JOURNAL OF CLINICAL MICROBIOLOGY, Nov. 2004, p. 5199–5204 Vol. 42, No. 11
0095-1137/04/$08.00+0 DOI: 10.1128/JCM.42.11.5199–5204.2004

Assessment of the Target-Capture PCR Hepatitis B Virus (HBV) DNA Quantitative Assay and Comparison with Commercial HBV DNA Quantitative Assays

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Received 13 February 2004/Returned for modification 11 May 2004/Accepted 11 August 2004

Recent clinical studies suggest that hepatitis B virus (HBV) load and genotype may be independent predictors of responses to antiviral therapies. However, it is difficult for clinicians to accurately determine viral loads in patient samples because results—both the values and the units of measure—can vary greatly among different tests. Accordingly, the World Health Organization (WHO) has produced the first international standard for HBV DNA for nucleic acid amplification technology (NAT) assays. In the present study, we describe the performance of the target-capture PCR HBV DNA quantitative assay for the quantitation of HBV DNA in clinical samples and reference panels. The range of quantitation was between 50 and 10^{10} IU/ml. The sensitivity and accuracy of the target-capture PCR assay were demonstrated by using the HBV panel from Quality Control for Medical Diagnostics (QCMD) and the WHO HBV DNA standard. The target-capture PCR assay quantitated the six genotype A members of the QCMD panel and dilutions of the WHO HBV DNA standard within an accuracy of 74 to 142%. Compared to current serological methods, the assay offers window period reductions of 19 days prior to HBV surface antigen and 26 days prior to HBV e antigen detection. The target-capture PCR assay was also compared with four commercially available NAT assays, and the various units of measure were standardized with respect to the international units of the WHO HBV DNA standard. The target-capture PCR assay is a sensitive, accurate, high-throughput, rapid, and reproducible assay for the determination of HBV loads.

Hepatitis B virus (HBV) presently infects 2 billion people and is the ninth leading cause of death worldwide. Each year there are estimated to be >350 million new cases of HBV infection worldwide, >100,000 of which occur in the United States alone (http://www.who.int/emc-documents/hepatitis/docs/whocdscsrlyo2002/index.html). The standard laboratory techniques for the diagnosis of HBV are serological assays, mainly modified enzyme immunoassay systems, for HBV surface antigen (HBsAg) and a secreted nonstructural HBsAg as well as for antibodies against HBsAg, HBV core antigen, and HBV e antigen. With the current limits of detection of serological tests for transfusion-transmitted infectious diseases, HBV has the greatest residual viral infection risk, at 1:63,000 (1, 3, 21), due to the long doubling time of 2 to 4 days and the persistence of low-level viral loads of 100 to 20,000 copies/ml for weeks without detectable HBsAg (3). Data from posttransfusion hepatitis cases has shown that the HBsAg assay becomes positive 50 to 60 days after infection but does not detect HBsAg during the preceding phase of 2 to 4 weeks of low-level viremia (3). Additionally, as few as 10 to 20 viral equivalents have been correlated with infectivity in chimpanzees, showing the necessity for early diagnosis (23).

Testing by nucleic acid amplification technology (NAT) can detect low viral loads, resulting in an earlier diagnosis of HBV infection (3, 4, 8, 9, 11, 14, 20). Recent clinical studies suggest that HBV load and genotype may be independent predictors of responses to antiviral therapies, including lamivudine, femcyclovir, ribavirin, and interferon (7, 13, 18, 24). As new anti-HBV therapies emerge, the ability to monitor viral loads in patients undergoing therapy to gauge responses has become important. Quantitative assays provide clinicians, testing laboratories, and blood banks with well-controlled, consistent, and reproducible tests for detecting viremia and monitoring disease progression (2, 6, 10, 13, 14, 16, 18).

Each of the tests for HBV has been developed by using proprietary HBV DNA standards and provides results in units of measure unique to that particular method. The inconsistency among various units of measure for HBV DNA assays has complicated the interpretation of viral load data and could have an impact on clinical decisions based on those results. In an attempt to address this concern, the World Health Organization (WHO) Collaborative Study Group and the WHO Expert Committee on Biological Standardization have recommended and made available the first international standard, 97/746, for HBV DNA for NAT assays (19). The standard was established by evaluating end-point dilutions of an HBV genotype A sample (ADW2) by several NAT assays in 22 laboratories worldwide. Since 1999, the European Union Quality Control Concerted Action has been evaluating available NAT assays by providing coded proficiency panels. The results for the HBV panel from the 2000 study indicated that only 51% of the qualitative tests could detect the lowest HBV concentration of 1,000 copies/ml and that just 65% could predict the concentration within ±0.5 log unit. These data indicate that

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the detection levels of many of the commercial quantitative assays are too high to allow adequate quantitation of viral titers in the clinical samples tested in routine diagnostic laboratories (24).

