DETECTION OF HEPATITIS B VIRUS DNA IN HEPATITIS B SURFACE ANTIGEN-NEGATIVE SERUM BY POLYMERASE CHAIN REACTION: EVALUATION OF DIFFERENT PRIMER PAIRS AND CONDITIONS

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Received May 28, 1996

Summary. – The presence of hepatitis B virus (HBV) DNA was investigated by polymerase chain reaction (PCR) in the serum of twenty Brazilian blood donors. All sera were negative for hepatitis B surface antigen (HBsAg), 17 of them presented antibodies to the hepatitis B core antigen (anti-HBc) as the unique serological marker of HBV infection, and 3 were positive for antibodies to HBsAg (anti-HBs) and anti-HBc. PCR assays were carried out using different pairs of oligonucleotides designed from conserved sequences of C, pre-S and S regions of the HBV genome. First, all oligonucleotide pairs were tested in PCR using plasmids carrying HBV genome from *ayw* or *adw* strains as templates. One-round PCR assays were able to detect 100 – 25,000 molecules of plasmid DNA, depending on the oligonucleotide pair, while semi-nested PCR assays detected 10 – 1000 molecules. The frequency of HBV DNA-positive results with $HBsAg^-$ sera varied from 0% to 50% depending upon the PCR assay. The results indicated that a number of both isolated anti-HBc and anti-HBs^+, anti-HBc^+ samples contained HBV DNA at a very low concentration, neighboring the limit of detection.

Key words: hepatitis B virus; HBsAg; anti-HBc; polymerase chain reaction; primer pairs, Brazil

Introduction

HBV infection is commonly diagnosed by the presence of HBsAg and anti-HBc in the serum using radioimmunoassay (RIA) or enzyme-linked immunosorbent assay (ELISA). Anti-HBc usually appear in the acute phase of HBV infection and can persist over a long period of time after virus clearance. The convalescent phase of the disease is confirmed by an anti-HBs-positive test. Anti-HBc coexisting with anti-HBs indicate a previous HBV infection whereas the simultaneous presence of HBsAg and anti-HBc reveals a current infection. Persistent isolated anti-HBc may present two possibilities with different clinical and epidemiological significance: (a) past HBV infection with undetectable levels of anti-HBs or (b) HBV chronic carriership with undetectable levels of HBsAg in the blood (Medrano *et al.*, 1991). Studies on post-transfusion hepatitis have shown that in some cases, "anti-HBc alone" serum or even a serum without any serological marker of HBV infection can transmit infection to transfusion recipients (Elghouzzi *et al.*, 1995; Hoofnagle *et al.*, 1978; Thiers *et al.*, 1988).

In a transfusion context, HBV, as retroviruses and hepatitis C virus, has been one of the viruses most extensively investigated through PCR analysis. The presence of

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Abbreviations: EDTA = ethylenediamine tetraacetate; HBsAg = hepatitis B surface antigen; ELISA = enzyme-linked immunosorbent assay; HBV = hepatitis B virus; nt = nucleotide; PCR = polymerase chain reaction; SDS = sodium dodecyl sulphate; Tris = tris-(hydroxymethyl)-aminomethane

HBV DNA in the serum of patients with anti-HBc as the sole HBV infection marker has been investigated in Canada (Scully *et al.*, 1994), China (Luo *et al.*, 1991,1992; Pao *et al.*, 1991; Shih *et al.*, 1990; Wang *et al.*, 1991), Finland (Ranki *et al.*, 1995), Japan (Iizuka *et al.*, 1992), The Netherlands (Kroes *et al.*, 1991), Switzerland (Joller-Jemelka *et al.*, 1994), The United States (Douglas *et al.*, 1993; Liang *et al.*, 1994), and Venezuela (Zabaleta *et al.*, 1992). The results of these studies were discrepant, with the ratio of positive HBV DNA samples varying from 0% (Douglas *et al.*, 1993; Scully *et al.*, 1994) to 90% (Zabaleta *et al.*, 1992).

