

**HEPATITIS B VIRUS INFECTION PROFILE IN DIFFERENT
HEMODIALYSIS UNITS IN RECIFE, PERNAMBUCO, BRAZIL.**

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ABSTRACT

Patients under hemodialysis are considered a high risk to acquire hepatitis B virus (HBV) infection. The aim of this study was to determine the serological and molecular profile and risk factors for HBV among hemodialysis patients in five clinics in Recife, Pernambuco, between August 2006 and August 2007. The study population (n = 781) was interviewed after signing a free and informed consent statement and the blood collected through the patient's fistula prior to hemodialysis. Serum samples underwent ELISA to investigate total anti-HBc, HBsAg and anti-HBs. The samples that were HBsAg-positive were subjected to the PCR to investigate viral DNA. The DNA-positive samples were sequenced to identify the genotype. The information was stored and analyzed in Epi-Info 6.0. The seroprevalences of total anti-HBc, HBsAg and anti-HBs were 29.4% (229/781), 3.3% (26/781) and 66.2% (135/203), respectively. Among the 26 HBsAg-positive samples, 14 were positive for HBV DNA; genotypes A and F were found. The variables of sex, age, length of time on dialysis and number of transfusions showed statistically significant association with HBV, thus corroborating other Brazilian studies. Even with the implementation of HBV infection control measures and vaccinations, dialysis units still present widespread virus circulation. All units need to rigidly follow the universal precautions, so that the virus circulation in these places can increasingly be minimized.

INTRODUCTION

Parenteral exposure is one of the transmission routes for the hepatitis B virus (HBV) and therefore hemodialysis patients whose blood has greater contact with the machines and reusable filters, that are used for their treatment, present a high risk of acquiring this virus (Ferreira et al. 2006). The general incidence of HBV infection among hemodialysis patients has been decreasing because of testing blood donors for HBsAg and total anti-HBc, use of human recombinant erythropoietin, HBV vaccination and implementation of infection control measures (Wong et al. 2005).

Hepatitis B is an important public health problem and, even with all these controls, cases among hemodialysis patients continue to occur (Boulaajaj et al. 2005, Chattopadhyay et al. 2005, Ferreira et al. 2006, Yakaryilmaz et al. 2006, Thanachartwet et al. 2007).

The epidemiology of HBV among hemodialysis patients in the less-developed world is not well known. The frequency of HBsAg ranges from 2 a 20% in countries such as Morocco, Brazil, Saudi Arabia, Thailand, India, Turkey and Romania. (Teles et al. 1998, Vladution et al. 2000, Busek et al. 2002, Souza et al. 2003, Qadi et al. 2004, Carrilho et al. 2004, Boulaajaj et al. 2005, Ferreira et al. 2006, Yakaryilmaz et al. 2006, Thanachartwet et al. 2007).

Studies have revealed that the HBV genotype may be associated with disease activity, prognosis and treatment response; however the distribution of genotypes in dialysis populations has not been addressed to date (Fabrizi et al. 2008).

Risk factors may contribute towards increasing the prevalence of HBV, for example: length of time on hemodialysis, number of blood units transfused, schooling level, sex, treatment in more than one unit or kidney transplantation (Cendoroglo et al.

1995, Teles et al. 1998, Busek et al. 2002, Souza et al. 2003, Carrilho et al. 2004, Ferreira et al. 2006).

The aims of the present study were: to determine the serological and molecular profile of HBV markers among hemodialysis patients at five units in the city of Recife, Pernambuco, Brazil; to sequence the viral DNA in the HBsAg-positive samples, using primers for the S and Core regions of the virus and to investigate associations between the total anti-HBc marker and the variables studied.

MATERIAL AND METHODS

Type of study.

A hybrid cross-sectional and case-control study was carried out. The cross-sectional part of the study allowed the formation of two groups (cases and controls) and thus it became possible to analyze the risk factors.

Sample calculation.

The calculation was performed with the Epi-Info 6.0 software. Based on risk factors for HBV risk within time of hemodialysis and number of transfusions, with confidence interval 95% and 80% power, it was found that a population of 336 controls and 84 cases would be needed.

Population.

A population of 781 hemodialysis patients was analyzed in five clinics between August 2006 and August 2007.

Definition of the variables.

A standardized questionnaire was used to gather information such as: sex, age, length of time on hemodialysis, kidney transplantation, reported vaccination against hepatitis B, blood and blood product transfusion and number of times, tattooing,

intravenous and intranasal drug administration, condom use and hemodialysis performed in other clinics.

Inclusion criteria.

All patients who were undergoing treatment systematically at the centers during the study period.

Exclusion criteria.

Patients who presented any difficulty in oral expression and patients who were possibly not in their native city were excluded.