The objective of the present study was to evaluate the in-house target-capture PCR HBV DNA quantitative assay against four commercial NAT assays for the quantitation of HBV DNA; the commercial assays were the COBAS Amplicor HBV Monitor assay (Roche Diagnostics), the SuperQuant HBV DNA assay (National Genetics Institute, Los Angeles, Calif.), the Quantiplex HBV version 2.0 branched-DNA (bDNA) assay (Bayer Reference and Testing Laboratories, Berkeley, Calif.), and the Hybrid Capture assay (Digene Laboratories). Three of the five assays—target-capture PCR, Amplicor, and SuperQuant—use target amplification methodology, while the Hybrid Capture and Quantiplex assays use signal amplification methodology.

Data from the four commercial assays were obtained from the laboratories that provide diagnostic services for the respective assays. All of the assays give results in proprietary units of measure. This investigation allowed us to compare those proprietary units to the international units (IU) established by WHO. The performance of the target-capture PCR assay was also independently tested with a coded 2003 HBV panel from the Blood-Borne Virus (BBV) program of Quality Control for Medical Diagnostics (QCMD; Glasgow, United Kingdom).

MATERIALS AND METHODS

Samples. A lyophilized aliquot of the WHO international standard for HBV DNA for NAT assays (National Institute for Biological Standards and Control code 97/746; 5×10^5 IU) was reconstituted with 0.5 ml of sterile water and then serially diluted to viral concentrations of 1,000, 135, and 45 IU/ml in serum negative for HBV, hepatitis C virus, and human immunodeficiency virus nucleic acids, i.e., in delipidized and defibrinated normal human serum (Seracare, Inc., Oceanside, Calif.). These dilutions of the international standard were used as the primary reference in the preparation of a 12-member, in-house secondary standard panel. The secondary standard panel was made from a sample containing HBV DNA at 20×10^6 IU/ml (OptiQuant panel 94-2012; NAP-HBV006; AcroMetrix, Inc., Benicia, Calif.). Serial dilutions of this sample were made to obtain a panel with a concentration range of 100,000 to 50 IU/ml and a negative control. The quantitative accuracy of the panel was determined by quantitating the WHO HBV dilutions against the 12-point standard curve obtained from the panel. A 14-member HBV seroconversion panel, 6282 (BioClinical Partners/Impath, Franklin, Mass.), was used to test the performance of each of the five assays. The QCMD BBV proficiency panel consisted of eight coded samples in the range of 200 to 10^6 copies/ml for two HBV genotypes.

Target-capture PCR HBV DNA quantitative assay. The target-capture PCR assay consists of two steps: target capture and amplification-detection. A 927-nucleotide HBV internal control (IC) single-stranded DNA, modified by replacement with the probe binding sequence, was prepared as a control for target isolation and amplification (22). The IC DNA was isolated by cloning into an M13 plasmid to obtain the negative strand. A total of 400 μl of capture reagent, consisting of 15 μg of Sera-Mag oligo(dT) microparticles (Seradyn, Indianapolis, Ind.) suspended in lysis buffer and also containing the IC, and 500 μl of each specimen were used per test. The target was isolated with capture oligonucleotides complementary to the negative strand, containing an HBV sequence contiguous with a stretch of poly(dA), and magnetic beads coupled with oligo(dT). The suspension was incubated in a water bath at 60°C for 20 min to effect viral lysis, solubilize the proteins, and release viral DNA. The samples were cooled to 25°C for 15 min to obtain the tertiary complex of viral DNA, capture oligonucleotides, and beads. The beads with the hybridized target were washed three times in a buffer containing HEPES, 0.3 M NaCl, and 0.5% NP-40 (pH 7.5) (wash buffer). Following removal of the wash buffer, 100 μl of universal PCR master mix (ABI, Foster City, Calif.) containing amplification-detection primers corresponding to region X was added to the beads. The beads then were transferred to a 96-well multititer plate that was placed in a Prism 7900 thermocycler (ABI) for PCR amplification. The samples were kept at 50°C for 2 min to degrade any preamplicons with uracil-*N*-glycosylase and then at 95°C for 10 min to activate the *Taq* polymerase enzyme. The samples then were cycled for 50 cycles of 95°C for 15 s and 60°C for 1 min. The HBV target and IC signals were monitored for 6-carboxy-fluorescein and tetrachloro-6-carboxy-fluorescein, respectively. The standard, the negative control, and the unknowns were tested in triplicate. A standard graph was generated by using SDS software (ABI). The target-capture PCR assay quantitates HBV DNA in the range of 50 to 10^{10} IU/ml.

