JOURNAL OF CLINICAL MICROBIOLOGY, Feb. 2005, p. 596–603 Vol. 43, No. 2
0095-1137/05/$08.00+0 doi:10.1128/JCM.43.2.596–603.2005

Evaluation of the COBAS Amplicor HBV Monitor Assay and Comparison with the Ultrasensitive HBV Hybrid Capture 2 Assay for Quantification of Hepatitis B Virus DNA

Eric Q. Konnick,[1]* Maria Erali,[1] Edward R. Ashwood,[1,2] and David R. Hillyard[1,2]

Associated Regional and University Pathologists Institute for Clinical and Experimental Pathology,[1] and Department of Pathology, University of Utah Medical Center,[2] Salt Lake City, Utah

Received 29 December 2003/Returned for modification 22 April 2004/Accepted 1 October 2004

Performance characteristics of the COBAS Amplicor HBV Monitor test (Roche Diagnostics), which measures hepatitis B virus (HBV) DNA quantitatively, were evaluated and compared with the Ultrasensitive HBV Hybrid Capture 2 (HC2; Digene Corporation) assay. Linearity and within-run precision were assessed for both methods by using eight HBV DNA-positive samples serially diluted to obtain a range of <100 to 500,000 HBV DNA copies/ml and run in triplicate. Agreement between the methods was studied with 100 clinical samples. HC2 assay performance near the limit of detection was investigated through repeat testing of 149 samples with HC2 and testing of 37 samples with HC2 results of <4,700 HBV DNA copies/ml by Amplicor assay and a qualitative PCR assay. The linearity experiment for Amplicor had regression of observed values compared to expected values ($y = 1.073x - 0.247$; $R^2 = 0.993$, $n = 32$; for HC2, $y = 0.855x + 0.759$, $R^2 = 0.729$, $n = 18$). Within-run standard deviation of log HBV DNA copies/ml ranged from 0.003 to 0.348 (Amplicor) and 0.027 to 0.253 (HC2). Agreement assessed by Deming regression was poor [Amplicor = 1.197(HC2) − 0.961; $R^2 = 0.799$, standard error of the estimate (SEE) = 0.710, $n = 94$]. Near the lower limit of detection, 32 of 149 repeat HC2 results were <4,700 HBV DNA copies/ml. Of the 37 samples with HC2 results of <4,700 HBV DNA copies/ml, HBV DNA was not detected in 15 samples, while HBV DNA was detected by at least one PCR method in 12 samples. Amplicor is linear from 200 to 200,000 HBV DNA copies/ml with undiluted samples, and this range can be expanded through dilution. Inconsistent HC2 results near the limit of detection justify use of a grey zone.

The incidence of new hepatitis B virus (HBV) infection in the United States has been dramatically reduced in large part due to the introduction of blood screening (12, 48, 51) and vaccination (13, 33), allowing the focus of HBV-related laboratory testing to shift from acute detection to the management of the chronically infected patient. In addition to the use of traditional antigen and antibody serologic assays, HBV DNA testing has been widely adopted as an important monitoring test to assess patient response to therapy (26–28, 31, 37) and activity of infection (8, 36, 54). The development of quantitative HBV DNA assays has contributed to an understanding of viremia at different stages of disease and in different populations (4, 20, 41). HBV DNA measurement has been used to demonstrate the effects of antiviral drugs on HBV replication (5, 7, 10, 17, 24–29, 38, 39, 50, 52, 54, 56). National Institutes of Health guidelines published in 2001 (31, 32) provide provisional recommendations for use of HBV DNA testing but raise questions regarding performance requirements. A challenge for any quantitative HBV DNA assay is the enormous dynamic range of HBV concentration observed in biological fluids (9), which ranges from 100 to 1,000,000,000 copies/ml. Extremely high levels increase the potential for contamination and compound the difficulty of accurately determining viral load in clinical samples. Hybridization methods including Genostics liquid hybridization (Abbott Diagnostics, Abbott Park, Ill.), bDNA (Bayer Diagnostics, Tarrytown, N.Y.), and the PCR-based Amplicor HBV Monitor (Roche Molecular Diagnostics, Indianapolis, Ind.) assays have been useful for measuring the upper range of HBV viremia and have been compared extensively (1, 3, 14, 16, 18, 19, 22, 23, 34, 39, 43) but have lacked sensitivity, precluding the analysis of some chronic carrier patients with low serum levels of HBV DNA (4, 20, 41). Improvements in the limit of HBV DNA detection afforded by advanced hybridization and amplification methods have resulted in the discoveries that HBV DNA can be detected in low levels in some patients years after clinical recovery and even when circulating anti-HBs are present (36, 47, 53). While the use of DNA testing in the research setting has yielded insights into the biological response of HBV to antiviral compounds and the natural course of the infection, these tests remain challenging for clinical laboratories.

