S2-5' GGGTTTAAATGTATACCCAAAGA 3' and PS4-5' CATCTCAGGCCATGCAGTG 3' (Pre-S2 and S) as previously described [16]. The reactions were performed in a final volume of 50  $\mu$ L containing 500 ng of extracted DNA, 5 pmol of each primer, 200  $\mu$ M of each dNTP, 50 mM KCl, 2.0 mM MgCl<sub>2</sub>, 10 mM Tris-HCl pH 8.3, and 0.5 IU of Taq DNA polymerase. After initial denaturation at 94°C for 5 minutes, each reaction consisted of 35 cycles of 60 seconds at 94°C, 50 seconds at 52°C and 1 min at 72°C, followed by a final extension of 10 minutes at 72°C.

A direct sequencing assay was used according to the protocol of the ABI Prism Dye Terminator Cycle Sequencing Ready Kit (Applied Biosystems, US), and the products of the reactions were loaded into the ABI Prism 377 DNA Sequencer (Applied Biosystems, US).

### Phylogenetic analysis

The phylogenetic analysis was based on the sequences obtained in the present study for the Pre-S1, Pre-S2 and S regions of the HBV (HQ646059–HQ646098), and those of other strains obtained from the GenBank. The sequences were aligned using the BioEdit sequence editor, version 7.0.5 [17] and used to construct a maximum-likelihood tree using the PAUP - Phylogenetic Analysis Using Parsimony - software package, version 4.0 Beta [18]. The statistical reliance of the neighbor-joining tree was evaluated using 2000 bootstrap replicates. The trees were drawn with the TreeView program, version 1.6.6 [19].

### Results

The subjects had a mean age of 42.2  $\pm$  15.9 years, and approximately

two-thirds were male (Table 1). The same proportion was originally from the Belém metropolitan area, while others were from the northeastern, southeastern, and western regions of the country. Clinical manifestations were divided almost equally between groups A (21 subjects, 52.5%) and B (19 subject, 47.5%). The vast majority (38 or 95.0%) of patients presented genotype A, with the remaining two returning genotype D (Figure 1). Most (92.1%) of the genotypes A were A1, the remainder being A2 (7.9%). The A1 subtypes were either ayw1 (51.4%) or adw2 (48.6%), while genotype A2 and D were represented by the adw2 and ayw3 subtypes, respectively (Table 2). Twenty-one of the 40 patients were inactive carriers, while 12 had chronic hepatitis but no cirrhosis, and seven had cirrhosis (Table 2). However, no relationship was found between genotype or subtype and the clinical manifestation of HBV. Similarly, no relationship was found between the viral load (Genotype A1, n=35, 17,616.24  $\pm$  17,691.41 UI/mL; Genotype A2, n=3, 19,438.50  $\pm$  26,249.92 UI/mL; Genotype D, n=2, 103.0  $\pm$  60.81 UI/mL), HBeAg positivity and genotypes (p>0,05).

### Discussion

In the present study in eastern Amazonia, the genotype A was predominant (95%) in a group of patients from an urban area in the Brazilian state of Pará. These results are similar to those of our previous study in the same region [6], where 89.1% of the infected subjects were A, 8.7% F, and 2.2% D, as well as in others Brazilian regions [7]. In

Characteristic	Group A (n=21)	Group B (n=19)	P
<b>Gender</b>			
Female	08 (38.1%)	06 (31%)	0.920*
Male	13 (61.9%)	13 (68.4%)	
Mean Age $\pm$ SD in years	44.3 $\pm$ 16.1	33.8 $\pm$ 15.8	0.381**
<b>Geographic origin (%)</b>			
Metropolitan Region of Belém	85.9	47.4	0.918***
Northeastern Brazil	4.7	10.5	
Southeastern Brazil	4.7	21.1	
Tocantins state/Marajó Island	0.0	10.5	
Western Brazil	4.7	10.5	