COBAS Amplicor HBV Monitor assay. The Amplicor assay was performed at ARUP Laboratories (Salt Lake City, Utah). Briefly, the method involves PCR amplification of precipitated nucleic acids with one biotinylated primer. The biotinylated amplicon is serially diluted three times, and the four resulting solutions are captured onto magnetic particles coated with an oligonucleotide probe. The bound amplicon is detected with an avidin-horseradish peroxidase conjugate. A quantitation standard of plasmid DNA, modified for the probe binding sequence at a known concentration, is added to each sample. The HBV DNA level in each test sample is calculated by comparing the target signal with the quantitation standard signal. A sample volume of 100 μl is processed, and DNA from the equivalent of a 50-μl sample is used for amplification. Results from the Amplicor assay are reported as copies per milliliter, and the nominal range of quantitation is 200 to 200,000 copies/ml.

SuperQuant HBV DNA assay. The SuperQuant assay was performed at National Genetics Institute. The method involves PCR amplification of extracted nucleic acids followed by Southern hybridization. Samples are centrifuged, and nucleic acids are isolated by proteinase K extraction. Following PCR amplification of the unknowns and the standard, the amplicons are electrophoresed and transferred to a membrane. Southern blotting is performed with a nonradioactive digoxigenin-labeled DNA probe. The profile of each Southern blot membrane is scanned into a computer by using an automated scanner-densitometer. The resulting electronic images are measured for band area and mean band density. A sample volume of 2 ml is used, and the results are reported as copies per milliliter. The nominal range of the SuperQuant assay is 100 to 10^8 copies/ml, requiring dilution of high-titer samples before analysis to obtain measurements in the linear range.

Quantiplex HBV version 2.0 bDNA assay. The Quantiplex assay was performed at Bayer Reference and Testing Laboratories. The method uses bDNA technology with signal amplification. The target is isolated by hybridization to specific complementary oligonucleotides. The bDNA assay achieves sensitivity by using target probes that bind to selective regions of the full viral nucleic acid sequence, which in turn hybridizes to the bDNA amplifiers. Alkaline phosphatase-labeled probes then hybridize to the bDNA amplifiers, and signal detection is accomplished by using chemiluminescence technology. Reference to an external standard curve with known concentrations of HBV DNA permits quantitation of the unknowns. A sample volume of 0.2 ml is used, and the results are reported as milliequivalents (mEq) per milliliter, with 1 mEq/ml $\approx 10^6$ copies/ml. The limits of quantitation of the assay are from 7×10^5 to 4.8×10^9 copies/ml.

Hybrid Capture assay. The Hybrid Capture assay was performed at Quest Diagnostics (San Juan Capistrano, Calif.). The method uses an RNA probe coupled with signal amplification. For ultrasensitive testing, 1.0 ml of sample is concentrated by centrifugation. HBV DNA in the samples is hybridized to an RNA probe. The resulting hybrids are captured on the surface of microplate wells coated with antibodies specific for RNA-DNA hybrids. Thus immobilized, the hybrids are reacted with alkaline phosphatase-conjugated antibodies specific for RNA-DNA hybrids. Bound alkaline phosphatase is detected with a chemiluminescent dioxetane substrate. The results are reported as picograms per milliliter, and the nominal range of the assay is 0.017 to 6,000 pg/ml (4,700 to 1.7×10^9 copies/ml).

Standardization of WHO IU per milliliter. The HBV DNA concentrations in the 14 seroconversion panel members were quantified by using the proprietary data reduction procedures unique to each assay system. Results from reference laboratories were provided in the units of measure established for each assay. For the target-capture PCR assay, the results were reported as IU, as with the WHO HBV DNA international standard.

