



## Article/Artigo

# Hepatitis B virus infection in children, adolescents, and their relatives: genotype distribution and precore and core gene mutations

Infecção pelo vírus da hepatite B em crianças, adolescentes e seus familiares: distribuição dos genótipos e mutações no gene *pré-core* e *core*

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### ABSTRACT

**Introduction:** The objectives of this study were evaluate hepatitis B virus (HBV) serological markers in children and adolescents followed up at the Child Institute of the *Hospital das Clínicas, Faculdade de Medicina de São Paulo, Universidade de São Paulo*; identify chronic HBV carriers and susceptible individuals in the intrafamilial environment; characterize HBV genotypes; and identify mutations in the patients and household contacts. **Methods:** Ninety-five hepatitis B surface antigen-positive children aged <19 years and 118 household contacts were enrolled in this study. Commercial kits were used for the detection of serological markers, and PCR was used for genotyping. **Results:** Hepatitis B e antigen (HBeAg) was detected in 66.3% (63/95) of cases. Three of the 30 HBeAg-negative and anti-HBeAg-positive patients presented with precore mutations and 11 presented with mutations in the basal core promoter (BCP). Genotype A was identified in 39 (43.8%) patients, genotype D in 45 (50.6%), and genotype C in 5 (5.6%). Of the 118 relatives, 40 were chronic HBV carriers, 52 presented with the anti-HBc marker, 19 were vaccinated, and 7 were susceptible. Among the relatives, genotypes A, D, and C were the most frequent. One parent presented with a precore mutation and 4 presented with BCP mutations. **Conclusions:** Genotypes A and D were the most frequent among children, adolescents, and their relatives. The high prevalence of HBV in the families showed the possibility of its intrafamilial transmission.

**Keywords:** Hepatitis B. Genotype. Mutation. Vertical transmission. Horizontal transmission.

### RESUMO

**Introdução:** Os objetivos deste estudo foram: avaliar os marcadores sorológicos nas crianças e adolescentes acompanhadas no Instituto da Criança do Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP); identificar portadores crônicos do VHB e indivíduos suscetíveis no ambiente intrafamiliar; caracterizar o genótipo do VHB; observar a presença de cepas mutantes entre os pacientes e familiares estudados. **Métodos:** Noventa e cinco crianças e adolescentes positivas para o antígeno de superfície do vírus da Hepatite B (AgHBs), menores de 19 anos, e 118 familiares foram envolvidos neste estudo. Foram utilizados kits comerciais para a pesquisa dos marcadores sorológicos e a PCR foi utilizada para genotipagem. **Resultados:** O antígeno e do vírus da hepatite B (AgHBe) foi detectado em 66,3% (63/95) dos casos. Três dos 30 pacientes AgHBe negativo e anti-HBe positivo apresentaram mutação na região *pré-core* e 11 na região BCP. Em 39 (43,8%) pacientes, foi identificado o genótipo A, 45 (50,6%) o genótipo D e cinco (5,6%) o genótipo C. Dos 118 familiares estudados, 40 eram portadores crônicos do VHB, 52 tinham marcador sorológico de contato prévio e sete eram suscetíveis. Dentre os familiares AgHBs positivos, os genótipos A, D e C foram os mais frequentes. Um familiar apresentou mutação na região *pré-core* e quatro apresentaram mutação na região do BCP. **Conclusões:** Os genótipos A e D foram os mais frequentes dentre as crianças adolescentes e seus familiares. Alta frequência do VHB nos familiares mostrou a possibilidade de transmissão intrafamiliar.

**Palavras-chaves:** Hepatite B. Genótipo. Mutação. Transmissão vertical. Transmissão horizontal.

### INTRODUCTION

Hepatitis B continues to be an important public health problem worldwide and more than 350 million people are estimated to be chronic carriers, with the occurrence of 1 million deaths yearly<sup>1</sup>.

Parenteral and sexual exposure seem to be the major routes of viral dissemination, and vertical transmission may occur from chronic hepatitis B virus (HBV)-carrier mothers to their children either in utero or around the time of delivery. The risk of mother-to-infant transmission is 85-90% if the mother is positive for the hepatitis B surface antigen (HBsAg) and hepatitis B e antigen (HBeAg), but infection is still possible even if the mother is HBeAg-negative<sup>2</sup>. Children who are not infected at birth are still at risk from infected households and community contacts, especially in subpopulations in which HBV infection is prevalent<sup>2</sup>. Horizontal transmission has been reported in regions of the world where the prevalence of hepatitis B infection is high, e.g., Southeast Asia, Middle East, and sub-Saharan Africa<sup>2,3</sup>.

