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# CHARACTERIZATION OF A HEPATITIS B VIRUS STRAIN IN SOUTHWESTERN PARANÁ, BRAZIL, PRESENTING MUTATIONS PREVIOUSLY ASSOCIATED WITH ANTI-HBs RESISTANCE

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## SUMMARY

The present study investigated if hepatitis B virus (HBV) mutants circulate in the southwestern region of the State of Paraná, Brazil, by analyzing samples from children who received immunoprophylaxis but were born to HBV carrier mothers. Samples from 25 children were screened for HBV serum markers and for HBV DNA by PCR. Only one sample was positive for HBsAg, anti-HBs and HBV DNA, although the child had been vaccinated. Analysis of the S gene sequence of this sample showed the presence of a proline at position 105, a serine at position 114, three threonines at positions 115, 116 and 140, and a glutamine at position 129. The presence of these amino acids, except for serine at position 114, has been related to monoclonal or polyclonal therapy with anti-HBs after liver transplantation, whereas the presence of threonine at position 116 has been described in immunized children from Singapore. This finding demonstrates the possible circulation of HBV strains resistant to hepatitis B immunoprophylaxis in southwestern Paraná, Brazil. The genotype of the sample was identified as genotype D, which is frequently found in the region studied. Since 36% of the children had received incomplete or no immunoprophylaxis, more extensive follow-up of children born to HBsAg-positive mothers is needed.

**KEYWORDS**: Hepatitis B; HBsAg, Vaccine; Hyperimmune gammaglobulin; Mutations.

## INTRODUCTION

Hepatitis B virus (HBV) belongs to the genus *Orthohepadnavirus*, family Hepadnaviridae. HBV infection is one of the major global public health problems. Among the approximately two billion people infected worldwide, more than 350 million are chronic HBV carriers[33]. Approximately 400,000 new cases are estimated to occur in Latin America each year[12].

HBV is characterized by its wide genetic variability. These genetic differences have led to the classification of the HBV into eight genotypes (A-H), which are divided into subgenotypes with distinct virological and epidemiological properties[26]. Few genotype data are available in Brazil, but it is known that A, D and F are the main genotypes circulating in the country. MELLO *et al.* (2007) identified genotypes A, D and F among 303 HBV isolates which were obtained from all geographic regions of Brazil, genotype D being the most prevalent in the south region (84.2%). SITNIK *et al.* (2004), studying 103 samples from patients with chronic hepatitis B originating from different regions of Brazil, found genotypes A (49.5%), B (2.9%), C (13.6%), D (24.3%), and F (9.7%). In Paraná, genotypes A (14.0%), C (1.3%), D (82.9%), F (1.3%) and H (0.4%) were identified in samples from HBsAg-positive blood donors (BERTOLINI *et al.,* unpublished data).

Hepatitis B is an important disease in the State of Paraná, Brazil[9,10,28]. An intermediate prevalence has been recently observed in Cascavel, Foz do Iguaçu and Francisco Beltrão[3]. Vaccination of risk groups started in 1991 but had no impact on infection control. Children under one year of age were vaccinated in 1995, when the hepatitis B vaccine was first introduced in the National Immunization Program throughout Brazil. Whereas in 1998 the vaccine was applied to children under 15 years of age only in the southwestern region of the State of Paraná, vaccination was extended to all states in 1999. In 2001, the age range comprised individuals under 21 years of age[18]. The measures mentioned above led to a rapid decline in the incidence of hepatitis B in the under-15-year-old population. Preliminary results show that vaccination strategies are effective in the control of hepatitis B in a certain age group[18].

The exposure of the host's immune system to viral surface antigen (HBsAg) during HBV infection provides an important basis for the protective response against the virus. However, HBsAg exposure, together with the fact that HBV has a mutation rate ten times higher than that of other DNA viruses[15], favors the emergence of mutations in the surface (S) gene. Data regarding the primary structure of HBsAg in different regions of the world are important for the understanding of failure of protection with current vaccines[21].