The Digene Hybrid Capture 2 (HC2; Digene Corporation, Gaithersburg, Md.) assay in the ultrasensitive format has an improved lower limit of detection (4,700 HBV DNA copies/ml) but requires the use of 1 ml of patient sample and an ultracentrifugation step prior to testing. The analytical measurement range (AMR) of the Ultrasensitive HC2 assay determined by the manufacturer and evaluated elsewhere (40) is 4,700 to 56,000,000 HBV DNA copies/ml (3.7 to 7.8 log HBV DNA copies/ml). According to the manufacturer, the AMR of the standard HC2 assay is 142,000 to 1,700,000,000 HBV DNA copies/ml (5.2 to 10.2 log HBV DNA copies/ml). The COBAS

* Corresponding author. Mailing address: ARUP Institute for Clinical and Experimental Pathology, 500 Chipeta Way, Salt Lake City, UT 84108. Phone: (801) 583-2787, ext. 2225. Fax: (801) 584-5207. E-mail: KonnicE@ARUPLab.com.

Amplicor HBV Monitor assay (Roche Molecular Diagnostics) offers a better lower limit of quantification (200 HBV DNA copies/ml) than non-PCR-based assays. However, like other endpoint detection assays, this assay has the disadvantage of a narrow AMR: 200 to 200,000 HBV DNA copies/ml (42) (range, 2.3 to 5.3 log HBV DNA copies/ml). We evaluated the COBAS Amplicor HBV Monitor test (Amplicor), compared it to the HBV HC2 assay, and examined the efficacy of diluting samples to expand the dynamic range of the Amplicor assay.

MATERIALS AND METHODS

COBAS Amplicor HBV Monitor assay. Samples were extracted as per the manufacturer's instructions and as previously described (35, 42, 46). Sample volumes of 100 μl were extracted using polyethylene glycol precipitation and alkaline lysis, yielding a final volume of 225 μl of processed sample. PCR was accomplished by using 50 μl of the processed patient sample with the PCR reagents provided with the Amplicor kit. Amplification, detection, and quantitation were performed by using a COBAS Amplicor instrument (6, 42). Samples with >200,000 HBV DNA copies/ml in the Amplicor assay were diluted 1:900, 1:27,000, or 1:810,000, as necessary, in HBV DNA-negative normal human serum (NHS), and the diluted material was reextracted, amplified, and detected to obtain a result within the analytical measurement range of the Amplicor assay. Final results for diluted samples were determined by multiplying the HBV DNA copies/milliliter obtained with the Amplicor assay by the dilution factor for that sample. Results were reported as HBV DNA copies/milliliter by the COBAS Amplicor instrument.