The genetic variability of HBV is based on variations in the sequence of protein S. Okamoto et al. proposed a classification system for the complete viral sequence, and HBV is currently classified into 8 genotypes (A-H)<sup>4</sup>. Genotypes A, B, C, D, and F have already been described in Brazil<sup>5</sup>. Mutations inhibiting the synthesis of HBeAg (i.e., in the precore and core regions) reportedly aggravate liver disease and cause fulminant hepatitis in children and adults<sup>6</sup>.

The objectives of this study were: I) evaluate HBV serological markers in children and adolescents followed up at the Child Institute of the *Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo (HC-FMUSP)*; II) identify chronic carriers of HBV and susceptible individuals in the intrafamilial environment; III) characterize HBV genotypes; and IV) identify mutations in the patients and household contacts.

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## METHODS

### Population

The study population consisted of children and adolescents aged <19 years who were seen at the Hepatology Unit of the Child Institute, HC-FMUSP, and who were HBsAg-positive for >6 months. In addition, the household contacts of these patients were invited to participate in this study. Blood samples were collected from the patients and their relatives between 1999 and 2004 after signing an informed consent form.

### Serological assays

Serological markers (HBsAg and total anti-HBc) were identified using a commercially available enzyme-linked immunosorbent assay (ELISA) (Diasorin™; Saluggia Mark, Vercelli, Italy).

### Polymerase chain reaction

A nested polymerase chain reaction (PCR) was carried out as described by Kaneko et al., with some modifications<sup>7-9</sup>. The S region was amplified according to the protocol reported by Sitnik et al. for the identification of the HBV genotype<sup>10</sup>.

### Sequencing reaction

For the characterization of viral strains, the samples were sequenced using PCR according to the method of Sanger et al., by using the ABI Prism BigDye™ Terminator Kit (PE Applied Biosystems, Foster City, CA, USA) in an automatic ABI Prism 377 sequencer<sup>11</sup>. Genotyping and basal core promoter (BCP) and precore mutations were analyzed by comparison with the sequences of different HBV genotypes deposited in GenBank, by using the EditSeq and MegAlign programs of the DNASTar package (LaserGene, Inc.).

### Viral load

Viral load was quantified in all HBsAg- and HBeAg-positive samples, employing the COBAS AMPLICOR HBV MONITOR test (Roche Molecular Systems, Inc., Branchburg, NJ, USA). Samples with a viral load higher than the detection limit were diluted in human HBV-negative plasma.

### Statistical analysis

For statistical analysis, the Kruskal-Wallis test was used. A p-value < 0.05 was considered significant.

### Ethical considerations

This project was approved by the Committees Scientific of the Division of Medical Biology (BM (04/04), for Ethics in Research at the Instituto Adolfo Lutz (CEPIAL) and Ethics of the Child Institute, HC-FMUSP, according to resolution no. 196/96 on research involving human beings published by the National Council of the Brazilian Ministry of Health (1996). It is noteworthy that all samples were collected only after the completion of the Free and Informed Consent form.

## RESULTS

### Serology

Ninety-five HBsAg-positive children and adolescents were enrolled in this study; 53 (55.8%) were male and 42 (44.2%) were female. Serological analysis showed that 63 (66.3%) patients were HBeAg-positive and 30 (93.7%) of the 32 negative samples were positive for anti-HBe (Table 1).

The relatives of 85 of the patients enrolled in this study could be contacted, for a total of 118 individuals. These serological data are shown in Table 2.

TABLE 1 - Frequency of hepatitis B virus serological markers in children and adolescents seen at the Child Institute, Hospital das Clínicas, Faculdade de Medicina da Universidade de São Paulo, according to age.

