

Rapid and Sensitive Method for the Detection of Serum Hepatitis B Virus DNA Using the Polymerase Chain Reaction Technique

SHUICHI KANEKO, STEPHEN M. FEINSTONE, AND ROGER H. MILLER*

Hepatitis Viruses Section, Laboratory of Infectious Diseases, National Institute of Allergy and Infectious Diseases, Bethesda, Maryland 20892

Received 9 February 1989/Accepted 31 May 1989

We have developed a rapid procedure for the detection of serum hepatitis B virus (HBV) DNA using the polymerase chain reaction (PCR) technique. HBV DNA is released from virions by incubating serum with 0.1 M NaOH for 60 min at 37°C. The mixture is brought to neutral pH with HCl, and the HBV DNA sequences are detected by agarose gel electrophoresis and ethidium bromide staining after PCR amplification with two successive sets of primer pairs. The detection limit of this method (i.e., 10^{-5} pg of HBV DNA) is equivalent to that previously determined by one round of PCR amplification and Southern blot hybridization analysis. The advantages are that the assay can be completed in 1 day, is very sensitive, and does not require the use of radiolabeled reagents.

In the past, two methods were available for detecting the presence of hepatitis B virus (HBV) DNA in serum, the endogenous DNA polymerase reaction (2, 7) and dot or slot blot hybridization analysis (1, 4, 8-11). Of the two techniques, slot blot hybridization analysis was demonstrated to be the more sensitive and has been shown to be able to detect HBV DNA in amounts as low as approximately 0.1 pg, which corresponds to approximately 3×10^4 virus genomes. However, a new technique, the polymerase chain reaction (PCR) assay, has been shown recently to detect 10^{-5} pg of HBV DNA (6). Thus, PCR is the most sensitive assay currently available for detecting serum HBV DNA.

Extraction of virion HBV DNA by incubation of serum with proteinase K in the presence of sodium dodecyl sulfate followed by phenol-chloroform extraction and ethanol precipitation of DNA is a standard technique used for the isolation of virus sequences for hybridization analysis (1, 4-6). HBV DNA in the sample can also be detected by PCR analysis (6). Briefly, PCR is performed by using repeated rounds of primer annealing and elongation by a heat-stable polymerase (Taq polymerase). Amplified HBV DNA is then detected by agarose gel electrophoresis and ethidium bromide staining (PCR-EB) by visualization under ultraviolet light. Samples containing an initial quantity of 10^{-2} pg of HBV DNA can be detected by this method. However, even greater sensitivity is possible with a combination of PCR-EB and Southern blot hybridization analysis (PCR-SBH). After transfer of the amplified HBV DNA sequences to a nylon membrane and hybridization with a ^{32}P -labeled HBV DNA probe, 10^{-5} pg of HBV DNA can be detected from the original sample. Although PCR-SBH offers a tremendous increase in the ability to detect serum HBV DNA, it is time-consuming, involves several critical steps, and utilizes a radiolabeled probe that must be prepared frequently. Therefore, this procedure is unlikely to be used routinely in a diagnostic setting. Thus, we attempted to develop a rapid and sensitive method for detection of HBV DNA by PCR.

MATERIALS AND METHODS

Patient serum. Serum was obtained from eight patients with chronic hepatitis who were admitted to Kanazawa

University Hospital, Japan. Five patients (sera 1 through 5) had hepatitis B virus surface antigen (HBsAg), hepatitis B virus e antigen (HBeAg), and antibody against hepatitis B core antigen (anti-HBc) in their sera for more than 1 year. Three patients (sera 6 through 8) had HBsAg, anti-HBc, and antibody against hepatitis e antigen (anti-HBe) in their serum for more than 6 months. Serological determinations of HBV markers (HBsAg, anti-HBs, anti-HBc, HBeAg, and anti-HBe) were performed with radioimmunoassays by using commercially available reagent kits (Ausria II for HBsAg and Corab for anti-HBc and HBeAg/anti-HBe; Abbott Laboratories, North Chicago, Ill.).

