Digoxigenin-Labeled Probes for the Detection of Hepatitis B Virus DNA in Serum

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A nonradioactive hybridization assay for the detection of hepatitis B virus (HBV) DNA in serum with a digoxigenin-labeled probe is described. The probe was sensitive, being able to detect 0.25 pg of homologous HBV DNA, equivalent to 7 × 10⁶ genome copies. After extraction of DNA from clinical samples, the probe detected HBV DNA in 11 of 12 hepatitis B e antigen-positive sera and did not react with 6 hepatitis B surface antigen-negative sera. This result was comparable to that obtained with a radiolabeled probe. When serum samples were treated by the alkaline denaturation method, some false-positive reactions were apparent with the digoxigenin-labeled probe, although their frequency could be reduced to around 8% by modifying the sample treatment with a centrifugation step. Overall, the sensitivity and specificity of the digoxigenin-labeled probe indicate that it is a viable alternative to the radiolabeled probe for the detection of HBV DNA in serum. The lack of radioactive reagents in the digoxigenin labeling and detection system and its long shelf-life make this system suitable for routine use in laboratories.

Hepatitis B virus (HBV) infection represents a major public health problem because of the ability of HBV to cause a chronic infection. While a chronic infection may remain asymptomatic, a significant number of infected individuals subsequently develop liver disease, including cirrhosis and primary hepatocellular carcinoma (5, 11). To determine whether active virus replication is taking place, one can assay a number of serum markers, such as hepatitis B e antigen (HBeAg) and HBV-specific DNA polymerase, or the presence of virions detected in serum by electron microscopy (6). Unfortunately, these assays suffer either from a lack of sensitivity or from being technically involved.

Nucleic acid hybridization assays have become useful tools for the diagnosis of many infectious diseases (15). The detection of HBV DNA in serum by hybridization techniques is a direct measure of the quantity of virus present and correlates closely with infectivity (1–3, 10, 14, 17). One of the major obstacles to the wider application of such assays has been the requirement for radiolabeled probes, usually 32P, to obtain maximum sensitivity. The use of radioisotopes is not desirable in most laboratories because of safety considerations, and the short half-life means frequent probe preparation. To overcome such limitations, researchers investigated a number of nonradioactive labeling procedures, the biotin-avidin system being the most commonly used. However, when biotinylated probes were used to assay HBV DNA in serum, the limit of detection was found to be significantly poorer than that for radiolabeled probes and, furthermore, a significant proportion of samples gave false-positive signals (4, 13). More recently, an alternative nonradioactive labeling and detection system has been developed by random primer labeling of DNA with digoxigenin-UTP. In a brief report, this system was shown to have a sensitivity comparable to that of radiolabeling in dot blot hybridization assays for HBV DNA from serum (4).

In the present study, we confirm that digoxigenin-labeled probes and radiolabeled probes have similar abilities to detect HBV DNA in serum. We also show that some false-positive reactions can occur but that these can be limited by technical strategies.

MATERIALS AND METHODS

Patient sera. The serum samples used in this study had been previously submitted for routine screening of HBV serological markers and were stored at −20°C. Samples were tested for hepatitis B surface antigen (HBsAg) and HBeAg with solid-phase radioimmunoassay kits obtained commercially from Abbott Laboratories (North Chicago, Ill.). From these samples, a panel of HBeAg-positive and HBsAg-negative sera were selected for the HBV DNA dot blot hybridization assays. Aliquots of fresh, unfrozen sera were also tested in the dot blot hybridization assays. These sera were all subsequently shown to be HBsAg negative.

Preparation of probe material. Full-length HBV DNA inserted into the PstI site of pBR322 was a gift from C. Burrell. Insert DNA was excised from the plasmid by restriction endonuclease digestion, separated by preparative agarose gel electrophoresis, and removed by electroelution (12). The insert DNA was purified by ion-exchange chromatography with an NACS PREPAC column (Bethesda Research Laboratories, Gaithersburg, Md.). HBV DNA was either radiolabeled with [α-32P]dATP with a hexamer-primer labeling kit (Bresatec, Adelaide, South Australia) or labeled with digoxigenin-11-dUTP with a kit supplied by Boehringer (Mannheim, Federal Republic of Germany). Labeling was carried out by following the manufacturers' instructions. In brief, both procedures are based on the annealing of random hexamers to the single strand of the denatured DNA, with the incorporation of either the radiolabeled nucleotide or the nucleotide analog (digoxigenin-11-dUTP) into the growing second strand in a Klenow enzyme-mediated reaction.

