

# Occult hepatitis B virus infection in hemodialysis patients in Recife, State of Pernambuco, Brazil

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## ABSTRACT

**Introduction:** Persistence of the hepatitis B virus (HBV) genome in individuals negative for the HBV surface antigen (HBsAg) reflects occult infection. The aim of this study was to identify occult HBV infection among hemodialysis patients at 5 clinics in Recife, State of Pernambuco, Brazil, between August 2006 and August 2007. **Methods:** Serum samples underwent enzyme-linked immunosorbent assay to investigate total antibodies against HBcAg (anti-HBc), HBsAg, and antibodies against HBsAg (anti-HBs). Samples that were HBsAg-negative were tested for total anti-HBc, and those that were positive for total anti-HBc were tested for anti-HBs. HBV DNA was investigated with an in-house PCR technique to identify samples positive for total anti-HBc. Subsequently, the samples positive for HBV DNA were sequenced to identify the genotype and mutations. **Results:** The study population (n = 752) had a mean age of 50 15.1 years and included both sexes. All samples analyzed were negative for HBsAg. The seroprevalence of total anti-HBc was 26.7% (201/752), while that of anti-HBs was 67.2% (135/201). Total anti-HBc alone was detected in 5.7% of the patients. Occult infection was found in 1.5%, comprising genotypes A (33.3%, 1/3) and D (66.7%, 2/3). No mutations were found. **Conclusions:** The study detected occult hepatitis B virus infection in hemodialysis patients. Molecular studies on HBV are of fundamental importance because they identify patients that had been considered virus-negative but who, in reality, host the virus and have the ability to transmit it to other patients and staff.

**Keywords:** Hepatitis B. Occult infection. Genotypes. Prevalence.

## INTRODUCTION

The hepatitis B virus (HBV), which is a species of hepatotropic virus in the genus *Orthohepadnavirus*, family *Hepadnaviridae*, is the most significant cause of morbidity and mortality among hemodialysis patients<sup>1</sup>. The clinical significance of occult HBV infection, i.e., absence of HBV surface antigen (HBsAg) with presence of HBV deoxyribonucleic acid (HBV DNA) in serum or liver tissue, remains controversial<sup>2</sup>.

Patients with liver disease and individuals at high risk of parenterally transmitted infections, such as drug users, hemophiliacs, and hemodialysis users, have been widely investigated for occult HBV<sup>3</sup>. Occult HBV infection rates of 45% and 51% have been found among drug users in Baltimore, USA, and hemophiliacs in Japan, respectively<sup>4,5</sup>. The rates of occult HBV infection among human immunodeficiency virus (HIV)-positive patients range from 0 to 89%<sup>6-10</sup>. In Brazil, a cross-sectional population-based study conducted on 1,536 individuals living in rural areas found that 11 individuals were positive for total antibodies to HBcAg (anti-HBc) alone, of which 2 (18%) were HBV DNA-positive<sup>11</sup>.

Occult HBV infection is a possible source of virus transmission in hemodialysis units, thus representing an infection risk for patients and staff. Therefore, certain precautions, including HBV DNA screening for all hemodialysis patients, have been suggested<sup>12</sup>. Some reports have investigated HBV viremia in HBsAg-negative hemodialysis patients<sup>2,12-19</sup>.

It has been observed that some mutations are caused by the high diversity of certain antigens for HBV<sup>20</sup>. In some cases, occult HBV infection is associated with mutant viruses that give rise to the suppression of S gene expression, such that the HBsAg marker becomes undetectable by the techniques used<sup>3,12</sup>. Therefore, the detection of mutations is important in hemodialysis centers as this may identify dialysis patients with occult infection, thereby determining whether they are HBV carriers and avoiding future complications such as cirrhosis and hepatocellular carcinoma. Hemodialysis patients with occult HBV infection are generally not identified by the dialysis clinic, as routine serological tests are unable to detect these patients.

The aims of the present study were to determine the prevalence of occult HBV infection among hemodialysis patients at 5 hemodialysis units in the City of Recife, State of Pernambuco, Brazil, between August 2006 and August 2007, to genotype the viral DNA in positive samples and to identify the presence of any mutations in the viral genome.

