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Real-Time PCR Assay for Detection and Quantification of Hepatitis B Virus Genotypes A to G

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The detection and quantification of hepatitis B virus (HBV) DNA play an important role in diagnosing and monitoring HBV infection as well as assessing therapeutic response. The great variability among HBV genotypes and the enormous range of clinical HBV DNA levels present challenges for PCR-based amplification techniques. In this study, we describe the development, evaluation, and validation of a novel real-time PCR assay designed to provide accurate quantification of DNA from all eight HBV genotypes in patient plasma specimens. A computer algorithm was used to design degenerate real-time PCR primers and probes based upon a large number ($n = 340$) of full-length genomic sequences including HBV genotypes A to H from Europe, Africa, Asia, and North and South America. Genotype performance was tested and confirmed using 59 genotype A to G specimens from two commercially available worldwide genotype panels. This assay has a dynamic range of at least 8 $\log_{10}$ without the need for specimen dilution, good clinical intra- and interassay precision, and excellent correlation with the Bayer Diagnostics VERSANT HBV DNA 3.0 (branched DNA) assay ($r = 0.93$). Probit analysis determined the 95% detection level was 56 IU/ml, corresponding to 11 copies per PCR well. The high sensitivity, wide linear range, good reproducibility, and genotype inclusivity, combined with a small sample volume requirement and low cost, make this novel quantitative HBV real-time PCR assay particularly well suited for application to large clinical and epidemiological studies.

Hepatitis B virus (HBV) is an enveloped DNA virus that belongs to the family *Hepadnaviridae* (4). Approximately 350 million people are chronically infected with HBV worldwide, placing them at high risk for developing cirrhosis, end-stage liver disease, and hepatocellular carcinoma (34). Detection and quantification of circulating HBV in plasma or serum play an important role in diagnosing and monitoring HBV infection as well as assessing response to therapy (27). Clinically, HBV DNA titers vary greatly, from levels as high as 10^{10} copies/ml during acute HBV infection (33), to very low levels in HBe antigen-negative chronic carriers and in patients undergoing antiviral therapy, and in those with occult HBV infection (30).

In addition to the wide range of HBV DNA levels likely to be encountered in patient specimens, there is great variability within the HBV genome. In combination, these present technical challenges for developing a quantitative HBV DNA assay. To date, eight different HBV genotypes (A to H) have been identified and defined by sequence divergence of greater than 8% throughout the genome (23, 24, 29). The various HBV genotypes have characteristic geographic distributions; genotype A is prevalent in North America, Europe, South Africa, and India; genotypes B and C are prevalent in Asia; genotype D is prevalent in Europe (Mediterranean area), the Middle East and Far East, India, and South Africa; genotype E is prevalent in West Africa; genotype F is prevalent in Central and South America and Alaska; and genotype G is prevalent in Europe, Mexico, and the United States. Genotype H was recently reported in Central America, the United States, and Japan (3, 13, 25). In addition, a number of common mutations have been described in HBV strains (17) and recombinant genomes have been reported. These include variability within the basic core promoter, precore/core region, and the pre-S1 gene, which displays great heterogeneity. A number of vaccine-induced mutations in the S gene major hydrophilic region, as well as the X gene, and, finally, drug-induced polymerase gene mutations are common in patients undergoing antiviral therapy.

Among the methods most commonly used for HBV DNA quantification, including hybrid capture and branched DNA (bDNA) signal amplification (19–21, 35), assays based on real-time PCR technology (12) offer the greatest sensitivity and broadest linear dynamic range (9, 32). This technology utilizes three oligonucleotides in the PCR mix: a probe labeled with a fluorescent reporter dye and a quenching dye, as well as two primers. During amplification, the reporter dye is cleaved from the probe by the 5′-exonuclease activity of a DNA polymerase, allowing real-time measurement of the fluorescent light emissions from the reporter dye. In real-time PCR, there is a direct relationship between the starting template copy number and the number of cycles needed to measure a positive signal from the reporter dye.

However, due to the great heterogeneity in HBV genomic sequences, designing primer-probe sets to detect and quantify all HBV genotypes by real-time PCR is challenging at best. A recent report suggested that at least two different primer-probe sets may be necessary for detection and amplification of all eight HBV genotypes (8). In the present study, we describe a

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novel, highly sensitive, and accurate real-time quantitative PCR assay capable of equally detecting and quantifying HBV genotypes A through G using a single degenerate primer-probe set within the X gene.

MATERIALS AND METHODS

Plasmid control template and standard curve preparation. The plasmid pAM6 (ATCC 45020D; ATCC, Manassas, VA) was used as the template to prepare the standard curve for the real-time PCR assay and also as a source of HBV DNA for initial testing of all primer-probe sets. This plasmid contains a full-length HBV genomic sequence (nucleotide positions 1 to 3020) inserted into the vector pBR322 (22). Plasmid pAM6 was propagated in *Escherichia coli* and purified using the QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA). The purified plasmid DNA was digested using endonuclease EcoRI (Invitrogen, Carlsbad, CA), and the insert size was confirmed by agarose gel electrophoresis. The pAM6 DNA was quantified by A_{260} measurement (Spectramax 384Plus, Sunnyvale, CA), and the copy number was calculated based on the plasmid molecular weight. For each experiment, a standard curve was prepared by serial end-point dilution of linearized pAM6 control template DNA. Typically, the standard curve ranged from 5×10^1 IU/ml to 5×10^6 IU/ml, with each dilution tested in triplicate reactions. To assess linearity of the assay over an extended range, a standard curve ranging from 5×10^1 IU/ml to 5×10^8 IU/ml was prepared and tested.

