JOURNAL OF CLINICAL MICROBIOLOGY, June 1990, p. 1411–1416
0095-1137/90/061411-06$02.00/0

Vol. 28, No. 6

Detection of Hepatitis B Virus DNA in Serum by Polymerase Chain Reaction Amplification and Microtiter Sandwich Hybridization

GEORGE H. KELLER,[1]* DAO-PEI HUANG,[1] J. WAI-KUO SHIH,[2] AND MARK M. MANAK[1]

Biotech Research Laboratories, Inc., 1600 East Gude Drive, Rockville, Maryland 20850,[1] and Department of Transfusion Medicine, Warren Grant Magnuson Clinical Center, National Institutes of Health, Bethesda, Maryland 20892[2]

Received 27 December 1989/Accepted 14 March 1990

We have developed a microtiter sandwich hybridization assay for the detection of polymerase chain reaction (PCR)-amplified hepatitis B virus (HBV) sequences. This assay utilizes an enzyme-linked immunosorbent assay-like format in which cloned DNA containing a sequence complementary to half of one PCR product strand is immobilized in microtiter wells. A biotin-labeled DNA sequence complementary to the other portion of the same PCR product strand is used as the probe. The DNAs from 69 hepatitis B surface antigen-positive serum samples and 16 antigen-negative control samples were amplified by the PCR procedure, and the product was detected by Southern and sandwich hybridization. Both detection procedures were capable of detecting as few as five copies of HBV DNA. Compared with Southern hybridization, the sandwich hybridization assay exhibited a sensitivity of 100% and a specificity of 95% for the detection of amplified HBV sequences. Unlike Southern hybridization, however, the sandwich hybridization assay employs a nonradioactive probe and allows easy handling of large numbers of samples. DNA was detected in 74% of the antigen-positive samples. All of the antigen-negative samples (healthy blood donors) were negative for HBV DNA by both procedures.

Hepatitis B virus (HBV) is a significant cause of posttransfusion hepatitis (1) and a major cause of chronic hepatitis and hepatoma in Southeast Asia and Japan. The incidence of HBV infection is increasing in the United States, partially because of increased intravenous drug abuse. Chronic infection by HBV usually results in persistent production of viral surface antigen (HBsAg) and e antigen (HBeAg) by infected hepatocytes (3). The level of antigen production in infected individuals can vary substantially and does not necessarily correlate with the release of complete viral particles.

Although many carriers exhibit high titers of infectious HBV in serum, others exhibit little or no HBV. It is important to be able to distinguish between these two classes of carriers, since viremic carriers have a poor prognosis and are a potential source of new infection. Screening for anti-HBs as an indicator of HBV infection is not as reliable as antibody tests for other viruses. In developed countries, many high-risk individuals have been immunized against HBsAg, and thus they are seropositive for anti-HBs; in endemic areas, the antibody prevalence is expectedly high (20), yet not all of these individuals are actively infected. In chronic hepatitis patients, blood tests for hepatitis B core and e antigens are often used as an index of active viral replication (14, 19), but these are indirect markers and are not entirely reliable (2, 13, 16, 17). The presence of HBV DNA in serum correlates best with viral replication and infectivity (5, 18); thus, the DNA detection method should be superior to serologic tests for screening donated blood or for monitoring the course of chronic hepatitis and the effectiveness of antiviral therapy.

It has previously been established that amplification with the polymerase chain reaction (PCR) followed by Southern hybridization with a ^{32}P-labeled probe provides the most sensitive detection of HBV DNA in serum (4, 5). The combination of PCR and Southern hybridization provides at least a 10^4-fold increase in sensitivity over slot blot detection of unamplified material (5) and allows the detection of as few as three HBV DNA molecules in a sample. The detection of the amplified product by Southern hybridization is not practical in a clinical laboratory because such laboratories are not equipped for handling ^{32}P and the total time required can be at least 4 days. We have developed a hybridization assay for a specific amplified HBV sequence that is comparable in sensitivity to Southern hybridization but is nonradioactive, less labor intensive, and more rapid. In this report, we describe the assay and demonstrate its usefulness in detecting HBV DNA in clinical samples.