## METHODS

A cross-sectional study was carried out from August 2006 to August 2007 to investigate a population of 752 hemodialysis patients at 5 clinics in Recife, Pernambuco. All patients were evaluated.

The study was conducted in 5 dialysis units in the City of Recife, northeastern Brazil, between August 2006 and August 2007. To maintain the anonymity of the clinics, they have been identified with the letters of the alphabet. The mean numbers of patients undergoing hemodialysis at these clinics every month were A (45), B (40), C (248), D (300), and E (250). Each patient received treatment 3 times a week. At clinics A and B, treatment was performed in 2 shifts, and in clinics C, D, and E, in 3 shifts. Each shift comprised 1 4-hour treatment period. Clinic A is public while clinic B is private.

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Clinics C, D, and E receive patients through both the public and private networks. The HBsAg-positive patients received their treatment and the filters (dialyzers) for these patients were processed in rooms that were separate from where the HBsAg-negative patients were treated.

A total of 752 blood samples were collected by nursing auxiliaries in the clinics studied. The samples were sent promptly (within 4 hours) to the laboratory for processing and storage at -80C. Blood was obtained from each patient before hemodialysis in order to avoid *Taq* polymerase inactivation by the anticoagulant used.

Serum samples from 752 patients were analyzed by enzyme-linked immunosorbent assay to investigate total anti-HBc and HBsAg. Patients who were positive for total anti-HBc and negative for HBsAg were investigated for anti-HBs. All tests were conducted in accordance with the manufacturer's instructions. The test kits used were Monolisa™ anti-HBc PLUS, Monolisa™ HBsAg ULTRA, and Monolisa™ anti-HBs PLUS (Bio-Rad, Irving, CA, USA).

Samples that were total anti-HBc-positive and HBsAg-negative (201 in total) underwent polymerase chain reaction (PCR) to investigate viral DNA. Nested PCR, as described by Kaneko<sup>21</sup>, was used to amplify the S and C regions of HBV, with some modifications<sup>20</sup>. In the phenol-chloroform method, 200µL of serum was added to 300µL of phenol and guanidine isothiocyanate solution and 50µL of chloroform. The solution was phenol-chloroform extracted, and the DNA was precipitated with ethanol and eluted with ultra-pure water<sup>21</sup>.

The primers FHBS1 (5'-GAG CTC TCT AGA GTG GTG GAC TTC-3') and RHBS1 (5'-AAA TKG CAC TAG TAA ACT GAG CCA-3') were used for the first round of amplification. For the second amplification, the primers FHBS2 (5'-CGT GGT GGA CTC CTT TCA ATT TTC-3') and RHBS2 (5'-CCG ARG AGA AAC GGR CTG AGG CCC-3') were used. The samples that produced a PCR result that was negative for the S region of the virus underwent a second test using primers for the core region: 2032R (5'-CTG ACT ACT AAT TCC CTG GAT GCT GGG TCT-3') and 1763 (5'-GCT TTG GGG CAT GGA CAT TGA CCC GTA TAA-3') for the first PCR amplification. For the second amplification, the primers 1778-E (5'-GAC GAA TTC CAT TGA CCC GTA TAA AGA ATT-3') and 2017R-B (5'-ATG GGA TCC CTG GAT GCT GCC TCT TCC AAA-3') were used. Water was used as the negative control for each reaction in order to exclude possible cross-contamination. HBV DNA-positive samples were used as the positive control. The detection limit of the PCR used was 300 copies/mL.

To characterize the virus strains, the PCR products of 3 samples were sequenced in accordance with the Sanger method<sup>22</sup>. The Applied Biosystems (ABI) PRISM BigDye Terminator™ kit (PE Applied Biosystems, Foster City, CA, USA) was used in an ABI PRISM 377 automatic sequencer. The genotypes were classified by comparing the alignments of the amino acid sequences obtained from the S gene nucleotide sequences in this study with other known sequences from different HBV genotypes in GenBank (**Table 1**) using EditSeq, MegAlign, and software in the DNASTAR package (Lasergene Inc., Madison, WA, USA). The genotype classifications were confirmed by the National Center for Biotechnology Information (NCBI) genotyping tool: <http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi><sup>23</sup>. Mutations of the S region were investigated using the EditSeq and MegAlign software from the DNASTAR package (Lasergene Inc.).