RESULTS

Evaluation of the 12-member secondary standard panel. The WHO international standard for HBV DNA for NAT assays, 97/746, is available in limited supply and is recommended for use only as the primary reference standard for the

TABLE 1. Cycle thresholds obtained for secondary standards in the target-capture PCR assay

Standard (IU/ml)	Cycle threshold[a]					Coefficient of variation (%)
	Sample 1	Sample 2	Sample 3	Mean	SD	
100,000	27.19	27.40	27.11	27.23	0.15	1
50,000	28.49	28.17	28.33	28.33	0.16	1
10,000	30.19	30.33	30.64	30.39	0.23	1
7,500	30.83	31.22	31.08	31.04	0.20	1
5,000	31.38	31.13	31.81	31.44	0.35	1
2,500	32.70	32.52	32.20	32.47	0.25	1
1,000	34.30	33.91	34.13	34.11	0.20	1
750	34.37	33.54	33.89	33.93	0.41	1
500	35.23	36.50	34.38	35.37	1.07	3
250	36.30	36.21	36.21	36.24	0.05	0
100	36.73	37.23	36.08	36.68	0.58	2
50	47.20	37.77	38.44	41.14	5.26	13
0	NS	NS	NS	NS	0	0

[a] NS, no signal.

calibration of secondary in-house standards (19). The performance of the in-house secondary standard panel was evaluated by quantitating serially diluted WHO international standard samples. The magnetic bead-based protocol used for the isolation of HBV DNA from samples in a single tube significantly simplifies the isolation of nucleic acids from samples compared to standard centrifugation and precipitation methods. The single-stranded 927-nucleotide IC serves as an efficient control for all steps except lysis and detection. All samples, including the standards, are tested in triplicate to derive an average value and to remove outliers. Table 1 shows the cycle thresholds obtained for the standards and demonstrates the close agreement among triplicate samples for all 12 members.

Table 2 shows the quantitation of the diluted WHO international standard with the secondary standard panel. The experimental results are in the range of 106 to 142% the predicted values, indicating the reliability of the 12-member panel for use as a standard in quantitation studies. Direct amplification of the target captured on the beads without the intermediate step of elution offers this high precision by avoiding the variability of yield of other methods and eliminating losses due to transfer.

Quantitation of the HBV seroconversion panel. With the 12-member standard panel, the 14 members of the seroconversion panel were tested in triplicate by the target-capture PCR assay and the four other assays described in Materials and Methods. The results are shown in Table 3. The Amplicor assay results were identical to the nominal HBV copy numbers provided by the seroconversion panel vendor; that vendor had used the Roche PCR assay to establish concentrations. Quantiplex results were converted to copies per milliliter from the proprietary mEq per milliliter, and Hybrid Capture results were provided by the vendor in copies per milliliter and in picograms per milliliter with a conversion factor of 250,000 to 280,000 copies/ml (24). Of the target amplification assays, the SuperQuant and target-capture PCR assays were quantitative for samples starting from bleed 1 and therefore were the most sensitive. The Amplicor assay had no reportable results for bleeds 1 and 2. Of the signal amplification assays, the Hybrid Capture assay yielded reportable results beginning with bleed 6, and the Quantiplex assay yielded reportable results beginning with bleed 10.

TABLE 2. Quantitation of WHO HBV DNA international standard by target-capture PCR assay with the 12-member secondary standard panel

Predicted concn (IU/ml)	Observed concn (IU/ml)[a]	Accuracy (%)
1,000	1,063	106
135	191	142
45	63	140

[a] Each result is the average for triplicate samples.

Based on the information provided by the vendor, the seroconversion samples were positive for HBsAg and HBV e antigen beginning from bleeds 6 and 8, respectively. These data represent window period closures of 19 and 26 days for the target-capture PCR and SuperQuant assays, respectively, and 7 days for the Hybrid Capture assay. With a predicted doubling time of 2.56 days in the ramp-up phase for HBV (3), these data indicate the ability of the assays to detect a virus prior to 7.4 to 10.1 logarithmic doublings of the virus.