MATERIALS AND METHODS

Sample preparation. HBsAg-positive sera were obtained from North Atlantic Biologicals, Inc. (Miami, Fla.). Sixteen control serum samples from healthy blood donors were obtained from the American Red Cross (Rockville, Md.). A 1 ml sample of extraction buffer (150 mM NaCl, 10 mM EDTA [pH 8.0], 10 mM Tris hydrochloride [pH 8.0], 2% sodium dodecyl sulfate, 20 μg of salmon sperm DNA per ml [Sigma Chemical Co., St. Louis, Mo.]) was added to 1 ml of serum. A 25-mg/ml proteinase K (Boehringer-Mannheim Biochemicals, Indianapolis, Ind.) stock solution (40 μl) was added, and the mixture was incubated at 55°C for 1 h. Phenol-chloroform-isoamyl alcohol (25:24:1) (2 ml) (all from Aldrich Chemical Co., Inc., Milwaukee, Wis.) was added, and the reaction was vortexed and incubated at 50°C for 10 min. After centrifugation, 750 μl of the supernatant was removed, leaving some of the supernatant and all of the interphase behind. The DNA was precipitated with a mixture of 150 μl of 4 M NaOAc and 600 μl of isopropanol at −20°C overnight. The DNA was recovered by centrifugation and dissolved in 37.5 μl of water. For most PCRs, we used 10 μl of this DNA solution, which was equivalent to 100 μl of the original serum and contained about 2 μg of carrier DNA.

DNA amplification. DNA samples (containing 2 μg of carrier DNA) in a volume of 10 μl were adjusted to PCR buffer conditions with 2× PCR buffer [1× PCR buffer contains 70 mM Tris, pH 8.8; 2 mM $MgCl_2$; 20 mM

* Corresponding author.

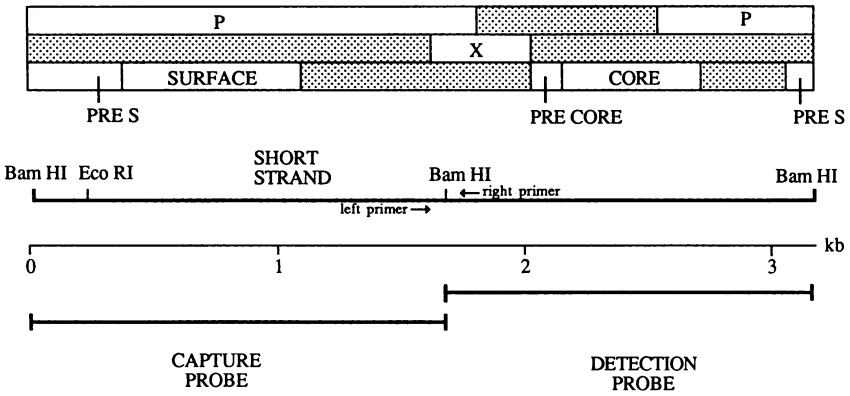

FIG. 1. Map of the HBV genome showing the amplified region and the location of the DNA fragments used in sandwich hybridization. The primers were chosen to amplify a region surrounding the *Bam*HI site in open reading frame X. The 1.7-kilobase *Bam*HI fragment was used for the capture probe, and the 1.5-kilobase *Bam*HI fragment was used for the detection probe.

$(NH_4)_2SO_4$; 100 μg of bovine serum albumin (Bethesda Research Laboratories, Gaithersburg, Md.) per ml; and 6 mM dithiothreitol]. This was followed by adding 10 μl of dimethyl sulfoxide and adjusting each deoxynucleoside triphosphate to 1 mM and each primer to 6.3 μg/ml (1 μM). The reaction was denatured at 95°C for 7 min and cooled to room temperature and 7 U of *Taq* DNA polymerase (New England BioLabs, Inc., Beverly, Mass.) was added. The final reaction volume of 100 μl was overlaid with 100 μl of mineral oil. The reactions were centrifuged in a microfuge for 10 s, and elongation was carried out at 70°C for 5 min. The reactions were taken through 25 cycles of alternating temperature with each cycle consisting of 92°C for 1 min, 37°C for 1 min, and 70°C for 3 min. The final 70°C incubation was for 10 min. Amplified samples were stored at −20°C until analyzed. Under these conditions, 25 PCR cycles resulted in an amplification of about 8×10^6-fold, as determined by slot blotting. Temperature cycling was performed by using a programmable robot arm (Zymark) and three oil baths.