Purification of virus DNA from serum particles. In the phenol-chloroform method, a 100- μl portion of serum was incubated at 70°C for 3 h in the presence of proteinase K (100 $\mu\text{g}/\text{ml}$), 0.5% sodium dodecyl sulfate, 5 mM EDTA, and 10 mM Tris (pH 8). The solution was phenol-chloroform extracted, and the DNA was precipitated with ethanol in the presence of carrier tRNA (100 $\mu\text{g}/\text{ml}$). The precipitate was dissolved in 100 μl of TE buffer (10 mM Tris [pH 8], 1 mM EDTA). In the NaOH extraction method, a 10- μl portion of serum was pipetted into a 0.5-ml microcentrifuge tube (Robbins Scientific) and incubated with NaOH at a final concentration of 0.1 M at 37°C for 60 min. The solution was centrifuged for 15 s in a microfuge and neutralized with HCl at a final concentration of 0.1 M.

Oligonucleotide primers. Oligonucleotide primers, specific for HBV DNA, were synthesized on a DNA synthesizer (no. 380A; Applied Biosystems) by the methoxy phosphoramidite method. Primer 1763, 5'-GCTTTGGGGCATGGACA TTGACCCGTATAA-3', and primer 109, 5'-ATACCACAG AGTCTAGACTCGTGGTGGACT-3', begin at map positions 1763 and 109 of the HBV genome (3), respectively. Primer 2032R, from the complementary or reverse (R) DNA strand, 5'-CTGACTACTAATTCCCTGGATGCTGGGTCT-3', and primer 585R, 5'-AAGCCCTACGAACCACTGAAC AAATGGCAC-3', begin at map positions 2032 and 585, respectively. Primer 1778-E, 5'-GACGAATTCCATTGACC CGTATAAAGAATT-3', begins at map position 1778 (15 base pairs [bp] 3' to the beginning of primer 1763) and was synthesized to contain an *EcoRI* recognition cleavage site at its 5' end. Primer 2017R-B, 5'-ATGGGATCCCTGGATGCT GGGTCTTCCAAA-3', begins at map position 2017 (15 bp 3'

* Corresponding author.

to the beginning of primer 2032R) and was synthesized to contain a *Bam*HI recognition cleavage site at its 5' end.

PCR amplification of HBV DNA. A 10- μ l sample of serum was treated with 0.1 M NaOH for 60 min at 37°C. The solution was brought to neutral pH by addition of HCl, and the sample was used directly for PCR amplification of HBV DNA sequences. The serum DNA sample was amplified in a 100- μ l reaction volume containing 2.5 U of Taq polymerase (Perkin Elmer Cetus), each of the four deoxynucleoside triphosphates at 200 μ M each, each primer pair (primers 1763 and 2032R, primers 109 and 585R, and primers 1778-E and 2017R-B) at 1 μ M each, 50 mM KCl, 10 mM Tris (pH 8.3), 1.5 mM MgCl₂, 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 (annealing to primer), and incubated for 3 min at 72°C (polymerase amplification reaction). Recombinant HBV DNA samples containing 1 to 10⁻⁷ pg were used as positive controls for PCR amplification and for establishing the lower detection limit of the assay. Recombinant pBRHBadr₄ DNA (3) was a generous gift of K. Matsubara, Osaka University, Japan. HBV DNA was isolated from plasmid vector sequences by digestion with restriction endonuclease *Bam*HI followed by agarose gel electrophoresis and electroelution. Negative serum and reagent controls were routinely included. For reamplification of the samples amplified with primers 1763 and 2032R, a 10- μ l portion of the primary amplification reaction was amplified as described above with primers 1778-E and 2017R-B (the PCR-PCR technique).

Analysis of amplified DNA. Amplified DNA reaction mixtures were centrifuged for 1 min in a microfuge. A 25- μ l sample of the 100- μ l reaction was fractionated by 2.0% agarose gel electrophoresis and visualized by UV fluorescence after staining with ethidium bromide (PCR-EB). The specificity of the amplified bands was confirmed by Southern blot hybridization using a radiolabeled HBV DNA probe (6).

RESULTS AND DISCUSSION

Preparation of virus DNA for PCR amplification by treatment of serum with NaOH. Patient serum 1, positive for HBsAg, HBeAg, and anti-HBc, was treated with various concentrations (0.05 to 0.2 N) of NaOH at 37°C for 60 min, and the HBV DNA was amplified in a PCR assay with primers 109 and 585R (see Materials and Methods). HBV DNA was fractionated by agarose gel electrophoresis, and DNA sequences were detected by UV fluorescence after ethidium bromide staining. The results demonstrated that PCR reaction mixtures from serum samples treated with NaOH concentrations of \geq 0.05 M contained a virus DNA band of expected size (i.e., 477 bp) that was visible by ethidium bromide staining. Overall, the sensitivity of the NaOH method compared favorably with that of the standard phenol-chloroform extraction method. The greatest sensitivity was observed when NaOH concentrations of 0.1 and 0.125 M were used. This finding is similar to that obtained by other investigators by using NaOH treatment of serum for detection of HBV DNA by dot blot hybridization analysis, in which virion DNA is readily detected after treatment of serum with NaOH concentrations of 0.1 to 1.0 M (8–11).