The data were analyzed using Epi Info 6.0. Descriptive analysis was performed on the total anti-HBc, HBsAg, and occult HBV infection results, and table of absolute and relative frequency distribution were produced.

### Ethical considerations

The study protocol received approval from the research ethics committee of the Health Sciences Center of the Federal University of Pernambuco under protocol 069/06, and patients signed an informed consent statement in accordance with the parameters of the Helsinki Declaration.

**TABLE 1 - Genotype-representative sequences from GenBank: accession numbers.**

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

## RESULTS

Of the 752 patients analyzed, 58.1% were men. The patients' mean age was 50 15.1 years. All the samples analyzed were HBsAg-negative. The seroprevalence of total anti-HBc was 26.7% (201/752), and the seropositivity to anti-HBs in anti-HBc-positive patients was 67.2% (135/201) (**Table 2**). Total anti-HBc alone was found in 5.7% (43/752), and 11.4% had indefinable anti-HBs status.

**TABLE 2 - Frequency of total anti-HBc and anti-HBs in hemodialysis population at 5 clinics in Recife, State of Pernambuco, Brazil, between August 2006 and August 2007.**

Serological markers	n	%
<b>Anti-HBc</b>		
positive	201	26.7
negative	551	73.3
<b>total</b>	<b>752</b>	<b>100.0</b>
<b>Anti-HBs</b>		
positive	135	67.2
negative	43	21.4
indefinite	23	11.4
<b>total</b>	<b>201</b>	<b>100.0</b>

**Anti-HBc:** Total antibodies against HBcAg; **Anti-HBs:** antibodies against HBsAg.

The prevalence of occult infection was 1.5% (3/201). No positive reactions were found among the samples when tested with primers for the core region. The patients who presented occult infection had the following HBV serological marker profiles: 2 patients were positive for total anti-HBc+ anti-HBs and 1 patient was positive for total anti-HBc alone.

The HBV DNA-positive samples were sequenced, and genotypes A and D were found in 33.3% (1/3) and 66.7% (2/3), respectively. No mutations relating to the S region were found in the HBV DNA-positive samples.

## DISCUSSION

The frequency of occult infection among the hemodialysis patients studied corroborates the results in the literature. Among hemodialysis patients, this rate ranges 0-58% in countries such as Canada, Turkey, Italy, Greece, Spain, Iran, and Brazil<sup>2,12-19</sup>.

In Brazil, data on the frequency of occult HBV infection among hemodialysis patients are scarce. Jardim<sup>19</sup> evaluated hemodialysis patients, HIV-positive patients, and blood donors in State of São Paulo and found frequencies of occult infection of 0% (0/34), 5% (8/159), and 4% (6/150), respectively. Thus, the findings from the present study are the first to come from State of Pernambuco.

Hemodialysis patients present high susceptibility to acquiring HBV if the clinics do not follow the universal precautions recommended in the Board Collegiate Resolution, *Resolução da Diretoria Colegiada* (RDC) No. 154, by the Brazilian Ministry of Health<sup>24</sup>. According to these norms, patients should be evaluated every 6 months for HBsAg and anti-HBs markers. Patients who are reactive to HBsAg are sent to the yellow room (reserved for patients with HBsAg), while the seronegative individuals are then tested for anti-HBs. If this marker is not present, the local health department recommends that the patient take doses of hepatitis B vaccine<sup>24</sup>.

Nonetheless, detecting such patients in hemodialysis clinics is of prime importance for avoiding dissemination of the virus inside these units, given that patients with unidentified occult HBV may transmit this infection to other patients as they undergo their treatment alongside other hemodialysis patients who are susceptible to HBV. The repeated exposure to body fluids during dialysis procedures predisposes dialysis patients to nosocomial transmission of HBV<sup>25</sup>. A molecular biological study on patients receiving treatment in the same hemodialysis units showed that there was relative homogeneity of HBV subtypes, which supports the notion that patient-to-patient transmission of HBV infection takes place in hemodialysis units<sup>25</sup>. HBV DNA is detectable in the serum and peripheral blood mononuclear cells of these patients, thus indicating that active virus replication is occurring<sup>26</sup>.

