JOURNAL OF CLINICAL MICROBIOLOGY, Aug. 1992, p. 1913–1916
0095-1137/92/081913-04$02.00/0

Vol. 30, No. 8

Direct Detection of Hepatitis B Virus from Dried Blood Spots by Polymerase Chain Reaction Amplification

BHAGWATI PRASAD GUPTA, N. JAYASURYAN, AND SHAHID JAMEEL*

Virology Group, International Centre for Genetic Engineering and Biotechnology, NII Campus, Shaheed Jeet Singh Marg, New Delhi 110067, India

Received 9 December 1991/Accepted 4 May 1992

The presence of hepatitis B virus DNA in the sera of individuals is the most definitive marker of an active viral infection. We have used polymerase chain reaction detection of hepatitis B virus DNA directly on whole blood dried as a spot on filter paper. The method is rapid, specific, and sensitive and has the ability to detect as little as 10 virus particles by ethidium bromide staining of the polymerase chain reaction-amplified products. The method is cost-effective, and the stability of the spots makes the collection and transportation of potentially infectious blood safe.

Hepatitis B virus (HBV) infections represent a major public health problem because of the ability of HBV to cause a chronic carrier state. Even though chronic carriers remain largely asymptomatic, a large number of these individuals subsequently develop cirrhosis and primary hepatocellular carcinomas (2, 8). Active viral replication is traditionally correlated with the serum markers hepatitis B e antigen (HBeAg) and HBV-specific DNA polymerase or direct visualization of virus in serum by electron microscopy (11). These techniques, however, lack the sensitivity to detect low levels of viremia. Nucleic acid hybridization and polymerase chain reaction (PCR) amplification methods have been used for the detection of HBV in serum, with a dramatic increase in sensitivity (5, 13, 16). These methods suffer from the problem of being technically involved, requiring the isolation of viral DNA before detection and the preparation of probes, isotopic or otherwise. Our aim was to develop a procedure that would be directly applicable to blood without the need to separate serum or to isolate DNA. Furthermore, we desired a procedure that could be performed in a single reaction tube and would be free of hybridization protocols that are time-consuming and require the use of radioisotopes for maximum sensitivity.

The technique of collecting blood on filter paper aroused our interest. Dried blood spots (4) are attractive, as they can be prepared with a finger prick rather than with venipuncture, thereby causing less discomfort to the donor. Furthermore, they require minimal storage space, pose little biohazard risk, and facilitate the shipment of specimens. Dried blood spots have been widely used to screen for genetic and metabolic disorders (9, 17, 18), to study polymorphisms in parasitic diseases (12), and to evaluate specimens for infectious diseases after shipment from areas of endemicity to a central laboratory (3, 6). They are ideally suited for large-scale population screening studies, whether for epidemiology or blood bank procedures.

In this study, we have linked the dried blood spot collection method to PCR for the detection of HBV. The entire procedure is carried out in a single reaction tube, is rapid, and is specific. Agarose gel electrophoresis of PCR products and ethidium bromide staining are capable of detecting as few as 10 virus particles in the blood spot.

* Corresponding author.

(This study represents part of the project work of B.P.G. towards fulfillment of the requirements of the M.Sc. Biotechnology Program at Jawaharlal Nehru University, New Delhi, India.)

MATERIALS AND METHODS

Sample collection. The samples used in this study had been submitted for routine HBV serological testing and were part of another study dealing with the transmission of HBV from hepatitis B surface antigen (HBsAg)-positive mothers to newborns. All sera were stored at −20°C. In the initial experiments, stored sera were used after being mixed with blood cells from a donor who was found negative for HBV DNA by PCR amplification with at least two separate sets of primers. Subsequently, whole blood from donors was collected by finger pricking directly onto a piece of Whatman 3MM paper, and the paper was air dried and stored sealed in a plastic bag at −20°C. Virus particles were purified by sucrose density gradient ultracentrifugation (15) and quantitated by dot blot hybridization with a ^{32}P-labelled, full-length HBV DNA probe (19). Standards containing known amounts of cloned HBV DNA were run on the same blot for comparison. The purified stock contained 10^6 virus particles per μl.