\* $\chi^2$ ; \*\*ANOVA; \*\*\*Hottelrig test

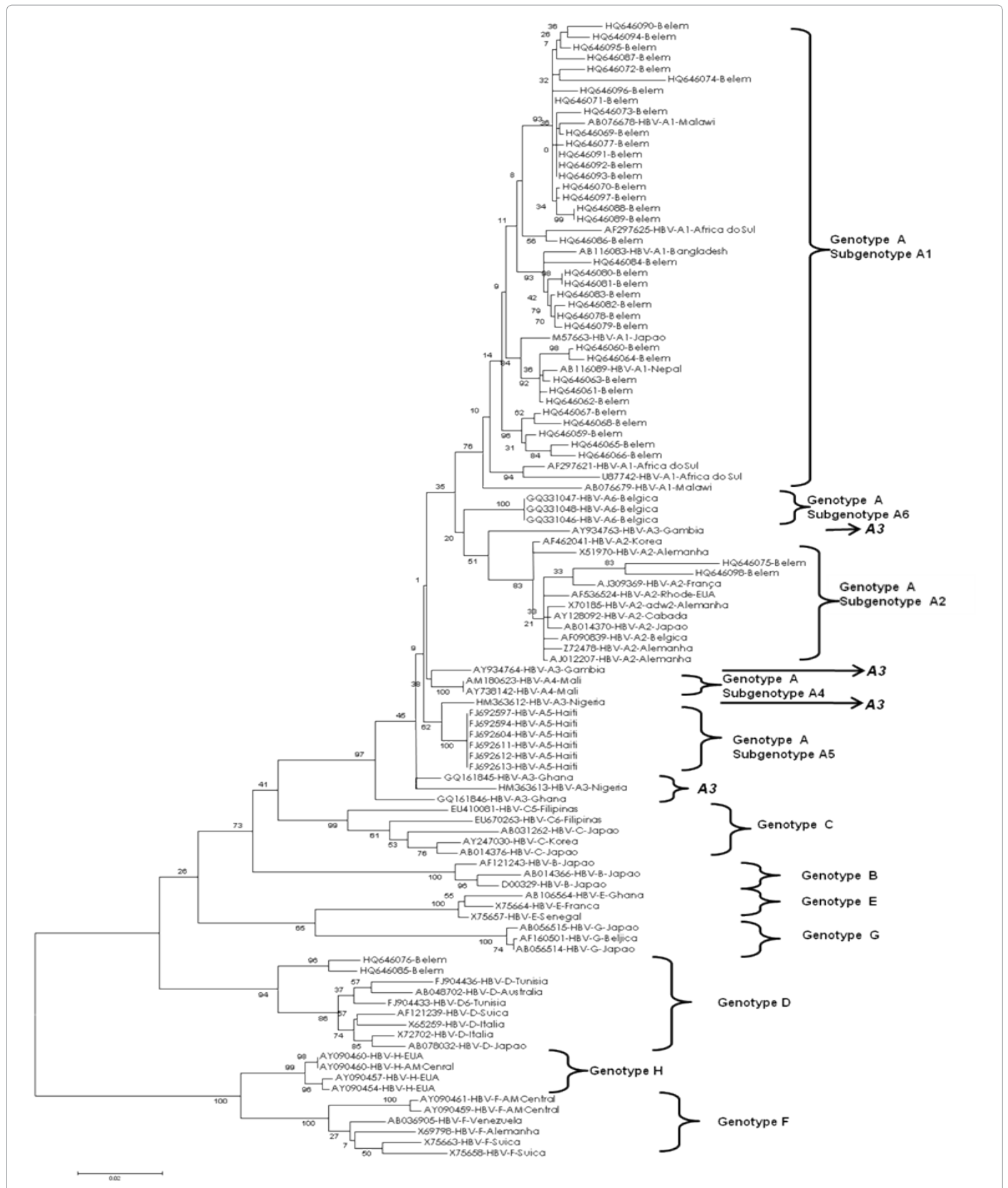
**Table 1:** Demographic characteristics of the chronic HBV patients by diagnostic group (A=inactive carrier; B=chronic hepatitis with or without cirrhosis).

