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Simple and Rapid Identification of Low Level Hepatitis B Virus DNA by the Nested Polymerase Chain Reaction

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A rapid and sensitive method has been developed to detect hepatitis B virus DNA (HBV) by nested polymerase chain reaction (PCR) technique with primers specific for the surface and core regions in capillary thermal cycler within 80 min. The lower limit for detection by present PCR method is 10^{-5} pg of recombinant HBV DNA which is equivalent to that determined by one round of PCR amplification and Southern blot hybridization analysis. When boiled HBV positive serum was serially diluted 10-fold, HBV DNA was successfully determined in 1 μl~10^{-3} μl of serum. HBV DNA was detected by present method in 69 clinical samples including HBsAg positives and negatives by enzyme-linked immunosorbent assay (ELISA). When serum samples were amplified by nested PCR using surface and core region primers, HBV DNAs were detected in 37 of 69 samples (53.6%) and 18 of 69 samples (26.1%), respectively. These results can inform the infectious state of HBsAg positive pateints. A simple and rapid nested PCR protocol by using boiled serum as DNA template has been described for the clinical utility to determine HBV DNA in human serum.

Key words : Hepatitis B Virus, Nested PCR, Boiling of serum, Sensitive detection, Rapid identification

INTRODUCTION

The amplification of hepatitis B virus (HBV) DNA sequences in sera for molecular epidemiology of HBV is an important application of the polymerase chain reaction (PCR) with regard to HBV. Immunoassays for hepatitis B surface antigen (HBsAg) and hepatitis B envelop antigen (HBeAg) are unable to detect viral associated antigen at the concentration at the limit of infectivity: only few viruses per ml. One step PCR for HBV DNA can only detect 0.1 to 1 pg (2,500 to 25,000 viruses). While some HBsAg positive patients may not be actively producing virus and are not infectious, it is difficult to distinguish these individuals from patients who have low levels of viral replication. For the sensitive detection, previous investigations with PCR technique have shown to detect 10^{-5} pg of HBV DNA (Kaneko *et al*, 1989a and b) by one round of PCR amplification and Southern blot hydridization analysis. A single step DNA extraction procedure using guanidium thiocyanate and phenol as protein denaturants for the detection of specific HBV DNA is described (Manzin *et al.*, 1991). For the simple procedure, whole blood samples pretreated with anticoagulants, freeze and thawing have been used as HBV templates without further purification of DNA (Burckhardt, 1994). In this study, boiled serum without DNA extraction was used as HBV DNA template for PCR, and various combinations of primer sets in core and surface region were employed for nested PCR using capillary thermal cycler. The results demonstrate that the fast and sensitive nested PCR method for detecting HBV DNA in the sera of HBsAg positive patients and negative normal blood donors can be a possible tool for detecting the infectious state in clinical measurement.

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MATERIALS AND METHODS

Materials

Serum samples were obtained from Blood Bank (Daejon, Korea) and Korea Research Institute of Standards and Science. Out of 69 sera, 46 were positives for HBsAg by an enzyme-linked immunosorbent assay (ELISA) (Organon Teknika B.V., Boxtel, Holland). All sera were stored at -70°C. Oligonucleotide primers, specific for the most conserved surface (surface primers) and core gene sequences (core primers) of HBV genome were synthesized using a DNA synthesizer (Oligos Etc. Inc., USA). These were chosen

Table I. Oligonucleotide sequences used for detection of serum HBV DNA

Region	Primer	Sequence (5' to 3')	Nucleotide position[a]	Product size
surface				
	outer primers			532 bp
	BSO1	-GACAAGAATCCTCACAATACC-	93-113	
	BSO2	-ACCACATCATCCATATAACTGAAAG-	625-601	
	inner primers			297 bp
	BSI1	-ACCTCCAATCACTCACCAACC-	196-216	
	BSI2	-AGGATGATGGGATGGGAATA-	493-474	
	probe			
	BSPB	-GTGTCTGCGGCGTTTTATCAT-	252-272	
core				
	BCO1	-TCTCTTGTTCATGTCCTACTGTTC-	1714-1737	
	BCO2	-AAGCTGGAGGAGTGCGAATC-	2166-2147	
	BCO3	-AAAAGACACCAAATACTCTAGAA-	2138-2116	
	BCO4	-TCGTCGTCTAACAACAGTAGT-	2228-2208	
	BCI1	-TTACTCTCTTTTTTGCCTTCTGAC-	1815-1838	
	BCI2	-AGACAAGAAATGTGAAACCAC-	2095-2075	

[a]Nucleotide positions are numbered as A. Fujiyama *et al.* (3).

by comparison with available nucleotide sequences from Hepadnaviruses (subtype *adr, adw,* and *ayw*) in order to define different sets of conserved DNA sequences to be use as primers (Table I).