Assay controls. The Accurun 325 HBV DNA positive control (series 370; BBI Diagnostics, West Bridgewater, MA) was used as the assay positive control. The value assigned to this HBV positive control by the manufacturer, based upon Roche Molecular Diagnostics COBAS AMPLICOR HBV MONITOR test results, was 7.25×10^3 copies/ml, corresponding to 1.38×10^3 IU/ml. Basematrix 53 diluent (BBI Diagnostics), a defribrinated human plasma product certified negative for HBV DNA, HBsAg, anti-HBc, human immunodeficiency virus (HIV) RNA, anti-HIV-1/2, anti-human T-cell leukemia virus type 1/2, hepatitis C virus (HCV) RNA, and anti-HCV was used as the assay negative control. For each experiment, the assay positive and negative controls were extracted concurrently with test specimens to serve as extraction controls that were included in triplicate on every 96-well PCR plate. The HBV positive control also served as a longitudinal control since the positive control data were used to track assay performance over time.

Clinical specimens. Clinical specimens (listed in Table 3) from HBsAg-seropositive, HBV DNA-positive patients from various countries were obtained as genotype panels from two commercial sources: Teragenix Corporation (Ft. Lauderdale, FL) and BBI Diagnostics (West Bridgewater, MA). The Teragenix HBV worldwide genotype panel (HBVGTP-002A) comprised 50 specimens with genotypes A to G, and the BBI Diagnostics HBV genotype performance panel PHD201(E) included 9 genotype A-to-F clinical samples. These 59 clinical specimens were used to investigate genotype inclusivity of the novel HBV real-time PCR assay. In addition, the clinical specimen quantitative results from our real-time PCR assay were compared to the Bayer Diagnostics VERSANT HBV DNA 3.0 (bDNA) assay results provided by the genotype panel manufacturers.

Two high-titer clinical HBV DNA specimens from HBsAg-seropositive patients, one genotype A and one genotype B specimen, were also used to measure assay characteristics such as sensitivity, linearity, and precision. The high-titer HBV genotype A specimen had 4.77×10^8 copies/ml (9.10×10^7 IU/ml), and the genotype B specimen had 1.59×10^9 copies/ml (3.02×10^8 IU/ml), based upon results of the Roche Molecular Diagnostics COBAS AMPLICOR HBV MONITOR test, v2.0 (supplied by the manufacturer, BBI Diagnostics).

Specimens from blood donors ($n = 100$) who tested negative for HBsAg by enzyme-linked immunosorbent assay (ELISA) were obtained from the National Heart, Lung, and Blood Institute Retrovirus Epidemiology Donor Study (REDS) (37) and were tested using the HBV real-time PCR assay to determine clinical specificity. All samples were stored at −70°C until processing and testing.

PCR primers and probe design. HBV sequences were selected from the GenBank nucleotide database using the search terms "human hepatitis B virus" and "complete genome." We obtained 544 HBV genomic sequences, but excluded incomplete sequences, redundant isolates, and those lacking clear genotype information. This left 340 complete HBV genomic sequences, including genotypes A to H from Europe, North and South America, Asia, and Africa. The sequences were aligned using AlignX (Informax Vector NTI suite of programs, version 9.0.0; Invitrogen, Carlsbad, CA), and a consensus nucleotide sequence was generated by requiring greater than 50% agreement among the sequences. All sequences, including the consensus sequence, were exported in MSF format and converted to a FASTA file using an in-house PERL script that preserved all alignment landmarks such as gaps, insertions, and deletions. For each nucleotide position of the HBV consensus sequence, the PERL script program calculated the absolute number and percentage agreement with the corresponding nucleotides of all the aligned sequences. This allowed identification of the most conserved nucleotide positions and regions across all 340 HBV genomes. For divergent positions, the program replaced the corresponding base in the consensus sequence with a degenerate or universal base (such as P and K nucleoside phosphoramitides to mimic a C/T and an A/G mix, respectively) as necessary to maximize concordance (15, 16). The program then recalculated both the absolute number and percentage of agreement of the individual nucleotide positions among the 340 sequences.

In selecting primer-probe candidates, the variability of the HBV genome and existence of common mutations were taken into account (17). Specifically, HBV variants such as drug-induced *pol* mutations, basic core promoter polymorphisms, pre-S and S gene vaccine escape mutants, mutations resulting in premature stop codons, and regions prone to recombination were all considered. A large number of potential primer-probe combinations were selected from the HBV S gene and core/X gene regions and analyzed for melting temperature (T_m), self-complementarity, and secondary structure using Northwestern University's Online Oligonucleotide Properties Calculator (http://www.basic.northwestern.edu/biotools/oligocalc.html). BLAST (Basic Local Alignment Search Tool) searches (2) were performed on all candidate primer and probe oligonucleotides to assess their potential for amplifying non-HBV sequences. No matches with any human or microbial nucleotide sequences were found for any of the selected oligonucleotides.

A total of 70 different primer-probe combinations were initially tested using a single concentration of plasmid pAM6 as the DNA template. Primer-probe sets that successfully amplified the stock concentration of pAM6 were subsequently tested using a standard curve dilution series of the plasmid. Regression analyses of the data were examined, and those primer-probe sets that met predetermined criteria (slope < -3.8 and y-intercept value $<$ a cycle threshold [C_T] of 50) were selected for further evaluation. The eight most promising sets were tested using the individual clinical plasma specimens with HBV genotypes A to G from the two commercial genotype panels described above. The single primer-probe pair that performed best was chosen for further development and full validation of the HBV real-time quantitative PCR assay. The selected HBV X gene primer and probe sequences are as follows: forward primer HBV-F3, 5′-GGCCATCA GCGCATGC-3′, and reverse primer HBV-R3M3, 5′-C [5-NitIdl] GCTGCGA GCAAAACA-3′; and probe HBV-P3, 5′-R-CTCTGCCGATCCATACTGCGG AACTC–Q-3′.