Age (years)	n	%	Male		Female		Anti-HBc (total)		HBeAgB		Anti-HBe	
			n	%	n	%	n	%	n	%	n	%*
0-4	23	24.2	10	13	23	100.0	16	69.5	7	30.4		
5-9	40	42.1	27	13	38	95.0	26	65.0	14	35.0		
10-14	26	27.4	14	12	26	100.0	18	69.2	6	23.0		
15-19	6	6.3	2	4	6	100.0	3	50.0	3	50.0		
<b>Total</b>	<b>95</b>	<b>100.0</b>	<b>53</b>	<b>55.8</b>	<b>42</b>	<b>44.2</b>	<b>93</b>	<b>97.9</b>	<b>63</b>	<b>66.3</b>	<b>30</b>	<b>93.7</b>

Anti-HBc total: total antibodies against the hepatitis B core antigen; HBeAg: hepatitis B e antigen; anti-HBe: antibodies against the hepatitis B e antigen. \*The presence of anti-HBe was only determined in HBeAg-negative samples.

TABLE 2 - Frequency in relatives according to the degree of kinship and serological markers.

Relationship	N	Serological markers									
		HBsAg		Anti-HBc (total)		HBeAg		Anti-HBe		Anti-HBs	
		n	%	n	%	n	%	n	%	n	%
Mother	58	30	51.7	52	89.7	13	22.4	16	29.3	22	37.9
Father	28	7	25.0	22	78.6	2	7.1	4	14.3	15	53.6
Sibling	25	1	4.0	13	52.0	0	0.0	0	0.0	13	52.0
Others	7	2	28.5	3	42.8	0	0.0	1	14.3	2	28.5
<b>Total</b>	<b>118</b>	<b>40</b>	<b>33.9</b>	<b>90</b>	<b>76.3</b>	<b>15</b>	<b>12.7</b>	<b>21</b>	<b>17.8</b>	<b>52</b>	<b>44.1</b>

HBsAg: hepatitis B surface antigen; anti-HBc total: total antibodies against the hepatitis B core antigen; HBeAg: hepatitis B e antigen; anti-HBe: antibodies against the hepatitis B e antigen; anti-HBs: antibodies against the hepatitis B surface antigen; N: number of individuals.

### Genotypes

The S region could be amplified in samples from 89 (93.7%) patients. Genotype A was identified in 39 (43.8%) patients, genotype D in 45 (50.6%), and genotype C in 5 (5.6%). Genotype D showed a significantly higher viral load in children ( $6.09 \times 10^7$  IU/mL;  $p = 0.01$ ).

The S region of the HBV genome could be amplified and genotyped in 27 samples from HBsAg-positive relatives. The distribution of the genotypes was as follows: genotype A, 13 (48.1%) samples; genotype D, 12 (44.4%); and genotype C, 2 (7.5%). Table 3 shows the genotypes of the patients and their relatives.

**TABLE 3 - Frequency in children, adolescents, and relatives according to viral load.**

Viral load (IU/mL)	Children and adolescents		Relatives		Total	
	n	%	n	%	n	%
	<60	6	6.7	1	3.8	7
60-10 <sup>2</sup>	4	4.5	3	11.1	7	6.0
10 <sup>2</sup> -10 <sup>4</sup>	18	20.2	7	25.9	25	21.5
10 <sup>4</sup> -10 <sup>6</sup>	3	3.4	5	18.5	8	7.0
10 <sup>6</sup> -10 <sup>8</sup>	19	21.4	5	18.5	24	20.7
>10 <sup>8</sup>	39	43.8	6	22.2	45	38.8
<b>Total</b>	<b>89</b>	<b>100.0</b>	<b>27</b>	<b>100.0</b>	<b>116</b>	<b>100.0</b>

### Mutations

Among the 95 children and adolescents studied, 30 were HBsAg/anti-HBe-positive and HBeAg-negative, and the precore and core regions could be amplified in 21 (70%). Of these, 13 patients harbored mutations, while 8 did not. Among the 21 relatives who participated in the study and had compatible profiles for the investigation of core and precore mutations, the samples of 9 individuals could be amplified and 4 (44.4%) harbored mutations.

The mean viral load was significantly higher among HBeAg-positive individuals ( $5.56 \times 10^7$  IU/mL) compared with HBeAg-negative individuals ( $1.51 \times 10^5$  IU/mL;  $p \leq 0.00001$ ). No significant difference was found between the distribution of the 2 genotypes and viral load ( $p = 0.0177$ ).

## DISCUSSION

Chronic hepatitis B infection is a global health problem, and transmission from mother to infant is a major route of acquisition throughout the world. Infections acquired in childhood are responsible for the largest majority of chronic HBV infections, with its attendant complications of cirrhosis and hepatocellular carcinoma<sup>2</sup>.

