Detection of Hepatitis B Virus in Plasma Using Flow Cytometric Analyses of Polymerase Chain Reaction-Amplified DNA Incorporating Digoxigenin-11-dUTP

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Blood donations are routinely screened by multiple serologic assays for antigens/antibodies associated with infection by blood-borne viruses, including hepatitis B virus (HBV), hepatitis C virus (HCV), human immunodeficiency viruses (HIV-1 and HIV-2), and human T-cell lymphotropic virus (HTLV-I and HTLV-II). A direct detection of these viruses would be more effective for the prevention of transfusion-transmitted infections than the indirect measurement of the variable host immune response to these agents. Because the polymerase chain reaction (PCR) for viral gene amplification offers the most sensitive and direct means of detecting viruses in blood, we have developed a nonisotopic PCR procedure for the detection of HBV, chosen as a prototype. The problems, common to previously described PCR methods, of nucleic acid extraction and inhibition of the PCR by plasma proteins were overcome by inhibition of HBV from plasma by means of 450-μm polystyrene beads covalently coated with monoclonal antibody to the Pre-S, region of the viral envelope protein. Detergent lysis and proteinase K digestion of the immunocaptured virions isolated from plasma released the HBV DNA. A modified PCR-amplification protocol, incorporating digoxigenin-labeled dUTP in the amplified gene products followed by hybridization with a specific biotinylated oligonucleotide probe bound to streptavidin-coated 2.8-μm magnetic beads, allowed flow cytometric analyses of HBV-specific PCR products by means of antibodies to digoxigenin labeled with fluorescein isothiocyanate. The endpoint serial dilutions of pedigreed human plasma samples containing chimpanzee infectious dose (CID₀) of 10⁷ for adw and CID₀ of 10⁷.8 for the ayw subtypes were compared in repeated testing of PCR products by our immunoreactive bead (PCR-IRB) assay. HBV DNA was consistently detected in a 5 × 10⁻⁶ dilution of each sample. In testing 20 coded specimens of blood donors, with or without serologic markers of HBV infection, the PCR-IRB was specific and more sensitive than the PCR analyses by slot blot hybridization with radioactive probe. The PCR-IRB assay can be adapted for simultaneous detection of multiple blood-borne viruses by an automated flow cytometric analysis system.

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MATERIALS AND METHODS

Sources of plasma with HBV. Two pedigreed human plasma samples containing HBV/adw and HBV/ayw, respectively, were re-

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ceived from the Food and Drug Administration Center for Biologic Research and Evaluation. The HBV/adsW was derived from an asymptomatic chronic carrier and had a chimpanzee infectious dose (CID_{50}) of 10^{-7.5} mL. The HBV/adsW was a pool of serial bleedings from a single patient with acute hepatitis B and had a CID_{50} of 10^{-7.5} mL. The hepatitis B surface antigen (HBsAg) by radioimmunoassay was detectable at dilutions 10 to 100 times lower than those proven infectious in chimpanzees. A third specimen was plasma from an asymptomatic chronic carrier (W.M.) of HBsAg. To evaluate the performance of the noisotopic detection of amplified HBV DNA, 20 specimens of blood donors, with or without serologic markers of HBV infection, were selected and coded for PCR tests. Serial 10-fold dilutions of each specimen were made in fetal bovine serum (FBS) and stored as 200-µL aliquots at -70°C.

**Coupling of antibodies to beads.** Blue-dyed polystyrene beads 400 to 450 µm in size (Polysciences, Inc, Waltham, PA) were rehydrated with phosphate-buffered saline (PBS) for 2 hours, washed three times with PBS, and 2.5 mg of the beads were resuspended in 5 mL of 8% glutaraldehyde in PBS. The beads were mixed on an end-to-end rocking mixer for 4 hours at room temperature, washed three times with PBS, and then 50 µg of the monoclonal antibody to Pre-S2 region of HBsAg (Abbott Laboratories, Chicago, IL) was added to 600 beads. After mixing overnight at room temperature, the beads were washed three times in PBS and then resuspended in 2.5 mL of 0.2 mol/L ethanolamine with gentle agitation for 30 minutes. To block nonspecific protein-binding sites, the beads were resuspended in 2.5 mL of PBS containing 0.5% bovine serum albumin (BSA; Polysciences Inc, Waltham, PA) for 30 minutes at room temperature. Similarly, monoclonal antibodies (MoAbs) to the pre-S2 region of HBsAg and to the common "a" determinant of HBsAg (Abbott Laboratories, Chicago, IL) also were used in the immunofluorimetry isolation of virions from the plasma (W.M.) for a functional evaluation of PCR amplification of HBV DNA.