The probe is labeled at the 5′end with the reporter dye (R) FAM (6-carboxyfluorescein) and at the 3′ end with the quencher dye (Q) TAMRA (6-carboxytetramethylrhodamine). A 5-nitroindole residue was incorporated in the reverse primer at a heterogeneous position to enable detection of variant HBV strains. The primers and probe were purified by high-pressure liquid chromatography by Operon Technologies (Alameda, CA). These primers amplify an 86-bp fragment of the HBV genome X gene. Agarose gel analysis of the PCR product confirmed that it migrated at the expected size (data not shown).

Real-time PCR. Real-time PCR was performed using an ABI Prism 7900HT or 7700 Sequence Detection System (Applied Biosystems, Foster City, CA) in a 96-well optical plate format. The standard curve, positive and negative controls, and samples were all tested in triplicate replicates. Each 50-μl reaction mixture consisted of 40 μl of PCR mix combined with 10 μl of standard, control, or specimen DNA. The 10 μl of specimen DNA was equivalent to 2% of the total DNA present in 1 ml of plasma. The PCR mix contained universal master mix (Applied Biosystems), primers HBV-F3 and HBV-R3M3 (each at 50 nmol/reaction), probe HBV-P3 (10 nmol/reaction), and 13 μl of RNase/DNase-free water (Brinkmann/Eppendorf, Westbury, NY). After 2 min at 50°C and 10 min at 95°C, there were 45 cycles (95°C for 15 s and 60°C for 1 min) of PCR amplification for HBV detection. AmpErase and dUTP within the master mix provided carryover contamination control.

Least-squares regression analysis, performed by the ABI Prism 7700 or 7900HT software, plotted C_T as a function of nominal input copy number. The measured raw copy number for each reaction was calculated using the C_T value of each PCR interpolated against the linear regression of the standard curve. For each DNA specimen, the triplicate average raw copy number per well was multiplied either by 50 to convert to copies/ml or 5 to convert to IU/ml.

Specimen processing/DNA extraction. Reportedly, recovery of virion-derived HBV DNA can vary with the method used for DNA extraction because HBV DNA is covalently linked to protein that remains bound to it unless removed by proteinase digestion. The bound protein can interfere with elution of HBV DNA from purification columns (11). To circumvent this problem, we investigated the effect of proteinase K (QIAGEN, Valencia, CA) digestion time on DNA recovery from an HBV genotype A clinical specimen (BBI Diagnostics). The specimen