Total anti-HBc is a marker for previous contact with the virus, but there are no recommendations regarding investigation of this marker in hemodialysis units<sup>24</sup>. The present study demonstrates the importance of investigating this marker, given that HBsAg-negative hemodialysis patients may nonetheless carry HBV and present total anti-HBc as the only serological marker. This was observed in the present study: 43 patients only presented the total anti-HBc marker and thus, they would not have been identified routinely in the hemodialysis units. Detection of anti-HBc alone may reflect unrecognized occult HBV infection in hemodialysis patients<sup>18</sup>. These findings corroborate the anti-HBc prevalence that was found in studies conducted in different regions of Brazil<sup>27,28</sup>. Ferreira<sup>28</sup> found that 2.5% of the patients at 15 hemodialysis units (28/1095) presented anti-HBc alone. Such patients have not yet developed anti-HBs production, so they are not immune to this infection. Out of the 201 patients tested

for anti-HBs in the present study, 23 were found to have indefinable status. These tests were then repeated, but the results remained indeterminate. It would be important to conduct an investigation at another time in order to define the status of such patients. The patients who were seronegative for anti-HBs were referred to receive new doses of the vaccine.

Studies have shown that patients who are positive for total anti-HBc and/or anti-HBs may present occult infection<sup>12,14,16,17,18</sup>. In a study on hemodialysis patients in Canada, Minuk<sup>12</sup> found occult infection in 3.8% (9239) of the patients; of these, 4 were positive for anti-HBs alone, 1 was positive for total anti-HBc+ anti-HBs, and 3 did not present any serological marker. Occult infection is more common in patients with HBV seropositivity<sup>12,29</sup>; therefore, it is also described in those who are seronegative for HBV<sup>12,30</sup>.

The number of patients with occult HBV infection in a given study may be influenced by the sensitivity of the molecular biological technique used, the presence of low viral loads, and the size of the population sample studied<sup>2</sup>. The greater the sensitivity of the PCR used, the greater the likelihood that viral DNA will be detected, even in the presence of low viral loads<sup>31,32</sup>. The majority of these infections are associated with low viral loads<sup>18</sup>.

The HBV level in serum is usually less than 10<sup>4</sup> copies/mL in patients with occult HBV infection<sup>33</sup>, which is significantly lower than in those who are HBsAg-positive<sup>34,35</sup>. The detection limit for HBV DNA through the in-house PCR technique used in this study was 300 copies/mL. Therefore, a sample with a viral load of less than 300 copies would not have been detected. Tests with low detection limits are ideal for detecting patients with occult HBV infection. In 1 study, the HBV DNA load in plasma was found to be less than 50IU/mL in all patients with occult HBV infection<sup>18</sup>.

We initially decided to use primers for the HBV S region because these primers present the same sensitivity in PCR as the primers for the core region do, and they allow differentiation of the genotypes of the virus<sup>20</sup>.

The high diversity of HBV antigenic determinants may be a cause for concern in hemodialysis centers. Patients may present mutations in the S region of the virus, such as that of type sG145R, in which a modification to antigenic determinant *a* occurs, and through this, anti-HBs is unable to neutralize the virus. This gives rise to patients who are seropositive for total anti-HBc and anti-HBs, but with free HBV DNA in their serum<sup>12</sup>. The present study investigated mutations in the S region of the virus because this region produces HBsAg, an important serological marker tested in dialysis units. Patients who are serologically negative for HBsAg are considered negative for HBV, but if the virus happens to undergo mutation in the S region, such individuals will no longer produce this surface protein, with consequences for their immunological status<sup>26</sup>. Patients undergo serological tests when they visit hemodialysis units, and most of them are sent to receive the vaccine<sup>24</sup>. Patients with kidney diseases receive 4 doses of hepatitis B vaccine, i.e., twice the dose recommended for healthy individuals within the same age range<sup>36</sup>. Booster doses are indicated whenever antibody titers fall by at least 10mIU/mL. Thus, hemodialysis patients receive large numbers of vaccine doses, and according to Barone<sup>37</sup>, isolated cases of mutation with changes in the S region have been selected both for vaccination and for immunoprophylaxis among transplant patients.