Digene Hybrid Capture 2 assay. Samples were assayed using the ultrasensitive protocol according to the manufacturer's instructions. The HBV in a sample volume of 1 ml was concentrated by using ultracentrifugation (33,000 × *g* for 110 min at 4°C), the supernatant was decanted, and 25 μl of buffer was added to residual supernatant and pellet. Thirty microliters each of concentrated calibrators, controls, and samples were denatured in a 96-well microtiter plate, and the RNA probes were added and incubated. Capture of the RNA-DNA hybrids was performed by transfer of the solution into microtiter plates coated with anti-RNA-DNA hybrid antibody. Following incubation and wash steps, the captured antibody hybrid complexes were reacted with anti-hybrid antibody conjugated to alkaline phosphatase and detected with the chemiluminescent substrate CDP-Star with Emerald II. The plate was read using a DML 2000 luminometer (Digene Corporation), and the results were analyzed by using software supplied by Digene. The AMR for this method is 4,700 to 56,000,000 HBV DNA copies/ml (3.7 to 7.8 log HBV DNA copies/ml). Results were reported as HBV DNA copies/milliliter and picograms of HBV DNA/milliliter.

Qualitative HBV DNA PCR. The qualitative assay consisted of HBV DNA extracted by QIAGEN (Valencia, Calif.) chemistry, amplified using hot-start PCR, and analyzed using agarose gel electrophoresis, ethidium bromide staining, and detection at 302 nm. HBV DNA extraction from clinical serum and plasma samples was achieved by using QIAamp DNA Blood Mini kit protease (QIAGEN Inc.), and buffers (AW1 and AW2) were reconstituted as per the manufacturer's instructions. Twenty-five microliters of QIAGEN protease was added to a labeled 1.5-ml microcentrifuge tube, to which 200 μl of patient sample and 200 μl of buffer AL were added. The mixture was then pulse vortexed and placed in a 60°C dry-heat bath for 15 min and then transferred to a 100°C dry-heat bath for 10 min. Tubes were removed from heat and allowed to cool for 2 min, and 200 μl of 96% ethanol was added to each tube. Samples were vortexed and then transferred to the appropriately labeled QIAamp column, which was then centrifuged at 6,000 × *g* for 1 min. The used collection tube was discarded, and a new collection tube was placed on the column. Five hundred microliters of buffer AW1 was added to each column, which was then centrifuged at 6,000 × *g* for 1 min. The used collection tube was discarded, and a new collection tube was placed on the column. Five hundred microliters of buffer AW2 was added to each column, which was then centrifuged at 20,000 × *g* for 3 min. The used collection tube was discarded, and a new collection tube was placed on the column and centrifuged at 20,000 × *g* for 1 min. The used collection tube was discarded, and the QIAamp column was placed in a 1.5-ml flip-cap tube, and 100 μl of molecular-grade water was added to each column, allowed to incubate at room temperature for 5 min, and then centrifuged for 1 min at 6,000 × *g*.

The master mix for each sample contained a total final volume of 50 μl and consisted of a final concentration of the following components per reaction: 1.25 U of AmpliTaq Gold DNA polymerase (PE Applied Biosystems, Foster City, Calif.), 1× GeneAmp 10× PCR Buffer II (PE Applied Biosystems), 3 mM (25 mM $MgCl_2$) solution (PE Applied Biosystems), 200 mM (400 mM dUTP) Gene Amp dNTP Blend (12.5 mM with dUTP; PE Applied Biosystems), 0.5 U of AmpErase UNG (PE Applied Biosystems), 1 mM S96-1 primer (50 μM [5′-GTG GCT CCA GTT CAG GAA CA-3′]), and 1 mM S96-3 primer (50 μM [5′-CAT CCA GCG ATA ACC AGG AC-3′]), with the remaining master mix volume of 40 μl made up with molecular-grade water. Ten microliters of processed sample was added to this master mix. Samples were amplified using a GeneAmp 9700 (PE Applied Biosystems) under the following conditions: a hold step for 10 min at 50°C, a hold step for 10 min at 95°C, and 45 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 30 s.