The HBV primers, specific for P (polymerase) and X (unknown function) gene sequences (Fig. 1), were synthesized on an Applied Biosystems 381A DNA synthesizer by the methoxyphosphoramidite method. The left primer, 5′-ATACTGCGGAACTCCTAGC, begins at position 1269 in the P gene; the right primer, 5′-CCGCGTAAAGAGAGGT GCG, begins at position 1546 in the X gene (15). The size of the amplified HBV product is 278 base pairs. The primer sequences are conserved in HBV subtypes adw, adr, and ayw. In some experiments, human β-globin sequences (in the form of human genomic DNA) were added to the sample as a positive control and amplified in a separate reaction with β-globin primers specific for regions in exons 1 and 2. The left primer, 5′-CAAGGTGAACGTGGATGAAG, begins at position 317; the right primer, 5′-CCTGAAGTTCTCAG GATCCACG, begins at position 711 of the β-globin sequence (10). The length of the amplified β-globin product is 395 base pairs. To compensate for the greater copy number of β-globin sequences in a sample relative to that expected for HBV sequences, we made the following adjustments. The β-globin primer concentration in the PCR was 100-fold lower than the HBV primer concentration (10 nM versus 1 μM). The β-globin amplification products were detected by gel electrophoresis and ethidium bromide staining. These adjustments were designed to make the reaction more sensitive to inhibitors or differences in DNA recovery.

In order to avoid carryover of PCR product into samples, a number of precautions were taken. Sample preparation and amplification were performed in a specially designated laboratory. After amplification, the reaction tubes remained unopened, and they were assayed in a separate laboratory. Special precautions were also taken in the detection laboratory where the Southern and sandwich hybridization assays were done. All hybridization reagents were prepared with pipettes and containers which never came in contact with amplified products. The reagents were divided into equal portions and tested before use. Our success in avoiding contamination was monitored with negative-control DNA from HBV antigen and antibody-negative sera. These DNAs were amplified in parallel with the clinical samples as controls for the contamination of reactions or reagents with amplified product. Positive controls contained amplified DNA from 10 pg of pAM6.

Probes. The sandwich hybridization probes were subcloned from pAM6 (ATCC 45020), a genomic clone of HBV, subtype adw (11). The plasmid was digested with *Bam*HI to release a 1.5-kilobase fragment containing the core antigen gene and a 1.7-kilobase fragment containing the surface antigen gene (Fig. 1). The surface fragment was cloned into M13mp18 to serve as the capture probe; the core fragment was cloned into pBR322 to serve as the detection probe. For hybridization to Southern blots, the entire pAM6 plasmid was used as the probe.

Immobilization of capture DNA. We developed a modification of the method of Nagata et al. (12) for the noncovalent attachment of the M13 capture DNA to microtiter wells. We have omitted UV irradiation because it does not improve the performance of the well strips. The capture DNA (M13 clone) was dissolved to 100 μg/ml in water. For the preparation of four eight-well strips (Costar, Cambridge, Mass.), 108 μl of DNA and 1.65 ml of binding buffer (25 mM KH_2PO_4, pH 7.2, containing 200 mM $MgCl_2$) were combined; 50 μl (300 ng) of the diluted DNA was added to each well. After shaking on a mechanical rotator for 2 h at room temperature, the wells were washed three times with 400 μl of wash buffer (25 mM KH_2PO_4, pH 7.2, containing 100 mM $MgCl_2$). Remaining DNA-binding sites were blocked with 400 μl of blocking buffer (1% bovine serum albumin dissolved in 1× phosphate-buffered saline [pH 7.2] containing 2.7 mM KCl and 100 mM $MgCl_2$) per well for 1 h at room temperature. The wells were emptied and washed three times with 400 μl of water per well. Well strips were dried

and used immediately or stored at room temperature in a heat seal bag.

Detection probe labeling. (i) Biotin labeling. The pBR322 core DNA was nicked by treatment with alkali before labeling (6). Nicked DNA (20 μg) dissolved in water was combined with 40 μg of photobiotin (Vector) in a total volume of 50 μl and irradiated under a sunlamp for 10 min on ice. The labeled DNA was recovered by adding 100 μl of 0.1 M Tris (pH 8.0), extracting twice with 100 μl of *sec*-butanol, and precipitating with ethanol. The pelleted probe DNA was redissolved in water to 50 μg/ml.