Sample preparation. For reconstitution, blood obtained by finger pricking or venipuncture was diluted immediately into 10 volumes of ice-cold saline and the cells were pelleted at 4°C in a microcentrifuge (Eppendorf 5415) at 4,000 rpm for 2 min. After one wash with ice-cold saline, 0.7 volume of serum (relative to the volume of blood originally used) was added to the packed-cell pellet, and the reconstituted blood was spotted immediately on precut 7-mm disks of Whatman 3MM filter paper or a nitrocellulose membrane (BA85; Schleicher & Schuell). Disks were punched out of the dried blood spots with a sterile punch. The disks were placed at the bottom of 0.5-ml microcentrifuge tubes, 75 μl water was added, and the tubes capped and subjected to radiation in a microwave oven (Sharp carousel; 650 W) at full power for 10 min. After brief centrifugation in the microcentrifuge, the PCR mixture was added.

The isolation of HBV DNA from serum was carried out by a modification of a published procedure (1). To 25 μl of serum was added 225 μl of a proteinase K solution containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 0.1 mg of gelatin per ml, 0.45% Nonidet P-40, 0.45% Tween

20, and 60 μg of proteinase K per ml (added fresh from a 10-mg/ml stock prepared in water). After incubation at 56°C for 2 h, proteinase K was inactivated at 95°C for 10 min, and 50 μl of the mixture was used for PCR amplification.

HBV serological testing. The HBV carrier state of each patient (donor) was determined by enzyme immunoassays for HBsAg, HBeAg, and antibody to HBeAg (anti-HBe) (Organon Teknika B.V., Boxtel, Holland) in accordance with the manufacturer's instructions.

DNA amplification and detection. PCR amplifications were carried out in a final volume of 100 μl containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% gelatin, 200 μM each deoxynucleotide triphosphate, 50 pmol each of the upstream and downstream primers, and 1.0 to 1.5 U of *Taq* polymerase (Stratagene, La Jolla, Calif.). After layering with 100 μl of mineral oil (Sigma, St. Louis, Mo.), amplifications were performed for the desired number of cycles on an automated DNA thermocycler (Coy Corp., Ann Arbor, Mich., or Cetus-Perkin Elmer, Norwalk, Conn.) in accordance with the following cycling program: denaturation at 94°C, annealing at 55°C, and extension at 72°C for 1 min each, with a final extension at 72°C for 7 min.

The primer set used, 27-25, was previously used for amplifying HBV DNA from sera and tissues (1, 16) and amplified a conserved region of the core gene. The sequences of these primers are as follows: 27 (sense), 5′-GCG GGATCCACTGTTCAAGCCTCCAAGCT-3′, and 25 (antisense), 5′-GCGAAGCTTAAGGAAAGAAGTCAGAAGG-3′. Oligonucleotide primers were synthesized on an automated DNA synthesizer (model 380B; Applied Biosystems Inc., Foster City, Calif.) by use of phosphoramidite chemistry. The crude oligonucleotides were precipitated with 2.2 M ammonium acetate and 2.5 volumes of ethanol prior to use in the PCR.

Following amplifications, 10 μl of the amplified mixture was run on a 2% agarose (Bio-Rad, Richmond, Calif.) gel and stained with ethidium bromide. For Southern blotting, the stained gel was transferred to a nylon membrane (Zeta probe; Bio-Rad) in transfer buffer, containing 10 mM NaOH and 1 mM EDTA. Following overnight transfer, the membrane was rinsed briefly in transfer buffer, and the DNA was denatured with 0.4 M NaOH for 10 min. The membrane was neutralized with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and baked under vacuum at 80°C for 1 h. Prehybridization, hybridization, and washes were performed in accordance with published procedures (19). For hybridization, a 3.2-kb linearized fragment of cloned HBV DNA was labelled with ^{32}P by use of a nick translation kit (GIBCO BRL, Gaithersburg, Md.). Slot blot hybridizations were carried out as described elsewhere (19).

To avoid contamination and false-positivity in the PCR results, we strictly applied published control measures (14). To prevent cross-contamination at the blood spot level, we applied the blood to precut circles of filter paper. When blood spots were punched out, the stainless steel punch was flamed with alcohol between samples. Spots were stored sealed in separate, disposable plastic bags. Every PCR amplification included positive and negative controls to ascertain faithful amplification.