Preparation of samples. Two procedures for sample preparation were used. (i) Phenol-chloroform extraction was done as follows. Aliquots of 25 μl of serum were made up to 100 μl with TE (Tris hydrochloride [pH 8.0], 1 mM EDTA) and treated with 200 μg of pronase per ml in 1% sodium...
dodecyl sulfate (SDS) at 37°C for 2 h. DNA was extracted with an equal volume of phenol-chloroform (1:1) and precipitated with ethanol, and the resultant pellet was resuspended in 100 µl of TE. The DNA was denatured by being boiled, rapidly cooled on ice, and mixed with an equal volume of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7.0]) prior to application to a nitrocellulose membrane. (ii) Alkaline denaturation was done as follows. Aliquots of 25 µl of serum were denatured with an equal volume of 1 M NaOH–2 M NaCl for 10 min at room temperature, and the reaction was neutralized by the addition of 50 µl of 0.5 M Tris hydrochloride (pH 7.4)–2.5 M NaCl. The treated serum was applied to a nitrocellulose membrane. This procedure was later modified by including a centrifugation step after the neutralization. In this case, the samples were clarified by being centrifuged at 10,000 × g for 5 min in an Eppendorf centrifuge before being applied to the membrane.

Hybridization conditions. Samples were applied to a prewetted (water and then 20× SSC) nitrocellulose membrane (Hybond-C extra; Amersham International, Amersham, England) with a dot blot manifold (Bio-Rad, Richmond, Calif.). The membrane was baked at 80°C for 2 h and prehybridized at 42°C for 3 h. For the radiolabeled probe, prehybridization solution was 50% deionized formamide–5× SSC, 2× Denhardt solution, 5 mM sodium phosphate (pH 6.5)–200 µg of denatured herring sperm DNA per ml. The heat-denatured radiolabeled probe was added to a concentration of at least 2 × 10^6 cpm/ml, and hybridization was done overnight at 42°C. For the digoxigenin-labeled probe, hybridization solution was prepared in accordance with the manufacturer’s recommendations and was 50% deionized formamide–5× SSC–0.1% N-lauroylsarcosine–0.02% SDS–5% blocking reagent (supplied by the manufacturer). The digoxigenin-labeled probe was added to a concentration of 20 to 30 ng/ml. The time and temperature of hybridization were the same as those used for the radiolabeled probe. After hybridization, membranes were washed twice in 2× SSC-0.1% SDS for 5 min each at room temperature and twice in 0.1× SSC-0.1% SDS for 30 min each at 50°C. Radiolabeled DNA was detected by exposure of the membrane to X-ray film at −70°C between intensifying screens. When required for reprobing, the radiolabeled probe was removed from the membrane by brief boiling in 0.1% SDS. Digoxigenin-labeled DNA was detected immunologically by following the manufacturer’s protocol. The detection system uses an antibody-enzyme conjugate, anti-digoxigenin–alkaline phosphatase, and the DNA is visualized by an enzyme-linked color reaction performed in the dark. The manufacturer recommends a further blocking step prior to the addition of the enzyme conjugate when nylon membranes have been used. We found this step necessary with nitrocellulose membranes as well.

RESULTS

The relative sensitivities of the radiolabeled probe and the digoxigenin-labeled probe were determined by hybridization with membranes containing dilutions of recombinant HBV DNA (Fig. 1). For the radiolabeled dot blot, autoradiography was carried out for 18 h (Fig. 1A), and for direct comparison, the color development of the digoxigenin-labeled dot blot was allowed to proceed for the same length of time (Fig. 1B). Under these conditions, the digoxigenin-labeled probe showed a sensitivity superior to that of radiolabeled probe, detecting as little as 0.25 pg of HBV DNA. The 32P-probed filter required a 4-day exposure to X-ray film to achieve the same level of sensitivity. Neither probe hybridized to 10 ng of nonspecific DNA of bacteriophage λ.

For the clinical samples, our initial evaluation of the probes was carried out after phenol-chloroform extractions were performed on 12 HBeAg-positive serum samples to isolate any HBV DNA present. Extractions were also performed on six HBsAg-negative serum samples which served as HBV DNA-negative controls. After immobilization of the target material, hybridization with the radiolabeled probe showed that HBV DNA could be detected in 11 of the 12 HBeAg-positive samples (Fig. 2A). The radiolabeled probe was removed from the membrane by boiling in 0.1% SDS; reexposure to X-ray film indicated that this technique was effective in removing the bulk of the label from the target DNA (results not shown). The two procedures were compared after hybridization of the filter with the digoxigenin-
labeled probe (Fig. 2B). Both probes detected HBV DNA in the same 11 HBeAg-positive samples, and no signals from the HBsAg-negative samples were observed with either method.

