Comparison of the Nucleic Acid-Based Crosslinking Hybridization Assay and the Branched DNA Signal Amplification Assay in the Quantitative Measurement of Serum Hepatitis B Virus DNA

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Quantitative measurement of hepatitis B virus (HBV) DNA has become important in the clinical diagnosis of patients with chronic hepatitis B, especially in patients with hepatitis B e antigen (HBeAg)-negative precore mutant and in patients who received treatment with interferon or antiviral agents. Two different hybridization assays for quantitative measurement of HBV DNA: Naxcor crosslinking assays and Chiron branched DNA signal amplification (bDNA) assay, were applied to 158 serum samples which were positive for HBV DNA by polymerase chain reaction. Among 158 serum samples, 135 samples (85.4%) were positive by the crosslinking assay and 129 samples (81.6%) were positive by the bDNA assay in the quantification of serum HBV DNA ($P > 0.05$). Serum HBV DNA levels obtained from both assays showed a good linear correlation ($r = 0.91$, $P < 0.001$). The sensitivity of both assays in HBeAg-positive samples was

90.5%, significantly higher than in HBeAg-negative samples (69.6% for the crosslinking assay and 56.5% for the bDNA assay, $P < 0.05$). In HBeAg-negative patients with elevated serum alanine transaminase levels, the so-called precore HBV mutant, the detection sensitivity for HBV DNA was better in the crosslinking assay (83%) than in the bDNA assay (61%). The crosslinking assay was less time consuming than the bDNA assay in performing the measurement of serum HBV DNA (6 hours vs. 20 hours). In conclusion, Naxcor crosslinking hybridization assay was equally as sensitive as Chiron bDNA assay in the quantitative measurement of serum HBV DNA. Less time-consuming procedures and better sensitivity in the detection of HBeAg-negative samples with elevated serum alanine transaminase levels may favor the clinical use of the crosslinking assay. *J. Clin. Lab. Anal.* 13:296–300, 1999. © 1999 Wiley-Liss, Inc.

Key words: branched DNA signal amplification assay; chronic hepatitis B; crosslinking assay; hepatitis B virus DNA

INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem worldwide, affecting millions of patients, especially in Southeast Asia and Africa (1,2). HBV is a partially double-stranded DNA virus. The diagnosis of HBV infection has traditionally relied on various serologic HBV markers. The presence of hepatitis B surface antigen (HBsAg) indicates a HBV infection but does not reflect its replicative activity. Hepatitis B e antigen (HBeAg), a secretory protein, has been thought to represent active viral replication in patients with chronic hepatitis B (3,4).

However, active viral replication without the presence of HBeAg has recently been identified in the so-called HBV precore mutant (5,6). The detection of serum HBV DNA, which actually reflects HBV replication activity, is important in determining the viremic stage in the precore HBV mutant and, most importantly, in evaluating the efficacy of interferon

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or antiviral agents in the treatment of patients with chronic hepatitis B (7,8). Advances in molecular biology have permitted the development of a specific nucleic acid probe for HBV DNA determination. However, direct membrane hybridization (dot-blot hybridization) and a liquid-phase hybridization assay, developed by Abbott Laboratories, were both too complicated and not sensitive enough to quantify HBV DNA (9,10). Polymerase chain reaction (PCR) is the most sensitive assay in measuring serum HBV DNA, but is limited in clinical use due to the qualitative nature of measurement results and the lack of a standardized procedure (11,12). In recent years, a solid phase hybridization assay using branched chain DNA (bDNA) molecules and signal amplification, which was developed by Chiron Corp., has been widely used in the quantitative measurement of viral genome (13). Recently, a new diagnostic assay using a photo-crosslinkable DNA probe for the quantitative measurement of HBV DNA has been developed by Naxcor Corp. (14–16). The aims of this study were to compare the Naxcor crosslinking and Chiron bDNA assays in the quantitative measurement of HBV DNA and to evaluate the clinical use of both assays.