RESULTS

Initial investigations were aimed at evaluating whether the PCR for HBV detection can be performed directly on whole blood and whether the use of a solid support interferes with such amplification. For this purpose, blood was obtained by finger pricking from one HBV-DNA positive donor and one HBV DNA-negative donor. After spotting of 5 μl of each blood sample on Whatman 3MM filter paper or a BA85 nitrocellulose membrane, PCR amplifications were performed directly with primers specific for the core region of HBV. An amplified product of 140 bp was observed (Fig. 1). That the amplified product was HBV specific was ascertained by Southern blot hybridization with a cloned full-length HBV probe (data not shown). The efficiency of the PCR amplifications did not vary with the type of solid support used (lanes 1 and 3) and appeared similar to that obtained directly with whole blood (without a solid support) (lane 5). For subsequent experiments, therefore, we used filter paper as the support of choice.

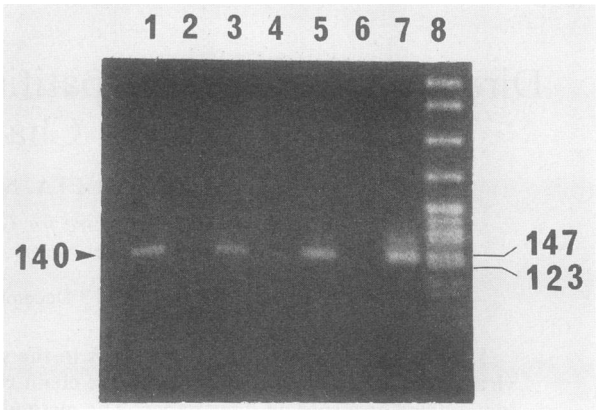

FIG. 1. PCR amplifications of HBV DNA from dried spots of blood from a positive donor (lanes 1, 3, and 5) and a negative donor (lanes 2, 4, and 6). Lanes 1 and 2, spots applied to disks of Whatman 3MM filter paper; lanes 3 and 4, spots applied to disks of a BA85 nitrocellulose membrane; lanes 5 and 6, whole blood (without a solid support); lane 7, cloned HBV DNA positive control; lane 8, *Msp*I-digested pBR322 DNA molecular weight markers. The sizes (base pairs) of the relevant markers are indicated on the right. The arrowhead on the left denotes the 140-bp amplified product.

Microwave treatment of HBV-positive sera has been claimed to increase the sensitivity of detection (7). We therefore used this method for our dried blood spot preparations. Microwave treatment prior to PCR amplifications increased the signal by about two- to threefold, and it did not matter whether the dried blood spots were subjected to microwave treatment directly or after the addition of 75 μl of water to the tube (data not shown). Since elution from the filter paper disk was more complete when microwaving was done with liquid, water was added to the tube for all subsequent investigations.

The sensitivity of the detection method was determined in reconstitution experiments. Serial 10-fold dilutions of purified virus particles were prepared in blood from an HBV DNA-negative donor, spotted on filter paper disks, and analyzed by the PCR. Using this reconstitution system, we were able to detect 10^4 virus particles in each 5-μl blood spot after 40 cycles of amplification and ethidium bromide staining (Fig. 2A). This sensitivity was increased to 10^2 virus particles in Southern blot or slot blot hybridization with a ^{32}P-labelled HBV DNA probe (data not shown). A similar sensitivity of 10 to 100 virus particles after ethidium bromide staining was also obtained when the PCR amplification was increased to 50 cycles (Fig. 2B). The sensitivity of ethidium bromide stain-based detection can be increased another three to four times by applying a larger sample size to the

