A Genotype-Independent Real-Time PCR Assay for Quantification of Hepatitis B Virus DNA[▽]

Ying Liu, Munira Hussain, Stephen Wong, Scott K. Fung, Hyung Joon Yim, and Anna S. F. Lok*

Division of Gastroenterology, University of Michigan Medical Center, Ann Arbor, Michigan

Received 4 April 2006/Returned for modification 14 June 2006/Accepted 22 November 2006

Accurate quantification of hepatitis B virus (HBV) DNA levels is important for monitoring patients with chronic HBV infection and for assessing their responses to antiviral therapy. This study aimed to develop a real-time PCR assay that is sensitive and can accurately quantify a wide range of HBV DNA levels across the known HBV genotypes. An “in-house” real-time PCR assay using primers and a TaqMan probe in a highly conserved region of the HBV surface gene was designed. The assay was standardized against a WHO standard and validated against plasmids of HBV genotypes A through H. The linear quantification range was approximately 5×10^0 to 2.0×10^9 IU/ml. Results of samples from patients infected with HBV genotypes A through H tested using our real-time “in-house” PCR assay showed an excellent correlation with those of the Cobas Amplicor HBV Monitor ($R^2 = 0.9435$) and the Cobas TaqMan HBV ($R^2 = 0.9873$) tests. We have established a real-time PCR assay that is genotype independent and can accurately quantify a wide range of HBV DNA levels. Further studies of additional samples are ongoing to validate the genotype independence of our assay.

The availability of PCR assays that can detect copy numbers as low as 1,000 copies/ml of hepatitis B virus (HBV) DNA led to the recognition that HBV persists, albeit at low levels, throughout the course of chronic HBV infection (6, 15, 16, 21). The level of viremia is highly variable among HBV carriers, and serum HBV DNA levels within the same person can fluctuate from undetectable to more than $10 \log_{10}$ IU/ml. Quantification of serum HBV DNA levels is important to determine the level of viral replication, to assess antiviral response, and to monitor antiviral resistance. However, HBV DNA assays used in clinical practice were not calibrated to WHO standards until recently, and the lower limit of detection as well as the range of linearity varies from one assay to another (5, 19).

The Amplicor assay is the most widely used commercial PCR assay for quantifying HBV DNA. The automated version, Cobas Amplicor HBV Monitor test, has a lower limit of detection of 60 IU/ml but a limited range of linearity, up to 38,000 IU/ml, based on the manufacturer's package insert. Given that many patients with chronic hepatitis B, particularly those who are hepatitis B e antigen (HBeAg) positive, have HBV DNA levels that are 10^9 IU/ml or higher, retesting serial dilutions up to 1:1,000,000 may be necessary. Thus, the possibility of errors in quantifying very high HBV DNA levels is tremendous. In addition, the Cobas Amplicor HBV Monitor test, like other conventional PCR assays, measures the amount of input DNA at the end of the amplification reaction and is, therefore, subject to errors caused by a “plateau” effect (3).

Various assays utilizing real-time PCR technology have been recently developed to quantify HBV DNA (1, 4, 18, 24, 26, 27). Real-time PCR technology is based on the evaluation of a

threshold cycle when the amplification products are detected for the first time. It is more precise than conventional PCR because results are obtained when the PCR is still in the exponential phase (10, 11). Many real-time PCR HBV DNA assays were developed in-house and have not been standardized or validated (1, 4, 18, 27). The Cobas TaqMan HBV test, the most commonly used commercial real-time PCR HBV DNA assay, has a lower limit of detection of 6 IU/ml and a range of linearity up to 780,000,000 IU/ml, based on the manufacturer's brochure. This assay is fully automated and incorporates an internal quantification standard, but it is expensive (8, 23, 25).

There are eight recognized HBV genotypes, designated A to H, based on a nucleotide difference of >8% in whole-genome sequencing (2, 7). Discrepancies in quantifying the hepatitis C virus (HCV) RNA in samples from patients infected with various HCV genotypes have been reported (9, 17, 20, 22). Data for the performance of HBV DNA assays across HBV genotypes are scanty.