Such discrepancy may reflect a large diversity of sample collections originating from populations of lower or higher prevalence rates. It can also be due to variations in the methodology: although PCR is a very sensitive method for detection of DNA, some factors, as the method of DNA extraction from the serum, the choice of oligonucleotides used as primers, and the experimental conditions of PCR, can affect the limit of detection of HBV DNA from positive biological samples.

In this study, we demonstrate the presence of HBV DNA in serum of HBsAg$^-$ Brazilian blood donors and find that the frequency of HBV DNA-positive results can vary considerably depending on the PCR assay used.

Materials and Methods

Serum samples were collected in 1994 from voluntary blood donors from the Brazilian states of Acre and Santa Catarina. Acre is a northern Brazilian state in the Amazon region where the prevalence of HBV carriers is high, 8–10% of HBsAg$^+$ (Fay, 1994) and 67% of anti-HBc$^+$ (Bensabath *et al.*, 1987). Santa Catarina, located in the south, presents a low HBV prevalence, 1% of HBsAg$^+$, and 13% of anti-HBc$^+$ (Vasconcelos *et al.*, 1994). Samples were tested for HBsAg, anti-HBs, and antiHBc using Hepanostika HBsAg Uniform II, Hepanostika anti-HBs, and Hepanostika anti-HBc Uniform, respectively, in microELISA system (Organon Teknika, The Netherlands) according to the manufacturer's instructions. The presence of anti-HBc IgM was also investigated by ELISA (Tedeschi *et al.*, 1989). Twenty samples (10 from Acre and 10 from Santa Catarina) were selected for this study. All were HBsAg$^-$ and anti-HBc$^+$ IgM. Seventeen samples presented anti-HBc as the unique serological marker of HBV infection and the remaining 3 (Ac1, Ac2, and Ac6) were anti-HBs$^+$/anti-HBc$^+$. Anti-HBc titer was established as the endpoint from two-fold serial dilutions (first dilution 1/5). Fifteen samples from blood donors with a high titer of HBsAg were used as a control group.

DNA extraction. Two DNA extraction methods were compared. In the first one, 150 µl of serum was treated with 0.5 mg/ml of proteinase K in the presence of 0.2 mol/l NaCl and 0.25% sodium dodecyl sulphate (SDS) for 4 hrs at 37°C. DNA was then extracted with phenol/chloroform and precipitated by ethanol. The pellet was dried and resuspended in 30 µl of distilled water. One µl was used for each PCR assay (method 1).

In the second method (Baginski *et al.*, 1990), 25 µl of serum was mixed with 25 µl of a buffer containing 250 µg/ml of proteinase K, 0.25% SDS, 5 mmol/l ethylenediamine tetraacetate (EDTA), 10 mmol/l tris-(hydroxymethyl)-aminomethane (Tris) pH 8 and incubated for 2 hrs at 56°C. After heat-inactivation of the proteinase K for 10 mins at 95°C, the volume was adjusted to 200 µl with TAQ buffer (10 mmol/l Tris-HCl pH 8.4, 50 mmol/l KCl, 3 mmol/l $MgCl_2$, 0.01% gelatin, 0.05% Tween 20, and 0.05% NP40). Forty µl were used for each PCR assay (method 2).

Plasmids. PCR assays were carried out with different oligonucleotide pairs. To determine the limit of detection for each PCR assay, two plasmids were used as HBV DNA templates. The first plasmid, designated as "plasmid *ayw3*", was constructed by insering into the pCRII vector (Invitrogen Co.) a PCR product containing 97% of the genome of HBV *ayw3* strain. The segment of HBV genome carried by plasmid *ayw3* extended from nucleotide (nt) 1935 to 3182 and from nt 1 (*Eco*RI site) to 1837. The second plasmid, designated "plasmid *adw4*", contained the complete genome of an HBV isolate that expressed the subtype *adw4* of HBsAg (Naumann *et al.*, 1993). Both plasmids were purified by ultracentrifugation in CsCl gradient and serially diluted when used as templates for PCR.

PCR. The following oligonucleotides were used as primers for PCR.