Point mutations in the S gene that result in amino acid substitutions in the common "a" antigenic determinant, which lies between amino acids 124 and 147, can alter B-cell epitopes of HBsAg, leading

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to variants that escape from the host immune response elicited by vaccination or previous infection[16]. WALLACE & CARMAN (1997) suggested increasing the area of investigation in the "S" region to include the entire major hydrophilic region (MHR) which comprises amino acids 99 to 169. The authors proposed the following system for the classification of mutations into five regions: HBs1, region upstream of amino acid 120; HBs2, region comprising amino acids 120 to 123; HBs3, region comprising amino acids 124 to 137; HBs4, region comprising amino acids 138 to 147, and HBs5, region comprising amino acids 148 to 169.

The most common HBsAg variant associated with resistance to vaccine-induced anti-HBs is a glycine-to-arginine substitution at amino acid 145 of the MHR of protein S[4,24,31]. Vaccine-associated variants have been demonstrated in Italy[4], the United Kingdom[20], the United States[19], Singapore[13], Taiwan[16,22], Senegal[11], India[29], Indonesia[5], and Japan[24]. The following vaccine-associated HBsAg mutations were identified: I110V, T/I126N, Q129H, M133L, K141E, P142S, D144A, G145R, and C149R[6,7,32]. OON *et al.* (1999) detected various previously unknown mutations outside the region of the "a" determinant in immunized children from Singapore (N116T, V118A, A159V, F183C, and V184A). Recently, ARAÚJO *et al.* (2008) reported the presence of a single amino acid substitution, L215Q, in genotype F which may affect the conformation of HBsAg, possibly causing defective insertion in the endoplasmic reticulum membrane and intracellular retention.

Vaccine-induced escape mutants of the HBV S gene pose a high risk to society since the use of hepatitis B immunoglobulins (HBIG) and vaccines are not effective in preventing infection. Blood units containing HBV mutants are not detected by routine screening for HBsAg and may transmit HBV infection to recipients of blood transfusions. Since these mutants may actively replicate even in the presence of a powerful anti-HBc response in the host, the exclusion of blood units with high anti-HBc titers is expected to be effective in the prevention of their transmission by transfusion[31].

Preliminary studies have detected mutations associated with vaccine resistance in HBV strains isolated from blood donors from the southeastern region of the State of Paraná[2,3]. This fact emphasizes the urgent need to determine the presence of HBV mutants in children. Progression of the disease in children has serious consequences since it maintains infection within the population.

Therefore, the objective of the present study was to investigate if HBV mutants associated with resistance to vaccine and HBIG circulate in the southwestern region of the State of Paraná.

## PATIENTS AND METHODS

**Patients**: A total of 123 children from the 8th Regional Health Division of the municipality of Francisco Beltrão were studied. These children were tested for HBIG between 1998 and 2004, but only 25 were located for the collection of biological material.

A blood sample (5-10 mL) was collected from each of the 25 children with disposable syringes under aseptic conditions. After serum separation the samples were divided into two tubes and stored in a freezer at -20 ºC until the time of use.

The study was approved by the Ethics Committee on Human Research of the State University of Maringá, and written informed consent was obtained from all mothers.

**Serology for HBV:** Serum HBV markers (HBsAg, total anti-HBc, anti-HBc IgM and anti-HBs) were analyzed by microparticle immunoassay using commercial kits (Abbott Laboratories, Abbott Park, Illinois, USA) in an AXSYM device (Abbott Laboratories) according to manufacturer specifications.

**Amplification of the HBV S gene region by the Polymerase Chain Reaction (PCR):** All samples were submitted to PCR amplification of the HBV S gene region, i.e., the region of the viral genome containing the MHR, thus permitting the identification of mutations that confer resistance to vaccine and HBIG.