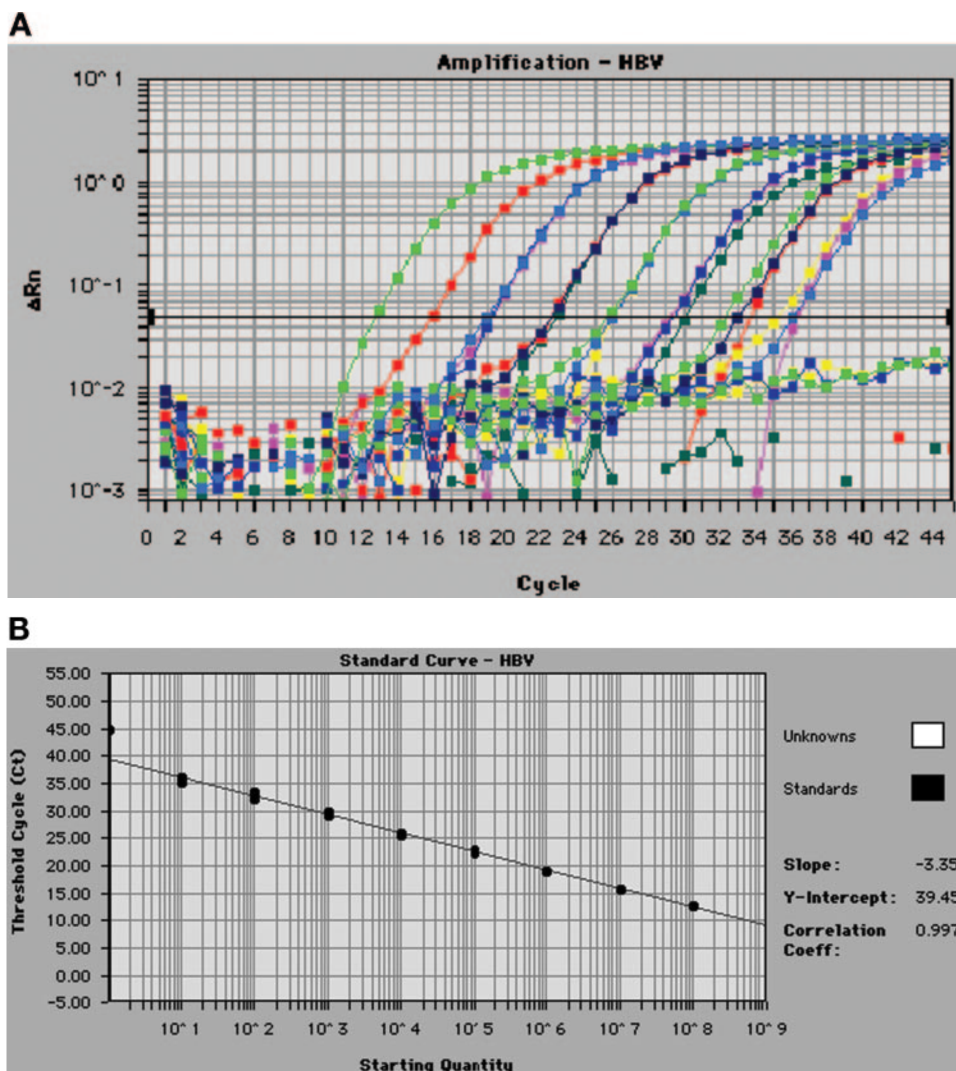

FIG. 1. (A) HBV real-time PCR assay amplification plot obtained from triplicates of 8-$\log_{10}$ serial dilutions of plasmid pAM6 ranging from a nominal concentration of 5×10^1 to 5×10^8 IU/ml. This figure was generated by the ABI Prism 7700 Sequence Detection Software (SDS) version 1.9.1. (B) HBV real-time PCR plasmid pAM6 standard curve showing linearity over an 8-$\log_{10}$ dynamic range. This standard curve was generated from the amplification plot displayed in Fig. 1A. The least-squares regression was calculated from plots of measured C_T (y axis) versus input plasmid DNA over a range of 5×10^1 to 5×10^8 IU/ml (x axis) tested in triplicate PCR wells per dilution. The correlation coefficient was 0.997, and the slope of the line was −3.36.

was incubated for 15, 30, 45, 60, and 90 min, as well as 24 and 48 h, in two separate experiments that included three aliquots of plasma specimen extracted at each time point. All aliquots were tested in triplicate with the HBV real-time PCR assay. Maximum HBV DNA recovery was seen with incubation times of at least 24 h (data not shown). Therefore, we included a 24-h proteinase K digestion in the protocol that was used for all experiments described herein.

HBV DNA was isolated from specimens using the QIAamp MinElute Virus Spin kit (QIAGEN, Valencia, CA) with adaptations to the manufacturer's protocol. Briefly, 200 μl of plasma, 30 μl of proteinase K (QIAGEN), and 200 μl of buffer AL lysis reagent were incubated overnight at 56°C with mixing. After proteinase K digestion, 6 μg of carrier RNA, followed by 250 μl of ethanol (Aaper Alcohol & Chemical, Shelbyville, KY), was added. The rest of the procedure was performed according to the manufacturer's instructions, except that HBV DNA was eluted in 100 μl of buffer AVE (provided with the kit).

WHO HBV International Unit Standard. The World Health Organization (WHO) International Standard for HBV Nucleic Acid Amplification Techniques (NAT), product code 97/746 (28), was obtained from the National Institute for Biological Standards and Controls (NIBSC; Hertfordshire, United Kingdom). This control is used as the benchmark reference for comparison of HBV assay results from a diverse range of nucleic acid detection technologies. The standard was tested according to the NIBSC protocol (version 6). The test results were used to calculate the factor for converting the HBV real-time PCR assay units from copies/ml to IU/ml.

Data analysis. All results were converted to IU/ml prior to analysis, and all statistical analyses were performed using SAS Enterprise Guide software, version 9.0 (SAS Institute, Cary, NC). The HBV real-time quantitative PCR assay and Bayer VERSANT HBV bDNA assay results for 30 genotype panel specimens within the linear range of both assays were correlated using Pearson's correlation. Probit statistics was performed to determine the limit of detection of our HBV real-time PCR assay. Poisson distribution statistics were used to assess the single-copy dilution level of the plasmid standard curve.

RESULTS

We tested the WHO HBV International Standard to allow direct comparison of our HBV quantitative assay results with those obtained by other assay methods. The amount of HBV in

TABLE 1. Probit analysis used to determine the 95% detection limit of the HBV real-time PCR assay using a genotype B specimen[a]

Measured HBV DNA concn (IU/ml)	Measured amt of HBV DNA (copies/PCR well)	No. (%) of positive wells/total wells
150	30	36/36 (100)
125	25	36/36 (100)
100	20	36/36 (100)
75	15	36/36 (100)
56	**11**	**(95)**
40	8	32/36 (89)
30	6	30/36 (83)

[a] The assay clinical sensitivity was calculated using 36 replicate measurements at each dilution obtained from 12 independent experiments. The data used for the analysis are shown in regular typeface, and the calculated 95% detection limit of 56 IU/ml (equivalent to 11 copies per PCR well) is shown in boldface.

each vial of NIBSC lyophilized WHO International Standard was defined as 1,000,000 IU/ml. Using our novel HBV real-time PCR assay, this WHO International Standard measured an average of 1×10^7 copies/ml. This yielded a calculated conversion ratio of copies/ml to IU/ml of 10:1, indicating that 10 HBV copies/ml measured by our real-time PCR assay is equivalent to 1 IU/ml. Data are presented as IU/ml followed by conversion to $\log_{10}$ values for statistical analyses.

The linear dynamic range of the HBV real-time PCR assay was initially assessed using an 8-$\log_{10}$ dilution series of plasmid pAM6 DNA. The assay was shown to be linear over the entire range of 5×10^1 to 5×10^8 IU/ml. A typical standard curve amplification plot and linear regression analysis of these data are shown in Fig. 1A and B. The regression analysis yielded a correlation coefficient of 0.997 and a y-intercept value of 39.5. The slope of -3.36 closely approximates the theoretical maximum amplification efficiency of 100% (-3.32 slope).

To evaluate assay reproducibility, three independent experiments were performed, in which each of the standard curve dilution levels was tested in triplicate reactions and each plate contained quadruplicate plasmid pAM6 standard curves. The standard curve inter- and intra-assay precision, calculated from a total of 36 replicates, ranged from 0.5% at 5.0×10^6 IU/ml to a maximum of 21% at the level of 5.0×10^1 IU/ml (data not shown).

