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# Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay

**(hepadnavirus/hybridization)**

SHUICHI KANEKO*, ROGER H. MILLER*, STEPHEN M. FEINSTONE*, MASASHI UNOURA†, KENICHI KOBAYASHI†, NOBU HATTORI†, AND ROBERT H. PURCELL*

*Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, MD 20892; and †First Department of Internal Medicine, Kanazawa University, Kanazawa, Japan

*Contributed by Robert H. Purcell, August 30, 1988*

ABSTRACT **We compared the sensitivity of the polymerase chain-reaction (PCR) assay to that of slot-blot hybridization for detecting hepatitis B virus (HBV) DNA in the serum of 31 patients with chronic hepatitis. Of 14 chronic hepatitis patients positive for both HBV surface and HBV e antigens, 9 were positive for HBV DNA by slot-blot hybridization and all 14 by PCR. Also, of 9 patients positive for HBV surface antigen and antibody against HBV e antigen, 2 were positive for HBV DNA by slot-blot analysis and 8 by PCR. Finally, in 8 patients positive for antibody against HBV surface antigen, none were positive for HBV DNA by slot-blot hybridization, but 4 were positive by PCR. We find that analysis by the PCR technique provides a $>10^4$-fold increase in sensitivity over the slot-blot hybridization assay. This result represents an important breakthrough in sensitivity because it is now possible to detect as few as three HBV DNA genomes per sample of serum.**

The polymerase chain reaction (PCR) technique is a method for amplifying nucleic acid by repeated cycles of high-temperature template denaturation, oligonucleotide primer hybridization, and polymerase extension. The thermostable polymerase of *Thermus aquaticus* (*Taq*) is often used because it can withstand the high denaturation temperatures without loss of enzymatic activity. Previous investigations with this technique have shown amplification of $>10^5$-fold over the input quantity of nucleic acid (1, 2). We have used PCR amplification, combined with agarose gel electrophoresis and Southern blot hybridization analysis, to detect extremely small quantities of hepatitis B virus (HBV) DNA in the serum of chronic hepatitis patients.

## MATERIALS AND METHODS

**Patient Population.** A group of 31 patients with chronic hepatitis were admitted to Kanazawa University Hospital (Kanazawa, Japan). Group A consisted of 14 patients with chronic hepatitis whose sera contained HBV surface antigen (HBsAg), antibody to HBV core antigen (anti-HBc), and HBV e antigen (HBeAg) for at least 1 yr. Group B consisted of 9 patients with chronic hepatitis whose sera tested positive for HBsAg, anti-HBc, and antibody against HBeAg (anti-HBe) for at least 6 months. Group C consisted of 8 patients with chronic hepatitis whose sera were positive for antibody against HBsAg (anti-HBs) (Table 1).

**Serological Methods.** Serological assays for HBV markers (HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe) were performed by RIA using commercially available reagent kits (Ausria II for HBsAg, Ausab for anti-HBs, and Corab for anti-HBc and HBeAg/anti-HBe, Abbott). The anti-HBc titer was considered negative when the percent inhibition in the Corab assay was <30%.

**Preparation of DNA Samples.** One hundred microliters of serum was incubated at 70°C for 3 hr in proteinase K (100 μg/ml)/0.5% $NaDodSO_4$/5 mM EDTA/10 mM Tris·HCl, pH 8. The solution was phenol/chloroform extracted, and the DNA was precipitated with ethanol in the presence of carrier tRNA (100 μg/ml). The precipitate was dissolved in 100 μl of 10 mM Tris·HCl, pH 8/1 mM EDTA.