Conversion of assay units to IU. The WHO international standard uses IU as the definition for HBV DNA. Therefore, results reported as copies per milliliter by other assays were correlated with the IU obtained from the target-capture PCR assay (Table 4). Because the Amplicor, SuperQuant, and target-capture PCR assays use PCR-based target amplification, several points of overlap could be established to obtain a ratio of copies per milliliter to IU. For the Amplicor and SuperQuant assays, the values in the early phase of seroconversion with low viral titers, resulting in an exponential phase of amplification, were used to calculate this ratio. For the Hybrid Capture and Quantiplex signal amplification assays, few such overlapping points were noted. Consequently, the values in the later phase of seroconversion with high titers were used to calculate the ratio of copies per milliliter to IU for these two assays. The mean ratios obtained from this experiment were as follows: target-capture PCR assay, 2.6; Amplicor, 7.3; SuperQuant, 6.2; Quantiplex, 4.5; and Hybrid Capture, 2.3. Similar observations of a failure to obtain unique ratios with different NAT assays have been reported by Pas et al. (16) and Pawlotsky et al. (17).

TABLE 3. Comparative HBV load estimates obtained with seroconversion panel 6282

Bleed no.	Day	Results obtained with:					
		Roche PCR (copies/ml)[a]	NAT assay[b]				
			Target-capture PCR (IU/ml)	Amplicor (copies/ml)	SuperQuant (copies/ml)	Quantiplex (copies/ml)	Hybrid Capture (copies/ml)
1	0	5×10^2	62	BLD	2×10^2	BLD	BLD
2	2	5×10^2	62	BLD	5.5×10^2	BLD	BLD
3	7	5×10^2	99	9.7×10^2	1.5×10^3	BLD	BLD
4	12	7.1×10^3	1.7×10^3	1.2×10^4	1.1×10^4	BLD	BLD
5	14	8.2×10^3	2.3×10^3	2×10^4	1.9×10^4	BLD	BLD
6	19	6.5×10^4	7.3×10^3	7.2×10^4	6×10^4	BLD	1.3×10^4
7	21	1.9×10^4	2.3×10^4	1.0×10^5	7.3×10^4	BLD	3.2×10^4
8	26	2×10^5	8.8×10^4	5.3×10^5	4.8×10^5	BLD	1.9×10^5
9	28	7.5×10^5	1.9×10^5	1.1×10^6	9.5×10^5	BLD	3.1×10^5
10	33	2.7×10^6	7.4×10^5	2.9×10^6	4.6×10^6	2.1×10^6	1.9×10^6
11	35	7×10^6	1.1×10^6	5.8×10^6	7.1×10^6	4×10^6	2.5×10^6
12	40	3×10^7	7×10^6	4.8×10^7	6×10^7	3.5×10^7	1.2×10^7
13	43	4.3×10^7	2.1×10^7	$>2 \times 10^8$	3×10^8	1.3×10^8	5.4×10^7
14	47	$>5 \times 10^7$	1.6×10^8	$>2 \times 10^8$	8.4×10^8	8.2×10^8	7.7×10^8

[a] Roche PCR assay results were provided by the panel vendor (BioClinical Partners/Impath).
[b] BLD, below the limit of detection: for Amplicor, <200 copies/ml; for Quantiplex, <700,000 copies/ml; for Hybrid Capture, <4,700 copies/ml.

Performance of the target-capture PCR assay with the QCMD panel. As part of the 2003 QCMD BBV program, an HBV proficiency panel consisting of eight coded members was tested by the target-capture PCR assay. The results are shown in Table 5. The results indicated no detection of HBV DNA in the HBV-negative sample. The assay detected and quantitated the genotype A sample with the lowest copy number, 200 copies/ml, demonstrating high sensitivity. All of the genotype A samples with higher copy numbers as well as genotype D samples were detected. For all of the quantitated genotype A samples, the conversion factor was 1 IU equals 2.6 copies.

TABLE 4. Conversion of copies to IU

Bleed no.	Factor for conversion of copies from the following assay to IU from the target-capture PCR assay[a]:			
	Amplicor	SuperQuant	Quantiplex	Hybrid Capture
1	NQ	3.23	NQ	NQ
2	NQ	8.40	NQ	NQ
3	9.76	15.10	NQ	NQ
4	**7.32**	**6.34**	NQ	NQ
5	**8.89**	**8.12**	NQ	NQ
6	**9.77**	**8.13**	NQ	**1.84**
7	**4.67**	**3.12**	NQ	**1.37**
8	**6.07**	**5.42**	NQ	**2.18**
9	5.84	4.87	NQ	**1.59**
10	3.89	6.17	**2.89**	**2.63**
11	5.31	6.42	**3.63**	**2.28**
12	6.92	8.56	**5.05**	**1.84**
13	NQ	13.71	**6.11**	**2.49**
14	NQ	5.03	**4.93**	**4.63**
Mean	7.3	6.2	4.5	2.3
SD	2.1	3.5	1.3	1.0
Coefficient of variation (%)	28	56	28	41

[a] NQ, not quantifiable. Bold type indicates the values used for calculating the ratios.