(ii) Radioactive labeling. The radioactively labeled probe for filter hybridization was prepared by nick translation of pAM6 with [^{32}P]dATP (3,000 Ci/mmol; Dupont, NEN Research Products, Boston, Mass.). Unincorporated nucleotides were removed by chromatography on Sephadex G-50 in 10 mM Tris (pH 8.0)–2 mM EDTA. The final specific activity of the probe was 1×10^8 to 3×10^8 cpm/μg.

Hybridization conditions. (i) Filter hybridization. Slot blotting, Southern transfers, and filter hybridization were performed as previously described (6, 7), with minor changes. For slot blot analysis, 10 μl of the extracted DNA equivalent to the DNA from 100 μl of the original serum was used. For Southern analysis, a 20-μl sample of the amplification reaction was separated on a 2% Nusieve GTG–0.5% Seakem HGT agarose gel (FMC Corp., Rockland, Maine) and transferred to a nylon membrane. The transfer was hybridized with ^{32}P-labeled pAM6 at 42°C overnight. Autoradiography was performed for 16 h at −70°C with screens.

(ii) Sandwich hybridization. Microtiter strips coated with capture DNA were prehybridized at 42°C for 30 min with each well containing 150 μl of the following hybridization buffer: 50% formamide (Aldrich), 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× FPG (0.02% Ficoll 400, 0.02% polyvinylpyrrolidone 360, 0.02% glycine [all from Sigma]), 25 mM KH_2PO_4 (pH 7.0), 0.2% sodium dodecyl sulfate, 5% dextran sulfate (Pharmacia LKB BioTechnology, Piscataway, N.J.) and 200 μg of salmon sperm carrier DNA (Sigma) per ml. During incubation, microtiter wells were sealed with Mylar tape (Flow Laboratories, Inc., McLean, Va.). A 5- to 10-μl sample of the PCR, diluted to a final volume of 10 μl with 10 mM Tris (pH 8.0) and 2 mM EDTA, was mixed with 2 μl of probe (100 ng) and 2 μl of carrier DNA (20 μg) in a separate microtiter well. The mixture was denatured by adding 2 μl of 2.5 M NaOH and incubating at room temperature for 10 min. The reaction was neutralized by adding 15 μl of 2 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, pH 6.5). Hybridization buffer (100 μl) was added to the denatured sample plus probe (hybridization reaction). The prehybridization buffer was removed from the wells, and the hybridization reactions were added to the wells. Samples were hybridized for 4 h at 42°C. After hybridization, the wells were washed four times with 200 μl of 2× SSC–0.1% sodium dodecyl sulfate at 42°C. Each well was blocked for 10 min with 200 μl of 3% bovine serum albumin in wash buffer (100 mM Tris [pH 7.4], 200 mM NaCl, 0.3% Tween 20) followed by 10 min with 100 μl of 1 μg of peroxidase-conjugated streptavidin (Kirkegaard & Perry, Inc., Gaithersburg, Md.) per ml diluted in 3% bovine serum albumin-wash buffer. Wells were washed four times with 200 μl of wash buffer and developed for 30 min with 100 μl of tetramethylbenzidine color reagent (Kirkegaard & Perry, Inc.). Then, 100 μl of 2 N H_2SO_4 was added to each well, and the resulting yellow color was measured at 450 nm. Results were expressed as net absorbance after the absorbance of the buffer blanks was subtracted. The specificity of all sandwich hybridization results was confirmed by Southern hybridization.