MATERIALS AND METHODS

Serum Samples

A total of 158 serum samples which were qualitatively positive for serum HBV DNA by PCR were collected for the quantitative measurement of serum HBV DNA by the crosslinking and bDNA assays. Among the 158 serum samples, 120 samples were collected consecutively from 54 patients (42 males, 12 females, mean age: 33 ± 2 years) with chronic hepatitis B who had received regular follow-up for a mean of one year at the Division of Gastroenterology, Veterans General Hospital-Taipei, Taiwan. All of these 54 patients were positive for serum HBsAg. Seventy-four of the 120 samples were positive for serum HBeAg and 46 samples were negative for serum HBeAg. Serum alanine transaminase (ALT) levels were elevated (> 45 IU/l) in 55 of the 74 HBeAg-positive samples and in 18 of the 46 HBeAg-negative samples. The remaining 38 serum samples were collected consecutively from 7 patients (6 males, 1 female, mean age: 30 ± 2 years) with chronic hepatitis B who received subcutaneous injection of interferon α -2a 4.5 million units, three times a week for 24 weeks. Serum HBsAg and HBeAg were positive and serum ALT > 90 IU/l before treatment in these 7 patients. All patients were negative for serum antibody to delta virus (anti-delta) and antibody to hepatitis C virus (anti-HCV). None of these patients had other causes of liver disease such as alcoholism or metabolic liver disease. All samples were stored in aliquots at -70°C and thawed once prior to testing.

Procedure for the Crosslinking Assay (NAXCOR, Menlo Park, CA)

Serum samples were prepared by addition 30 μl of lysis reagent (proteinase K and sodium dodecyl sulfate) to 300 μl of serum and were then incubated for 30 min at 65°C . Nucleic acid in the samples was denatured with the addition of 6.3 μl of alkaline denaturation reagent and boiling for 15 min. The cooled samples were centrifuged at 12,000g for 5 min. Each processed sample was aliquoted (125 μl) into two wells of a 96-well polypropylene microtiter plate. In addition to the samples, two negative controls and three different HBV quantitative standards (alkaline diluent containing different concentrations of cloned HBV DNA, ranging from 0.5 to 6,000 million genome equivalent [Meq]/ml) were also put into the microtiter plate. HBV probes containing photoactive coumarin crosslinking agent (biotin-modified capture probes and fluorescein-modified reporter probes), which were complementary to the conserved regions within the entire HBV genome, were then added into each well along with neutralization reagents for hybridization. Next, the mixture was irradiated with a UV light source for 30 min to crosslink the probe-target hybrids. Following irradiation, streptavidin-coated magnetic beads (Dynabeads M-280, Dynal Inc., Lake Success, NY) were added to each well to capture the crosslinked probe-target hybrids via the biotin residue attached to the capture probes. After 30 min, the beads in the microwells were incubated with anti fluorescein antibody-alkaline phosphate conjugate. The wells were washed to remove unbound conjugate and then 100 μl AttophosTM (Attophos, JBL Scientific, San Luis Obispo, CA) were added to each well and the plate was incubated at 37°C for 60 min. Finally, fluorescent products were detected by measuring the fluorescent signal with a microplate reader. The concentration of HBV DNA in samples were calculated by comparing the fluorescent signal produced from each sample to the standard curve constructed from the results of the negative and positive standards. The quantification range of the assay was 0.5 to 6,000 Meq/ml. Each of the samples was run in duplicate and was retested if the coefficient of variation was greater than 20%.

Procedure for the bDNA Assay (Quantiplex, Chiron Corp. Emeryville, CA)

This sandwich nucleic acid hybridization procedure was followed according to the manufacturer's instructions. Briefly, 10 μl of serum samples were first added to a microwell coated with oligonucleotides derived from conserved parts of the HBV genome. The oligonucleotides were complementary to the minus-sense strand of HBV DNA. A lysis reagent was added to release and denature DNA. If present, HBV DNA hybridized to the coated oligonucleotides. The target probes (complementary to the conserved regions of the minus-sense strand of the HBV genome) were added, binding to the captured HBV DNA, then alkaline phosphatase-labeled probes

hybridized to the bDNA molecules. The detection scheme utilized alkaline phosphatase-catalyzed chemoluminescent emission from a Dioxetane substrate, and the results were recorded as luminescent counts on a plate luminometer. Light emissions were proportional to the amount of HBV DNA present in each sample. A standard curve was defined by light emissions from known quantities of HBV DNA target. Quantities of HBV DNA in specimens, expressed as Meq/mL, were determined from the standard curve. Each sample was run in duplicate and results were consistent (coefficient of variation less than 20%). The quantification range of this assay was 0.7 to 6,300 Meq/mL.

Measurement of HBV DNA by Polymerase Chain Reaction

A nested-PCR procedure was applied to amplify HBV DNA. The primer pairs used in the procedure were from the highly conserved HBV DNA polymerase region as previously described (17). These included MD03: 5'-CTC AAG CTT ATC ATC CAT ATA-3'; MD06: 5'-CTT GGA TCC TAT GGG AGT GG-3'; SMD09: 5'-GGC CTC AGT CCG TTT CTC TTG-3'. The SMD09 primer was 26 bases shortened from the 3' end of the original MD09 primer and was used for the second PCR amplification procedure.