Here we describe the development and evaluation of a TaqMan-based real-time in-house PCR assay validated for HBV genotypes A through H. The lower limit of detection and the dynamic range of the assay were determined, and the performance results of this assay of clinical samples was compared to those of two commercially available assays, Cobas Amplicor HBV Monitor test and Cobas TaqMan HBV test.

MATERIALS AND METHODS

DNA extraction, PCR amplification, and detection. A QIAamp DNA blood minikit (QIAGEN, Valencia, CA) was used to extract nucleic acids. Two hundred microliters of serum was used, and DNA was eluted in 50 μ l of DNase/RNase-free water and stored at -20°C until used. Real-time PCR was performed in a 50- μ l reaction mixture containing 25 μ l TaqMan Universal PCR master mix (Applied Biosystems) with 0.2 μ M primers, 0.1 μ M probes, and 10 μ l extracted DNA. An initial 2-min incubation at 50°C to activate uracil-N-glycosylase and to destroy potential amplicon carryover was followed by a 10-min incubation at 95°C , which activates the thermostable DNA polymerase and denatures the uracil-N-glycosylase. Subsequently, 50 cycles of 95°C for 15 s and 60°C for 1 min were performed with an iCycler iQ multicolor real-time PCR detection

* Corresponding author. Mailing address: Division of Gastroenterology, University of Michigan Medical Center, 1500 East Medical Center Dr., 3912 Taubman Center, Box 0362, Ann Arbor, MI 48109-0362. Phone: (734) 615-4628. Fax: (734) 936-7392. E-mail: aslok@umich.edu.

[▽] Published ahead of print on 20 December 2006.

TaqMan probe (5'-FAM and 3'-TAMRA) (367-391bp, A, adw)
 5'-TATCGCTGGATGTGTCTGCGCGT-3' (A, B, D, E and G)
 5'-----T-----3' (C, F and H)

HBV-S-Forward (333-352bp, A, adw)
 5'-ACTCACCAACCTCTTGTCT-3' (C, D1 and E)
 5'-----C-----3' (A, D2-4, F, G and H)
 5'-----G-----3' (B)

HBV-S-Reverse (476-456bp, A, adw)
 5'-GACAAACGGGCAACATACCT-3' (A-G)
 5'-----C-----3' (H)

FIG. 1. Comparison of real-time PCR TaqMan probe and primer sequences with GenBank database sequences for HBV genotypes (accession numbers X02763, X51970, and AF090842), B (accession numbers D00329, AF100309, AB033554, D00330, and AB073858), C (accession numbers X04615, AB014381, M12906, and AB033556), D (accession numbers X65259, M32138, X85254, and X02496), E (accession numbers X75657 and AB032431), F (accession numbers X69798, AB036910, and AF22396), G (accession numbers AF160501, AB064310, and AF405706), and H (accession numbers AY090454, AY090457, and AY090460).

system (Bio-Rad Laboratories, Hercules, CA) and the results analyzed with sequence detector software (version 3.0) according to the manufacturer's instructions.

The primers and a fluorogenic 5'-end nuclease probe (TaqMan) were selected from a highly conserved region of the S gene after careful analysis of 27 full-length HBV sequences belonging to genotypes A through H deposited in the GenBank database and synthesized and purified by Integrated DNA Technologies (Coralville, IA). Comparisons of the sequences of the primers and probe to consensus sequences of each genotype are shown in Fig. 1.

Preparation of plasmids and calibration of the in-house real-time PCR assay against a WHO standard and plasmids of HBV genotypes A through H. DNA extracted from 200- μ l serum samples from patients who were previously determined to be infected with HBV genotypes A through H and DNA from a WHO international HBV DNA standard (NIBSC code 97/746, genotype A, serotype adw2) were PCR amplified. The PCR was conducted with a 50- μ l reaction mixture containing AmpliTaq Gold DNA polymerase (0.25 U; Applied Biosystems, Foster, CA) and buffer, MgCl₂ (5 mM), 800 nM of PCR nucleotide mix (10 mM of each deoxynucleoside triphosphate) (Promega, Madison, WI), 200 nM of