**Isolation of HBV DNA from plasma.** Two hundred-microliter aliquots of 10-fold dilutions of the test plasma were mixed with 50 beads coated with antibodies and incubated overnight at 37°C with gentle agitation to capture HBV. After three washes with PBS, the beads were resuspended in 2.5 mL of 0.2 mol/L ethanolamine with gentle agitation for 30 minutes. To block nonspecific protein-binding sites, the beads were resuspended in 2.5 mL of PBS containing 0.5% bovine serum albumin (BSA; Polysciences Inc, Waltham, PA) for 30 minutes at room temperature. Similarly, monoclonal antibodies (MoAbs) to the pre-S2 region of HBsAg and to the common "a" determinant of HBsAg (Abbott Laboratories, Chicago, IL) also were used in the immunofluorimetry isolation of virions from the plasma (W.M.) for a functional evaluation of PCR amplification of HBV DNA.

**Capture of amplified DNA to beads.** The 5′ biotinylated oligonucleotide probe, BGY3 (ACTGGTCCAAGCTTCAAGCTG-TGCCCCTGTTGGTGGCTTTGGG), was obtained from Clontech (Palo Alto, CA). Fifty picomoles of BGY3 were added to 10⁶ streptavidin-coated, superparamagnetic polystyrene beads (2.8 µm in diameter; Dynal, Inc, Great Neck, NY) in 100 µL of PBS and incubated at room temperature for 30 minutes with gentle agitation. The beads were washed three times with PBS, separated with a magnetic particle concentrator (Dynal), and resuspended in 100 µL of PBS.

**Isolation of HBV DNA from plasma.** Two hundred-microliter aliquots of 10-fold dilutions of the test plasma were mixed with 50 beads coated with antibodies and incubated overnight at 37°C with gentle agitation to capture HBV. After three washes with 300 µL of 1× Taq buffer (50 mmol/L KCl, 10 mmol/L Tris-hydroxymethylaminomethane [TRIS], pH 8.3, 2.0 mmol/L MgCl₂, 0.5% Nonidet P-40, 0.5% polyoxyethylene sorbilan monolaurate [Tween 20; Sigma, St Louis, MO] containing 250 µg/mL of proteinase K, and incubated at 60°C for 1 hour. Proteinase K was then inactivated at 95°C for 20 minutes. Twenty-microliter aliquots of the supernatant were stored at -70°C. For each experiment, 200 µL of FBS and PCR grade water were used as negative controls. For conventional PCR, 200-µL aliquots of serial 10-fold dilutions of serum specimens were digested with lysis buffer (50 mmol/L KCl, 10 mmol/L TRIS pH 8.3, 2.5 mmol/L MgCl₂, 0.5% Nonidet P-40, 0.5% Tween 20, and 0.5% SDS) containing 200 µg/mL proteinase K and incubated overnight at 37°C, after which the proteinase K was denatured at 100°C for 10 minutes, followed by phenol/chloroform/isopropanol extraction and precipitation as previously described. The pellet was resuspended in 50 µL of Tris-EDTA (TE) buffer, pH 8.0, half of which was used for PCR.

**PCR.** Conventional PCR, followed by slot blot hybridization, and autoradiography were performed, as previously described by Urich et al. For development of PCR-IRB, the following combinations of dTTP and digoxigenin-11-dUTP (dig-11-dUTP; Boehringer Mannheim Biochemicals, Indianapolis, IN) were used in PCR: dTTP 167 µmol/L and dig-11-dUTP 33 µmol/L (5:1); dTTP 182 µmol/L and dig-11-dUTP 18 µmol/L (10:1); dTTP 191 µmol/L, and dig-11-dUTP 9 µmol/L (20:1). The concentrations of dATP, dCTP, and dGTP were 200 µmol/L. A 100/µL reaction mixture contained a total of 50 µmol/L KCl, 10 mmol/L TRIS pH 8.3, 2.0 µmol/L MgCl₂, 0.01% gelatin, 2.5 U of Taq DNA polymerase (Cetus; Emeryville, CA), 0.5 µmol/L of the primer P55 (GAGGGAGGATTAGTTAA, nucleotide no. 1747 to 1764; Fig 1), 0.5 µmol/L of the primer GY2 (GAGGTGCGAATCCACACTC, nucleotide no. 2377 to 2360), and 10 µL of the sample. The primers were selected on the basis of sequence alignment of five HBV isolates and annealed to conserved regions of the HBV genome for amplifying a 630-bp fragment (Fig 1). The reaction was run through 40 cycles of amplification in a DNA Thermal Cycler (Perkin-Elmer Norwalk, CT). After the initial denaturation at 94°C for 1 minute, each cycle comprised denaturation at 94°C for 30 seconds, annealing at 40°C for 30 seconds, and extension at 72°C for 1 minute. Final extension was at 72°C for 5 minutes.