**PCR Amplification of Serum DNA.** Oligonucleotide primers, specific for HBV core gene sequences, were synthesized on a 380A DNA synthesizer (Applied Biosystems, Foster City, CA) by the methoxyphosphoramidite method. Primer 1763, 5′-GCTTTGGGGCATGGACATTGACCCGTATAA-3′, begins at map position 1763, and primer 2032R [from the complementary, or reverse (R), DNA strand], 5′-CTGAC-TACTAATTCCCTGGATGCTGGGTCT-3′, begins at map position 2032 of the HBV genome (3). Amplification using the *Taq* polymerase was performed according to a modification of the procedure described by Saiki *et al.* (1). Briefly, target sequences were amplified in a 100-μl reaction volume containing 10 μl of the serum DNA sample, 2.5 units of *Taq* polymerase (Perkin–Elmer Cetus), 200 μM each dNTP, 1 μM each primer (primer 1763, primer 2032R), 50 mM KCl, 10 mM Tris·HCl (pH 8.3), 1.5 mM $MgCl_2$, and 0.01% (wt/vol) gelatin. The reaction was performed for 30 cycles in a programmable DNA thermal cycler (Perkin–Elmer Cetus). Samples were heated to 94°C for 1.5 min (denaturation of DNA), cooled to 42°C for 1.5 min (hybridization to primer), and incubated for 3 min at 72°C (polymerase reaction). Contamination of laboratory equipment, material, samples, and reagents with minute amounts of recombinant HBV DNA represents a considerable problem because of the exquisite sensitivity of PCR analysis. To eliminate sources of DNA contamination, all reagents were prepared and stored in new disposable containers. Reagents were aliquoted and reaction mixtures were prepared with the use of disposable syringes (Combitips, Eppendorf) and pipets (Microman, Gilson). All reagents were assayed for the presence of HBV DNA, and all experiments were performed in parallel with positive and negative control sera. Authenticity of positive signals was confirmed by DNA sequence analysis.

**Analysis of Amplified DNA.** For each sample, a 25-μl aliquot of the amplified DNA reaction mixture was fractionated by 2.0% agarose gel electrophoresis, and DNA was visualized by UV fluorescence after staining with ethidium bromide (PCR-EB). The DNA was transferred to a Hybond-

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Abbreviations: HBV, hepatitis B virus; PCR, polymerase chain reaction; PCR-EB, polymerase chain reaction-ethidium bromide staining; PCR-SBH, polymerase chain reaction-Southern blot hybridization; HBsAg, HBeAg, and HBcAg, HBV surface, e, and core antigens, respectively; the respective antibodies are anti-HBs, anti-HBe, and anti-HBc.

N nylon membrane (Amersham) for Southern blot-hybridization analysis (PCR-SBH). The membranes were prehybridized in 50% (vol/vol) formamide/5× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate)/2.5× Denhardt's solution (1× Denhardt's solution is 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin)/0.1% $NaDodSO_4$/1 mM EDTA/5 mM $NaH_2PO_4$ containing denatured, sonicated calf thymus DNA (200 μg/ml) at 42°C for 4 hr (4, 5). Hybridization, with recombinant HBV DNA labeled to high specific activity ($2–4 \times 10^8$ cpm/μg), was performed in the above buffer at 42°C for 24 hr. After hybridization, membranes were washed twice in 1× SSC and 0.1% $NaDodSO_4$ for 5 min at room temperature and three times in 0.1× SSC/0.1% $NaDodSO_4$ at 65°C for 30 min per wash. Membranes were exposed to x-ray film (XAR, Kodak) for autoradiography. For slot-blot hybridization, 2.5 μl of serum DNA was denatured with 0.1 M NaOH in a final volume of 20 μl for 5 min, mixed with 180 μl of 1.0 M ammonium acetate, and the DNA was fixed to a nylon membrane. Hybridization analysis was as described above.