FIG. 2. Calibration of the HBV genotype A plasmid DNA against that of the WHO HBV international standard. Amounts of WHO HBV standard used in each reaction were 20, 2 $\times$ 10², 2 $\times$ 10³, 2 $\times$ 10⁴, 2 $\times$ 10⁵, and 2 $\times$ 10⁶ IU/ml. (A) The threshold cycle was plotted against input HBV DNA concentrations (standard curve). (B) Standard curve obtained from serial dilutions of HBV genotype A plasmid: 5, 10, 20, 2 $\times$ 10³, 2 $\times$ 10⁵, 2 $\times$ 10⁷, and 2 $\times$ 10⁹ IU/ml.

FIG. 3. Coamplification of plasmids of HBV genotypes A through H and the WHO standard at dilutions of 20, 2×10^3 , 2×10^5 , and 2×10^7 IU/ml. Correlation coefficient, 0.995; PCR efficiency, 111.9%. Genotype A, azure; B, pink; C, crimson; D, blue; E, purple; F, yellow; G, red; H, grass green; WHO standard, green.

each primer, and 5 μ l of DNA extract. Thermal cycling was performed using the following conditions: an initial incubation at 95°C for 10 min and then 45 cycles of 95°C for 30 s, 55°C for 30 s, and 72°C for 60 s. The amplified region corresponded to a 681-bp region of the S gene (nucleotide [nt] 1564 to 2244; genotype A, serotype adw2). The PCR products were cloned into a pGEM-T-Easy vector (Promega, Madison, WI). The recombinant plasmids were purified, and the concentration of HBV DNA was determined using a spectrophotometer.

Serial dilutions of the WHO standard, with concentrations ranging from 20 to 2.0×10^6 IU/ml, were tested with the real-time PCR assay to obtain a standard curve. Subsequently, serial dilutions of HBV S gene plasmid DNA from a genotype A sample with concentrations ranging from 2.0×10^2 to 2.0×10^5 IU/ml were calibrated against the WHO standard. Finally, serial dilutions of the WHO standard and S gene plasmids of HBV genotypes A through H with concentrations ranging from 2.0×10^2 to 2.0×10^7 IU/ml were tested in parallel with the real-time PCR assay to compare the amplification efficiencies across these genotypes.

Patients and serum samples. A total of 169 serum samples collected from patients with chronic HBV infection seen in the Liver Clinics at the University of Michigan Hospital were studied. These patients were participants in one of three studies: the natural history of chronic HBV infection, antiviral resistance surveillance, and prevention of recurrent HBV infection after liver transplantation. All three studies were approved by our institutional review board, and all patients provided written informed consent for sample collection. Sera were divided into aliquots and stored at -70°C until used.

HBV DNA quantification. All samples were tested using our in-house real-time PCR assay, without knowledge of the results of commercial assays. Each assay included the following: HBV S plasmid standards (genotype A, concentrations of 5×10^0 to 2.0×10^9 IU/ml), two human serum samples that had previously tested positive for HBV DNA using a Cobas Amplicor HBV Monitor test (positive controls), water, and two serum samples from healthy subjects who were HBsAg negative (negative controls).

All samples were also tested with a Cobas Amplicor HBV Monitor version 2 (Roche Molecular Diagnostics, Branchburg, NJ), which has a linear dynamic range from 60 to 38,000 IU/ml. All samples with values of $\geq 38,000$ IU/ml were diluted 1:1,000 to 1:100,000 to obtain a valid result. Forty-nine samples collected between June and December 2005 were further tested using a Cobas TaqMan HBV test (Roche Molecular Diagnostics, Branchburg, NJ), which has a linear dynamic range from 6 to at least 780,000,000 IU/ml.

HBV genotyping. The HBV genotypes of samples with HBV DNA detectable by a Cobas Amplicor Monitor assay were determined using a line probe assay, INNO-LiPA HBV Genotyping (Innogenetics NV, Ghent, Belgium), as described previously (14).