DNA preparation by boiling method

For obtaining the template DNA from human serum, 20 μl of serum was boiled at 94°C for 3 min, cooled rapidly in ice, and diluted with 40 μl of sterile water. After centrifugation at 12,000 rpm for 5 min, the supernatant was employed as DNA template. The 1st PCR amplifications were carried out in 10 μl reaction mixtures containing 3 μl of template, 200 μM each dNTP, 0.5 μM each outer primer, 0.4 U of *Taq* DNA polymerase and 1x RT-PCR buffer (10x buffer consists of 500 mM Tris-HCl (pH 8.3), 200 mM KCl, 30 mM $MgCl_2$, 5% dimethylsulfoxide, and 2.5 mg/ml bovine serum albumin). The reactions were performed in FTC 2000 capillary thermal cycler (Daehan Medical Co., Korea) which was developed for use in clinical application because of its fast PCR reaction (30~35 min for 30 cycles) using glass capillary with air flow system without a mineral oil overlay. The 1st PCR reaction proceeded with 30 cycles at 94°C for 5 s, 50°C for 10 s and 72°C for 15 s. The 2nd PCR amplifications (30 cycles) were carried out in 10 μl reaction mixtures containing 1 μl of the 1st PCR product, 200 μM each dNTP, 1.0 μM each inner primer and 0.4 U of *Taq* DNA polymerase. The PCR cycling conditions were same as those described in 1st PCR. Recombinant HBV DNA was used as positive control for PCR amplificaiton and for establishing the lower detection limit of the assay. Recombinant plasmid pHBV-315 containing cloned HBV gene was a gift from Dr. M.H. Yu (Korea Research Institute of Bioscience & Biotechnology, Korea) (Kim and Kang, 1984). HBV DNA was isolated from pHBV-315 by digestion with *Bam*HI followed by agarose gel electrophoresis and electroelution. For 2nd PCR, a 1 μl portion of the 1st PCR product was amplified as described in above boiling method. To eliminate sources of DNA contamination, reagents were all aliquoted and stored in new disposable containers. All experiments were performed in parallel with serum samples from positive, negative, and the reagent control without DNA.

Conventional DNA preparation method

To obtain the template DNA, 100 μl of human serum were incubated at 70°C for 3 hr in the presence of proteinase K (100 μg/ml), 0.5% sodium dodecyl sulfate (sos), 5 mM EDTA, and 10 mM Tris-HCl (pH 8.0). The solution was phenol/chloroform extracted, and the DNA was precipitated with ethanol in the presence of ammonium acetate (2.5 M) at -20°C for overnight. The precipitate was dissolved in 100 μl of sterile distilled water. The PCR amplifications were carried out in 10 μl reaction mixtures containing 5 μl of template. The other conditions were same as described in above boiling method.

Analysis of amplified DNA

For each sample, a 5 μl aliquot of the amplified DNA reaction mixture was analyzed by ethidium bromide staining after separation on 2% agarose gel electrophoresis. The specificity of the amplified bands was confirmed by Southern blot hybridization analysis using a [γ-^{32}P] labeled BSPB probe. The DNA transferred to a nitrocellulose membrane was prehy-

bridized in 6x SSC (1x SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/5x Denhardt's solution/0.5% SDS containing denatured, fragmented salmon sperm DNA (100 μg/ml) at 42°C for 4 hr. The prehybridized filter was hybridized with labeled BSPB probe (10^9 cpm/μg) at 42°C for overnight. After hybridization, the membrane filter was washed twice in 2x SSC/0.1% SDS for 5 min at room temperature, two times in 0.1x SSC/0.1% SDS at 42°C for 30 min, and then two times in 0.1x SSC/0.1% SDS at 65°C for 5 min per wash. The filter was exposed for overnight at -70°C with X-ray film for autoradiography.