HBV DNA was extracted from 100 µL serum in a 300 µL guanidine isothiocyanate solution. Two primer sets were used for nested PCR: FHBS1 (5' GAG TCT AGA CTC GTG GTG GAC TTC 3') and RHBS1 (5' AAA TKG CAC TAG TAA ACT GAG CCA 3') for the first reaction, and FHBS2 (5' CGT GGT GGA CTT CTC TCA ATT TTC 3') and RHBS2 (5' GCC ARG AGA AAC GGR CTG AGG CCC 3') for the second reaction. The primers occupy the following positions in the HBV genome (strain HBVADW; GenBank accession number V00866): HBS1F (positions 244 to 267), HBS2F (positions 255 to 278), HBS2R (positions 648 to 671), and HBS1R (positions 668 to 691). The two reactions were carried out in a thermocycler (TPersonal Thermocycler, Biometra) programmed as follows: one cycle at 94 ºC for 30 s for initial denaturation, 35 cycles at 94 ºC for 20 s (denaturation), 56 ºC for 20 s (annealing), and 72 ºC for 30 s (extension), and one cycle at 72 ºC for five min. The samples were stored at 4 ºC for an indeterminate period of time[27].

After amplification in duplicate, the nested PCR product was identified by electrophoresis on 1.0% agar gel stained with ethidium bromide and photographed with a Polaroid camera. Samples were classified as positive when they presented a band with a molecular weight close to 450 bp.

**Sequencing of the S gene region**: The nested PCR products were sequenced in duplicate using dideoxyribonucleotide triphosphates (ddNTPs) labeled with fluorescent markers. Sequencing reactions were performed using the ABI Prism BigDye™ Terminator Cycle Sequencing kit (PE Applied Biosystems, Foster City, CA) in an automatic ABI Prism 377 sequencer (PE Applied Biosystems). Sequences were analyzed in both directions (forward and reverse) using primers FHBS2 and RHBS2.

**HBV genotyping:** The EditSeq and Megalign programs of the DNAstar package (Lasergene Inc., USA) were used for the analysis, correction and alignment of nucleotide and amino acid sequences. The accession number of the strain in GenBank is FJ613840.

The genotype was classified by alignment of the nucleotide sequence of the S gene with 53 already known sequences of 8 HBV genotypes (A-H), deposited at DDBJ/EMBL/GenBank (Table 1). Sequences were aligned using the CLUSTAL method[14] and the PAM250 weight residue table.

Identification of mutations in the S gene region causing resistance

**TABLE 1**
Partial S gene sequences used for alignment to distinguish between the different HBV genotypes

| Genotype | Sequence (GenBank accession number) |
|---|---|
| A | X75666, X75669, X65258, M54898, J02205, X51970, and M74498 |
| B | X75660, D23677, D23678, D00329, D00330, D00331, M54923, S74815, and S74867 |
| C | X75667, X75665, X75792, X75656, X01587, D23680, D23682, D23681, D16665, S62754, S75184, S81945, S81946, and U19777 |
| D | X75668, X75662, M32138, X77309, X65259, X59795, X68292, X77308, J02202, V01460, X02496, and X77310 |
| E | X75657, X75664, and L24071 |
| F | X75658, X75661, and X69798 |
| G | AF369533 and AF160501 |
| H | U91819, U91827, and AY090460 |

to vaccine and HBIG: Mutations were identified and compared with mutations described in the literature as resistant to vaccine and to HBIG.

## RESULTS

Of the 25 children born to HBsAg-positive mothers, five (20%) were from the municipality of Capanema, four (16%) were from Cascavel, five (20%) from Francisco Beltrão, three (12%) from Salto do Lontra, two (8%) from Santo Antônio do Sudoeste, one (4%) from Verê, three (12%) from Dois Vizinhos, and two (8%) from Enéas Marques. Fifteen (60%) children were female and 10 (40%) were male. Although the municipality of Cascavel does not belong to the 8th Regional Health Division, the 10th Regional Health Division participated voluntarily in the present study. The mean age of the children was 2.4 years.