Effect of the length of time and incubation temperature on the efficiency of the NaOH extraction method. First, we held both the concentration of NaOH (i.e., 0.1 M) and the time of incubation (i.e., 60 min) constant and varied the temperature

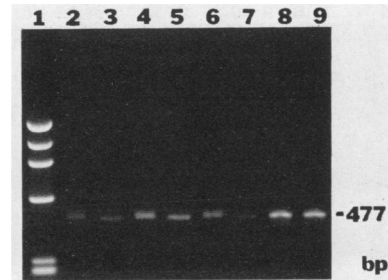


FIG. 1. Comparison of the effectiveness of two techniques for the extraction of serum HBV DNA for detection by PCR analysis. The NaOH (lanes 2, 4, 6, and 8) and phenol-chloroform (lanes 3, 5, 7, and 9) extraction methods were compared with serum samples 2 (lanes 2 and 3), 3 (lanes 4 and 5), 4 (lanes 6 and 7), and 5 (lanes 8 and 9). Lane 1, Molecular weight standard ϕ X174 (*Hae*III digest).

of the NaOH-serum mixture between room temperature (approximately 25°C) and 100°C. Analysis by PCR amplification showed that maximum HBV DNA was detected by PCR-EB in samples incubated at temperatures equal to or higher than 37°C. We selected 37°C for further use in the procedure because of several advantages associated with this temperature: less sample evaporation occurs at lower incubation temperatures, and 37°C incubators are readily available in research and diagnostic facilities.

Next, we tested the effect of incubation time of the NaOH-serum mixture at 37°C on the sensitivity of detection of HBV DNA by PCR-EB. Serum samples incubated for 20 min yielded lower amounts of HBV DNA after PCR amplification than samples incubated for 60 min or longer. However, equivalent amounts of HBV DNA were detected when 60- and 120-min incubation times were used. Thus, incubation at 37°C for 60 min was selected for future experiments.

It should be mentioned that HBV DNA released from serum particles incubated in NaOH at room temperature can be used in the PCR-EB assay. Incubation of a serum-NaOH mixture at room temperature for at least 180 min was roughly equivalent to an incubation of 60 min at 37°C. Thus, if time is not a factor in the experiment, incubation at room temperature yields satisfactory results.

Direct comparison of the NaOH and phenol-chloroform extraction methods. We analyzed four serum samples, positive for HBsAg, HBeAg, and anti-HBc, by PCR-EB after extraction of virion HBV DNA by both the NaOH and phenol-chloroform methods. In the NaOH extraction method, a 10- μ l portion of each serum sample was incubated in 0.1 M NaOH at 37°C for 60 min and amplified by PCR with primers 109 and 585R (see Materials and Methods). In the phenol-chloroform extraction method, a 100- μ l portion of each serum sample was incubated with proteinase K in the presence of sodium dodecyl sulfate, and the DNA was phenol-chloroform extracted and ethanol precipitated by using carrier tRNA. The precipitate was dissolved in 100 μ l of TE buffer. A 10- μ l portion of the sample was amplified by PCR as described above. A 25- μ l portion (from the 100- μ l reaction mixtures) was subjected to agarose gel electrophoresis, and HBV DNA was visualized with UV light after ethidium bromide staining. Interestingly, the NaOH extraction method was either as effective as or slightly more effective than the phenol-chloroform method for release of HBV DNA from serum particles for amplification in the PCR assay (Fig. 1).