RESULTS

The Sensitivity of the nested PCR technique

The detection limit of HBV DNA was determined after PCR amplificaion using 10 fold serial dilutions of recombinant HBV DNA. First PCR products were separated by agarose gel electrophoresis and visualized under UV light by ethidium bromide fluorescence. Samples containing an initial quantity of recombinant HBV DNA being $\geq 10^{-2}$ pg produced a visible DNA band of expected size (532 base pairs, bp) (Fig. 1A). When 1 μl of each amplified reaction mixture was reamplified with inner primers, a DNA band of 297 bp was detected in samples containing $\geq 10^{-5}$ pg of HBV DNA before the 1st PCR amplification reaction (Fig. 1C). On the other hand, the 1st PCR products transferred to a nitrocellulose paper after agarose gel electrophoresis and ethidium bromide staining were hybridized to a [γ-^{32}P] labeled HBV DNA probe (BSPB) to compare the sensitivity of 1st PCR-Southern blot hybridization with nested PCR. The 1st PCR-Southern blot hybridization analysis could detect 10^{-5} pg of HBV DNA in the original sample (Fig. 1B). Thus, the sensitivity of nested PCR is equivalent to that of 1st PCR-Southern blot hybridization.

When boiled HBV positive serum was serially diluted 10-fold, HBV DNA was successfully determined in 1 μl~10^{-3} μl of serum using either surface primers (Fig. 1) or core primers (Fig. 2). This indicates that the sensitivity using boiled serum as DNA template without DNA extraction allows the detection of HBV DNA in a wide range of concentration.

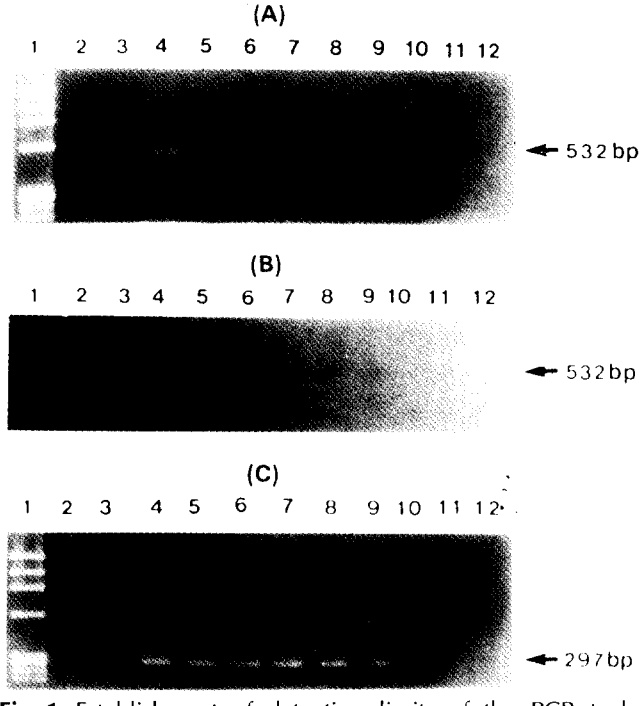

Fig. 1. Establishment of detection limits of the PCR technique with recombinant HBV DNA. Cloned HBV DNA were serially diluted and amplified with surface primers. The 1st PCR product with BSO1 and BSO2 was detected with (A) agarose gel electrophoresis and ethidium bromide staning or (B) Southern blot hybridization using [γ-^{32}P] labeled probe, and (C) nested PCR products with BSO1-BSO2/BSI1-BSI2 were detected with agarose gel electrophoresis and ethidium bromide staining. Lane 1, ΦX174 DNA/*Hae*III digest; lane 2, reagent control without DNA; lane 3, negative control; lane 4-12, serial 10-fold dilution of recombinant DNA ranging from 1 pg to 10^{-8} pg DNA, respectively

Detection of HBV DNA amplified from serum samples

HBV DNA was detected by nested PCR using boiled serum in 69 clinical samples including HBsAg negatives (23 samples), and positives (46 samples) by ELISA test. Negative control sample and reagent control (no nucleic acid) were included in each set of amplification reaction to test the contamination (Fig. 3).

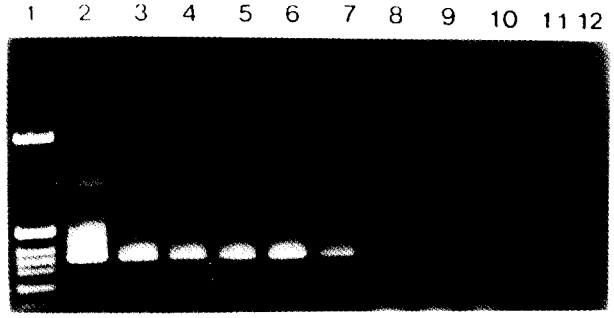

Fig. 2. HBV DNA detected with nested PCR using BCO1-BCO4/BCI1-BCO2 when the various amounts of boiled HBV positive serum were used as DNA template after dilution with sterile distilled water. A DNA band of 351 bp was detected in samples containing $\geq 10^{-3}$ μl of boiled serum. Lane 1, pBR322/*Hin*fI digest; lane 2, 1 μl serum; lane 3, 0.5 μl serum; lane 4, 0.2 μl serum; lane 5-12, serial 10-fold dilutions of boiled serum ranging from 10-1 to 10^{-8} μl, respectively