## RESULTS AND DISCUSSION

**Establishing the Sensitivity of the PCR Technique by Amplification of Recombinant HBV DNA.** The presence of serum HBV DNA in chronic hepatitis patients indicates active virus replication. The most sensitive method for detecting serum HBV DNA currently in use is molecular hybridization analysis. Slot-, or dot-, blot hybridization analysis can detect <0.1 pg of HBV DNA (6–8). However, PCR amplification of virus DNA is a more sensitive assay, potentially, than molecular hybridization. The PCR assay amplifies nucleic acid sequences $>10^5$-fold with the use of specific oligonucleotide primers. Therefore, PCR amplification, coupled with molecular hybridization analysis, is theoretically capable of detecting attogram ($10^{-18}$ g) levels of HBV DNA in the original sample.

To determine the lower limit of detection of HBV DNA in our experiments, 10-fold serial dilutions of cloned HBV DNA were analyzed by PCR with oligonucleotide primers specific for the core gene sequence of HBV. After PCR amplification, DNA was fractionated by agarose-gel electrophoresis and visualized under UV light by ethidium bromide fluorescence (the PCR-EB technique). Samples containing an initial quantity of $\geq 10^{-2}$ pg of recombinant HBV DNA produced a visible DNA band of expected size [i.e., 270 base pair (bp)] by ethidium bromide fluorescence (Fig. 1*A*, lanes 2–4). Thus, the sensitivity of the PCR-EB assay was 10-fold greater than the slot-blot hybridization assay for detecting HBV DNA. In addition, because PCR-EB analysis is faster and potentially more convenient than molecular hybridization, it may become useful for routine clinical diagnosis of virus DNA.

The sensitivity of PCR-SBH was found to be ≈1000-fold greater than that of PCR-EB for detecting HBV DNA. After gel electrophoresis and ethidium bromide staining, PCR-amplified HBV DNA was transferred to a nylon membrane and hybridized to a $^{32}$P-labeled HBV DNA probe (Fig. 1*B*). PCR-SBH analysis could detect as little as $10^{-5}$ pg (10 ag) of HBV DNA in the original sample (Fig. 1*B*, lane 7). Overall, we find that the recombinant HBV DNA in the samples was amplified $>10^5$-fold, which is in good agreement with the levels of amplification achieved by others (1, 2) and that PCR-SBH was $10^4$-fold more sensitive than slot-blot hybridization for detecting HBV DNA.

**Detection of HBV DNA in the Serum of Patients with Chronic Hepatitis Positive for HBsAg, Anti-HBc, and HBeAg.** The presence of HBsAg in serum, detected by RIA, is used to identify the chronic carrier state of HBV infection. In the past, endogenous DNA polymerase activity in serum was indicative of active virus replication in the host. Recently, slot-blot hybridization analysis, because of its greater sensitivity, has replaced the endogenous DNA polymerase reaction as the assay of choice for detecting serum HBV DNA. Analysis of sera by slot-blot hybridization has demonstrated that most, but not all, sera positive for HBeAg and HBsAg contain HBV DNA (6–15). In our study, the sera of 14 chronic hepatitis patients positive for HBeAg and HBsAg were assayed for the presence of HBV DNA by slot-blot hybridization and by PCR-EB and PCR-SBH analysis (Table 1). Although only 9/14 (64%) sera tested positive for virus DNA by slot-blot hybridization, 13/14 (93%), and 14/14 (100%) sera tested positive for HBV DNA by PCR-EB and PCR-SBH, respectively (Fig. 2). Therefore, PCR-SBH analysis was able to detect HBV DNA in all sera that were positive for HBeAg and HBsAg.