Sensitivity of the assay was assessed by comparing the observed and expected frequencies of single-copy detection using the plasmid pAM6 dilution series. The observed single-copy frequency of plasmid pAM6 was 28% (10 wells out of 36 wells were positive), in good agreement with the expected single-copy detection probability of 37% calculated by Poisson distribution (data not shown).

The clinical sensitivity of the quantitative real-time PCR assay was determined using a low-titer dilution series of an HBV genotype B specimen. HBV DNA was diluted to concentrations of 150, 125, 100, 75, 40, and 30 IU/ml, corresponding to 30, 25, 20, 15, 8, and 6 copies per reaction, respectively (Table 1). Twelve independent experiments were run, with each dilution tested in triplicate, yielding a total of 36 replicate results for each DNA concentration level. The 95% clinical detection limit of the assay, calculated by Probit statistics, was 11 copies per reaction, which corresponds to 56 IU/ml.

The clinical linear range of the assay was determined by diluting and testing high-titer HBV genotype A (9.10×10^7 IU/ml) and genotype B (3.02×10^8 IU/ml) plasma specimens. Eight independent experiments were performed for each genotype; each HBV DNA concentration level was tested in triplicate, resulting in a total of 24 replicate measurements per dilution. The results for the genotype A specimen are shown in Fig. 2. The clinical linear dynamic range was at least 7 $\log_{10}$ (2.88×10^0 to 2.69×10^7 IU/ml), since the results were linear over the entire range of dilutions tested.

The clinical HBV genotype A specimen dilution series was also used to assess the clinical intra- and interassay precision of the real-time PCR assay. The statistical analysis results for each concentration level are shown in Table 2. As expected, the percent CV increased as the HBV DNA level in the sample decreased with each dilution. Since the assay's 95% detection limit is 56 IU/ml (1.75 $\log_{10}$), the percent coefficient of variation (%CV) increases greatly in the two dilutions beyond this limit. The %CV for clinical specimens is greater than that for purified plasmid DNA since the patient specimens require the additional step of DNA extraction.

Clinical specificity was determined using 100 blood donor samples from the REDS study. All of these blood donor sam-

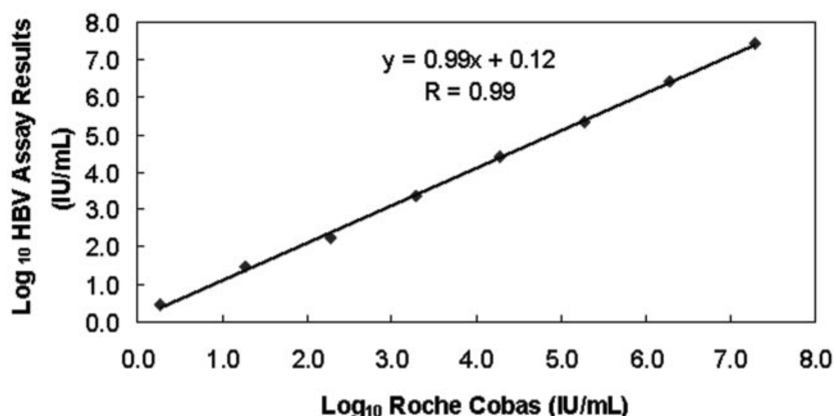

FIG. 2. Clinical linear dynamic range of the HBV real-time PCR assay. These data were obtained using a 7-$\log_{10}$ dilution series of a high-titer HBV genotype A plasma specimen in eight independent experiments. Each dilution was tested in triplicate, resulting in a total of 24 replicate measurements. The assay is linear over a range of 2.88×10^0 to at least 2.69×10^7 IU/ml.

TABLE 2. Precision of the novel HBV real-time PCR assay[a]

Predicted HBV concn (log_{10} IU/ml)	Measured HBV concn (log_{10} IU/ml)	Intra-assay		Interassay	
		SD	%CV	SD	%CV
7.28	7.43	0.014	0.19	0.07	1.0
6.28	6.43	0.014	0.22	0.15	2.3
5.28	5.34	0.019	0.35	0.15	2.9
4.28	4.42	0.032	0.72	0.07	1.5
3.28	3.37	0.039	1.17	0.17	5.1
2.28	2.22	0.077	3.56	0.22	10.1
1.28	1.47	0.196	16.6	0.26	18.0
0.28	0.46	0.369	97.2	0.30	64.1

[a] A clinical HBV genotype A plasma specimen was serially diluted in Basematrix (HBV-negative human plasma) and tested in triplicate in eight independent experiments. The SD and %CV were calculated using data from 24 replicates per dilution.

ples were negative for HBV DNA by our real-time PCR assay, resulting in a clinical specificity of 100%.

Genotype inclusivity was evaluated using a total of 59 clinical plasma specimens, including genotypes A to G. The novel real-time PCR assay successfully detected HBV DNA in 58 of these specimens, demonstrating an ability to detect all of these HBV genotypes (Table 3). Since the primer-probe set we selected has 100% homology to the genotype H sequence in the databank, it is likely to detect this genotype as well.

The 59 genotyped HBV clinical specimens were also used to compare results from our real-time PCR assay to those obtained with a commercial assay, the Bayer Diagnostics VERSANT HBV 3.0 (bDNA) assay. Of the 59 specimens, 21 members were below the limit of detection (<357 IU/ml) and 7 members were above the cutoff (>17,857,140 IU/ml) of the Bayer VERSANT HBV bDNA assay. Twenty of the 21 specimens that were undetectable with the Bayer VERSANT HBV bDNA assay tested positive with our novel real-time PCR assay, with values ranging from 1.91 to 525 IU/ml (0.28 to 2.72 log_{10} IU/ml) HBV DNA (Table 3). Values below the lower quantitative limit of 56 IU/ml are usually defined as qualitative positives and are not assigned a numerical value. However, to allow comparison with bDNA results, we assigned values (Table 3).