Dialysis centers.

To maintain the anonymity of the clinics studied, they have been identified by means of letters of the alphabet. The mean numbers of patients who were undergoing hemodialysis at these clinics every month were: A (45), B (40), C (248), D (300) and E (250). Each patient was receiving treatment three times a week. At centers A and B, treatment was performed in two shifts and in centers C, D and E in three shifts. Each shift comprised one four-hour period of treatment. 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.

Serum samples.

Blood samples were taken from 780 patients. These samples were separated into three aliquots, one for serological tests and another for PCR, while the third was held in reserve for repetitions and counter-sample tests. The serum samples were stored at -20°C and -70°C.

HBsAg, total anti-HBc and anti-HBsAg serological tests.

To study the serological profile of HBV infection in this population, all the serum samples were analyzed by means of immunoenzymatic assaying (ELISA) to

investigate total anti-HBc and HBsAg. The total anti-HBc positive, HBsAg-negative and indeterminate samples were investigated for anti-HBs. All the tests were conducted in accordance with the manufacturer's instructions for the Monolisa Plus kits (BioRad).

PCR.

The Nested PCR was performed as described by Kaneko et al. (1989), with some modifications (Sitnik et al. 2004). HBV DNA detection using PCR was performed on 26 HBsAg-positive and indeterminate samples. The DNA was extracted from serum using a solution of phenol and guanidine isothiocyanate. 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 presented 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') at first PCR. 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 samples were used as negative controls for each reaction, in order to exclude possible occurrences of cross-contamination.

Sequencing reaction.

To characterize the virus strains, the samples were sequenced using the PCR product, in accordance with the method of Sanger et al. (1977), using the ABI Prism BigDye Terminator™ Kit (PE Applied Biosystems, Foster City, California, USA), in an ABI Prism 377 automatic sequencer. The genotyping was analyzed by comparing the

sequences obtained with other known sequences from different HBV genotypes kept in the Gene Bank, using Edit Seq MegAlign and software in the DNASTAR package (LaserGene, Inc.). The genotype classification was confirmed by means of the Genotyping tool, which is available on the NCBI website: (<http://www.ncbi.nlm.nih.gov/projects/genotyping/formpage.cgi>) (Rozanov et al. 2004).

Statistical analysis.

For univariate analysis, the X^2 test was used to verify distribution of total anti-HBc frequency between different groups. For multivariate analysis, logistic regression was used to evaluate the significance obtained in the univariate analysis, in relation to the study variables. This procedure made it possible to evaluate the contribution of each variable towards the diagnosis of HBV infection. The significance level established was 5%. All the calculations were performed using STATA (Cary 1989).

Ethical matters.

The protocol for this study was submitted to and was granted approval by the research ethics committee of the Health Sciences Center of the Federal University of Pernambuco under registration number CEP/CCS/UFPE 069/06.

RESULTS

Among the 780 patients interviewed, 58.1% were male and the mean age was 50 ± 15.1. Stratification into age groups showed that 26.9% were 60 years old or over.

The seroprevalences of total anti-HBc, HBsAg and anti-HBs were 29.4% (229/781), 3.3% (26/781) and 66.5% (135/203), respectively (Table 1). Total anti-HBc was found alone in 21.2% (43/203) patients. Among HBsAg-positive samples, 53.8% (14/26) were HBV DNA-positive. These samples were sequenced and genotypes A and F were found.

Table 1. Serological markers for hepatitis B among the hemodialysis population at five clinics in Recife, Pernambuco, between August 2006 and August 2007.

Serological markers	N	%	CI (95%)
Anti-HBc			
Positive	229	29.4	26.2 – 32.7
Negative	551	70.6	67.3 – 73.8
HBsAg			
Positive	26	3.3	2.18 – 4.84
Negative	752	96.3	94.7 – 97.5
Indefinite	03	0.4	0.08 – 1.04
Total	781	100.0	
Anti-HBs			
Positive	135	66.5	59.5 – 72.9
Negative	43	21.2	15.8 – 27.4
Indefinite	25	12.3	8.1 – 17.4
Total	203	100.0	

N = Number of cases; a = Confidence Interval

Table 2 shows the results of the univariate analysis that were statistically significant associations with seropositivity for the total anti-HBc marker.

Table 2. Distribution of hemodialysis patients at 05 centers in Recife, between August 2006 and August 2007, according to the age, sex, length of time on hemodialysis, number of blood transfusions, hepatitis B vaccination and the seropositivity to anti-HBc total.