Based on medical chart seven (28%) children had received vaccine only, two (8%) had received HBIG, only 15 (60%) had received the two types of immunoprophylaxis, and one child (4%) had not received any immunoprophylaxis. Thus, nine (36%) children received incomplete immunoprophylaxis and one child (4%) did not receive any immunoprophylaxis, although HBIG is requested at the 8th Regional Health Division.

Only one (5.3%) sample was simultaneously positive for HBsAg and anti-HBs. Another sample (5.3%) was not positive for any serum marker, although this child had been vaccinated. Three (15.8%) children who were positive for total anti-HBc and anti-HBs probably contracted HBV during intrauterine life and the infection was resolved.

All 25 children (100%) were investigated for the presence of HBV DNA by PCR and only one sample (4%) was positive (sample ALMLHBS), corresponding to the sample positive for HBsAg and anti-HBs. This child was born on May 31, 2004 and was vaccinated on June 1, 2004. The blood sample was collected on August 9, 2006. The PCR product of this sample was sequenced and genotyped. Analysis of mutations that might interfere with vaccine and HBIG immunogenicity showed the presence of a proline (P) at position 105, a serine (S) at position 114, three threonines (T) at positions 115, 116 and 140, and a glutamine (Q) at position 129 (Fig. 1). Substitution of glycine (G) with arginine (R) at position 145 (G145R) in the MHR of protein S, the most common HBsAg variant associated with resistance to vaccine-induced anti-HBs, was not found.

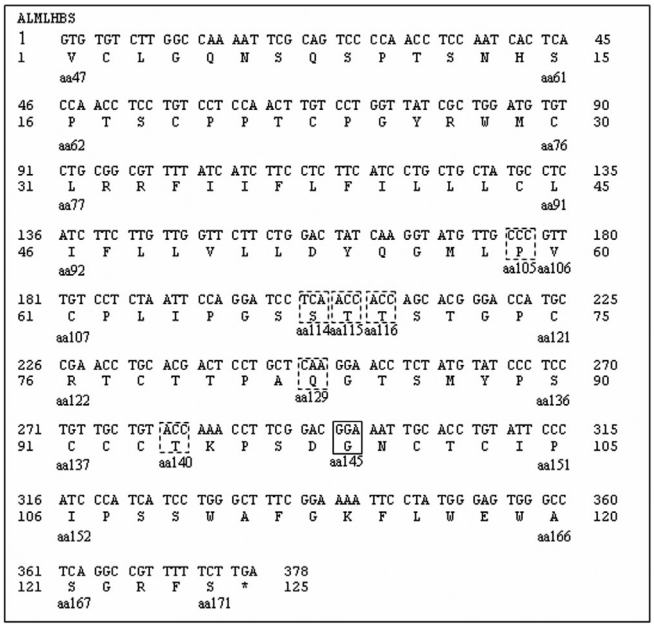


**Fig. 1 -** Nucleotide (378 bp) and amino acid sequence (125 aa) of the S gene region of a sample obtained from a PCR HBV-positive child from the municipality of Salto do Lontra, Paraná, Brazil, showing the positions of possible mutations associated with resistance to vaccine and HBIG (ALMLHBS - sample; box - position of aa 145; dotted box - mutations found).

## DISCUSSION

Although the amino acids found at positions 115 (T), 116 (T) and 129 (Q) are considered to be conserved residues and amino acids located at positions 105 (P/HAS) and 140 (T/S) are non-conserved residues (SCHAEFER *et al*. 2005), the presence of a P at position 105, three T's at positions 115, 116 and 140 and a Q at position 129 observed in the present study have been associated with monoclonal or polyclonal therapy with anti-HBs after liver transplantation, with these mutations tending to accumulate within and around the HBs2 and HBs4 regions[6,7,8,25,32]. Although mutation T114R has also been associated with monoclonal or polyclonal anti-HBs therapy after liver transplantation, a serine (S) was found at this position, which is a neutral polar amino acid like threonine (T). However, this finding does not imply tertiary structure alterations causing resistance to vaccine and HBIG. The presence of a T at position 116 has been identified in immunized children from Singapore[23].