For HBV DNA extraction, serum samples were treated with microwave irradiation as previously described with minor modifications (18). Briefly, 10 µl of the patient's sera were irradiated in a microwave oven (Sharp 900 watt, model R-5A83) for 3 min. After briefly spinning down, 45 µl of the PCR master mixture containing primer MD03 and MD06 were added. PCR was carried out by initial denaturation for 3 min at 94°C, followed by 40 cycles of denaturation at 94°C for 15 sec, annealing at 55°C for 15 sec and extension at 72°C for 15 sec. For the second round of PCR, 5 µl of the first-round PCR product was subjected to another 35 cycles of amplification using the same procedures with fresh components and a new set of primers (MD03 and SMD09).

After the second PCR amplification, 10 µl of the final PCR product was analyzed by electrophoresis on an agarose gel, stained with ethidium bromide, and photographed under ultraviolet light. The expected size of the final PCR product was 105 base pairs.

The methods we used in the measurement of serum HBV DNA, including Chiron bDNA assay, Naxcor crosslinking assay, and PCR have not currently been certified by the Food Drug Administration of the United States.

Measurement of Serum ALT and Serologic Markers of HBV

Serum ALT was measured by an autoanalyzer (Technicon SMA-II; Technicon Instruments, Tarrytown, NY). HBsAg, HBeAg and anti-delta were measured by radioimmunoassay (Austria II, Abbott Laboratories, North Chicago, IL). Anti-

HCV was measured by using a second-generation enzyme immunoassay (Abbott Laboratories).

Statistical Analysis

Data in the text are expressed as mean $\pm$ standard error of mean (s.e.m.). Results were compared using Chi-square test, Fisher exact test, and Student *t*-test according to the data analyzed. Linear regression (BMDP software, 1990 version, Los Angeles, CA) was used to determine the correlation between two different assays. A *P* value less than 0.05 was considered statistically significant.

RESULTS

Among the total 158 serum samples positive for serum HBV DNA by PCR, 135 samples (85.4%) were found to be positive by using the crosslinking assay and 129 samples (81.6%) were found to be positive by using the bDNA assay in the quantification of serum HBV DNA ($P > 0.05$). Of these samples, 119 (75%) were positive for HBV DNA in both assays, and 13 (8%) were below the detection limit for both assays. Of the remaining 26 samples, 16 contained measurable levels of HBV DNA by using the crosslinking assay alone and 10 other samples contained measurable levels of HBV DNA by using the bDNA assay (Table 1). Serum HBV DNA titers in 119 samples obtained from both assays showed a good linear correlation ($r = 0.91$, $P < 0.001$, Fig. 1).

Among the 120 samples collected from chronic hepatitis B patients who did not receive interferon treatment, the sensitivity of both assays in HBeAg-positive samples was 90.5%, significantly higher than in HBeAg-negative samples (69.6% for crosslinking assay and 56.5% for bDNA assay, both $P < 0.05$, Table 2). The mean serum HBV DNA titer in the quantifiable samples by both assays were significantly higher in HBeAg-positive patients than in HBeAg-negative patients. In HBeAg-negative patients, those with elevated serum ALT levels had a significantly higher HBV DNA positive rate and higher mean serum HBV DNA titer detected by both assays than in patients with normal serum ALT levels (Table 2). In HBeAg-negative patients with elevated serum ALT levels, the detection sensitivity for HBV DNA was better in the crosslinking assay (83%) than in the bDNA assay (61%). The crosslinking assay was less time consuming than the bDNA

TABLE 1. Comparisons of the results of the crosslinking assay and the branched DNA signal amplification (bDNA) assay in detecting serum HBV DNA in 158 PCR-positive samples

	Crosslinking	
	HBV DNA positive	HBV DNA negative
bDNA assay		
HBV DNA positive	119	10
HBV DNA negative	16	13

Fig. 1. Linear regression analysis of the quantitative measurement of serum HBV DNA detected by the Chiron branched DNA signal amplification (bDNA) assay and the Naxcor crosslinking assay. The dotted lines indicate the 95% confidence intervals.

assay when performing measurements of serum HBV DNA (6 hours vs. 20 hours).