C1 5'CTGTGGAGTTACTCTCGTTTTTGC3' (nt 1935-1958)
C2 5'CTAACATTGAGATTCCCGAGATTG3' (nt 2458-2432)
PS1 5'CCATATTCTTGGGAACAAGA3' (nt 2826-2845)
PS2 5'GGTCCCCAGTCCTCGAGAAG3' (nt 143-124)
PS4 5'ACACTCATCCTCAGGCCATGCAGTG3' (nt 3194-3218)
S2 5'GGGTTTAAATGTATACCCAAAGA3' (nt 841-819)

These primers were designed from highly conserved segments of the HBV genome. PCR assays were carried out in a volume of 50 µl in the presence of 0.2 mmol/l of each dNTP, 3 mmol/l $MgCl_2$, and 1 unit of *Taq* DNA polymerase (Gibco-BRL). After an initial denaturation for 3 mins at 94°C, DNA was amplified in 35 cycles (94°C for 30 secs, 52°C for 1 min and 72°C for 2 mins) followed by a final elongation for 7 mins at 72°C. Ten µl were loaded on a 2% agarose gel containing ethidium bromide and electrophoresed. DNA was visualized under UV light. The semi-nested PCR was done in the same conditions using 1 µl of the product of the first round of PCR.

To avoid contamination, pre-PCR reagent preparation, DNA extraction, DNA amplification, and gel electrophoresis of PCR products were performed in four separate rooms. In each series of experiments, five samples, two negative and one positive controls were subjected to PCR. Negative

Table 1. Limit of detection of plasmid HBV DNA in different PCR assays

Primer pairs used for PCR	Type of PCR	Size of DNA fragment[a] (bp)	Limit of detection (number of molecules)	
			Plasmid *ayw3*	Plasmid *adw4*
C1-C2	One round	524	5,000	2,500
C1-PS2	One round	1,430	5,000	2,500
C1-PS2 -> PS1-PS2	Semi-nested	539	150	25
PS1-PS2	One round	539	500	100
PS1-S2	One round	1,235	1,500	25,000
PS1-S2 -> PS4-S2	Semi-nested	867	10	1,000

[a]The sizes are given for plasmid *adw4*. Due to a deletion at the 5'-end of the PS1 region, the DNA fragments from plasmid *ayw3* were by 33 bp smaller with primer pairs C1-PS2, PS1-PS2, and PS1-S2.

Table 2. DNA positivity of HBsAg$^-$ and HBsAg$^+$ serum samples in PCR

Sample	Anti-HBc titer	PCR					
		PS1-PS2	PS1-S2	PS1-S2 ->PS4-S2	C1-PS2	C1-PS2 ->PS1-PS2	C1-C2
Ac1[a]	1:5	–	–	+	+	+	+
Ac2[a]	1:20	–	–	–	–	–[b]	–
Ac3	1:320	–	–	–	–	–[c]	–
Ac4	1:40	–	–	+	–	–	+
Ac5	1:1	–	–	+	–	–	–
Ac6[a]	1:1	–	–	+	ND	ND	+
Ac7	1:10	–	–	–	ND	ND	–
Ac8	1:10	–	–	+	ND	ND	+
Ac9	1:80	–	–	–	ND	ND	–
Ac10	1:5	–	–	–	ND	ND	–
SC1	1:20	–	–	–	–	–	+
SC2	1:1	–	–	–	–	–	–
SC3	1:20	–	–	–	–	–	+
SC4	1:20	–	–	+	–	–[b]	+
SC5	1:160	–	–	–	–	–	+
SC6	1:20	–	–	–	–	–	–
SC7	1:20	–	–	–	–	–[c]	+
SC8	1:40	–	–	+	–	+	+
SC9	1:10	–	–	–	–	–	–
SC10	1:20	–	–	–	–	–	–
HBsAg / samples (%)	0/20	0/20 (0%)	7/20 (0%)	1/15 (35%)	2/15 (7%)	10/20 (13%)	(50%)
HBsAg$^+$/ samples (%)	15/15	13/15 (100%)	15/15 (87%)	15/15 (100%)	15/15 (100%)	15/15 (100%)	(100%)

[a]Anti-HBs$^+$ samples.
[b]Presence of a DNA band of higher molecular mass than expected.
[c]Presence of a DNA band of lower molecular mass than expected.
ND = not done.

controls were sera of individuals without any serological marker of HBV infection, processed in the same manner as the samples. Positive control was a serum from an individual with high level of HBsAg. Additionally, the nucleotide sequence of a segment of pre-S2 region from PCR amplification product of each positive sample was determined. As no sequence was identical to each other (data not shown), the possibility of cross-contamination was eliminated.