We had both Bayer VERSANT bDNA and real-time PCR assay results for 30 plasma specimens with genotypes A to G within the shared linear range of both assays (Table 3). We compared the results for these 30 samples from both assays and calculated a correlation coefficient of 0.88. After removal of a single genotype C outlier (Table 3), the correlation coefficient increased to 0.93 (see Fig. 3 for a graphical representation).

Each experiment was assessed for quality control (QC) based upon the following QC data: standard curve performance, positive/negative control performance, and acceptable replicate PCR coefficient of variation (%CV). If the standard curve regression R^2 value was <0.985, the slope value was outside the range of -3.2 to -3.7, or the y-intercept was outside the C_T range of 37 to 43, the plate failed QC criteria, the experiment was invalidated, and all data were nonreportable. In this case, the assay was repeated in its entirety.

As an exogenous DNA extraction control and to monitor

TABLE 3. Detection of HBV genotypes A through G in specimens from all over the world by the HBV real-time PCR assay[a]

HBV genotype	Country of origin	Result (log_{10} IU/ml) by:	
		Versant HBV bDNA	HBV real-time PCR
A	**United States**	**2.92**	**2.33**
A	Senegal	ND	2.23
A	Senegal	ND	2.51
A	Senegal	ND	1.86
A	Senegal	ND	1.81
A	Senegal	ND	1.57[b]
A	**Senegal**	**3.75**	**1.81**
A	United States	ND	1.70[b]
A	**United States**	**3.30**	**2.26**
A	United States	ND	1.40[b]
A	**United States**	**2.80**	**1.80**
A	United States	7.25+	5.70
A	United States	ND	1.78
A*	**United States**	**3.99**	**3.90**
A*	**United States**	**3.97**	**3.72**
A*	**United States**	**3.37**	**3.12**
B	**United States**	**3.72**	**2.74**
B	United States	ND	1.60[b]
B	United States	ND	1.76
B*	**Eastern Asia**	**3.08**	**3.44**
C	**United States**	**4.06**	**3.29**
C	United States	7.25+	6.51
C	**United States**	**4.59**	**4.44**
C	**United States**	**4.50**	**4.20**
C	United States	ND	1.40[b]
C	United States	7.25+	7.88
C	United States	ND	1.48[b]
C outlier	*United States*	*5.81*	*3.44*
C*	**Eastern Asia**	**3.43**	**1.99**
D	**United States**	**6.47**	**5.81**
D	United States	7.25+	7.70
D	United States	ND	2.54
D	**United States**	**3.09**	**2.26**
D*	**Mediterranean**	**3.82**	**3.57**
D*	**Middle East**	**3.17**	**2.57**
E	Cote D'Ivoire	ND	1.51[b]
E	Cote D'Ivoire	7.25+	7.88
E	United States	ND	1.11[b]
E	Venezuela	ND	2.72
E	**Cote D'Ivoire**	**6.57**	**6.48**
E	**Cote D'Ivoire**	**2.70**	**0.75**[b]
E	Cote D'Ivoire	7.25+	7.38
E	Venezuela	ND	ND
E	**Venezuela**	**3.05**	**1.40**[b]
E*	**Africa**	**3.59**	**3.54**
F	Venezuela	ND	1.57[b]
F	**Venezuela**	**3.21**	**2.20**
F	**Venezuela**	**3.24**	**2.20**
F	Venezuela	ND	2.05
F	**Venezuela**	**2.56**	**1.70**[b]
F	**Venezuela**	**3.12**	**2.02**
F	**Venezuela**	**2.74**	**1.98**
F	**Venezuela**	**3.45**	**1.83**
F	Venezuela	ND	0.28[b]
F	Venezuela	ND	1.81
F	Venezuela	3.40	1.40[b]
F	Venezuela	7.25+	5.83
F*	**South America**	**3.76**	**1.97**
G	**United States**	**6.06**	**6.48**

[a] The clinical plasma specimens were from two commercially available worldwide genotype panels. Asterisks indicate specimens that were purchased from Boston Biomedica; all other samples were from Teragenix. The Bayer Diagnostics VER SANT HBV DNA 3.0 (bDNA) assay quantitative results provided by the manufacturers were converted to log_{10} IU/ml for comparison. Samples in boldface type were within the linear range of both the real-time PCR and Bayer bDNA assays. The data in italics are for an outlier that was omitted from the graph in Fig. 3. ND, not detected. +, value was greater than the bDNA assay upper limit of detection.

[b] Qualitative positive.