**Capture of amplified DNA to beads.** Relative performance of the following four different procedures was evaluated: (1) Ten-microliter aliquot of amplified HBV DNA was added to 10 µL of probe-coated beads (4×10⁵) in hybridization buffer (HB, 60 mmol/L NaCl, 40 mmol/L EDTA). After 2 minutes at 94°C for denaturation, the samples were hybridized for 30 minutes at 60°C. (2) Ten microliters of amplified DNA were denatured at 94°C for 2 minutes, chilled on ice, and then 10 µL of probe-coated beads (4×10⁵) in HB were added. Hybridization was carried out at 60°C for 30 minutes. (3) Ten microliters of amplified product were denatured at 94°C for 2 minutes, chilled on ice, 2 µmol/L of the oligonucleotide probe added, and hybridized at 60°C for 30 minutes. Then magnetic streptavidin-coated beads (4×10⁵) were added and incubated at room temperature for 30 minutes. (4) Ten microliters of amplified HBV DNA were added into 10 µL of HB containing 2 µmol/L of the oligonucleotide probe, denatured at 94°C for 2 minutes, and hybridized at 60°C for 30 minutes. Then the magnetic streptavidin-coated beads (4×10⁵) were added and incubated at room temperature for 30 minutes. After capture of amplified DNA to beads, the beads were washed three times with buffer (100 mmol/L TRIS, pH 8.0, 10 mmol/L EDTA, 50 mmol/L NaCl, 0.01% NP-40, and 0.01% Tween 20) at room temperature with gentle agitation for 5 minutes.

**Immunofluorescent detection of amplified DNA by flow cytometry.** Fluorescein isothiocyanate–conjugated antibody to digoxigenin (Boehringer Mannheim Biochemicals, Indianapolis, IN) was diluted (1:1; 1:10; 1:100; 1:500; 1:1000; 1:5000) in PBS containing 0.5% BSA, 0.01% NP-40, and 0.01% Tween 20 at room temperature with gentle agitation for 5 minutes. After the incubation, the beads were washed three times with buffer and suspended in 300 µL of Haema Line 2 (Serrano-Baker Instruments Corp, Allentown, PA). The mean channel fluorescence (MCF) of the beads was determined by flow cytometry using a FACScan (Becton Dickinson, San Jose, CA).

**RESULTS**

**Immunocapture of HBV from plasma.** Each group of blue-dyed polystyrene beads was coated with specific MoAbs to (1) HBsAg, (2) Pre-S2, and (3) Pre-S1 (components of the
envelope protein). Each of the three MoAbs was evaluated for relative efficiency of the immunoaffinity procedure in yielding HBV DNA amplifiable in a conventional PCR procedure using hybridization with a $^{32}$P-labeled oligomer probe. The anti-HBs and anti-Pre-S2 yielded amplifiable HBV DNA from $10^{-6}$ and $10^{-9}$ dilutions of W.M. plasma, respectively. However, the anti-Pre-S1 yielded amplifiable HBV DNA from $10^{-12}$ dilution of W.M. plasma. After three independent repeat experiments yielded similar results of amplifiable HBV DNA, the anti-Pre-S1 was selected as the immunoaffinity substrate for further experimental work on the development of PCR-IRB procedure. Because normal human serum and FBS gave comparable results in preliminary work and because FBS was used as a diluent for plasma samples, we used FBS as an HBV-negative control in standardization of PCR-IRB assay.