Results

The sensitivity of the PCR assay to detect the presence of HBV DNA in serum may be influenced by several parameters, such as the method of DNA extraction, the sequence of the oligonucleotides chosen as primers of the reaction, and the experimental conditions of PCR. Moreover, the nested PCR is known to be more sensitive than the one-round PCR.

Two methods of extraction of HBV DNA from serum, with and without phenol (methods 1 and 2, see Materials and Methods), were compared in detection of HBV DNA in HBsAg$^+$ serum. First, five HBsAg$^+$ samples from control group were treated with both methods. In all five samples, a HBV DNA band appeared after treatment with either method followed by PCR with oligonucleotide pair C1-C2 and gel electrophoresis (data not shown). After treatment with the method 1, four of ten HBsAg$^+$ samples gave positive results in PCR with C1-C2 primers pair while all remained negative when treated with the method 2 (data not shown). The method 1 was therefore more sensitive than the method 2 and was consequently selected to extract serum DNA to be used as template for PCR.

Preliminary PCR assays were carried out to ensure the validity of each oligonucleotide pair and to determine its capability to amplify low amounts of DNA. This was done using plasmids carrying the HBV genome either of an *ayw* strain or of an *adw* isolate. *Ayw* and *adw* were chosen because they are the two most common subtypes in Brazil (Gaspar and Yoshida, 1987). All five oligonucleotide pairs (C1-C2, C1-PS2, PS1-PS2, PS1-S2, and PS4-S2) used in this study were able to detect *ayw3* and *adw4* plasmid DNA. To determine the limit of detection of DNA in our PCR assays, serial dilutions of both plasmids were analyzed by PCR. Table 1 shows that the limit was very low, always inferior to 0.1 amole (attomole) (6×10^4 molecules). For one-round PCR assays, it was between 100 and 25,000 molecules. Semi-nested assays were more sensitive, decreasing the limit of detection 25-150 times, to reach 10 to 1000 plasmid molecules.

Twenty HBsAg$^+$ samples were tested in this study (Table 2). Samples Ac1 to Ac10 were from Acre state (high HBV prevalence region) while samples SC1 to SC10 were from Santa Catarina state (low HBV prevalence region). All had undetectable levels of HBsAg and anti-HBc IgM and only three (A1, A2, and A6) were anti-HBs$^+$. The anti-HBc titers of all samples were low (Table 2, column 2), between 1:320 and 1:1.

HBV DNA was detected by PCR in more than 85% of HBsAg$^+$ samples, regardless of the PCR assay (Table 2). Two negative control serum samples from individuals without HBV serological marker used in each series of DNA extraction/amplification did not show any DNA band in any PCR assay (data not shown). When subjected to PCR with oligonucleotide pairs PS1-PS2 and PS1-S2, all 20 HBsAg$^+$ samples were found negative for HBV DNA. However, semi-nested PCR (PS4-S2) following PCR (PS1-S2) allowed to detect HBV DNA in seven (35%) samples, two of them being anti-HBs$^+$/anti-HBc$^+$ and other five anti-HBc$^+$ only. One anti-HBs$^+$/anti-HBc$^+$ sample generated a visible DNA band with oligonucleotide pair C1-PS2. Semi-nested PCR (PS1-PS2) permitted to detect DNA in one more sample (anti-HBc$^+$ only). Moreover, four more samples, Ac2, Ac3, SC4, and SC7, gave a DNA band of unexpected molecular mass on agarose gel. Ac2, SC4, and SC7 gave a DNA band of higher molecular mass whereas DNA produced from sample Ac3 was of lower size. When transferred by Southern blot and hybridized against a HBV DNA probe, those DNAs of unexpected size did not present any positive hybridization signal (data not shown). Last, the largest number of positive samples (ten of twenty, 50%) was reached with primer pair C1-C2. Notably, two of three anti-HBs$^+$/anti-HBc$^+$ samples were also HBV DNA-positive.