Variables	Anti-HBc total				OR ^a (CI) ^b	p-value
	Positive		Negative			
	n	%	n	%		
Sex						
Female	73	22.3	255	77.7	1.0	-
Male	156	34.5	296	65.5	1.84 (1.31 – 2.59)	0.0002
						0.0004
Age group						
< 30	07	9.7	65	90.3	1.0	-
30 - 39	36	25.2	107	74.8	3.12 (1.24 – 8.20)	0.0076
40 - 49	58	36.5	101	63.5	5.33 (2.18 – 13.7)	0.0000
50 - 59	58	29.1	141	80.9	3.82 (1.57 – 9.71)	0.0009
>=60	70	33.8	137	66.2	4.74 (1.97 – 11.9)	0.0000
Time of hemodialysis						
< 5 years	105	24.2	329	75.8	1.0	-
≥ 5 years	124	35.8	222	64.2	1.75 (1.26 – 2.42)	0.0003
						0.0004
Number of transfusions						
0	64	26.1	181	73.9	1.0	-
1 - 5	115	27.5	303	72.5	1.07 (0.74 – 1.56)	0.6974
6 - 10	13	43.3	17	56.7	2.16 (0.93 – 5.01)	0.0479
>=11	24	54.5	20	65.5	3.39 (1.67 – 6.90)	0.0001
Hepatitis B vaccination						
						0.0000
No	54		33		1.0	-
Yes	129	22.7	440	77.3	0.18 (0.11 – 0.30)	0.0000
Unknown	46	37.1	78	62.9	0.36 (0.20 – 0.66)	0.0003

* age (as a continuous variable): OR = 1.02 (1.00 – 1.04) p = 0.045

a:Odds Ratio

b: Confidence Interval

The risk of acquiring the total anti-HBc marker in males was 1.84 times greater than the risk among females.

Applying age as a continuous variable, its association with seropositivity for total anti-HBc was significant, with an odds ratio of 1.017, i.e. for every one-year increase in age, there was a 1.7% increase in the risk of HBV infection.

The length of time on hemodialysis presented a statistically significant association. Patients who had been undergoing this treatment for five years or more had a risk of acquiring the virus that was 1.62 times greater than the risk among patients who had been on hemodialysis for shorter times.

Situations in which patients had undergone blood or blood product transfusion up to five times during their lifetimes did not show a statistically significant association with seropositivity for total anti-HBc. But from six transfusions onwards, the association was significant, with a risk of infection 2.82 times greater than in cases with up to five transfusions.

Reported vaccination against hepatitis B had a protective effect against seropositivity for the total anti-HBc marker, with an odds ratio of 0.18, i.e. protection of 82%.

With regard to drug use, only one patient used injectable drugs and two patients used cocaine, and thus no calculations on these associations were possible. Tattooing ($p = 0.370$), condom use ($p = 0.742$), kidney transplantation ($p = 0.453$) and undergoing hemodialysis in more than one center ($p = 0.179$) did not present statistically significant associations.

The multivariate model was composed of the variables that presented associations with seropositivity for hepatitis B with a significance level of less than 20% ($p < 0.200$). The variables of age, sex, length of time on dialysis and number of transfusions remained in the final model. The model did not modify the associations found in the univariate analysis, and thus the odds ratio was adjusted according to the other variables that made up the model (Table 3).

Table 3. Final model of the multiple logistic regression between the age, sex, length of time on hemodialysis, number of transfusions and seropositivity to total anti-HBc in the hemodialysis patients at 5 centers in Recife, between August 2006 and August 2007.

Variables	OR ^a	CI ^b (95%)	p-value
Sex			
Female	1.00	-	-
Male	2.13	1.50 – 3.05	0.000
Age			
Age	1.02	1.01 – 1.03	0.000
Time of hemodialysis			
< 5 years	1.0	-	-
≥ 5 years	1.62	1.15 – 2.29	0.006
Number of transfusion			
0	1.0	-	-
1 - 5	1.19	0.82 – 1.73	0.346
6 - 10	2.82	1.23 – 6.41	0.014
>=11	4.04	2.00 – 8.19	0.000

a:Odds Ratio

b: Confidence Interval

DISCUSSION

The present study found an overall HBV prevalence of 29.3%. This data corroborates with the findings from other studies conducted in Brazil, which ranged

from 29.8 to 55.7% among hemodialysis patients (Cendoroglo et al. 1995, Teles et al. 1998, Souza et al. 2003, Carrilho et al. 2004, Ferreira et al. 2006). These numbers show, even with the advent of the vaccine, that hemodialysis units need to avoid virus dissemination in itself.