Incorporation of digoxigenin-11-dUTP into amplified HBV DNA. In order to obtain optimal conditions for PCR amplification and dig-11-dUTP incorporation, we tested three different proportions of dTTP and dig-11-dUTP on HBV DNA as a template. HBV was isolated from a $5 \times 10^{-7}$ dilution of the pedigreed adw plasma by immunocapture with anti-Pre-S1, whereas normal human serum, FBS, and water were used as three negative controls. After amplification, the PCR product was incubated with probe-coated beads, followed by reaction with 1:100 dilution of fluorescent antidigoxigenin, and then analyzed by flow cytometry. For each of the three dTTP:dig-11-dUTP proportions (5:1, 10:1, 20:1), five independent experiments were performed and the results showed no significant difference. The assay is highly reproducible, as judged by the small standard deviation of the MCF values. The MCF for normal human serum and FBS were very similar, and hence FBS was consistently used as the negative control. The specimen to control ratio (S/C) of the MCF for each of the 5:1, 10:1, and 20:1 proportions were calculated as 2.29, 2.52, and 2.34, respectively. Therefore, we selected 10:1 proportion of dTTP:dig-11-dUTP for further work on PCR-IRB.

Hybridization of digoxigenin-labeled, amplified DNA to probe-coated beads. Four different experimental methods (described above) were tested to determine the optimal hybridization conditions for digoxigenin-labeled, amplified HBV DNA binding with the biotinylated probe. Methods (1) and (2), where the oligonucleotide was immobilized on the beads prior to hybridization, gave higher absolute MCF values and also higher S/C ratios than methods (3) and (4) (Table 1). In methods (3) and (4), hybridization of the oligonucleotide and amplified DNA was performed in solution prior to capturing the hybrids onto streptavidin beads. These results suggest that the binding between streptavidin-coated beads...
and the hybrids consisting of biotinylated probe and amplified DNA is impaired in these latter methods. Based on the results, method (1) was the optimal hybridization procedure because it gave the highest absolute MCF value, the highest S/C ratio, and was methodologically easier to perform.

**Optimization of fluorescent antidigoxigenin concentration.** We tested six different dilutions of fluorescein-labeled antidigoxigenin for the detection of digoxigenin-labeled, amplified HBV DNA. The results of three independent experiments with each antibody concentration are shown in Table 2. The S/C MCF ratio was optimal with 1:100 dilution of the antidigoxigenin and the nonspecific MCF values for both HBV DNA and negative controls increased at higher concentrations of the fluorescein-labeled antidigoxigenin. The 1:100 dilution was optimal in regard to the specific and nonspecific binding of the antibody; ie, the optimal S/C ratio was 2.53.

**Evaluation of nonradioactive detection of amplified DNA by flow cytometry.** Three well-characterized HBV-positive human plasma samples (adw, ayw, and W.M.) were used for the evaluation of our PCR-IRB assay standardized as above. We analyzed, in duplicate, serial 10-fold dilutions of these samples. Six independent measurements of FBS were used as a negative control and gave a mean MCF of 278 ± 30. Therefore, using a seven times standard deviation of the mean MCF for FBS, we calculated 488 as the cut-off value for PCR-IRB positivity. Table 3 shows the mean of four measured MCF values ± SD for the serial 10-fold dilutions ranging from 5 × 10^-7 to 5 × 10^-13 for each of the adw, ayw, and W.M. plasma specimens. The data show that PCR-IRB detected HBV DNA in both adw and ayw plasma specimens at 5 × 10^-10 and lower dilutions. The 5 × 10^-10 and greater dilutions of W.M. plasma gave results close to the cut-off value of 488 but were considered negative; all lower dilutions of the W.M. plasma were positive for amplifiable HBV DNA.

Consecutive 20 plasma specimens of healthy blood donors, with or without serologic markers of HBV infection, when tested under code, showed that PCR-IRB consistently performed as well or better than the conventional PCR slot blot hybridization with radioisotope-labeled probe, as shown in Table 4. After PCR amplification, both slot blot hybridization and the PCR-IRB detected HBV DNA in all HBsAg-positive specimens and also in an anti-HBc reactive specimen (no. 17) from an HBsAg-negative blood donor. Excepting specimen no. 10, PCR-IRB detected HBV DNA in each plasma at dilutions 10- to 1,000-fold higher than that for slot blot hybridization.