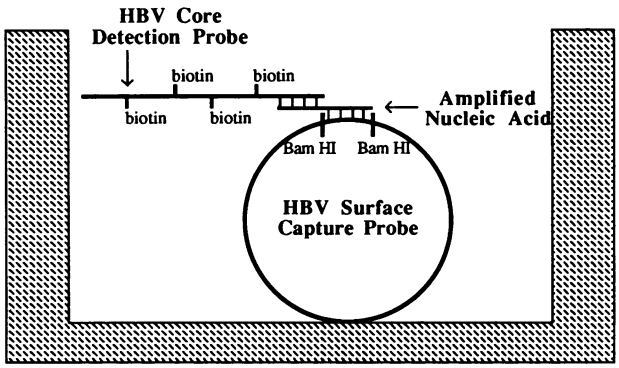

FIG. 2. Schematic diagram of the HBV sandwich hybridization assay. The capture probe, cloned into M13, was attached to the microtiter wells, and the detection probe, cloned into pBR322, was labeled with biotin. Each probe is homologous to half of the amplified HBV sequence, allowing formation of a sandwich structure which immobilizes the labeled detection probe in the microtiter well. After washing, the immobilized detection probe was quantified by using streptavidin-peroxidase and tetramethylbenzidine.

Assay for HBeAg and anti-HBe. The presence of HBeAg or antibody to HBeAg (anti-HBe) was determined with Abbott HBeAg enzyme immunoassay (Abbott Laboratories, North Chicago, Ill.) kits according to the instructions of the manufacturer.

RESULTS

Our assay for HBV DNA combines PCR amplification of a 278-base-pair viral sequence with nonradioactive detection in microtiter wells. A diagram of the assay is shown in Fig. 2. The use of two probes (i.e., the surface capture and the core detection probes) provides a high level of specificity, since both must hybridize to the amplified product to generate a signal. Bound biotin-labeled detection probe is detected by incubation with peroxidase-conjugated streptavidin and a colorimetric peroxidase substrate.

In order to determine the sensitivity of the assay, HBV DNA was extracted from a strong-positive serum sample and serially diluted 10^6-fold. The concentration of HBV DNA in the sample was determined by slot blot hybridization, as were HBV plasmid DNA dilutions. A sample of each dilution was amplified by the PCR, and the product was detected by Southern and sandwich hybridization (Table 1). The amount of hybridized detection probe and the signal intensity are proportional to the amount of specific PCR product added to the assay over the target DNA range of 5 to 5,000 copies. The sample from the highest dilution contained about 5 molecules of HBV DNA, which, after PCR amplification, was detectable by both hybridization assays (using a sandwich assay cutoff value of 0.20 optical density units). These results indicated that sandwich hybridization should be as sensitive as Southern hybridization at detecting amplified HBV sequences from patient samples.

We next compared sandwich hybridization with Southern hybridization using amplified products from the DNA of 69 HBsAg-positive serum samples (Fig. 3). Samples that were PCR negative were reamplified with twice the quantity of extracted DNA (20 μl), and two additional samples were

TABLE 1. Sensitivity of HBV sandwich hybridization assay[a]

Sample	PCR dilution	No. of copies	Net A_{450}	Result of Southern assay[b]
HBsAg+ serum no. 18	$1/10^2$	5×10^4	2.132	+++++
	$1/10^3$	5×10^3	1.720	++++
	$1/10^4$	5×10^2	1.538	+++
	$1/10^5$	5×10^1	0.640	++
	$1/10^6$	5	0.221	+
Negative control[c]		0	0.011	−
Positive control[d]		1×10^6	1.290	+++++
Reagent blank		0	0.008	−

[a] An HBsAg-positive serum sample containing a high copy number of HBV DNA (as determined by slot blotting) was extracted, and serial 10-fold dilutions of the DNA were prepared. A sample of each dilution containing 1 μg of carrier DNA and from 5 to 5×10^4 copies of HBV DNA was amplified by PCR and assayed by Southern and sandwich hybridization.
[b] −, Negative; + to +++++, weakly to very strongly positive.
[c] Amplified serum DNA sample from a healthy blood donor.
[d] Contained 10 pg of pAM6 which was PCR amplified; diluted 1/100 before being assayed by sandwich hybridization.

found to be PCR positive. Thus, the DNA from 200 μl of serum must be amplified in order to detect weak-positive samples. The sandwich assay results were grouped as Southern positive or Southern negative and arranged in order of decreasing signal. In addition, 16 control serum samples from healthy blood donors (negative controls) were tested, and all were HBV DNA negative by both assays (data not shown). The mean absorbance value of these negative controls was 0.032 with a standard deviation of 0.048. We chose an assay cutoff value of 0.20, which is more than 3 standard deviation units above the mean. When an assay cutoff value of 0.20 was used, all PCR-Southern blot-positive samples were also scored as positive by the sandwich assay (no false-negatives). Of the 18 PCR-Southern blot-negative samples, 17 were scored as negative by the sandwich assay, but one sample was scored as positive (false-positive). Overall, 74% of the HBsAg-positive samples were PCR positive. Unamplified DNAs from these same samples were also assayed for HBV sequences by slot blotting, and only 23% were positive for HBV DNA. Thus, PCR amplification allowed us to detect more positive samples than filter hybridization alone did.