Postamplification, 6 μl of 10× BlueJuice gel loading buffer (Gibco BRL, Rockville, Md.) was added to each reaction mixture as a loading dye. The samples and controls mixed with loading dye were loaded onto agarose gel (Latitude precast 2% Seakem LE plus agarose–1× Tris-borate-EDTA buffer plus ethidium bromide; Biowhittaker Molecular Applications, Rockland, Maine) including one lane with a BioMarker Low 50- to 1,000-bp ladder (Bioventures Inc., Murfreesboro, Tenn.). Electrophoresis was performed at 80 V for ~1 h, and the gel was viewed under UV light at 302 nm, digitally photographed, and labeled. A sample was considered positive for HBV DNA if a 340-bp PCR product was observed. The limit of detection of this assay is ~200 HBV DNA copies/ml as determined by replicate testing of serial dilutions of HBV DNA-positive samples previously quantitated by using the HC2 assay, and assay specificity was determined to be 100% (60 out of 60 positive by HC2 assay and 60 out of 60 negative from low-risk donor samples).

Sample selection. EDTA plasma and serum samples submitted to Associated Regional and University Pathologists (ARUP) Laboratories for the determination of HBV DNA levels by HC2 or Amplicor assay were systematically selected for use in this study by identifying samples with results within the range of interest and with ample volume remaining for additional testing. Samples tested with the Amplicor and in-house PCR assays were deidentified prior to testing. Clinical samples tested by HC2 assay with results less than 12,000 HBV DNA copies/ml were retested by HC2 assay. The retesting results were compiled by the testing technologists, removing patient identifiers from the data.

Linearity. Four HBV DNA-positive samples were serially diluted five times in NHS to obtain a range of HBV DNA levels from <100 to 40,000 HBV DNA copies/ml as measured with the HC2 assay. An additional four samples with HBV DNA levels determined by HC2 assay to be >200,000 copies/ml were serially diluted five times in NHS to obtain a range of HBV DNA levels from 20,000 to 500,000 copies/ml. All dilution samples were tested by using both the Amplicor and HC2 assays in triplicate on the same day. Results were reported as log HBV DNA copies per milliliter and observed-versus-expected plots were created. Expected results for each dilution series were back calculated by using the result within the claimed analytical measurement range of the assay with the best precision, and the value chosen was not used in the analysis, thereby yielding four expected values for each dilution series. This method of calculation was used to determine the internal linearity of the assays.

Precision. The triplicate measurements from the dilution series were used to evaluate the within-run precision of the methods. Duplicate values from the comparison study were used to evaluate the between-run precision of the Amplicor assay. Precision was expressed as the percent coefficient of variation (CV) (standard deviation [SD] divided by the mean of the replicates multiplied by 100%). In addition, the average within-run variance (SD^2) of replicates in both the Amplicor and HC2 assays was calculated. The average SD was determined by taking the square root of the average within-run variance. Average SDs were compared by using the *F* test.

Correlation. Fifty-eight clinical samples with HBV DNA levels of 5,000 to 300,000 HBV DNA copies/ml as measured in the HBV HC2 assay were split and tested in duplicate with the Amplicor assay. The aliquots of the duplicate specimens were submitted on separate weeks. Forty-two samples with HC2 HBV DNA levels of >200,000 copies/ml were run undiluted in the Amplicor assay. Previous studies indicated that high-titer HBV DNA-containing samples could be diluted to obtain quantitative results (21, 42) in the Amplicor assay, so samples with undiluted results greater than 200,000 HBV DNA copies/ml were diluted 1:900, 1:27,000, or 1:810,000 as needed to obtain results within the analytical measurement range of the Amplicor assay. Final results with the Amplicor and HC2 assays were compared by using Deming regression analysis. In addition, distribution patterns of HBV DNA copies/milliliter for patient results from 12,400 HC2 and 12,329 Amplicor samples were extracted from a central result database. The number and proportion of samples falling in various ranges for both assays were compared.