Statistical analyses. iCycler iQ™ sequence detector software (version 3.0) (Bio-Rad Laboratories, Hercules, CA) was used to determine real-time PCR results. Precision was calculated with the use of JMP statistical software (SAS Institute, Cary, NC). Assay reproducibility was assessed by the coefficient of variation (CV). Pearson's correlation coefficient was used to assess the linear regression between the log-transformed values of HBV DNA using our in-house real-time PCR assay and Cobas Amplicor and Cobas TaqMan assays.

RESULTS

Serial dilutions of the WHO HBV DNA standard were tested along with serial dilutions of HBV genotype A plasmid DNA. The amplification efficiency of the HBV genotype A plasmid DNA was the same as that of the WHO HBV DNA standard, as judged by the identical slope of the superimposed calibration curves (Fig. 2A). Triplicate testing confirmed these results, enabling us to use our panel of genotype A plasmid DNA dilutions, ranging from 5×10^0 to 2.0×10^9 IU/ml, as standards for future assays.

To determine the range of linearity, eight duplicate serial dilutions from the HBV genotype A plasmid DNA standard, with concentrations from 1×10^0 to 2.0×10^9 IU/ml, were tested. A linear relationship was observed over the range of 5×10^0 to 2.0×10^9 IU/ml (Fig. 2B). To determine the lower limit of detection, DNA was extracted from HBV genotype A plasmid at concentrations of 1, 2, 5, 10, 20, and 200 IU/reaction on four occasions, and each DNA extract was tested in duplicate in three different assays. Plasmid DNA concentrations above 5 IU/ml were detected on all 24 occasions, and concentrations at 5 IU/ml were detected on 20 of 24 occasions.

The precision of our in-house real-time PCR assay was determined by assessing the intra- and interassay CV. For intraassay variability, serum samples from 12 patients with chronic HBV infection including virus genotypes A through H and two samples from healthy subjects who were HBsAg neg-

FIG. 4. Comparison of HBV DNA levels of 32 serum samples with HBV genotypes A through H tested with our in-house real-time PCR assay using DNA from plasmids of HBV genotypes A through D as standards.

ative were tested in triplicate in the same assay. The mean CV was 0.082 (range, 0.051 to 0.099). For interassay variability, DNA was extracted from each of 10 serum samples on three separate occasions. Each DNA extract was tested in duplicate in three different PCR assays. The HBV DNA values were found to be normally distributed on a logarithmic scale. The mean CV for all 10 samples tested was 0.16 (range, 0.036 to 0.216).

To determine whether plasmid HBV DNA can be used as the standard for testing serum samples, we tested three dilutions of the WHO standard in human serum at concentrations of 3×10^6 , 3×10^4 , and 3×10^2 IU/ml, three serum samples from HBV patients that had the same HBV DNA concentrations based on Cobas Amplicor HBV Monitor test results, and three serum samples, which were spiked with the same concentrations of HBV genotype A plasmid DNA, from HBsAg-negative subjects. DNA was extracted and HBV DNA quantified with our in-house real-time PCR assay using HBV genotype A plasmid DNA as the standard. DNA extraction from serum samples versus that from plasmids had a negligible effect on the results.

To determine if our in-house real-time PCR assay is equally accurate in quantifying HBV genotypes A through H, HBV genotype A through H plasmid DNA at concentrations from 2×10^1 to 2.0×10^7 IU/reaction were coamplified with the WHO standard. Figure 3 shows that amplification efficiencies were comparable for these eight genotypes. We then tested 32 patient samples (HBV genotype A, 5 samples; B, 5 samples; C, 5 samples; D, 5 samples; E, 3 samples; F, 2 samples; G, 2 samples; and H, 1 sample) with our in-house real-time PCR assay using genotype A through D plasmids as standards. Figure 4 shows that the results of samples from all eight genotypes (A through H) were similar regardless of which genotype (A through D) plasmid was used as the standard. These results indicate that the genotype A plasmid can be used as the standard for testing patient samples with other HBV genotypes.