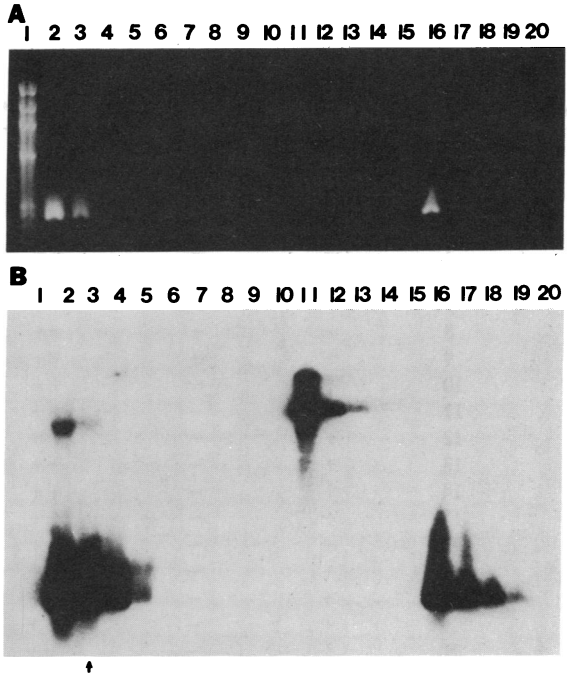


FIG. 1. Establishing the detection limits of the PCR technique with recombinant HBV DNA. Serial 10-fold dilutions of cloned HBV DNA, ranging from 1 pg to $10^{-7}$ pg, were amplified by PCR using primers 1763 and 2032R. (*A*) PCR-EB analysis. A 25-μl aliquot from a 100-μl amplification reaction mixture was fractionated by agarose-gel electrophoresis, and the nucleic acid was detected by ethidium bromide staining and visualization under UV light. DNA bands with an electrophoretic mobility of 270 bp, the distance between primers 1763 and 2032R, were seen in the amplified mixtures with the following initial amounts of recombinant HBV DNA: 1 pg (lane 2), $10^{-1}$ pg (lane 3), and $10^{-2}$ pg (lane 4). It should be noted that some positive signals are visible only in the original photograph or autoradiogram and may not be visible in figures due to a loss of sensitivity during photographic reproduction of the data. Also shown are serial dilutions of amplified linear recombinant HBV DNA: 100 ng (lane 16), 10 ng (lane 17), 1 ng (lane 18), 100 pg (lane 19), and 10 pg (lane 20). The bands are 270 and 2728 bp in size. The 2728-bp band arises due to amplification of sequences beyond the limits of one primer (i.e., from one primer to the end of the linear molecule) and is detected when relatively large amounts of DNA are amplified. A $M_r$ standard φX174 (*Hae* III digest) is shown in lane 1. (*B*) Autoradiogram from PCR-SBH analysis. The gel shown in *A* was transferred to a nylon membrane and hybridized with a $^{32}$P-labeled HBV DNA probe. A 1-day exposure of the blot permits detection of HBV DNA sequences in the $10^{-3}$ pg (lane 5), $10^{-4}$ pg (lane 6), and $10^{-5}$ pg (lane 7) samples (visible in the original autoradiogram). In our analysis, the $10^{-6}$ pg (lane 8) and $10^{-7}$ pg (lane 9) dilutions of HBV DNA did not score as positive, even after long exposure of the blot (i.e., >1 week). Also shown are the positive hybridization signals from serial dilutions of unamplified recombinant HBV DNA (i.e, 3214 bp): 1 ng (lane 11), 100 pg (lane 12), 10 pg (lane 13), and 1 pg (lane 14). The arrow depicts the detection limit of HBV DNA by slot-blot hybridization (i.e., 0.1 pg) after an exposure time of 3 days.