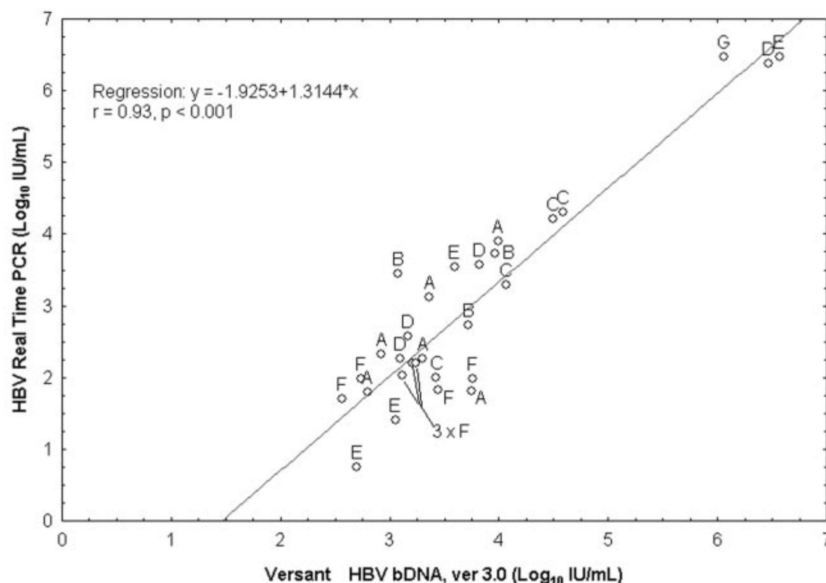

FIG. 3. Graph showing correlation of the HBV real-time PCR assay results with the Bayer Diagnostics VERSANT HBV DNA 3.0 (bDNA) assay results for 29 HBsAg-positive clinical specimens (numerical values are listed in boldface in Table 3). The HBV genotype of each specimen is adjacent to the data point on the graph. There was excellent correlation (r = 0.93) between the two assays for all HBV genotypes (A to G).

interassay precision to detect longitudinal assay drift, a positive control specimen was processed concurrently with the experimental specimens and tested in triplicate on every PCR plate. The longitudinal HBV control data from 78 independent experiments are shown as a bar graph in Fig. 4. The positive control running average was 2.78 $\log_{10}$ IU/ml, the standard deviation (SD) was 0.15, and %CV was 5.4%. The plate failed QC if the measured IU/ml of this HBV positive control was more than 2 SD greater or less than the HBV positive control running average. Three positive control results out of 78 (3.8%) exceeded these limits, invalidating those three plates.

Furthermore, an individual specimen failed QC criteria and was retested if the %CV of the PCR replicates for that sample exceeded 30% (for HBV DNA concentrations >500 IU/ml). Positive DNA measurements below the lower quantitative limit (56 IU/ml) of the real-time PCR assay were defined as qualitative positives and were not assigned a numerical value.

DISCUSSION

In this study, we described the development and validation of a novel, highly accurate quantitative real-time PCR assay that allows equal detection and quantification of HBV DNA (genotypes A to G) in human plasma specimens. This assay has good clinical sensitivity at 56 IU/ml using an initial sample volume of only 200 μl, at least an 8-$\log_{10}$ linear dynamic range without the need for specimen dilution, as well as good clinical reproducibility. Quantitative HBV DNA measurements for specimens with genotypes A to G obtained using this novel real-time PCR assay showed excellent correlation with Bayer VERSANT HBV DNA 3.0 (bDNA) assay results (r = 0.93).

Commercially available assays for detection and quantification of HBV DNA in human serum or plasma include the Bayer VERSANT HBV DNA 3.0 (bDNA) assay; the Digene hepatitis B virus (HBV) DNA assay, the Roche Molecular

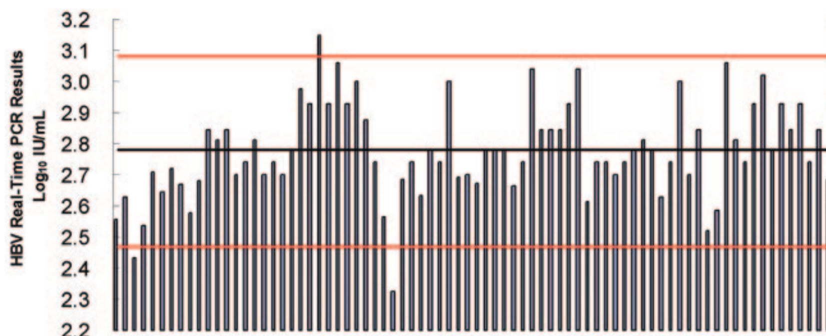

FIG. 4. Bar graph showing QC tracking of HBV real-time PCR positive control results over time. The BBI ACCURUN positive control was extracted and tested (in triplicate) concurrently with samples in 78 independent experiments over the course of 1 year. Each vertical bar represents the average positive control result for a single experiment. The overall average is shown as a horizontal black line. The positive control QC upper and lower limits, shown as horizontal red lines, were calculated as 2 SD above and below the average, respectively. Only 3 of the 78 positive control results (3.8%) exceeded these limits.

TABLE 4. Comparison of commercially available assays with the novel HBV real-time PCR assay described in this study

Assay name	Methodology	Target region	Sample vol (μl)	Conversion factor[a]	Sensitivity (95% level of detection in IU/ml)	Dynamic linear range (IU/ml)	Genotypes detected
Bayer Diagnostics VERSANT HBV DNA 3.0 (bDNA) assay	bDNA signal amplification	Entire genome[b]	50	5.60	<357	$357–1.79 \times 10^7$	A-F
Roche Diagnostics COBAS AMPLICOR HBV MONITOR test v2.0	Target amplification with biotinylated primers then amplicon hybridization[c]	Not disclosed	100	5.26	<38	$38.0–3.80 \times 10^4$	A-E
Roche Diagnostics COBAS TaqMan HBV test (32)	Real-time PCR	Precore/core region	500	5.82	<6	$54.0–1.46 \times 10^8$	A-G
Real-time PCR	Real-time PCR	X gene	200	10.0	<56	$56.0–5.00 \times 10^8$	A-G

[a] Divide by this factor to convert from copies/ml to IU/ml.
[b] Targets conserved sequences throughout the entire genome.
[c] Requires use of the COBAS AMPLICOR Analyzer instrument.