Forty-nine samples (HBV genotype A, 9 samples; B, 6 samples; C, 9 samples; D, 8 samples; F, 2 samples; and not available, 15 samples) previously tested by a Cobas TaqMan HBV

test were retested using our in-house real-time PCR assay. Correlation between the two assays was excellent, with an R^2 value of 0.9873 over the shared 9 $\log_{10}$ dynamic range (Fig. 5). All 46 samples with detectable HBV DNA according to the Cobas TaqMan HBV test results had detectable values in our real-time PCR assay. Similarly, none of the three samples with undetectable HBV DNA in the Cobas TaqMan HBV test had detectable values in our assay. Of the eight samples with HBV DNA values of $>7 \log_{10}$ IU/ml determined with the Cobas TaqMan HBV test results, our in-house real-time PCR assay reported values that were within 0.5 $\log_{10}$ IU/ml in six samples, a 0.5- to 1.0- $\log_{10}$ -IU/ml-lower value in one sample, and a 0.5- to 1.0- $\log_{10}$ -IU/ml-higher value in one sample.

One hundred sixty-nine samples (HBV genotype A, 38 samples; B, 20 samples; C, 35 samples; D, 25 samples; E, 8 samples; F, 7 samples; G, 6 samples; H, 1 sample; and not available, 29 samples) previously tested by the Cobas Amplicor HBV Monitor were retested using our in-house real-time PCR assay. The overall correlation of the two assays was very good, with an R^2 of 0.9435, regardless of HBV genotype (Fig. 6), and the correlation was excellent for HBV DNA concentrations from 2 to 5 $\log_{10}$ IU/ml ($R^2 = 0.9754$). Correlations between the two

FIG. 5. Correlation of HBV DNA values from 49 serum samples measured by our in-house real-time PCR assay and by the Cobas TaqMan HBV test.

FIG. 6. Correlation of HBV DNA values of 169 serum samples measured by our in-house real-time PCR assay and by the Cobas Amplicor HBV Monitor test.

assays were comparable for all the genotypes, including genotype F. Six of 13 samples with undetectable HBV DNA according to the Cobas Amplicor Monitor test had detectable HBV DNA with our in-house real-time PCR assay, with values from 60 to 70 IU/ml (Table 1). All eight samples that had detectable but nonquantifiable results with the Cobas Amplicor Monitor test had quantifiable results with our in-house real-time PCR assay, with values from 25 to 60 IU/ml. Of the 16 samples with HBV DNA results of $>7 \log_{10}$ IU/ml as determined with the Cobas Amplicor Monitor test, our in-house real-time PCR assay reported values that were within 0.5 $\log_{10}$ IU/ml in 12 samples, a 0.5- to 1.0- $\log_{10}$ -IU/ml-lower value in 1 sample and a $>1\text{-}\log_{10}$ -IU/ml-lower value in 3 (19%) samples.

DISCUSSION

We have established a sensitive “in-house” real-time PCR assay for the quantification of HBV DNA, with a lower limit of detection of 5 IU/ml and a dynamic range of up to 2.0×10^9 IU/ml. Our assay is reproducible, with limited intra- and inter-assay variability and similar amplification efficiencies across HBV genotypes A through H. We demonstrated that the results of our assay correlated very well with the two commonly used commercial assays, with an R^2 of 0.9435 for the Cobas Amplicor HBV Monitor test and an R^2 of 0.9873 for the Cobas

TaqMan test. Our assay was 1 $\log_{10}$ IU more sensitive and had a dynamic range of 5 $\log_{10}$ IU wider than the Cobas Amplicor HBV Monitor test. Furthermore, our assay costs approximately \$60 per sample, which is less than one-third of the charge for the Cobas TaqMan HBV test.

The availability of HBV DNA assays with an improved sensitivity and a wider dynamic range will improve our ability to assess viral response to antiviral therapy for chronic hepatitis B. One-step testing not only saves time but also eliminates the risk of errors introduced by the dilution process. The potential for errors when samples need to be diluted 100,000-fold or more is substantial. The tendency of the Cobas Amplicor HBV Monitor test to overestimate samples with high HBV DNA levels may explain why recent clinical trials of antiviral therapy for chronic hepatitis B reported baseline serum HBV DNA levels as high as 13 $\log_{10}$ IU/ml (13, 14). Falsely high pretreatment serum HBV DNA levels may inflate the potency of antiviral therapies.