TABLE 2. HBeAg and anti-HBe presence in DNA-positive and -negative HBsAg-positive sera[a]

Sample status[b] (no. tested)	Presence of: anti-HBe	Presence of: HBeAg	No. (%) of samples positive for HBeAg
DNA positive (37)	−	+	18 (49)
	+	−	9 (24)
	−	−	10 (27)
DNA negative (6)	−	+	0
	+	−	5 (83)
	−	−	1 (17)

[a] Of the 69 HBsAg-positive serum samples, 43 were tested for the presence of HBeAg and anti-HBe by enzyme immunoassay, as described in Materials and Methods.
[b] Determined by PCR-Southern hybridization.

False-negative PCR results can occur if the DNA recovery is poor or if the DNA contains impurities (salts or phenol) which interfere with the amplification process. To confirm that the 18 HBsAg-positive PCR-negative samples were truly DNA negative, a control PCR was performed on each sample. Human genomic DNA (20 μg) was added to each 1-ml serum sample instead of salmon sperm DNA, as described in Materials and Methods. The serum samples were extracted, and the DNA was amplified using both HBV and β-globin primers in separate reactions. All samples were β-globin positive, indicating that DNA recovery was consistent and that no PCR-inhibitory material was isolated with the DNA. These samples remained negative for HBV DNA (data not shown).

We also assayed the HBsAg-positive sera for HBeAg and for anti-HBe, since these markers are used clinically as an index of hepatitis B viral replication. A comparison of HBeAg, anti-HBe, and DNA results is shown in Table 2. Note that all samples positive for HBeAg were also PCR positive. However, only 18 of 37 PCR-positive samples were positive for HBeAg. Thus, many PCR-positive samples would have been scored as virus negative by HBeAg detection. These results are similar to those reported by Kaneko et al. (5). Thus, we do not find a clear correlation between

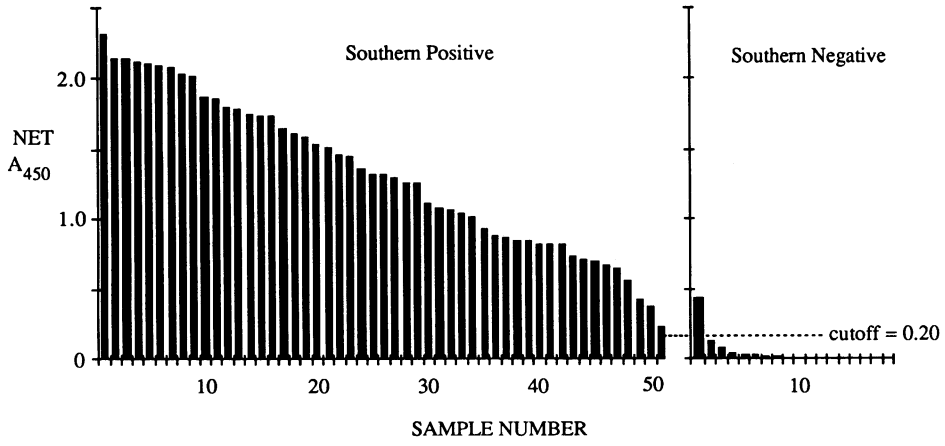

FIG. 3. Comparison of microtiter sandwich and Southern hybridization data on HBsAg-positive sera. DNA was extracted from 69 antigen-positive serum samples and amplified by 25 cycles of the PCR. The amplified product was detected by Southern hybridization and microtiter sandwich hybridization. The sandwich results have been grouped according to whether the samples were Southern positive or Southern negative, and they are arranged in order of decreasing absorbance values.

HBeAg or anti-HBe status and the presence of HBV DNA in serum, probably because both assays are less sensitive than DNA amplification.