Diagnostics COBAS AMPLICOR HBV MONITOR test, v2.0; and the COBAS TaqMan HBV test (32). These assays use various molecular methods for HBV DNA quantification. The Bayer assay is based on branched DNA signal amplification, the Digene assay uses chemiluminescent signal amplification, the Roche AMPLICOR HBV MONITOR test depends upon target amplification with biotinylated primers followed by amplicon hybridization within a microwell plate containing bound detection probe, and the Roche COBAS TaqMan utilizes real-time PCR.

Although these commercial assays are routinely used in diagnostic laboratories, some have limitations in sensitivity, linear range, genotype inclusivity, or sample volume. A comparison of these commercially available assays with our novel real-time PCR assay is shown in Table 4. For example, the sensitivity of the Bayer HBV bDNA and Digene HBV DNA assays might be insufficient for detecting very low HBV DNA levels typical in patients undergoing antiviral therapy and those with occult HBV DNA infection. In fact, 21 of 59 samples in the Teragenix and BBI worldwide genotype panels were below the detection limit of the Bayer VERSANT HBV DNA 3.0 (bDNA) assay (Table 3), whereas our assay detected HBV DNA in 58 of the 59 specimens. The limited dynamic range of the Roche Diagnostics COBAS AMPLICOR HBV MONITOR assay requires dilution of high-titer HBV specimens. In addition, two of the commercial assays do not detect all genotypes and some may show genotype bias.

The HBV COBAS TaqMan, recently introduced by Roche Molecular Diagnostics (32), is a real-time PCR assay reported to equally amplify HBV genotypes A to G. The linear range of at least 8 logs and good reproducibility are comparable to those of the real-time PCR assay described herein. The large specimen volume used for the Roche COBAS TaqMan (500 μl), as compared to 200 μl for our real-time PCR assay, results in greater clinical sensitivity (6 IU/ml compared to 56 IU/ml, respectively). However, the clinical sensitivity of our assay could also be easily increased by a similar scale up to a larger specimen volume (5, 10). In addition, the larger specimen volume required for the Roche COBAS TaqMan HBV assay may be problematic for studies with limited samples. The primer-probe set of the Roche COBAS TaqMan assay targets the precore/core region, while the real-time PCR assay we developed targets the HBV X gene.

Although a variety of in-house real-time PCR assays have been described (1, 6, 14, 18, 26, 31, 32, 36), genotype inclusivity has only been reported in one recent study (9). Compared to this previous study, our primer-probe set design was based on a much larger number ($n = 340$) of complete HBV DNA sequences. We used more than twice as many sequences and designed primer-probe sets within the HBV S and X genes. The sequences we used included HBV genotypes A to H obtained from North, Central, and South America; Europe; Asia; Africa; and Australia. In addition, we developed a unique algorithm and software program for primer-probe set design that enabled us to incorporate degenerate bases to increase detection of genotypic variants. We felt this was necessary due to the great variability seen in HBV genomic sequences. Furthermore, performance of primer-probe sets was tested on a large number ($n = 59$) of genotyped clinical specimens rather than linearized plasmid DNA containing cloned inserts of HBV genotypes A to G, as described by Weiss and coworkers (32).

A recent nationwide epidemiological study of HBV genotypes in the United States revealed that genotypes A to G are present in the United States. The prevalence of genotypes differed by region and was strongly associated with place of birth and ethnicity of the study participants (7). These results indicate that immigration from areas of HBV endemicity can alter country or region-specific HBV epidemiology. As a consequence, genotype-inclusive assays for HBV DNA quantification are not only relevant in epidemiological studies involving specimens from all over the world, but also in routine diagnostic laboratories, particularly in countries where immigration is common.

Quantitative HBV DNA measurements can vary widely with the quantification method used and among different laboratories performing the testing. To enable comparison of results from our novel assay to those obtained with other assays, we determined the conversion factor from HBV copies per milliliter to international units per ml by testing the World Health Organization International Standard for HBV (28). We compared our novel real-time PCR assay with the Bayer VERSANT HBV DNA 3.0 (bDNA) assay and found excellent correlation for results within the linear range of both assays ($r = 0.93$; Fig. 3).

Interestingly, the apparent homogeneous distribution of HBV genotypes with respect to the regression line (see Fig. 3) suggests there is no bias in detection of one genotype over another by either our real-time PCR assay or the Bayer VERSANT HBV DNA 3.0 (bDNA) assay. This is despite the fact

that our real-time PCR assay targets only an 86-bp sequence within the X gene while the bDNA targets conserved sequences across the entire HBV genome. Note that the regression line is skewed to the right in Fig. 3, reflecting the difference in sensitivities between the two assays.

In summary, the real-time quantitative PCR assay described in this study uses an X gene primer-probe set to provide accurate detection and quantification of HBV DNA from all genotypes over a wide dynamic range of levels. Specific advantages of this assay include the following: (i) good clinical sensitivity (56 IU/ml); (ii) excellent clinical specificity (100%); (iii) broad linear dynamic range (56 to 5.0×10^8 IU/ml); (iv) equal detection/quantification of HBV genotypes A to G; (v) excellent within- and between-run precision; (vi) excellent correlation with the Bayer VERSANT HBV bDNA assay ($r = 0.93$); (vii) low sample volume requirement (200 μl); (viii) significantly lower cost than commercial assays; and (ix) rigorous QC, including longitudinal assay tracking.

These characteristics make the novel quantitative real-time HBV PCR assay described herein particularly well suited for clinical and epidemiological studies for which good sensitivity and genotype inclusivity are mandatory, and the low sample volume and low cost are advantageous.

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This technology as described herein is subject to a pending patent application.

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