Discussion

Despite the progress made in the last years, post-transfusion hepatitis remains a significant health care problem in the world. HBV DNA has been detected in anti-HBs$^+$ sera (Coursaget *et al.*, 1991; Loriot *et al.*, 1992) and even in individuals without any HBV serological marker (Luo *et al.*, 1992). Persistent isolated anti-HBc reactivity may be a relatively common serological pattern for hepatitis B occult infection, at least in patients with chronic liver disease (Sanchez Quijano *et al.*, 1993). To evaluate the potential infectivity of blood from individuals with anti-HBc as the unique serological HBV infection marker, the presence of HBV DNA in the serum of such individuals has been investigated by PCR. Several studies, both in developed countries and in third world, have indicated a large percentage of DNA positivity. For example, amon 153 Swiss blood donors presenting a serological status as mentioned above, 39% were DNA-positive (Joller-Jemelka *et al.*, 1994). The fact that nine of ten "anti-HBc alone" individuals were HBV DNA-positive in Venezuela (Zabaleta *et al.*, 1992) led the authors to suggest that anti-HBc screening should be maintained and expanded to all the blood banks of less industrialized countries where the rate of HBV infection in apparently healthy people tends to be high. In other studies, the percentage of HBV DNA-positive samples was lower (Iizuka *et al.*, 1992; Kroes *et al.*, 1991; Liang *et al.*, 1994; Pao *et al.*, 1991; Wang *et al.*, 1991). In inquiries made in the United States (Douglas *et al.*, 1993) and Canada (Scully *et al.*, 1994), no HBV DNA-positive sample had been found, suggesting that the addition of anti-HBc testing for all blood

donors for detection of low level HBV replication would not be indicated in those countries. Such discrepancies may not only be due to differences in the studied populations, but also to technical factors, such as DNA extraction methods, experimental conditions of PCR, and choice of the oligonucleotides used as primers.

Although it is estimated that more than ten milion people are HBV-carriers in Latin America (Fay, 1994), few data are available on this area. In this study, twenty samples, seventeen of them isolated anti-HBc, from blood donors of two geographically distant states of Brazil were investigated. Ten samples were from Acre, which is a high HBV prevalence state of the Amazon region located in the north of Brazil, and ten samples were from Santa Catarina, a low prevalence state in the south of Brazil. Two methods of DNA extraction from serum were used and the phenol/chloroform method was found to be more efficient. Although a previous study has shown that HBV DNA could be detected by PCR using a simplified DNA extraction procedure without phenol (Norder *et al.,* 1990), our results corroborate another report showing that the phenol/chloroform stage was essential for the removal of nucleases and polymerase inhibitors present in sera (Nicholson *et al.,* 1992). Use of phenol may be indispensable for detection of very low amounts of DNA.

Several oligonucleotide pairs were used in six different PCR assays. These oligonucleotides were designed from conserved segments of the HBV genome and all had a high sensitivity in the detection of plasmid templates. Although all samples had a low titer of anti-HBc, a number of them were found DNA-positive. However, the ratio of positivity varied greatly, from 0 to 50%, depending upon the PCR assay. This showed that a number of samples contained HBV DNA at a concentration close to the limit of detection. As expected, the number of samples found positive with the semi-nested PCR was higher than that found with the one-round PCR. The largest level of positivity ratio was obtained using oligonucleotides from the HBV core region. No significant difference was observed between the positivity ratio of samples from the states of Acre and Santa Catarina.

Acknowledgements. We are grateful to Ms L. Lewis for critical reading of the manuscript. This work was supported by grant No. 10/4662-0 from the Banco do Brasil and by CNPq.

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