DISCUSSION

The presence of HBV DNA in serum is the best direct marker available for active HBV replication. Amplification of this DNA by PCR allows the detection of as few as five viral genomes in a 100-μl blood sample (Table 1), providing an extremely sensitive viral assay. We have previously reported the use of a microtiter sandwich hybridization assay for the detection of PCR-amplified human immunodeficiency virus type 1 DNA from peripheral blood lymphocytes (7). In this study, we developed a similar assay to detect PCR-amplified HBV DNA from patient sera. The assay is nonradioactive, requires only 4 h of hybridization time, and provides essentially the same detection sensitivity and specificity as Southern hybridization with a ^{32}P-labeled probe.

In order for the PCR to become a routine procedure in large clinical labs, at least two drawbacks must be overcome. The first is the carryover of amplified product to solutions and equipment through pipetting devices, fingers, and aerosols, which leads to false-positive results (9). This problem is best prevented by physical separation of work areas, by use of gloves, screw-cap tubes, and specialized pipetting devices and accessories, and by careful work habits. The second is the need for a simplified, nonradioactive hybridization assay for specific detection of the amplified product. It is this need that our microtiter sandwich hybridization assay addresses.

Our results indicate that the microtiter sandwich hybridization assay is as sensitive for the detection of amplified HBV DNA as traditional Southern hybridization but is faster and more convenient and does not employ radioisotopes. The microtiter plate format allows the assay to be compatible with standard enzyme-linked immunosorbant assay pipetting, washing, and reading equipment which is in use in clinical laboratories. An additional advantage is the semiquantitative nature of the assay results (Table 1), so that the relative amount of viral DNA in serum can be monitored. We believe that the ability to detect HBV replication at very low levels outweighs the need for exact quantitation. Sample preparation was accomplished by using traditional phenol extraction, but it is not absolutely necessary. We have obtained excellent results with rapid DNA isolation methods as well (8).

We have demonstrated the utility of the microtiter sandwich hybridization assay using clinical samples and have shown that a large percentage of HBeAg-negative samples are positive for HBV DNA. This is presumably due to the greater sensitivity of the PCR-based assay and is consistent with the results reported by Kaneko et al. (5). All samples which were positive by Southern hybridization were also positive by the sandwich hybridization assay (100% sensitivity). Of 18 Southern hybridization-negative samples, only 1 was positive by the sandwich hybridization assay (95% specificity).

Kaneko et al. (4) have also described a simplified approach to the detection of HBV DNA by the PCR using nested primers and fluorescence detection. Thirty PCR cycles were performed with one primer set; then, a second primer pair (within the sequence amplified by the first pair) was added, and the PCR was continued for another thirty cycles. The product was detected by agarose gel electrophoresis and ethidium bromide staining. The sensitivity was equivalent to that obtained using 30 PCR cycles and detection of the product by Southern hybridization with a ^{32}P-labeled probe. These additional PCR cycles require additional sample handling and could actually magnify any contamination problems. Furthermore, unlike enzyme-linked immunosorbent assay technology, the gel electrophoresis and staining are not routine clinical laboratory procedures and yield only qualitative results. In contrast, our hybridization assay is sensitive without the additional PCR cycles, and it yields numerical data.

ACKNOWLEDGMENTS

We acknowledge the excellent technical assistance of Catherine Moore, David Petersen, and Cathleen Overholt during the course of this work.