A major problem with HBV DNA assays is the lack of standardization. This has prompted recommendations to standardize HBV DNA assays against WHO or other international standards and to express results in international units (IU). Our assay was initially standardized against the WHO international HBV DNA standard (NIBSC code 97/746; genotype A, serotype adw2). However, the WHO standard was available only in concentrations of up to 2.0×10^7 IU/ml; therefore, we calibrated it against an HBV genotype A plasmid, which was used to establish a standard dilution panel with concentrations of up to 2×10^9 IU/ml for testing clinical samples. We showed that similar results were obtained for plasmid DNA or negative serum spiked with plasmid DNA, indicating that plasmid DNA can be used as the standard for testing patient samples despite potential differences in DNA extraction. Furthermore, we demonstrated that results were similar when a panel of genotype A through H patient samples was tested using genotype A through D plasmids as standards, indicating that our genotype A plasmid can be used as a standard for serum samples from patients infected with various HBV genotypes.

Several studies in the 1990s reported that some PCR assays used to quantify HCV RNA do not have the same accuracy across all HCV genotypes (16–19). One study reported the validation of the Cobas TaqMan HBV test against plasmids of HBV genotypes A through G (12). In that study, 57 HBsAg-

TABLE 1. Comparison of results of 169 serum samples tested by our in-house real-time PCR assay and the Cobas Amplicor HBV Monitor test

Concn detected by Cobas Amplicor HBV Monitor (IU/ml) ^a	No. of serum samples in which HBV DNA was detected by in-house real-time PCR at indicated concn (IU/ml)						
	Not detected	<60	60 to 2×10^2	2×10^2 to 2×10^4	2×10^4 to 2×10^7	2×10^7 to 2×10^9	$>2 \times 10^9$
Not detected ($n = 13$)	7	5 ^b	1 ^b				
<60 ($n = 8$)		8					
60 to 2×10^2 ($n = 8$)			6	2			
2×10^2 to 2×10^4 ($n = 83$)				81	3		
2×10^4 to 2×10^7 ($n = 41$)				2	39		
2×10^7 to 2×10^9 ($n = 11$)					2	9	
$>2 \times 10^9$ ($n = 5$)						2	3

^a n , number of samples.

^b Six of 13 samples with undetectable HBV DNA according to Cobas Amplicor Monitor results had detectable HBV DNA with our in-house real-time PCR assay, with values from 60 to 70 IU/ml.

positive specimens were tested using both a Cobas TaqMan HBV test and a Cobas Amplicor HBV Monitor test and results correlated well, with an R^2 of 0.9165, but it was observed that the Cobas Amplicor HBV Monitor test underestimated HBV DNA levels for four genotype F specimens.

Our assay has similar amplification efficiencies across all known HBV genotypes, including genotype H; and intra- and interassay variabilities were also comparable across these eight genotypes. Correlation between the results of our assay and those of the Cobas TaqMan HBV test was excellent for the genotypes tested (A, B, C, D, and F). Unfortunately, due to a limitation in sample availability, we were not able to compare these two assays for HBV genotypes E, G, and H samples. Likewise, the correlation between our assay and the Cobas Amplicor HBV Monitor test was excellent across genotypes A through H for samples with results that fall within the dynamic range of the Amplicor test. Of the seven genotype F samples tested with our assay and with the Cobas Amplicor HBV Monitor test, all seven had values within 0.5 log₁₀ IU/ml in the two assays. Two of these samples were also tested with the Cobas TaqMan HBV test, and results were within 0.5 log₁₀ IU/ml in all three assays.