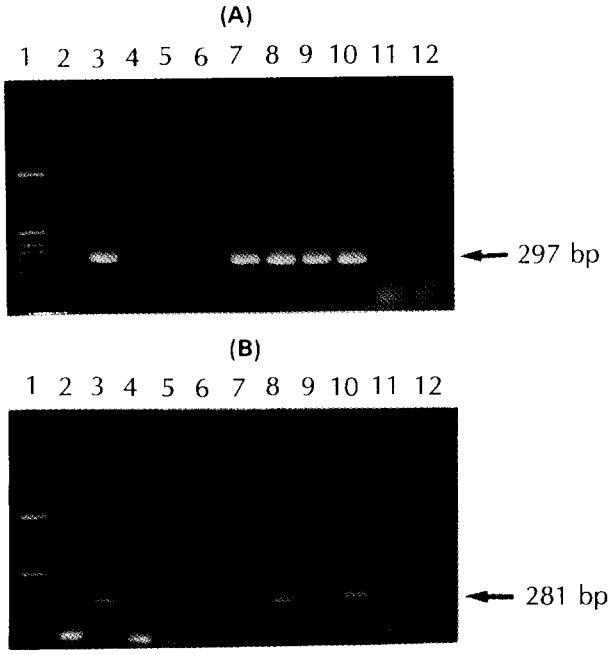

Fig. 3. Detection of nested PCR products from 3 μl of boiled serum from HBsAg positive or negative serums using surface primers (A) and core primers (BCO1-BCO2/BCI1-BCI2) (B). Lane 1, pBR322/*Hin*fI digest; lane 2, reagent control without DNA template; lane 3, positive control serum; lane 4-6, HBsAg negative serums; lane 7-11, HBsAg positive serums; lane 12, negative control serum

HBV DNAs amplified using surface primers (Fig. 3A) were detected by PCR in 4 of 23 HBsAg negative samples (17.4%), 33 of 46 positive samples (71.7%). On the other hand, HBV DNAs amplified using core primers (Fig. 3B) were detected by PCR in 3 of 23 HBsAg negative samples (13.0%) and 15 of 46 positive samples (32.6%). Table II summarizes the results using various primer sets. When HBV DNA extracted with conventional method was detected by nested PCR in 69 clinical samples, the results were well agreed with those of the HBV DNA prepared by boiling method (data not shown).

DISCUSSION

Application of the nested PCR technique requires the selection of well conserved target sequences. Target sequences of the surface and core regions in HBV DNA were amplified with air-heated capillary based thermal cycler, FTC 2000. FTC 2000 has several advantages over the pre-existed thermal cyclers, i.e., 10-fold shorter thermal cycling time with hot air system (30~35 min *versus* 2~4 hr) and reducing the amounts of PCR reaction components (including primers, each dNTP, *Taq* DNA polymerase, *etc*) because of its small reaction volume. Use of shorter annealing and ramping times increase the specificity of priming and therefore raise the overall quality of the PCR reaction. The