Genetic Variability	Frequency (%)		
	Group A	Group B	Total
<b>Variable</b>			
Genotype*			
A	19 (90.5)	19 (100)	38 (95)
D	2 (9.5)	0	2 (5)
Total	21 (100)	19 (100)	40 (100)
Sub-genotype**			
A1	17 (89.5)	18 (94.7)	35 (92.1)
A2	2 (10.5)	1 (5.3)	3 (7.9)
Total	19 (100)	19 (100)	38 (100)
Subtype***			
ayw1 (genotype A1)	8 (38.1)	10 (52.6)	18 (45.0)
adw2 (genotype A1)	9 (42.9)	8 (42.1)	17 (42.5)
adw2 (genotype A2)	2 (9.5)	1 (5.3)	3 (7.5)
ayw3 (genotype D)	2 (9.5)	0	2 (5.0)
Total	21 (100)	19 (100)	40 (100)

\* $\chi^2$  with Yates' correction: p=0.5133; \*\* $\chi^2$  with Yates' correction: p=0.5475;

\*\*\*Hottelrig test: p=0.3797

**Table 2:** Genotypes, sub-genotypes and subtypes of HBV DNA identified in 40 patients with chronic hepatitis B by diagnostic group.



**Figure 1:** Unrooted phylogenetic tree showing the evolutionary relationships of the hepatitis B virus strains described to date, including the new sequences isolated in the present study (HQ646059–HQ646098). The tree was constructed using the maximum-likelihood approach after alignment of 1200 nucleotides of the Pre-S1, Pre-S2 and S regions. The statistical support refers to 2.000 bootstrap replicates.

western Amazonia, while F increased to 26.5%, genotype A was still the most prevalent, being recorded in 73.5% of cases [8,9].

The genetic makeup of the Brazilian Amazon region conforms to a trihybrid model, with European, Amerindian, and African populations contributing 47%, 41%, and 12% of the gene pool, respectively [20]. This heterogeneity may have contributed to imbalances in the distribution of HBV genotypes and subtypes. The relative predominance of A1 (92.1%) over A2 reinforces the conclusion of Campos et al. [4] with regard to the role of the African slave trade, between the sixteenth and eighteenth centuries, as a route for the transfer of the virus to the continent.

The genotypes A2 and D found in 12.5% of the patients are the most prevalent in Europe [11], which is consistent with the contribution of European populations to the local gene pool [20]. The absence of patients with the genotype F was unexpected, however, given the Amerindian contribution (41%) to the gene pool of the target population, although the small size of the sample and factors such as the founder effect may have been important here. While the patients appeared to be representative of the genetic admixture of the local population, the absence of the genotype F may be the absence of patients from the region's most isolated communities. Given this, more adequate sampling of rural communities may be necessary in order to estimate the local prevalence of this genotype more reliably.

The A (adw2) and D (ayw3) subtypes recorded here were also among the more prevalent in a previous study of the same region [21], although a third subtype - adw4 - was also relatively common. This subtype is related to the genotype F, which was absent from the present study. In a recent study in southeastern Brazil, Tonetto et al. [22] recorded high frequencies of genotypes A (55%) and D (38%), and subtypes adw2 (48.2%) and ayw3 (44.6%).

No association was found between the HBV genotype and the clinical symptoms, primarily because the vast majority of subjects were infected with genotype A. In regions such as Asia, where the genotypes B and C predominate, a number of studies have demonstrated associations with certain types of disease. In the case of the genotype C, in particular C1, for example, an increased tendency for the development of cirrhosis and hepatocellular carcinoma (HCC) has been found for patients of over 50 years of age [23,24]. For the genotype B, higher rates of seroconversion of HBeAg and HCC have been found in young patients [25,26].

In Europe, the genotype D has been associated with higher HBeAg viral loads and a greater prevalence of cirrhosis in comparison with A [27,28]. In a previous study in Pará, however, no relationship was found between HBV genotype and clinical symptoms, although the results may have been influenced by the predominance of A - 89.1% [6]. Additional research with a larger sample of patients will be required for a more conclusive assessment of the possible association between the genotype A and the therapeutic response in hepatitis patients.

## Conclusion

In the present study most of the patients were infected by genotype A, of which A1 and A2 sub-genotypes were reported. Two patients were genotype D, but no association was found between genotype and the clinical outcomes of the HBV infection.

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