DISCUSSION

NAT assays used for screening donated blood include target amplification and signal amplification methods (for a review, see reference 18). PCR-based target amplification assays have been used extensively for the detection of HBV DNA (7, 12, 13, 15–17, 25). This report presents comparative results for HBV quantitation from five different assays—three based on target amplification and two based on signal amplification. Reproducible quantitation of low copy numbers of a target relies on target isolation from substantial volumes, region of amplification, range of standards, and amplification-detection technology. Here we discuss and compare the assay results with respect to these and other features.

Sensitivity of detection related to target isolation. A quantitative assay should cover a wide range of concentrations because the absolute viral load, along with the time course, are two valuable prognostic and therapeutic parameters (25). High sensitivity to enable the detection of low copy numbers is one of the most important goals for HBV detection systems. Additionally, HBV replicates to very high titers of $>10^9$ viral particles/ml of serum in patients with acute or chronic infection, a property which requires accurate quantitation at the high end as well (25). As amplification and detection have become more sensitive, the techniques for isolating viral nucleic acids have become the limiting factor for the detection threshold of diagnostic test systems. Of the assays described in this report, the target-capture PCR and Quantiplex assays use oligonucleotide-based capture of specific targets; the Amplicor, SuperQuant, and Hybrid Capture assays use techniques for the isolation of all nucleic acids. The SuperQuant and Hybrid Capture assays use 2.0- and 1.0-ml sample volumes, respectively; the target-capture PCR assay can process 0.5- to 1.5-ml sample volumes; but the Amplicor and Quantiplex assays suffer from decreased sensitivity due to their limited sample volumes of $\leq$200 μl. The SuperQuant and Hybrid Capture assays require concentration of samples by centrifugation,

TABLE 5. Performance of the target-capture PCR assay with the QCMD panel

QCMD code	Subtype	HBV concn			Accuracy (%)
		Calculated by target-capture PCR		Published by QCMD (copies/ml)	
		IU/ml	Copies/ml[a]		
HBV-01	A	547,239	1.4×10^6	1×10^6	140
HBV-02	A	288	0.74×10^3	1×10^3	74
HBV-03		0	0	0	100
HBV-04	D	14,265	0.37×10^5	1×10^5	37
HBV-05	A	4,077	1×10^4	1×10^4	100
HBV-06	A	1,155	3×10^3	3×10^3	100
HBV-07	A	87	226	200	113
HBV-08	A	39,445	1×10^5	1×10^5	100

[a] Each result is the average for triplicate samples in IU per milliliter converted to copies per milliliter with the following formula: 1 IU = 2.6 copies.

which is not recommended or practical for high-throughput testing. The target-capture PCR assay isolates nucleic acids in a single tube, minimizing sample transfer losses and accompanying contamination problems, with a quantitation limit as low as 50 IU/ml (22) (Tables 1 and 2). Additionally, the format of semiautomated target isolation from 100 samples per set in the target-capture PCR assay permits high-throughput testing.

Sensitivity of detection related to amplification-detection. The target-capture PCR, Amplicor, and SuperQuant assays utilize PCR for amplification of the target, with significant differences in the three detection mechanisms. Both the Amplicor and SuperQuant assays require processing of the amplicons and elaborate technology for quantitation; they estimate the initial amount of DNA by measuring the amount of amplified PCR product at the end of the amplification reaction. This end-point analysis is prone to error at high target concentrations, because the concentrations of the reaction components (primers and nucleotides) can become limiting, not resulting in the expected logarithmic increase in the amplicon for each cycle of amplification. The Amplicor assay involves serial dilution of the amplicon, followed by hybridization, washing, and enzyme-associated color development, and requires at least 4 h following amplification to obtain results. The SuperQuant assay involves gel separation, transfer to a membrane, hybridization, washing, and scanning and takes more than 24 h to generate results. Processing of amplicons in an open system, such as that required in the SuperQuant assay, can result in contamination and is generally considered undesirable.