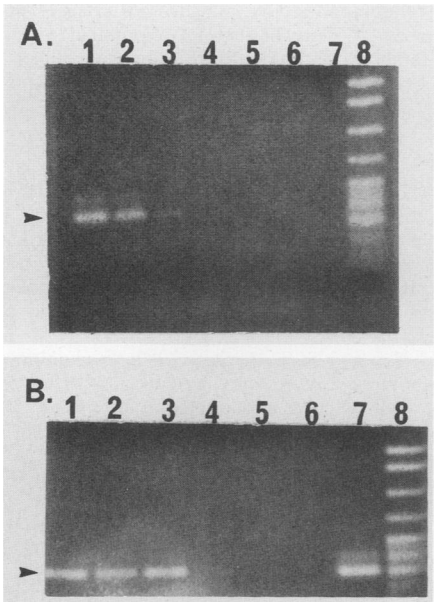

FIG. 2. PCR amplifications of HBV DNA from virus particle dilutions, showing the sensitivity of the method. Purified virus particles from a stock of 10^6 virus particles per μl were diluted into blood from an HBV DNA-negative donor, and 5 μl was applied to filter paper disks. Arrowheads indicate the amplified product. (A) Results of amplifications carried out for 40 cycles. Lanes 1 through 7, decreasing numbers of virus particles (10^6, 10^5, 10^4, 10^3, 10^2, 10^1, and 1, respectively); lane 8, molecular weight markers. (B) Results of amplifications carried out for 50 cycles. Lanes 1 through 5, decreasing numbers of virus particles (10^4, 10^3, 10^2, 10^1, and 1, respectively); lane 6, reagent control; lane 7, cloned HBV DNA-positive control; lane 8, molecular weight markers.

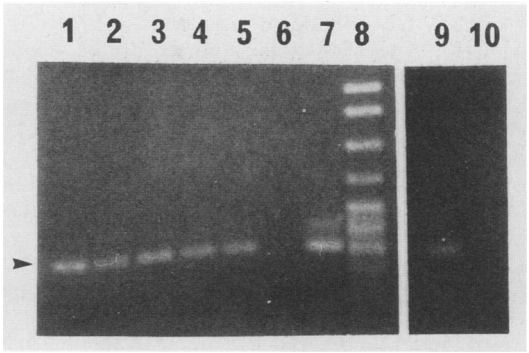

FIG. 3. Stability of the dried blood spots. Five microliters of blood obtained by finger pricking from an HBV DNA-positive patient was applied to each precut 7-mm filter paper disk and stored at 37°C for various times. Amplifications were carried out for 50 cycles. Lanes 1 through 5, dried blood spots stored for 0, 1, 3, 5, and 7 days, respectively; lane 6, reagent control; lane 7, cloned HBV DNA-positive control; lane 8, molecular weight markers; lanes 9 and 10, dried spots of blood obtained from a positive donor (lane 9) or a negative donor (lane 10) and stored for 5 months. The arrowhead indicates the amplified product.

filter paper. In our experience, a 7-mm disk of Whatman 3MM filter paper can be saturated with about 20 μl of blood.

The dried blood spot technique is ideally suited for the collection of samples in the field and for safe transportation. Therefore, we wanted to evaluate analyte stability in the dried blood spots for PCR detection. Spots were stored at 37°C for different times; complete stability was maintained under these conditions for up to 5 months (Fig. 3). Gel analysis of amplifications done on spots stored at 37°C also revealed fewer problems from interference due to denatured proteins. Although there was no difference between DNA amplifications (elutions) for spots stored at 37 versus −20°C, significantly fewer denatured proteins were present in the former after thermal cycling. It may therefore be helpful to routinely store dried blood spots at room temperature prior to analysis.

Finally, the suitability of this method of HBV DNA detection was tested with spots of blood obtained from mothers who were chronic HBV carriers (HBsAg positivity, >6 months) and who were attending the maternity clinic of a local hospital. A total of 60 such patients were analyzed for their HBV DNA status either by PCR amplification of DNA isolated from serum or by PCR amplification of dried blood spots. The negative controls included five laboratory personnel who were healthy, asymptomatic individuals without any previous episode of HBV infection and negative for HBsAg. All five negative controls were found negative for HBV DNA by the PCR.

Among the 60 HBsAg-positive carriers tested, three distinct subgroups emerged—HBeAg positive (m = 12), anti-HBe positive (m = 23), and HBeAg/ and anti-HBe negative (m = 25). All 12 carriers who were found HBeAg positive were also found HBV DNA positive by the conventional serum DNA PCR as well as our technique. Among the anti-HBe-positive carriers, 14 and 13 were found positive for HBV DNA by the serum DNA PCR and our technique, respectively; the remaining 8 carriers in this subgroup were found negative for HBV DNA by both methods. Among the HBeAg-negative and anti-HBe-negative carriers, 19 and 20 were found positive for HBV DNA by the serum DNA PCR and our technique, respectively; 5 carriers were found negative for HBV DNA by both methods. Of the 60 carriers tested, 56 (43 positives and 13 negatives) matched perfectly when analyzed by either method. Among the four carriers that did not match, two were found positive by the serum DNA PCR and negative by the blood spot PCR, whereas the other two were found positive by the blood spot PCR and negative by the serum DNA PCR. However, for all four discrepant carriers, the amplified bands were very weak, although reproducible. Thus, overall there was good agreement between the conventional serum DNA PCR and our blood spot PCR technique in assessing HBV DNA positivity in chronic carriers.