LITERATURE CITED

1. **Aach, R. D., and R. A. Khan.** 1980. Posttransfusion hepatitis: current perspectives. Ann. Intern. Med. **92**:539–546.
2. **Brechot, C., F. Degos, C. Lugassy, V. Thiers, S. Zafrani, D. Franco, H. Bismuth, C. Trepo, J.-P. Benhamou, J. Wands, K. Isselbacher, P. Tiollais, and P. Berthelot.** 1985. Hepatitis B virus DNA in patients with chronic liver disease and negative tests for hepatitis B surface antigen. N. Engl. J. Med. **312**:270–276.
3. **Hindman, S. H., C. R. Gravelle, B. L. Murphy, D. W. Bradley, W. R. Budge, and J. E. Maynard.** 1976. e-Antigen, Dane particles and serum DNA polymerase activity in a HBsAg carrier. Ann. Intern. Med. **85**:458–460.
4. **Kaneko, S., S. M. Feinstone, and R. H. Miller.** 1989. Rapid and sensitive method for the detection of serum hepatitis B virus DNA using the polymerase chain reaction technique. J. Clin. Microbiol. **27**:1930–1933.
5. **Kaneko, S., R. H. Miller, S. M. Feinstone, M. Unoura, K. Kobayashi, N. Hattori, and R. H. Purcell.** 1989. Detection of serum hepatitis B virus DNA in patients with chronic hepatitis using the polymerase chain reaction assay. Proc. Natl. Acad. Sci. USA **86**:312–316.
6. **Keller, G. H., C. U. Cumming, D. P. Huang, M. M. Manak, and R. Ting.** 1988. A chemical method for introducing haptens onto DNA probes. Anal. Biochem. **170**:441–450.
7. **Keller, G. H., D. P. Huang, and M. M. Manak.** 1989. A sensitive nonisotopic hybridization assay for HIV-1 DNA. Anal. Biochem. **177**:27–32.
8. **Keller, G. H., and M. M. Manak.** 1989. DNA probes, p. 220–221. Stockton Press, New York.
9. **Kwok, S., and R. Higuchi.** 1989. Avoiding false positives with PCR. Nature (London) **339**:237–238.
10. **Lawn, R. M., A. Efstratiatis, C. O'Connell, and T. Maniatis.** 1980. The nucleotide sequence of the human β-globin gene. Cell **21**:647–651.
11. **Moriarity, A. M., B. H. Hoyer, J. W.-K. Shih, J. L. Gerin, and D. H. Hamer.** 1981. Expression of the hepatitis B virus surface antigen gene in cell culture by using a simian virus 40 vector. Proc. Natl. Acad. Sci. USA **78**:2606–2610.
12. **Nagata, Y., H. Yokota, O. Kosuda, K. Yokoo, K. Takemura, and T. Kikuchi.** 1985. Quantification of picogram levels of specific DNA immobilized in microtiter wells. FEBS Lett. **183**:379–382.
13. **Nalpas, B., P. Berthelot, V. Thiers, G. Duhamel, A. M. Courouce, P. Tiollais, and C. Brechot.** 1985. Hepatitis B virus multiplication in the absence of usual serological markers: a study of 146 chronic alcoholics. J. Hepatol. **1**:89–97.
14. **Okada, K., I. Kamiyama, M. Inomata, M. Imai, Y. Miyakawa, and M. Mayumi.** 1976. E antigen and anti-e in the serum of asymptomatic carrier mothers as indicators of positive and negative transmission of hepatitis B to their infants. N. Engl. J.

Med. **294:**746–749.
15. **Ono, Y., H. Onda, R. Sasada, K. Igarashi, Y. Sugino, and K. Nishioka.** 1983. The complete nucleotide sequence of the cloned hepatitis B virus DNA; subtype adr and adw. Nucleic Acids Res. **11:**1747–1757.
16. **Schafritz, D. A., H. M. Lieberman, K. J. Isselbacher, and J. R. Wands.** 1982. Monoclonal immunoassays for hepatitis B surface antigen: demonstration of hepatitis B virus DNA or related sequences in serum and viral epitopes in immune complexes. Proc. Natl. Acad. Sci. USA **79:**5675–5679.
17. **Scotto, J., M. Hadscouel, C. Hery, J. Yvart, 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.
18. **Sun, C.-F., C. C. Pao, S.-Y. Wu, and Y.-F. Liaw.** 1988. Screening for hepatitis B virus in healthy blood donors by molecular DNA hybridization analysis. J. Clin. Microbiol. **26:** 1848–1852.
19. **Takahashi, K., M. Imai, F. Tsuda, T. Takahashi, Y. Miyakawa, and M. Mayumi.** 1976. Association of Dane particles with e antigen in the serum of asymptomatic carriers of hepatitis B surface antigen. J. Immunol. **117:**102–105.
20. **Wu, J. S., C. H. Chen, Y. H. Chiang, Y. C. Lee, M. H. Lee, Y. C. Ko, and H. T. Hu.** 1980. Hepatitis B virus infection in Taiwan: with reference to anti-HBc versus HBsAg and anti-HBs. J. Formosan Med. Assoc. **79:**760–767.