There are limitations with our in-house assays. The lack of an internal control does not allow us to rule out false-negative results due to the presence of inhibitors to PCR amplification. The limit of detection and the upper limit of the dynamic range are approximates, as a lot more replicates and lot-to-lot testing would be necessary to verify these values. Sequence variations in the primer target and probe regions that are not present in the 27 sequences from our GenBank search can affect quantification accuracy. There are also multiple challenges in maintaining quality control, including the prevention of cross-contamination, the maintenance of a dilution panel calibrated against the WHO standard, and the validation of genotype independence as new genotypes and new variants within known genotypes are identified.

In summary, we have developed an in-house real-time PCR assay that is sensitive, has a wide dynamic range, and has comparable amplification efficiencies across all known HBV genotypes. Our assay results have excellent correlation with those of the Cobas TaqMan HBV test and the Cobas Amplicor HBV Monitor test, regardless of HBV genotypes. Further studies to test additional samples with more replicates to confirm the genotype independence of our assay of clinical samples are ongoing.

ACKNOWLEDGMENTS

This study was supported in part by NIH grant U01-DK57577 (to A.S.F.L.). Cobas Amplicor HBV Monitor test kits were provided by Roche Molecular Diagnostics and Inno-Lipa kits for HBV genotype were provided by Innogenetics.