DISCUSSION

The quantitative measurement of serum HBV DNA has become important in the clinical diagnosis of patients with chronic hepatitis B, especially in patients with HBeAg-negative pre-core mutant and in patients who received treatment with interferon or antiviral agents. Chiron bDNA assay has been shown to be more sensitive than dot-blot hybridization assay, Abbott liquid hybridization, and Digene hybrid-capture assay in the quantitative measurement of serum HBV DNA and has been widely used in recent years (19,20). We compared the newly developed crosslinking assay with the bDNA assay, and results showed an equal sensitivity in the measurement of serum HBV DNA between both assays. How-

ever, the crosslinking assay was less time consuming than the bDNA assay when performing measurements of serum HBV DNA and may be more convenient when handling a large number of serum samples.

The nucleic acid-based crosslinking diagnostic assay offers advantages over conventional hybridization assays. By the irreversible covalently binding of the hybridized probe and target, the crosslinker guarantees that the maximal target will be firmly captured throughout the assay for maximal signal generation. In addition, crosslinking allows the use of stringent wash conditions for effective removal of nonspecific background signals. Also, the oligonucleotides probes used in the crosslinking assay were synthesized from the conserved regions within the entire HBV genome and may thereby increase the detection sensitivity in the measurement of HBV DNA with minor genome mutation (14–16). This may explain our findings that the crosslinking assay has a better serum HBV DNA detection rate than the bDNA assay in HBeAg-negative patients with elevated serum ALT levels, the so-called precore mutant.

Different clinical and virological manifestations may be encountered in Asian patients with positive serum HBsAg who contracted HBV infection mostly in peri-natal and pre-school period (21,22). Chronic hepatitis B patients with positive HBeAg or negative HBeAg with elevated serum ALT (precore mutant) levels usually indicated active viral replication. Our results showed high viremia in HBeAg-positive patients (mean quantifiable titer: 897 and 1,005 Meq/ml by the crosslinking assay and bDNA assay, respectively) and in HBeAg-negative patients with elevated serum ALT levels (mean quantifiable titer: 534 and 539 Meq/ml by the crosslinking assay and bDNA assay, respectively). These findings indicate how highly infectious these patients are. In addition, the prognosis of these patients is usually poor and some will develop chronic sequelae such as cirrhosis and hepatocellular carcinoma (23–25). Treatment of these patients is urgently needed if serum ALT levels are elevated. Chronic

TABLE 2. Comparison of the sensitivity of the crosslinking assay and the branched DNA signal amplification (bDNA) assay in 120 PCR-positive samples with different subgroups^a

	HBeAg positive samples (n = 74)	HBeAg negative samples		
		Total cases (n = 46)	Normal ALT (n = 28)	ALT elevation (n = 18)
Crosslinking assay				
No. of positive (%)	67 (90.5)	32 (69.6) ^b	17 (60.7)	15 (83.3)
Mean value (Meq/ml)	897 ± 176	251 ± 142 ^c	1.0 ± 0.1 ^d	534 ± 291
bDNA assay				
No. of positive (%)	67 (90.5)	26 (56.5) ^c	15 (53.6)	11 (61)
Mean value (Meq/ml)	1,005 ± 178	229 ± 114 ^f	1.7 ± 0.3 ^d	539 ± 245

^aData are expressed as mean ± s.e.m.; HBeAg, hepatitis B e antigen; ALT, alanine transaminase.

^bP = 0.007 when compared with HBeAg-positive samples.

^cP = 0.01 when compared with HBeAg-positive samples.

^dP < 0.02 compared with HBeAg-positive sample and HBeAg-negative samples with ALT elevation.

^eP < 0.001 when compared with HBeAg-positive samples.

^fP = 0.02 when compared with HBeAg-positive samples.

hepatitis B patients with negative HBeAg and persistent normal serum ALT levels usually indicates healthy HBV carriers. Our results showed that patients in this group were usually in the low-HBV viremic stage (mean quantifiable HBV DNA titer: 1–1.7 Meq/ml by both assays) and were less infectious. In this group of patients, 40 to 50% of serum HBV DNA were positive by PCR and negative by quantitative assays. Some of these patients may show no signs of ongoing liver disease, while others may have active hepatitis with low viremia. Increased sensitivity of a commercial quantitative HBV DNA assay in detecting low viremic samples is therefore awaited.

In conclusion, the Naxcor crosslinking assay was as equally sensitive as the Chiron bDNA assay in quantitative measurement of serum HBV DNA. Less time-consuming procedures and better sensitivity in the detection of HBeAg-negative samples with elevated serum ALT levels may facilitate the clinical use of the crosslinking assay.

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