Some studies have demonstrated the relationship between HBV genotypes and the clinical course of the infection; however, these data are still scarce and inconsistent, and the number of eligible patients is still small, especially with respect to childhood data. Therefore, the present study was of extreme importance since it was possible to analyze a large number of serum samples from children and adolescents who were chronic hepatitis B carriers. In studies conducted on children from Argentina and Belgium, the number of patients was ~25<sup>12,13</sup>. In addition, it was possible to investigate most of the household contacts of the patients, and we were only unable to contact the relatives of 10 patients. Analysis of the frequency of

HBsAg-positive children and adolescents according to age showed that most children with chronic HBV infection were aged 0-14 years, and only 6.3% of the population studied were adolescents. This fact might be related to the characteristics of the health service in which the study was carried out, which mainly cares for children.

Among the 118 familial contacts studied, 58 were mothers and 30 of them were chronic carriers of HBV. This finding indicates the importance of serological screening for HBV during pregnancy, since this route of transmission is associated with a high (90%) risk of chronic HBV infection and a low incidence of acute symptomatic hepatitis. Moreover, 13 of these 58 mothers were HBeAg-positive. This is an extremely relevant finding since the risk of vertical transmission increases with increasing maternal viral load, when the levels of HBsAg are high, and in the presence of HBeAg<sup>2</sup>.

Twenty-two fathers were found to be anti-HBc total-positive, and these individuals might be responsible for the intrafamilial transmission of the virus. The same may apply to the other 32 relatives, with 16 (50%) of them presenting with this marker, supporting the hypothesis for the intrafamilial dissemination of HBV.

Low vaccine coverage was observed among the relatives of chronic HBV carriers (19/118). This finding is a matter of concern since, despite the availability of the vaccine within the public health network for all children at birth, vaccine coverage is still low for adolescents and patients who are at an increased risk, especially among adolescents<sup>14</sup>. Thus, adolescent vaccination should be encouraged since the vaccine is the most important and efficient tool for the prevention of hepatitis B, especially for adolescents at a high risk of infection.

The present results are similar to those reported in the few studies published in Brazil, i.e., genotypes A and D were the most frequent among adolescents. Genotype C was detected in families of Asian origin, in agreement with studies showing the occurrence of genotype C in Asian countries<sup>15,16</sup>. Sitnik et al., studying the prevalence of HBV genotypes in chronic carriers from different regions of Brazil, also found genotype C in individuals of Asian origin<sup>5,17</sup>.

Our results agree with other Brazilian studies<sup>18-22</sup> showing that genotypes A and D were the most frequent in the general population. It is interesting to observe that viral load was higher in genotype D carriers than in the other carriers. This increases the possibility of the transmission of this strain, especially in countries with a high prevalence of this genotype, like Brazil.

A genotype discrepancy between individuals of 2 families was observed. In family 1, the patients had genotype A, whereas the father had genotype D. However, it was not possible to amplify the S region from the mother's sample, which could have elucidated this difference. In family 2, divergence between the patient's and mother's genotypes could not be elucidated using only serological evidence since the father had been vaccinated. In this case, both the mother and the child could have been infected by different sources at different times.

Only 2 patients had the G1896A mutation and 1 patient had mutations in the precore and core regions (G1899A) as well as in the BCP, all of which were characterized as genotype D. The same genotype was observed in a relative who harbored mutations in the precore and core regions.

In the present study, a high percentage of precore and core mutations were observed in genotype D strains. The low prevalence of mutations in genotype A strains was expected, as reported by other

investigators<sup>5,19,23</sup>. Mutations are more frequent in the BCP region than in the precore and core regions. This might be related to the fact that BCP mutations are associated with genotypes that present with a cytosine at position 1858, as is the case for genotype A<sup>24</sup>.

Our study also showed that HBeAg-positive individuals presented with the highest viral load. The determination of viral load, together with HBeAg detection, could also contribute to the choice of drugs and length of hepatitis B treatment. These data agree with those reported by other authors<sup>22</sup>.

A low frequency of precore and core mutations were observed in the population studied due to the high frequency of genotype A. The rate of susceptible individuals was still high, confirming the need for preventive and control measures for HBV infection, especially for individuals sharing the same intrafamilial environment in view of the high propagation rate of this virus.

## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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