Table 1. Detection of serum HBV DNA by PCR analysis

| Group | Patient | HBsAg | Anti-HBs | Anti-HBc* | HBeAg | Anti-HBe | HBV DNA | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | Slot-blot hybridization | PCR-EB | PCR-SBH |
| A | 1 | + | − | ++ | + | − | ++ | ++ | +++ |
| | 2 | + | − | ++ | + | − | ++ | ++ | +++ |
| | 3 | + | − | ++ | + | − | + | ++ | +++ |
| | 4 | + | − | ++ | + | − | +/− | ++ | +++ |
| | 5 | + | − | ++ | + | − | ++ | ++ | +++ |
| | 6 | + | − | ++ | + | − | ++ | ++ | +++ |
| | 7 | + | − | ++ | + | − | ++ | ++ | +++ |
| | 8 | + | − | ++ | + | − | +/− | ++ | +++ |
| | 9 | + | − | ++ | + | − | +/− | ++ | +++ |
| | 10 | + | − | ++ | + | − | − | + | ++ |
| | 11 | + | − | ++ | + | − | − | + | ++ |
| | 12 | + | − | ++ | + | − | − | + | + |
| | 13 | + | − | ++ | + | − | − | + | ++ |
| | 14 | + | − | ++ | + | − | − | − | + |
| B | 15 | + | − | ++ | − | + | ++ | ++ | +++ |
| | 16 | + | − | ++ | − | + | +/− | ++ | +++ |
| | 17 | + | − | ++ | − | + | − | + | ++ |
| | 18 | + | − | ++ | − | + | − | − | + |
| | 19 | + | − | ++ | − | + | − | − | − |
| | 20 | + | − | ++ | − | + | − | − | + |
| | 21 | + | − | ++ | − | + | − | − | + |
| | 22 | + | − | ++ | − | + | − | − | + |
| | 23 | + | − | ++ | − | + | − | − | + |
| C | 24 | − | + | + | − | − | − | − | + |
| | 25 | − | + | + | − | + | − | − | + |
| | 26 | − | + | + | − | + | − | − | + |
| | 27 | − | + | + | − | + | − | − | − |
| | 28 | − | + | + | − | − | − | − | + |
| | 29 | − | + | + | − | − | − | − | − |
| | 30 | − | + | + | − | + | − | − | − |
| | 31 | − | + | + | − | + | − | − | − |

*Anti-HBc: ++, high titer; +, low titer.

**Detection of HBV DNA in the Serum of Patients with Chronic Hepatitis Positive for HBsAg, Anti-HBc, and Anti-HBe.** Seroconversion from HBeAg to anti-HBe during the course of acute hepatitis is usually accompanied by the resolution of clinical and biochemical evidence of liver disease and the loss of detectable serum and liver HBV DNA. On the other hand, HBV DNA is often detected in the liver of patients with chronic hepatitis (8, 10–14). In one study, 0/9 chronic carriers were positive for serum HBV DNA, and 4/9 were positive for HBV DNA in the liver (9). Thus, it is likely that circulating virions were present in at least 4/9 patients but were below the detection limit of current technology. Therefore the presence of anti-HBe in the serum of chronic hepatitis patients is not an accurate marker for cessation of virus replication in the host.

In our study, the sera of 9 chronic hepatitis patients positive for HBsAg, anti-HBc, and anti-HBe were analyzed for the presence of HBV DNA by the slot-blot hybridization and PCR techniques. Although only 2/9 (22%) patients tested positive for serum HBV DNA by slot-blot hybridization analysis, 3/9 (33%) and 8/9 (89%) tested positive by PCR-EB and PCR-SBH, respectively (Table 1). Thus, nearly all of the patients positive for HBsAg, anti-HBc, and anti-HBe in our study possessed circulating virus particles because we detected the presence of serum HBV DNA. This result suggests that seroconversion from HBeAg to anti-HBe can be accompanied by a decrease, but not a total disappearance, of virus particles in the serum.

With PCR-SBH nearly all (i.e., 22/23) of the HBsAg-positive chronic hepatitis patients in our study group possessed serum HBV DNA, irrespective of their HBeAg/anti-HBe status. This finding supports the hypothesis that HBV continues to replicate in the liver of HBsAg-positive patients after seroconversion to anti-HBe, and that complete virions, in addition to HBsAg particles, continue to be released into the serum. However, we found that 1/9 patients positive for HBsAg and anti-HBe lacked detectable serum HBV DNA. There are at least two explanations for this result: (*i*) Virus replication is occurring in tissue but the titer of virus in the serum is low, and the amount of HBV DNA present is below our detection limits. (*ii*) Virus replication may not be occurring with HBsAg expressed from HBV genomes that are integrated into the host genome, analogous to HBV DNA-containing hepatoma cell lines (16). Overall, 96% of all HBsAg-positive chronic hepatitis patients tested possessed serum HBV DNA that was detectable by PCR-SBH.