The seroprevalence for HBsAg among hemodialysis patients varies between different localities and presents a correlation with the endemicity of the virus among the general population of the region (Wong et al. 2005). In Brazil, the prevalence of HBsAg ranges from 2.4 to 12% (Teles et al. 1998, Busek et al. 2002, Souza et al. 2003, Carrilho et al. 2004, Ferreira et al. 2006). The present study found 21.2% of the patients with total anti-HBc alone, i.e., these patients no longer presented the surface antigen of the virus, although they presented an immunological footprint that reflected previous contact. These patients would not yet be immune to the virus. Total Anti-HBc -positive found alone may be caused by low-levels of viremia, loss of anti-HBs many years after recovery, or a false positive result (Júnior & Gonçalves 2007). The prevalence of total anti-HBc alone in the Brazilian regions is from 2 to 6.7% (Teles et al. 1998, Souza et al. 2003, Carrilho et al. 2004, Ferreira et al. 2006). On the other hand, in other localities of the world, such as northeastern and southeastern Italy, it has a higher prevalence - 20.8% (Fabrizi et al. 2005).

In this research, the samples positive for total anti-HBc alone were retested to check the possibility of methodological error, but the result remained the same. It is important to investigate other markers like anti-HBe to analyze the prognosis and the presence of HBV DNA in these patients. Although such patients are considered seronegative, they may host the virus and spread it to the hemodialysis unit.

On entering the hemodialysis unit, all patients are tested for HBsAg and anti-HBs. If they are positive for anti-HBs, vaccine doses are not administered. On the other

hand, if these patients are negative, they receive doses in accordance with the flow diagram for hemodialysis patients and, subsequently, their production of antibodies is checked in other laboratory analyses (Wong et al. 2005).

The test for anti-HBs was performed on the patients that were total anti-HBc - positive but HBsAg-negative, and was positive in 66.5% (135/203). However, the presence of total anti-HBc was not related to unit or patient, therefore the anti-HBs produced must be a product from immunization. The information from patients about their vaccination history of hepatitis B showed that it presented a protective effect, with the odds ratio less than one. Nevertheless, the patients who denied vaccination against hepatitis B also showed odds ratio less than one. This may constitute memory bias about vaccination history.

The use of primers for the S and Core regions shows similar efficiency. Moreover the S region primers can distinguish the genotypes (Sitnik et al. 2004). The HBV viral load in these patients tends to be low and stable over time and this may consequently influence on DNA detection in the tests (Fabrizi et al. 2008). In this study it was observed that twelve HBsAg-positive patients did not present positive findings by PCR (S and Core regions). The ideal would be to carry out monitoring through investigating HBeAg and HBV DNA in these patients to assess level of active viral replication. The results found corroborated with literature which showed HBsAg seropositivity in hemodialysis patients without detectable serum HBV DNA were 11.8% to 58% (Teles et al. 1998, Fabrizi et al. 2003, Souza et al. 2003, Carrilho et al. 2004, Ferreira et al. 2006, Moutinho et al. 2006).

The genotypes A and F found in this present study were similar to those found in the other Brazilian studies. Carrilho et al. (2004) found genotypes A, D and F, all in hemodialysis patients and in the health professionals who were caring for them,

suggesting a nosocomial transmission. Nonetheless, this study found only genotypes A and F among hemodialysis patients. A single patient presented genotype F according to Teles et al. (2002) and Ferreira et al.(2006).

There are few studies reporting the risk factors associated with HBV. Multivariate analysis from this work revealed that gender, age, length of time on hemodialysis and the number of transfusions presented statistically significant association with HBV. Male gender presented a statistically significant association according to Ferreira et al. (2006) with a risk of 1.8 for acquiring HBV, although Carrilho et al. (2004) found an association close to the significance level ($p = 0.06$).

The length of time on hemodialysis has been shown to be statistically associated with markers for HBV in several studies (Cendoroglo et al. 1995, Teles et al. 1998, Busek et al. 2002, Souza et al. 2003, Carrilho et al. 2004, Ferreira et al. 2006). The present research found that patients who had been on hemodialysis for five years or more presented a risk of acquiring HBV 1.62 times greater than among those with shorter times. Carrilho et al. (2004) observed a risk of 1.47 for acquiring the virus every month. And two other studies conducted in Goiás found risks of acquiring HBV that were 10.1 and 2.6 times greater among patients who underwent treatment over periods of three years or more (Teles et al. 1998, Ferreira et al. 2006). Although, it was not found in other Brazilian studies, this showed that the risk of acquiring HBV is higher when the number of blood transfusions increases.

Considering that 29.2% of the hemodialysis patients positive for the total anti-HBc marker were not using condoms, it is important to point out the need to give sexual advice, because hepatitis B is a sexually transmittable infection (STI).

In conclusion, the HBsAg prevalence in hemodialysis studies was low compared with other Brazilian studies. The finding that length of time of hemodialysis is

associated with total anti-HBc seropositivity suggests nosocomial transmission. All units need to rigidly follow universal precautions to reduce viral circulation.

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