DISCUSSION

We have developed a method for the detection of HBV DNA directly in a dried blood spot by using PCR methodology. The procedure is sensitive, is specific, requires minimal sample handling, and is rapid, taking about 5 h from start to finish.

The detection of HBV DNA is traditionally carried out by dot blotting or PCR amplification of serum (7, 19) or DNA isolated from serum (13, 20). Since all presently available methods require serum as the starting material, blood must be obtained by venipuncture. In this study, we have successfully used PCR for HBV detection in dried spots of blood collected by the finger prick method. The method is capable of routinely detecting 10 to 100 virus particles without the use of hybridization methods. In comparison, direct dot blotting of serum coupled with hybridization to a ^{32}P-labelled probe is capable of detecting as little as 0.1 to

0.01 pg of HBV DNA, which corresponds to 10^4 to 10^3 virus particles (19). The PCR was reported to be capable of detecting 10^3 virus particles with ethidium bromide staining and as few as 3 virus particles with Southern blotting (13). Another report (20) claimed that the PCR with ethidium bromide staining detected as few as 1 to 10 virus particles. However, both of these reports are difficult to evaluate, as cloned HBV DNA and not known amounts of virus were used for the sensitivity analysis.

A potential problem with direct testing of blood by the PCR is the presence of erythrocyte contaminants, particularly the porphyrin moiety of heme, which is a strong inhibitor of the PCR (10). Besides aiding in the disruption of HBV particles, microwave treatment of sera prior to the PCR was suggested to be capable of denaturing such inhibitory factors (7). Therefore, we introduced a microwave treatment step to our protocol. The sensitivity of detection of HBV in serum was estimated to be 10 virus particles with the microwave treatment PCR method (7). Our lower limit of sensitivity of 10 virus particles compares well with that in this method, considering that significantly larger amounts of potential erythrocyte contaminants are present in blood spots than in serum.

Our quantitation experiments indicate that 10 virus particles can be detected after 50 amplification cycles. This means that about 25×10^{-6} pg of HBV DNA (one virus particle contains about 2.5×10^{-6} pg of DNA) can be amplified 2^{50} times, or about 10^{15} times. After amplification of 10 virus particles, 10% of the total material is barely visible on an agarose gel. With a lower limit of detection of 10 to 20 ng, the final amplified mixture contains about 200 ng of DNA. This corresponds to an amplification of about 10^{10} times. The average efficiency of each PCR cycle, therefore, under the conditions of our assay is greater than 75%. The limits of detection were found to be 10^4 and 10 particles after 40 and 50 amplification cycles, respectively. This 10-fold increase in sensitivity agrees well with the relative increase in theoretical product yield obtained with an increase from 40 to 50 amplification cycles.

When the blood spot PCR technique was used to assess HBV DNA positivity in HBsAg-positive carriers, good agreement was found with the established method of HBV DNA detection by PCR following preparation of DNA from serum. However, because the blood spot method is a single-step procedure, it is more convenient and less prone to contamination in a clinical laboratory setting. When this technique was used to assess HBV DNA positivity in HBeAg- or anti-HBe-positive carriers the data support previous observations (13, 20) that (i) HBV DNA positivity is generally high in HBsAg- and HBeAg-positive sera, compared with that in HBsAg- and anti-HBe-positive sera, and (ii) seroconversion from HBeAg to anti-HBe may be accompanied by a decrease in, but not a total disappearance of, circulating levels of virus particles.

Thus, we have developed a rapid, specific, and sensitive direct test for HBV DNA in blood obtained from patients by simple finger pricking. Whether such a method can be used for the simultaneous detection of more than one blood-borne pathogen remains to be seen and is the subject of current investigations.

ACKNOWLEDGMENTS

We thank S. K. Panda for clinical material, the purified virus preparation, and the use of the thermocycler and A. Bhardwaj for oligonucleotide synthesis.

This work was supported by funds from ICGEB. Assistance from the Department of Biotechnology, Government of India, for an M.Sc. Scholarship to B.P.G. and from the Rockefeller Foundation for a Biotechnology Career Fellowship to S.J. is acknowledged.

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