**Detection of HBV DNA in the Serum of Patients with Chronic Hepatitis Positive for Anti-HBs.** The presence of anti-HBs in the serum of chronic hepatitis patients indicates a previous HBV infection. In such patients, HBV DNA is rarely detected in the serum but is often found in liver tissue. One striking example is a study by Brechot *et al.* (17) in which only 1/19 anti-HBs-positive patients was found to possess serum HBV DNA, but 17/22 such patients were found to have virus DNA in the liver. In our study, the sera of 8 chronic hepatitis patients with anti-HBs were examined by PCR analysis. The patients were diagnosed as chronic active hepatitis (patients 25, 26, 28–30) or chronic persistent hepatitis (patients 24, 27, and 31) by histologic examination of liver tissue. The sera of all 8 patients tested negative for HBV DNA by slot-blot hybridization and PCR-EB analysis. However, 4/8 (50%) sera contained low levels of HBV DNA as

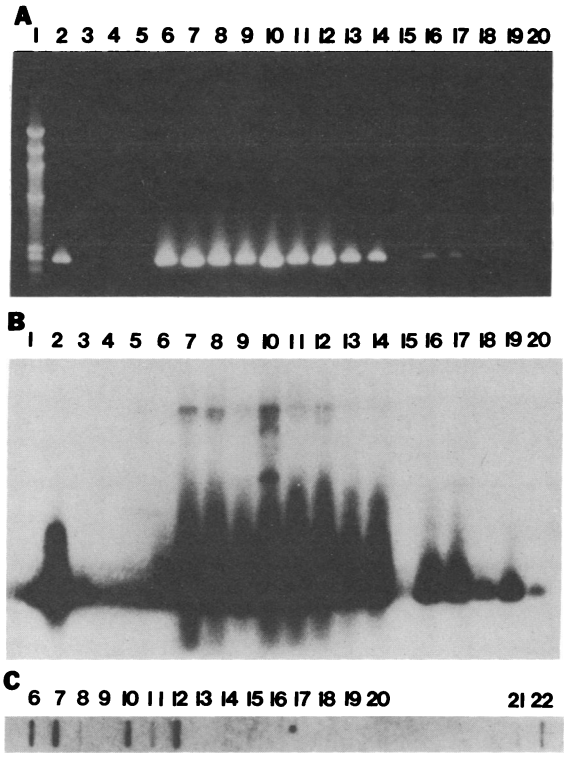


FIG. 2. HBV DNA in the serum of patients positive for HBsAg, anti-HBc, and HBeAg. The serum of patients 1–9 (lanes 6–14) and 10–14 (lanes 16–20) was analyzed for HBV DNA by PCR-EB and PCR-SBH. (*A*) PCR-EB analysis. DNA was extracted from 10 $\mu$l of serum and amplified by PCR as described. A 25-$\mu$l aliquot from the 100-$\mu$l reaction mixture was subjected to agarose-gel electrophoresis, and DNA was visualized under UV light after staining with ethidium bromide. DNA bands of 270 bp were seen for patients 1–9 (lanes 6–14). The intensity of the amplified DNA bands in these samples is rated as ++ (Table 1). DNA bands from patients 10–13 (lanes 16–19), with a weaker intensity of the amplified signal, are rated as + (Table 1). Serial dilutions of amplified 270-bp HBV DNA are shown: 100 ng (lane 2), 1 ng (lane 3), and 10 pg (lane 4). Lane 1 is the $M_r$ standard as described in the legend to Fig. 1. (*B*) PCR-SBH analysis. Southern blot hybridization was performed using the gel shown in *A*. The intensity of the hybridization is rated as +++ for the samples in lanes 6–14; as ++ for the samples in lanes 16, 17, and 19; and as + for the samples in lanes 18 and 20 (Table 1). (*C*) Slot-blot hybridization analysis of serum HBV DNA. A 2.5-$\mu$l aliquot of serum DNA was denatured and applied to a nylon membrane for analysis. Hybridization was performed as described for PCR-SBH. Autoradiography (3-day exposure) demonstrated the presence of HBV DNA in the serum of patients 1, 2, 3, 5, 6, and 7 rated + or ++ (Table 1). Patients 4, 8, and 9 exhibited a trace, rated as +/−, of virus DNA (Table 1). Also shown are positive controls of 0.1 pg (lane 21) and 1 pg (lane 22) of amplified 270-bp HBV DNA.