**DISCUSSION**

PCR has been reported to detect a single copy of a specific DNA sequence out of the total DNA sequences from 10^6 cells. However, purification of viral DNA from the infected plasma is necessary because of an inhibitory effect of plasma proteins on the performance of PCR. By coupling the high-affinity anti–Pre-S1 monoclonal antibody onto polystyrene beads for immunoaffinity isolation of HBV particles from the test plasma, we have simplified the recovery of viral DNA and overcome the inhibitory effect of the human plasma proteins on PCR. The yield of HBV DNA amplifiable by PCR was relatively greater when anti–Pre-S1 was used (in comparison with anti–Pre-S2 or anti-HBs) for immunoaffinity isolation of the virions. This is consistent with the fact that the pre-S1 region contains the viral receptor for hepatocytes, thus enabling the anti–Pre-S1 to preferentially capture the virions over the excess of DNA-free HBsAg circulating in the blood of infected persons. We have demonstrated this improved yield of HBV DNA amplifiable in both the conventional PCR with radioactive probe hybridization as well as our PCR-IRB.

| Table 1. Results of Four Different Hybridization Procedures for the Flow Cytometric Detection of HBV DNA Amplified From 5 × 10^-7 Dilution of W.M. Plasma |
|-----------------|----------|----------|----------|----------|
|                | Hybridization Method |
| HBV DNA        | 1        | 2        | 3        | 4        |
| FBS            | 143.5 ± 14.3 | 276.2 ± 58.7 | 290.7 ± 27.4 | 292.0 ± 28.3 |
| S/C ratio      | 4.60     | 2.20     | 1.35     | 1.32     |

Data indicate mean MCF ± SD of five independent experiments, and the specimen/control (S/C) ratio represents relative performance of each of the hybridization procedures. See Materials and Methods for procedures 1, 2, 3, and 4.

| Table 2. Influence of Different Concentrations of Fluorescenated Antidigoxigenin on the Flow Cytometric Detection of HBV DNA |
|---------------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Dilutions of Antidigoxigenin | 1:1 | 1:10 | 1:100 | 1:500 | 1:1,000 | 1:5,000 |
| HBV DNA             | 824.0 ± 14       | 777.3 ± 3.1     | 772.0 ± 12.3    | 654.0 ± 18.2    | 529.0 ± 20.7    | 441.0 ± 23.3    |
| FBS                 | 623.0 ± 25.4     | 470.0 ± 32.7    | 306.0 ± 7.1     | 313.0 ± 17.5    | 288.3 ± 13.1    | 278.3 ± 8.5     |
| S/C ratio           | 1.32             | 1.65            | 2.53            | 2.08            | 1.83            | 1.58            |

Data indicate mean MCF ± SD of three independent experiments. The specimen/control (S/C) ratio was used as a guide for selection of optimal antibody dilution.
Our data demonstrate that (1) consistent with previous reports, HBV DNA can be released from the bead-captured virions by detergent lysis and digestion with proteinase K for successful amplification by PCR; (2) digoxigenin-labeled dUTP can be used for nonradioactive labeling of amplified HBV DNA sequences during PCR; (3) digoxigenin, linked to dUTP via an 11-atom linear spacer, does not have any adverse effect on the hybridization of the PCR products with biotinylated probe prebound to streptavidin-coated magnetic beads; and (4) the hybridized products of PCR can be detected by flow cytometric analysis of the magnetic beads after reaction with FITC-labeled antidigoxigenin.

HBsAg is only detectable by immunoassays in the plasma at dilutions 10 to 100 times lower than the proven infectivity of dilutions of $10^{-5}$ for $\text{adw}$ and $10^{-2.5}$ for $\text{ayw}$ following inoculation in chimpanzees. The conventional PCR and hybridization with radiolabeled probes could detect HBV DNA in the same dilutions or a 10-fold greater dilution than the infectious dose in chimpanzees. Similarly, the previously reported PCR procedures with immunocapture of HBV have detected HBV DNA in a 10-fold greater dilution than the infectious dose in chimpanzees.

In this study, the PCR-IRB could consistently detect HBV DNA in a 1,000-fold greater dilution than the infectious dose in chimpanzees. In this study, the PCR-IRB could consistently detect HBV DNA in a 1,000-fold greater dilution than the infectious dose in chimpanzees.

In conclusion, the higher sensitivity, specificity, and non-radioactive solid-phase probe that is comparable in cost with the conventional radioactive detection procedure, render PCR-IRB assay potentially useful for clinical diagnosis and therapeutic monitoring of viremia in HBV-infected patients. We have recently adapted the IRB test used for the detection of anti-HIV by Scillian et al. to design a totally automated blood-screening system (manuscript in preparation). Similarly, the PCR-IRB lends itself to automation, with possible simultaneous amplification of the viral nucleic acids for direct detection of various blood-borne viruses.

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Detection of hepatitis B virus in plasma using flow cytometric analyses of polymerase chain reaction-amplified DNA incorporating digoxigenin-11-dUTP

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