The amino acid at position 140 (T/S) contributes to the classification of genotypes E and F, a fact that does not impair the analysis of the present results since genotype D was identified.

The isolate was classified as genotype D by alignment of its amino acid sequence with 53 known sequences from 8 HBV genotypes (A-H)

deposited in GenBank . Genotype D is the most prevalent in the southern region of Brazil[17,27] and in the State of Paraná[2].

The Technical Committee of the National Program of Immunization, National Health Foundation, Brazilian Ministry of Health[30], has suspended the supply of HBIG to children born to HBsAg-positive mothers between July 2000 and October 2002. It was reported that the vaccine itself would provide the same protection. However, the nine (36%) children who received incomplete or no immunoprophylaxis were not born during this period.

Positivity for HBsAg and anti-HBs serum markers might be observed when the patient is in the resolution phase of the infection. The child with this serum marker profile was born on May 31, 2004 and only received the hepatitis B vaccine on June 1, 2004, a fact that might have contributed to infection. The child may have been infected during the period of sample collection for the present study. When HBV positivity was confirmed by analysis of serum markers and PCR, the Epidemiological Service of the municipality of Salto do Lontra was notified to guarantee medical follow-up.

One child from the municipality of Cascavel, born on November 9, 1997, was not positive for any of the serum markers analyzed, although the child had been vaccinated on February 3, 1998. One child (5.3%) was positive for hepatitis B by analysis of serum markers and PCR. The presence of amino acids P, T, T, Q and T at positions 105, 115, 116, 129 and 140, respectively, may indicate the possibility of resistance to vaccine and HBIG. This fact shows that immunoprophylaxis-resistant HBV strains may circulate in the southwestern region of the State of Paraná, Brazil,

Most children (n = 12, 63.2%) received complete hepatitis B immunoprophylaxis, including vaccine and HBIG. However, nine (36%) children received incomplete or no immunoprophylaxis, although they were born during a period other than that from July 2000 to October 2002, a fact that may have exposed them to the HBV infection. Immunoprophylaxis of these children should be better controlled by health authorities to prevent the consequences of the disease and transmission of the infection.

## RESUMO

### Caracterização de uma cepa de hepatite por vírus B no sudoeste do Paraná, Brasil, apresentando mutações previamente associadas à resistência anti-HBs

O presente estudo investigou se mutantes do vírus da hepatite B (HBV) circulam na região Sudoeste do Estado do Paraná, Brasil, analisando amostras de crianças que receberam a imunoprofilaxia por terem nascido de mães portadoras do HBV. Amostras de 25 crianças foram analisadas para os marcadores sorológicos do HBV e para o DNA-HBV por PCR. Somente uma amostra foi positiva para AgHBs, anti-HBs e DNA-HBV, apesar da criança ter sido vacinada. Análises da seqüência do gene S desta amostra mostrou a presença de uma prolina na posição 105, uma serina na posição 114, três treoninas nas posições 115, 116 e 140, e uma glutamina na posição 129. A presença destes aminoácidos, exceto para Serina na posição 114, tem sido relacionada a terapia monoclonal ou policlonal com anti-HBs após transplante de fígado, enquanto a presença da treonina na posição 116 tem sido descrita em crianças imunizadas de Singapura. Este achado demonstra a possível circulação de cepas do HBV resistentes a imunoprofilaxia para hepatite B no Sudoeste do Paraná, Brasil. O genótipo da amostra foi identificado como genótipo D, o qual é frequentemente encontrado na região estudada. Desde que 36% das crianças tinham recebido incompleta ou nenhuma imunoprofilaxia, um seguimento mais intensivo das crianças nascidas de mães AgHBs positivo é necessário.

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