determined by PCR-SBH analysis (Table 1). Thus, using a method with high sensitivity, we found that 50% of the anti-HBs-positive chronic hepatitis patients had HBV DNA in their serum. This finding agrees with the observation that HBV replication continues in cells after seroconversion to anti-HBs.

**Authenticity of Positive Results.** Contamination of laboratory equipment and reagents with minute amounts of recombinant HBV DNA represents a possible source of false positive results because of the exquisite sensitivity of PCR analysis. Precautions were taken to eliminate DNA contamination (see *Materials and Methods*), all reagents were tested to verify the absence of HBV DNA, and all experiments were performed in parallel with positive and negative control sera. Authenticity of HBV DNA in patients was verified by DNA sequence analysis of selected amplified DNA sequences (Table 2). The amplified HBV DNA sequences from patient sera were significantly different from the recombinant HBV DNA used in our laboratory. The differences ranged from 2–15% over a nucleotide length of 198 bp. However, the DNA from one patient, patient 26, possessed a nucleotide sequence that was identical to the recombinant HBV DNA used in our laboratory and, therefore, may represent exogenous recombinant HBV DNA that was amplified in the serum sample. Overall, the sequences of HBV DNA amplified from the sera of chronic hepatitis patients were specific, which suggests that they did not arise from contaminating recombinant HBV DNA.

Table 2. Sequence analysis of PCR-amplified HBV DNA

| Group | Patient or sample | Changes per 198 bp (%) |
|---|---|---|
| A | 2 | 29 (15) |
| | 4 | 4 (2) |
| | 5 | 8 (4) |
| | 6 | 7 (4) |
| C | 24 | 22 (11) |
| | 25 | 3 (2) |
| | 26 | 0 (0) |
| pBRHBadr4 (cloned HBV DNA) | a | 0 (0) |
| | b | 0 (0) |
| | c | 0 (0) |
| | d | 0 (0) |

In summary, we find that 26/31 (84%) of the chronic hepatitis patients in our study possess serum HBV DNA that is detected by PCR analysis. This suggests that such patients continue to support active HBV replication. In addition, it is clear that serological analysis is not sufficient for predicting the course of chronic liver disease. PCR analysis offers several advantages over other methods for detecting serum HBV DNA. PCR-EB analysis is 10-fold more sensitive, can be performed faster, and, under stringent conditions, can be more specific than slot-blot hybridization analysis. Further, PCR-SBH offers even more advantages over slot-blot hybridization—it is $10^4$-fold more sensitive and is a more specific assay (i.e., because it uses hybridization of an amplified DNA fragment of specific size with a virus-specific radiolabeled probe). However, care must be exercised to eliminate sources of contaminating HBV DNA from samples and reagents. It is clear that the PCR technique represents a significant advance in analyzing serum samples because it is possible to detect HBV DNA isolated from samples containing only three virions. This technique should revolutionize diagnostic assays for detecting virus DNA and yield valuable information on the biology of HBV.

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