Specificity. Three clinical samples with alternating HC2-positive and -negative (discrepant) results on the same specimen and 34 samples reported with <5,000 HBV DNA copies/ml in the HC2 assay were assayed singly in the following

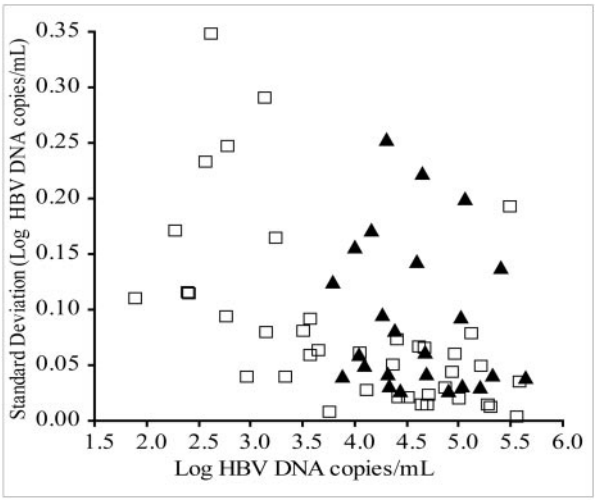

FIG. 1. Within-run precision of COBAS Amplicor HBV Monitor and Ultrasensitive Digene HC2 assays. Eight samples were serially diluted in HBV-seronegative NHS and tested in the Amplicor and HC2 assays in triplicate on the same day. The means and standard deviations of triplicate values (log HBV DNA copies per milliliter) were calculated and plotted (HC2 [▲], $n = 24$; Amplicor [□], $n = 40$).

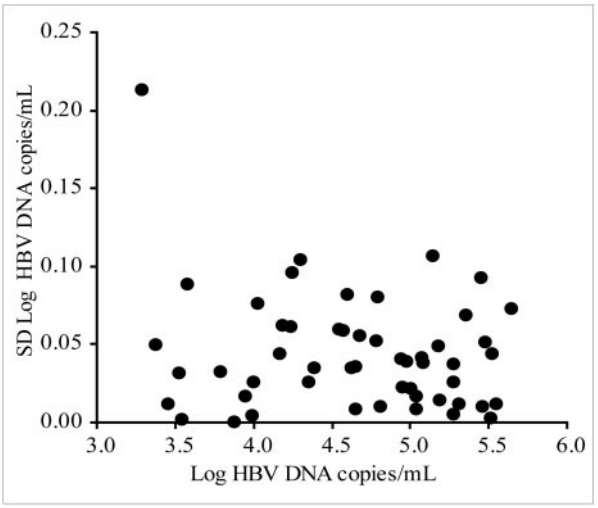

FIG. 2. Between-run precision of COBAS Amplicor HBV Monitor assay. Fifty-two clinical samples were tested undiluted in duplicate in the Amplicor assay on separate days. The means and standard deviations of log HBV DNA copies/milliliter of the duplicate values were calculated and plotted.

assays: COBAS Amplicor HBV Monitor, Digene HBV Hybrid Capture 2, and qualitative HBV PCR assays. In addition, over a 4-month period from October 1999 to February 2000, 149 samples quantitated with between 4,700 and 30,000 HBV DNA copies/ml in the Ultrasensitive HC2 assay were observed. These specimens were retested with the Ultrasensitive HC2 assay when enough sample was available. Paired Student's *t* test analysis was used to compare the means of the initial results against the means of repeated results.

Data analysis. The results from the Amplicor and HC2 assays in HBV DNA copies/milliliter were converted to log units for linearity, comparison, and limit-of-detection analysis. The observed-versus-expected graph was analyzed by using linear regression, and all other comparisons were evaluated by using Deming regression.

RESULTS

Precision. The triplicate measurements from the dilution series were used to evaluate the within-run precision of the methods. The percent CVs of the log HBV DNA copies/milliliter of samples measured with the Amplicor assay ranged from 0.06 to 13.24% at mean log HBV DNA levels of 5.6 and 2.6, respectively. The percent CVs of the log HBV DNA copies/milliliter of samples measured with the Ultrasensitive HC2 assay ranged from 0.56 to 5.88% at mean log HBV DNA levels of 4.9 and 4.3, respectively. Data comparing standard deviations versus log HBV DNA copies/milliliter for the within-run precision of the Amplicor and HC2 assays are presented in Fig. 1. *F* test analysis of the average within-run variances of samples that were quantified with both the Amplicor and HC2 assays indicated that the Amplicor assay is more precise than the HC2 assay ($P < 0.001$). The between-run percent CVs of the log HBV DNA copies/milliliter of duplicate determination of samples measured with the Amplicor assay ranged from 0.01 to 6.47% at mean log HBV DNA levels of 3.9 and 3.3, respectively. A plot comparing between-run standard deviations and log HBV DNA copies/milliliter for the Amplicor assay is presented in Fig. 2.