REFERENCES

1. Abe, A., K. Inoue, T. Tanaka, J. Kato, N. Kajiyama, R. Kawaguchi, et al. 1999. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. *J. Clin. Microbiol.* **37**:2899–2903.
2. Bartholomeusz, A., and S. Schaefer. 2004. Hepatitis B virus genotypes: comparison of genotyping methods. *Rev. Med. Virol.* **14**:3–16.
3. Becker-Andre, M., and K. Hahlbrock. 1989. Absolute mRNA quantification using the polymerase chain reaction (PCR). A novel approach by a PCR aided transcript titration assay (PATY). *Nucleic Acids Res.* **17**:9437–9446.
4. Chen, R. W., H. Piipainen, M. Seppanen, P. Koskela, S. Sarna, and M. Lappalainen. 2001. Real-time PCR for detection and quantitation of hepatitis B virus DNA. *J. Med. Virol.* **65**:250–256.
5. Chu, C. J., and A. S. Lok. 2002. Clinical utility in quantifying serum HBV DNA levels using PCR assays. *J. Hepatol.* **36**:549–551.
6. Chu, C. J., M. Hussain, and A. S. Lok. 2002. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. *Hepatology* **36**:1408–1415.
7. Fung, S. K., and A. S. Lok. 2004. Hepatitis B virus genotypes: do they play a role in the outcome of HBV infection? *Hepatology* **40**:790–792.
8. Gordillo, R. M., J. Gutierrez, and M. Casal. 2005. Evaluation of the COBAS TaqMan 48 real-time PCR system for quantitation of hepatitis B virus DNA. *J. Clin. Microbiol.* **43**:3504–3507.
9. Hawkins, A., F. Davidson, and P. Simmonds. 1997. Comparison of plasma virus loads among individuals infected with hepatitis C virus (HCV) genotypes 1, 2, and 3 by quantiplex HCV RNA assay versions 1 and 2, Roche Monitor assay, and an in-house limiting dilution method. *J. Clin. Microbiol.* **35**:187–192.
10. Heid, C. A., J. Stevens, K. J. Livak, and P. M. Williams. 1996. Real time quantitative PCR. *Genome Res.* **6**:986–994.
11. Holland, P. M., R. D. Abramson, R. Watson, and D. H. Gelfand. 1991. Detection of specific polymerase chain reaction product by utilizing the 5'-3' exonuclease activity of *Thermus aquaticus* DNA polymerase. *Proc. Natl. Acad. Sci. USA* **88**:7276–7280.
12. Huang, Y. H., J. C. Wu, T. T. Chang, I. J. Sheen, P. C. Lee, T. I. Huo, et al. 2003. Analysis of clinical, biochemical and viral factors associated with early relapse after lamivudine treatment for hepatitis B e antigen-negative chronic hepatitis B patients in Taiwan. *J. Viral Hepat.* **10**:277–284.
13. Lai, C. L., N. Leung, E. K. Teo, M. Tong, F. Wong, H. W. Hann, et al. 2005. A 1-year trial of telbivudine, lamivudine, and the combination in patients with hepatitis B e antigen-positive chronic hepatitis B. *Gastroenterology* **129**:528–536.
14. Lau, G. K., T. Piratvisuth, K. X. Luo, P. Marcellin, S. Thongsawat, G. Cooksley, et al. 2005. Peginterferon alfa-2a, lamivudine, and the combination for HBeAg-positive chronic hepatitis B. *N. Engl. J. Med.* **352**:2682–2695.
15. Loeb, K. R., K. R. Jerome, J. Goddard, M. Huang, A. Cent, and L. Corey. 2000. High-throughput quantitative analysis of hepatitis B virus DNA in serum using the TaqMan fluorogenic detection system. *Hepatology* **32**:626–629.
16. Noborg, U., A. Gusdal, E. K. Pisa, A. Hedrum, and M. Lindh. 1999. Automated quantitative analysis of hepatitis B virus DNA by using the Cobas Amplicor HBV monitor test. *J. Clin. Microbiol.* **37**:2793–2797.
17. Ohno, T., and J. Y. Lau. 1996. The “gold-standard,” accuracy, and the current concepts: hepatitis C virus genotype and viremia. *Hepatology* **24**:1312–1315.
18. Pas, S. D., E. Fries, R. A. De Man, A. D. M. E. Osterhaus, and H. G. M. Niesters. 2000. Development of a quantitative real-time detection assay for hepatitis B virus DNA and comparison with two commercial assays. *J. Clin. Microbiol.* **38**:2897–2901.
19. Pawlotsky, J. M. 2002. Molecular diagnosis of viral hepatitis. *Gastroenterology* **122**:1554–1568.
20. Pockros, P. J., V. G. Bain, E. B. Hunter, A. Conrad, A. Balart, F. B. Hollinger, and D. Albert for the Consensus Interferon Study Group. 1999. A comparison of reverse transcription-polymerase chain reaction and branched-chain DNA assays for hepatitis C virus RNA in patients receiving interferon treatment. *J. Viral Hepat.* **6**:145–150.
21. Rehmann, B., C. Ferrari, C. Pasquinelli, and F. V. Chisari. 1996. The hepatitis B virus persists for decades after patients' recovery from acute viral hepatitis despite active maintenance of a cytotoxic T-lymphocyte response. *Nat. Med.* **2**:1104–1108.
22. Seme, K., and M. Poljak. 1995. Use of a commercial PCR kit for detection of hepatitis C virus. *Eur. J. Clin. Microbiol. Infect. Dis.* **14**:549–552.
23. Sum, S. S., D. K. Wong, J. C. Yuen, C. L. Lai, and M. F. Yuen. 2005. Comparison of the COBAS TaqMan HBV test with the COBAS Amplicor monitor test for measurement of hepatitis B virus DNA in serum. *J. Med. Virol.* **77**:486–490.
24. Weinberger, K. M., E. Wiedenmann, S. Bohm, and W. Jilg. 2000. Sensitive and accurate quantitation of hepatitis B virus DNA using a kinetic fluorescence detection system (TaqMan PCR). *J. Virol. Methods* **85**:75–82.
25. Weiss, J., H. Wu, B. Farrenkopf, T. Schultz, G. Song, S. Shah, and J. Siegel. 2004. Real time TaqMan PCR detection and quantitation of HBV genotypes A-G with the use of an internal quantitation standard. *J. Clin. Virol.* **30**:86–93.
26. Yeh, S. H., C. Y. Tsai, J. H. Kao, C. J. Liu, T. J. Kuo, M. W. Lin, W. L. Huang, S. F. Liu, J. Jih, D. S. Chen, and P. J. Chen. 2004. Quantification and genotyping of hepatitis B virus in a single reaction by real-time PCR and melting curve analysis. *J. Hepatol.* **41**:659–666.
27. Zella, I., A. Rossini, D. Domenighini, A. Albertini, and E. Cariani. 2002. Quantitative analysis of hepatitis B virus DNA by real-time amplification. *Eur. J. Clin. Microbiol. Infect. Dis.* **21**:22–26.