The study did not identify any mutations in the viral sequences studied. However, occult infection may be determined not only by the presence of mutations, but also by the formation of HBsAg-anti-HBs immunocomplexes, HBV integration, and HBV infection of peripheral blood mononuclear cells, thus leading to decreased levels of this marker<sup>26</sup>.

Few studies have identified the HBV genotypes among hemodialysis patients with occult infection. In the present study, the genotypes encountered were A and D, and these were similar to the findings of Weinberger<sup>33</sup>, who reported frequencies of 30.3% and 60.6% for genotypes A and D, respectively, in a study on different sources within a German population with occult HBV infection, such as dentists, blood donors, and prisoners. These same genotypes have also been reported in another European population of blood donors with occult HBV infection<sup>38</sup>. The genotypes identified in the present study exhibited similarities to previous findings from the general population<sup>20</sup>, as well as to findings from other HBsAg-positive Brazilian hemodialysis patients<sup>27,39,40</sup>. However, genetic analysis on adequate numbers of samples from patients with occult infection would be needed for better comparison.

It can be concluded that, in accordance with current studies in the literature, in which no patients with occult infection have been found, it was to be expected that the present study would find a small number of such patients. The serological markers evaluated the HBV infection well, but these should always be backed up with molecular tests to investigate possible occult infections and virus genotypes. Identification of patients with occult HBV infection should be expanded through further studies using molecular biology tests with lower detection limits in order to have better understanding of the clinical, laboratory, and epidemiological characteristics of such infection. Molecular biology tests such as PCR are not implemented in routine clinical hemodialysis, but patients may harbor the virus and remain undetected by the clinic, as seen in the results of this study. Hence, serial studies should be conducted to evaluate optimal cost-effective deployment of PCR and its inclusion in the universal precautions used by hemodialysis clinics.

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## CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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## ABSTRACT IN PORTUGUESE

### Infecção oculta para o vírus da hepatite B em pacientes hemodialisados de Recife, Estado de Pernambuco, Brasil

**Introdução:** A persistência do genoma do vírus da hepatite B (HBV) em indivíduos com o antígeno de superfície para o HBV (HBsAg) negativo,

caracteriza-se como infecção oculta. O objetivo desse trabalho foi identificar infecção oculta pelo HBV em hemodialisados provenientes de cinco clínicas de Recife, Estado de Pernambuco, Brasil, de agosto de 2006 a agosto de 2007. **Métodos:** Os soros foram submetidos à pesquisa do HBsAg, anticorpos totais para o AgHBc (anti-HBc total) e para os anticorpos contra o antígeno de superfície do HBV (anti-HBs) pelo ensaio imunoenzimático (ELISA). As amostras negativas para o HBsAg foram testadas para o anti-HBc total e os soros reativos, foram testados para o anti-HBs. O DNA-HBV foi analisado pela reação em cadeia da polimerase (PCR) *in house* nas amostras anti-HBc total reagentes. As amostras DNA-HBV positivas foram sequenciadas para a identificação do genótipo e pesquisa de mutação. **Resultados:** A população estudada foi de 752 hemodialisados, com média de idade de 50±15,1 e ambos os sexos. Todos os soros analisados foram HBsAg negativos. A soroprevalência para o anti-HBc total e anti-HBs foi de 26,7% (201/752) e 67,2% (135/201), respectivamente. O anti-HBc total foi encontrado em 5,7% dos pacientes. A infecção oculta foi identificada em 1,5% (3/201) das amostras, sendo 33,3% com o genótipo A e 66,7% com o D. Nenhuma mutação foi observada. **Conclusões:** O estudo encontrou infecção oculta na amostra analisada. A infecção oculta encontrada mostra a importância de estudos moleculares, pois pacientes HBsAg negativos podem albergar o vírus disseminando-o para os demais pacientes.

**Palavras-chaves:** Hepatite B. Infecção oculta. Genótipos. Prevalência.

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