Amplicor linearity. Of the 120 individual replicate samples tested, all samples were quantitated with the Amplicor assay. Linear regression analysis comparing the observed-versus-expected relationship for the average log HBV DNA copies/milliliter for the Amplicor assay was as follows: Observed = 1.073(Expected) − 0.247; $R^2 = 0.993$, $n = 32$ (96 individual determinations), and data are presented in Fig. 3. These data indicate that HBV DNA levels above 200,000 (log 5.3) HBV DNA copies/ml as determined by the Amplicor assay are slightly overestimated and confirm the manufacturer's analytical measurement range of the assay as 200 to 200,000 HBV DNA copies/ml.

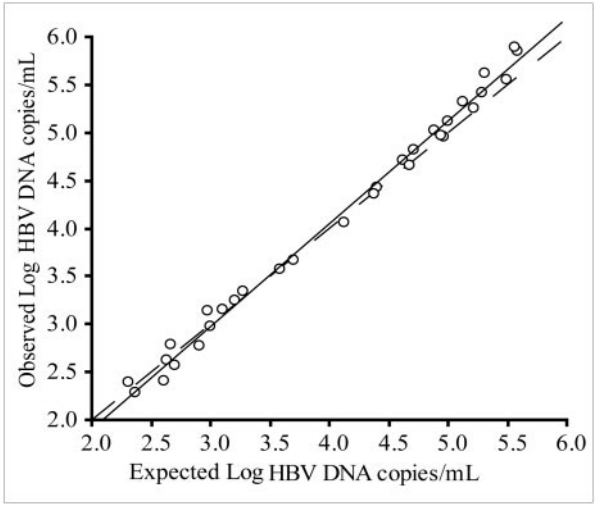

FIG. 3. COBAS Amplicor HBV Monitor assay linearity. Eight samples were serially diluted in HBV-seronegative NHS and tested by the Amplicor assay in triplicate on the same day. The means of triplicate values were calculated and plotted as log HBV DNA copies/milliliter. The equation of the linear regression (solid line) is as follows: Observed = 1.073(Expected) − 0.247; $R^2 = 0.993$, $n = 32$. The dashed line represents identity.

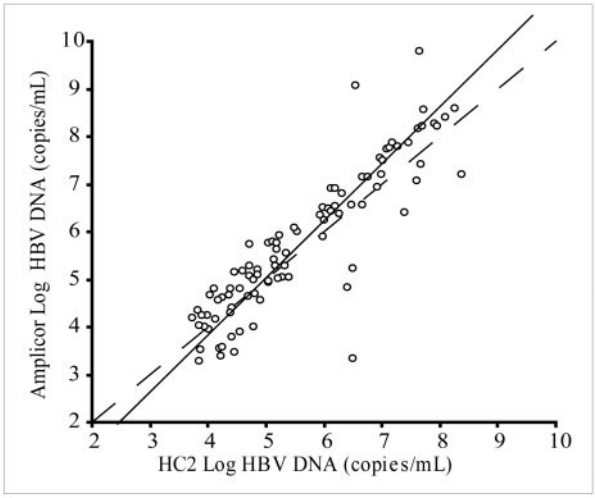

FIG. 4. Deming regression comparison of Digene HC2 assay and COBAS Amplicor HBV Monitor assay. One-hundred samples previously tested using the HC2 assay were tested undiluted in the Amplicor assay. Samples with results greater than 200,000 HBV DNA copies/ml were diluted and retested. Deming regression (solid line) was as follows: Amplicor = 1.197(HC2) − 0.961; $R^2 = 0.799$, SEE = 0.710, $n = 94$. The dashed line represents identity.