The alkaline denaturation treatment of serum for the detection of HBV DNA is a rapid and more convenient procedure than individual DNA extractions when multiple samples are to be assayed and, as a consequence, it is more commonly used. Hence, the specificity and sensitivity of the digoxigenin-labeled probe were tested on alkaline-denatured target material. For the serum samples previously tested, four of the HBeAg-positive samples shown to contain HBV DNA and three of the HBsAg-negative samples were subjected to alkaline denaturation. Hybridization with the radiolabeled probe revealed four positive and three negative signals, as expected (Fig. 3A, row 1). In contrast, reprobing with the digoxigenin label showed that under alkaline denaturation conditions, some HBV DNA-negative samples can produce a false-positive signal (Fig. 3B, row 1, e to g). Increasing the concentration of the blocking reagent used prior to the binding of the antibody conjugate and decreasing the color development time only succeeded in reducing the intensity of the false-positive signal.

In an attempt to reduce this interference, we introduced a short centrifugation step after neutralization. When samples pretreated in this manner were probed with the digoxigenin label, most of the false-positive reactions were eliminated (Fig. 3B, row 2), but one of the samples still gave a positive signal (Fig. 3B, row 2, g). The frequency of false-positive signals was shown to be about 8%, because the assay of a further 50 HBsAg-negative serum samples under the same conditions produced another four false-positive signals. Interestingly, when an aliquot of each of the 50 serum samples was assayed prior to being frozen and stored, no false-positive reactions were encountered (results not shown).

The extra centrifugation step resulted in a negligible loss of sensitivity for the HBV DNA-positive samples (Fig. 3A, compare row 2 to row 1), but neither of the alkaline denaturation protocols gave a signal as strong as that obtained with the phenol-chloroform-extracted DNA (Fig. 3A and B, rows 3).

**DISCUSSION**

We compared the radiolabeled and digoxigenin-labeled DNA hybridization assays for their ability to detect homologous HBV DNA; both showed a similar detection limit of 0.25 pg. With the digoxigenin system, however, the result was obtained more rapidly, because the time required for color development was much shorter than the autoradiography exposure time. In all of the hybridization assays, the digoxigenin-labeled probe reproducibly gave a more rapid result than did the radiolabeled probe, and we found that 3 h of color development was roughly equivalent to overnight autoradiography. This finding correlates closely with that of Kimpton et al. (8), who found the digoxigenin assay to be more rapid than radiolabeling for probing homologous cytomegalovirus DNA and cytomegalovirus DNA extracted from cells or urine.

The digoxigenin-labeled probe did not react with bacteriophage λ DNA, and it demonstrated the same specificity as did the radiolabeled probe with partially purified DNA from serum specimens. Unfortunately, the use of the digoxigenin-labeled probe for the detection of HBV DNA by the direct alkaline denaturation method could not be recommended because false-positive signals were obtained. A brief clarifying centrifugation of the serum after neutralization reduced the frequency of nonspecific binding, but this procedure was still not as reliable as that of extracting DNA from serum with phenol-chloroform and the positive signals were not as strong. The interference was probably due to protein or protein-lipid binding to the membrane that reacted later in the immunological detection of digoxigenin. The proteinaceous material responsible for the false-positive reactions was only present in some sera that had been frozen and thawed, because no false-positives were detected when fresh sera were used.

The detection of HBV DNA in serum is probably the most accurate guide to the presence of active viral replication and ideally should be used in the routine screening of all HBsAg-positive patients. The use of molecular hybridization assays for routine HBV DNA detection in clinical samples would be enhanced if a suitable alternative to radiolabeled probes were available. Our results suggest that digoxigenin-labeled probes may represent such an alternative. These probes have a long shelf-life, can be safely reused several times (thus conserving probe), and showed a detection limit of 0.25 pg or about $7 \times 10^4$ HBV genome copies, similar to the limits that have been claimed with other conventional hybridization assays.

This limit of detection with the direct hybridization assay presents a problem because false-negative results can still
occupy, particularly with chronic active hepatitis, in which there may be a low level of viral replication. To overcome such problems, researchers have developed the polymerase chain reaction to selectively amplify sequences of HBV DNA in serum, and an increase in sensitivity of 10^2- to 10^4-fold over that of standard hybridization assays has been claimed (7, 9, 16). The sensitivity and specificity of the polymerase chain reaction assays were demonstrated by Southern blot hybridization with radiolabeled probes. Again, the use of a radiolabel and its inherent disadvantages may limit the availability of this technique. Our results suggest that digoxigenin-labeled probes could be equally useful in the detection of polymerase chain reaction-amplified sequences, and the availability of such sensitive and specific nonradioactive probes may allow greater access to this new technology.

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