Table II. Detection of serum HBV DNA by different primers

Serum sample number	HBsAg	Primer to :Surface gene BSO1-BSO2/ BSI1-BSI2	BCO1-BCO2/ BCI1-BCI2	Core gene BCO1-BCO3/ BCI1-BCI2	BCO1-BCO4/ BCI1-BCO2
1	+	+	-	-	-
2	+	+	-	-	-
3	+	+	+	+	+
4	+	+	+	+	+
5	+	+	+	+	+
6	+	+	+	+	+
7	+	+	-	-	-
8	+	+	-	-	-
9	+	+	-	-	-
10	+	+	-	-	-
11	+	+	-	-	-
12	+	+	-	-	-
13	+	+	+	+	+
14	+	+	-	-	-
15	+	+	+	+	+
16	+	+	+	+	+
17	+	+	-	-	-
18	+	+	+	+	+
19	+	+	+	+	+
20	+	+	-	-	-
21	+	+	+	+	+
22	+	+	-	-	-
23	+	+	-	-	-
24	+	+	-	-	-
25	+	+	-	-	-
26	+	+	-	-	-
27	+	+	+	+	+
28	+	-	+	+	+
29	+	-	+	+	+
30	+	-	+	+	+
31	+	-	+	+	+
32	+	-	-	-	-
33	+	-	-	-	-
34	+	-	-	-	-
35	+	-	-	-	-
36	+	-	-	-	-
37	+	-	-	-	-
38	+	-	-	-	-
39	+	-	-	-	-
40	+	-	-	-	-
41	+	-	-	-	-
42	+	-	-	-	-
43	+	-	-	-	-
44	+	-	-	-	-
45	+	-	-	-	-
46	+	-	-	-	-
47	-	-	-	-	-
48	-	-	-	-	-
49	-	-	-	-	-
50	-	-	-	-	-
51	-	-	-	-	-
52	-	-	-	-	-
53	-	-	-	-	-
54	-	-	-	-	-
55	-	-	-	-	-
56	-	+	+	+	+
57	-	+	+	+	+
58	-	+	+	+	+
59	-	-	-	-	-
60	-	-	-	-	-
61	-	-	-	-	-
62	-	-	-	-	-
63	-	-	-	-	-
64	-	+	-	-	-
65	-	-	-	-	-
66	-	-	-	-	-
67	-	-	-	-	-
68	-	-	-	-	-
69	-	-	-	-	-

NOTE: +, positive result; -, negative result

lower limit of detection of recombinant HBV DNA was determined as 10^{-5} pg by nested PCR with surface primers. This sensitivity is equivalent to that by detection the 1st PCR product with radio labeled Southern blot hybridization (Kaneko *et al.*, 1989a and b).

HBV DNA in 1 μl~10^{-3} μl of serum prepared by present boiling method were amplified using either surface or core region primers. The detection limit of HBV DNA in 10^{-3} μl serum corresponding to the 10^{-5} pg was determined with recombinant HBV DNA.

When serum samples were amplified using surface and core region primers, HBV DNA were detected in 37 of 69 tested samples (53.6%) and 18 of 69 tested samples (26.1%), respectively. Not all HBsAg positive serums are HBV DNA positives similar to those reported by others (Zeldis *et al.*, 1989; Liang *et al.*, 1989; Zaaijer *et al.*, 1994; Scully *et al.*, 1994). On the other hand, HBV DNA detection (percentage of positives) using core primers are relatively low compared with those of surface primers as shown in Table II. This discrepancy might be caused either by a lower rate of viral replication leading to a reduced number of circulating HBV DNA or modifications of the viral genome (Chemin *et al*, 1991; Okamoto *et al.*, 1990; Wright *et al.*, 1992; Liang *et al.*, 1991; Omata *et al*, 1991; Persing *et al.*, 1986; Koh *et al.*, 1995) rather than the problem of the sensitivity (both 10^{-3} μl of serum). The core region primer sets BCO1-BCO2/BCI1-BCI2, were used initially and two other core region primers, BCO3 and BCO4, covering a wide range of the core gene were employed to solve this difference. By adopting various combination of six core region primers as shown in Table II, the identical results for 69 serum samples were acquired. The deviations between serological method and nested PCR method using surface and core region primers can be explained by further investigation on the correlation between HBV serological markers (anti-HBs, anti-HBc, HBeAg, Anti-HBe, etc) and clinical traits of the HBV infection as a function of infectious stage, and the nucleotide sequence determination. In this respect, primers used in this experiments can be utilized for differentiating HBV genotypes (Norder *et al.*, 1990). The results demonstrate that HBV DNA detection by nested PCR should be done for the different regions on the HBV viral genome, for example, surface and core region, to obtain the information on the infectious state of HBV similar as those of the serological determination of HBV infection.

Despite its powerful technique as a diagnostic tool for clinical samples, a major problem in PCR is the ease of contaminating target sequences from samples and reagents by carryover in PCR (Kwok and Higuchi, 1989). The precaution should be exercised to control contamination risks. The reagent control and negative control were included in each set of PCR amplification. When performing nested PCR with surface and core region primers, contaminations by carryover were not occured in the various conditions of MATERIALS AND METHODS. Nested PCR eliminating the carryover is a sensitive method to detect low level of HBV DNA (few viruses). Rapid protocols of nested PCR for the clinical laboratory are possible to adopt air-heated capillary thermal cycler (FTC 2000) and to use boiled serum as DNA template without DNA extraction. The rapidity and high sensitivity of the nested PCR using various primer sets for detecting HBV DNA in clinical samples should facilitate diagnostic assays for detecting viral DNA in various infectious conditions and may give information on the biology of HBV.

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