If the sensitivities of the two procedures are considered equivalent, there are several advantages in using the NaOH

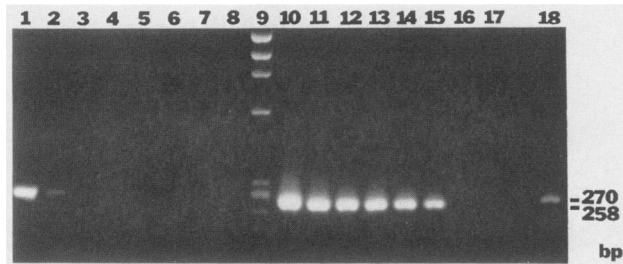


FIG. 2. Establishing the sensitivity of the PCR-PCR technique by amplification of recombinant HBV DNA. Serial 10-fold dilutions of recombinant HBV DNA, ranging from 1 to 10^{-7} pg (lanes 1 through 8, respectively), were amplified by PCR with primers 1763 and 2032R (see Materials and Methods). PCR-PCR amplification was performed on samples containing the following initial amounts of recombinant HBV DNA: 1 pg (lane 10), 10^{-1} pg (lane 11), 10^{-2} pg (lane 12), 10^{-3} pg (lane 13), 10^{-4} pg (lane 14), and 10^{-5} pg (lane 15). Bands were not detected for samples with an initial amount of HBV 10^{-6} pg (lane 16) or 10^{-7} pg (lane 17). For standardization, 0.1 μ g of 270-bp HBV DNA is shown (lane 18). Also shown is a molecular weight marker, ϕ X174 (*Hae*III digest) (lane 9). It should be noted that some positive signals are visible only in the original photograph because a loss of sensitivity during photographic reproduction of the data.

method. The procedure is both rapid and easy to perform. The fact that few steps are required reduces the possibility of contaminating the sample with exogenous HBV DNA. This eliminates a potential source of error in the PCR assay. Also, variation between samples is virtually eliminated in the NaOH method. (Sources of variation in the phenol-chloroform method are losses of sample at the organic-aqueous interphase during extraction and during ethanol precipitation.) Therefore, the accuracy of PCR analysis is enhanced by using the NaOH extraction method.

Increased detection limits with the PCR-PCR technique. We demonstrated previously that the detection limits for HBV DNA were 10^{-2} pg by PCR-EB and 10^{-5} pg by PCR-SBH (6). However, PCR-SBH analysis is a time-consuming procedure and requires hybridization with a radiolabeled nucleic acid probe. Overall, the entire PCR-SBH procedure can take 3 to 7 days to complete. Therefore, we attempted to devise a procedure that would permit rapid detection of 10^{-5} pg of HBV DNA without the use of radioisotopes. Attempts to reamplify the HBV DNA produced in a PCR reaction mixture with the same primer pairs produced unsatisfactory results. However, the use of a second set of primer pairs, internal to the original pair, produced excellent results. Tenfold serial dilutions of recombinant HBV DNA were amplified by PCR with oligonucleotide primers specific for the core gene sequence of HBV (primers 1778 and 2032R). After PCR amplification, DNA was fractionated by agarose gel electrophoresis and visualized under UV light by ethidium bromide fluorescence. 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 bp) in this experiment (Fig. 2, lanes 1 through 3). When 10 μ l of each amplified reaction mixture was reamplified (PCR-PCR) with internal primers (i.e., primers 1763-E and 2017R-B), a DNA band of 258 bp was detected in samples containing $\geq 10^{-5}$ pg of HBV DNA before the first PCR amplification reaction. Thus, the sensitivity of PCR-PCR is equivalent to that of PCR-SBH. In addition, PCR-PCR can be completed in one day and is convenient because it does not involve the use of radioisotopes. Thus, this method may become useful for routine detection of virus DNA.

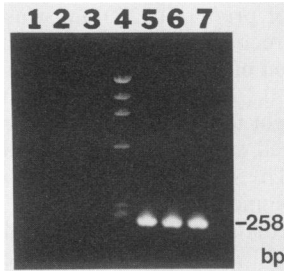


FIG. 3. Combination of the NaOH extraction method and the PCR-PCR technique for detection of HBV DNA. Serum samples 6 (lanes 1 and 5), 7 (lanes 2 and 6), and 8 (lanes 3 and 7), positive for HBsAg, anti-HBe, and anti-HBc, were used for the detection of HBV DNA by a combination of the NaOH extraction method and PCR/PCR analysis. Samples were assayed either in a single PCR reaction (lanes 1 through 3) or in double PCR reactions (lanes 5 through 7). Lane 4, Molecular weight standard ϕ X174 (*Hae*III digest).