The turnaround times for results from the various assays are as follows: Amplicor assay, 5 h; Hybrid Capture assay, 4 h; Quantiplex assay, 18 h; and target-capture PCR assay, 4 h. The total numbers of test results per run vary, with the Quantiplex, Hybrid Capture, and target-capture PCR assays being performed in 96-sample formats and the Amplicor assay being performed in a 24-sample format.

The target-capture PCR and Amplicor assays yield a signal that is target specific through the binding of a target-specific internal probe; the SuperQuant assay relies on Southern hybridization to ascertain specificity. The target-capture PCR assay uses fluorescence-labeled probes for continuous automated quantitation of the amplicons and allows the real-time observation of target amplification in a thermocycler. Real-time PCR quantitation is based on the evaluation of the threshold cycle when amplification of a PCR product is first detected. Real-time PCR has greatly improved precision in DNA quantitation due to the fact that the threshold cycle observed when PCR is still in the exponential phase is a more reliable measurement than an end-point measurement of the amplified PCR product (25). The benefits of the real-time assay format also include elimination of postamplification processing in order to detect products and reduction of the risk of amplicon contamination in subsequent PCRs. With real-time PCR, it is possible to quantitate HBV DNA in a dynamic range of 10 to 10^{10} copies ($r = -1.0$) without having to dilute high-titer samples.

Presence of an IC. One prerequisite for the PCR-based quantitative approach is to avoid PCR inhibitory substances, such as hemoglobin or heparin, in clinical samples; such substances can adversely affect the amplification reaction. External controls do not adequately control for these conditions; consequently, if the PCR is inhibited, false-negative test results are obtained. ICs that are thought to exhibit the most accurate control are designed to be coamplified with the target nucleic acid in a competitive PCR within the same reaction vessel and with the same set of primers as those used for the target nucleic acid. The SuperQuant assay does not include a coamplifying IC and therefore does not control for false-negative results or suboptimal amplifications. The target-capture PCR and Amplicor assays include an IC, which controls for target isolation and amplification.

Use of standards for quantitation. With the exception of the Amplicor assay, all assays, including the target-capture PCR assay, use a range of standards external to the sample to obtain the standard curve. This strategy assumes equal amplification and detection efficiencies for the standards and the samples, which need not be the case. The Amplicor assay uses an IC which also serves as an internal quantitative standard for virus-derived nucleic acids.

Signal amplification. Signal amplification assays tend to yield more accurate and linear quantitation than target amplification assays. However, these assays typically lack sensitivity and have lower limits of detection that are too high to allow the detection of low-level virus replication. Thus, the target-capture PCR assay, which uses half the sample volume that is required for the Hybrid Capture assay, is nevertheless >100-fold more sensitive (Table 3). The advantages of signal amplification assays include better quantitation at high viral titers.

Conversion of copies to IU based on the QCMD panel. The conversion of proprietary units specific to each assay to copies resulted in a wide range of ratios (Table 4). Because different

standards and methods were used in developing the units of measure for each of these various assays, such a divergence in units of measure is not surprising (16, 17). The means for target amplification assays were higher (6.2 to 7.5) than those for signal amplification assays (2.3 to 4.5). The results from the QCMD panel indicated conversion factors of 2.6 copies per IU for genotype A and 7.0 copies per IU for genotype D. This high conversion factor for genotype D is unlikely to be a genotype difference but more likely to be an error of quantitation of the stock. Based on the results obtained during the collaborative study, a conversion factor of 2.6 copies per IU was proposed (5). The higher ratios obtained for the Amplicor and Super-Quant assays could be due to overquantitation by those assays. Despite attempts to unify conversion units, it is difficult to generate a consensus, and it is hoped that the WHO international standard will be adopted universally as a reference and that consistent reporting of results in IU per milliliter will emerge.

In summary, we have developed a sensitive, accurate, user-friendly, high-throughput target-capture PCR assay and compared its performance with those of four commercial NAT assays. It has high sensitivity, since it amplifies and detects a target isolated from 0.5 ml of sample. It is accurate, because the 12-member secondary standard panel provided confidence in quantitation. The addition of a target-related IC to each sample to control for target isolation and amplification offers the highest confidence for true-negative results. The assay is user-friendly because of semiautomation of target isolation and the use of uracil-*N*-glycosylase to decrease false-positive results from contaminating amplicons. The high detection sensitivity offered by the target-capture PCR assay permits detection in pools and in chronic late-stage HBV infections characterized by low copy numbers. The target-capture PCR assay can be used effectively to monitor HBV DNA levels that might be undetectable by other commercial assays.

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