Combination of the NaOH extraction method and PCR-PCR for the detection of HBV DNA. We combined the NaOH extraction method and PCR-PCR analysis to determine the HBV DNA content of three serum samples (no. 6 through 8) which were positive for HBsAg, anti-HBe, and anti-HBc. HBV DNA was not detectable in these sera by slot blot hybridization analysis (data not shown). The sera were incubated in 0.1 M NaOH at 37°C for 60 min, and HBV DNA sequences were amplified by PCR with primers 1763 and 2032R. The samples from the first round of amplification were negative for HBV DNA by PCR-EB (Fig. 3, lanes 1 through 3). However, when 10 μ l of the amplified mixtures was amplified a second time with primers 1778-E and 2017R-B, HBV DNA was detected in all three samples (Fig. 3, lanes 5 through 7). Thus, NaOH extraction of serum HBV DNA coupled with PCR-PCR analysis is capable of detecting extremely low levels of HBV DNA and can be accomplished by a 1-day procedure. This technique should be useful for detecting sequences of other DNA viruses and with some modifications may be adapted to detect sequences of RNA-containing viruses. Thus, this detection method may become a valuable tool for the detection of virus nucleic acid in serum.

ACKNOWLEDGMENTS

We thank K. Matsubara for pBRHBadr₄ DNA; M. Unoura, K. Kobayashi, and N. Hattori for patient serum samples; and J. Sninsky for helpful discussions.

This work was supported in part by Cetus, Inc.

LITERATURE CITED

- Berninger, M., M. Hammer, B. Hoyer, and J. Gerin. 1982. An assay for the detection of the DNA genome of hepatitis B virus in serum. *J. Med. Virol.* 9:57-68.
- Bonino, F., B. Hoyer, J. Nelson, R. Engle, G. Verme, and J. Gerin. 1981. Hepatitis B virus DNA in the sera of HBsAg carriers: a marker of active hepatitis B virus replication in the liver. *Hepatology* 5:386-391.
- Fujiyama, A., A. Miyanohara, C. Nozaki, T. Yoneyama, N. Ohtomo, and K. Matsubara. 1983. Cloning and structural analyses of hepatitis B virus DNAs, subtype adr. *Nucleic Acids Res.* 11:4601-4610.
- Kam, W., L. Rall, E. Smuckler, R. Schmid, and W. Rutter. 1982. Hepatitis B viral DNA in liver and serum of asymptomatic carriers. *Proc. Natl. Acad. Sci. USA* 79:7522-7526.
- Kaneko, S. 1985. State of hepatitis B virus DNA in liver of patients with hepatitis B surface antigen positive liver diseases:

- its variation and its correlation with HBV serological markers. *Acta Hepatol. Jpn.* **26**:846-855.
6. Kaneko, S., R. Miller, S. Feinstone, M. Unoura, K. Kobayashi, N. Hattori, and R. Purcell. 1988. Detection of serum hepatitis B virus DNA in patients with chronic hepatitis utilizing the polymerase chain reaction assay. *Proc. Natl. Acad. Sci. USA* **86**:312-316.
 7. Landers, T., H. Greenberg, and W. Robinson. 1977. Structure of hepatitis B Dane particle DNA and nature of the endogenous DNA polymerase reaction. *J. Virol.* **23**:368-376.
 8. Lieberman, H., D. LaBrecque, M. Kew, S. Hadziyannis, and D. Shafritz. 1983. Detection of hepatitis B virus DNA directly in human serum by a simplified molecular hybridization test: comparison to HBeAg/anti-HBe status in HBsAg carriers. *Hepatology* **3**:285-291.
 9. Matsuyama, Y., M. Omata, O. Yokosuka, F. Imazeki, Y. Ito, and K. Okuda. 1985. Discordance of hepatitis B e antigen/antibody and hepatitis B virus deoxyribonucleic acid in serum. *Gastroenterology* **89**:1104-1108.
 10. Scotto, J., M. Hadchouel, C. Hery, J. Yvert, P. Tiollais, and C. Brechot. 1983. Detection of hepatitis B virus DNA in serum by a simple spot hybridization technique: comparison with results for other viral markers. *Hepatology* **3**:279-284.
 11. Walter, E., H. Blum, W.-B. Offensperger, C. Zeschnigk, S. Offensperger, and W. Gerok. 1987. Spot-blot hybridization assay for the detection of hepatitis B virus DNA in serum: factors determining its sensitivity and specificity. *Hepatology* **7**:557-562.