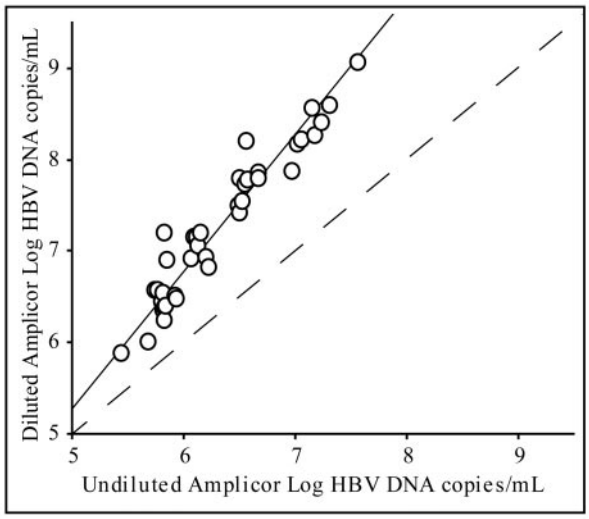

FIG. 5. Deming regression comparison of samples run undiluted and diluted in the COBAS Amplicor HBV Monitor assay (Amplicor). Forty-one clinical samples tested undiluted and diluted in the Amplicor assay are shown. Deming regression comparing undiluted and diluted samples (solid line) was as follows: Diluted = 1.50 (Undiluted) − 2.23; SEE = 0.22, $n = 38$, $R^2 = 0.922$. The dashed line represents identity.

HC2 linearity. Of the 120 individual replicate samples tested, 69 results were above the limit of detection (4,700 HBV DNA copies/ml), yielding mean values for 24 sets of triplicates. The calculated linear regression for the observed-versus-expected relationship for the average log HBV DNA copies/milliliter in the Ultrasensitive HC2 test was as follows: Observed = 0.855(Expected) + 0.759; $R^2 = 0.729$, $n = 18$ (52 individual determinations).

Correlation. One hundred clinical samples previously tested by HC2 assay were tested with the Amplicor assay. The Deming regression was as follows: Amplicor = 1.197(HC2) − 0.961; standard error of the estimate (SEE) = 0.710, $n = 94$, $R^2 = 0.799$. A Deming regression analysis comparing Amplicor and HC2 results is presented in Fig. 4. Paired Student's t test analysis indicated that the slope (1.197 log) and intercept (−0.961 log) are statistically different from 1.000 ($P = 0.001$) and 0 ($P = 0.004$), respectively. Paired Student's t test analysis of the means of the Amplicor and HC2 assays indicates that the means are not equivalent ($P = 0.04$, $n = 94$). Note that in addition to the poor correlation noted above, six samples which had >5,000 HBV DNA copies/ml by the HC2 assay were found to have <200 HBV DNA copies/ml by Amplicor assay. These samples were tested by the qualitative PCR method and were found to be negative for HBV DNA.

Clinical samples, which required dilution to obtain a result in the linear range of the Amplicor assay, were evaluated to determine the difference between diluted and undiluted results. These data were plotted, and Deming regression analysis was performed. The Deming regression was as follows: Diluted = 1.50(Undiluted) − 2.23; SEE = 0.22, $n = 38$, $R^2 = 0.922$. These data are presented in Fig. 5 and show that as the HBV DNA level in samples increases, the Amplicor assay cannot accurately measure the HBV DNA titer without dilution. In a separate subset of clinical samples tested undiluted in the Amplicor assay, it was noted that occasionally the quantitation standard value was so low that the test was considered invalid, but the HBV-specific probe had a very strong optical density (OD) signal. Upon dilution (1:1,009) and retesting, it was found that 95% of samples (64 of 67 samples) with this pattern had HBV DNA levels greater than 200,000,000 HBV DNA copies/ml (data not shown).

In order to determine if Amplicor and HC2 tests resulted in similar patient population patterns, we compared 12,391 HC2 results and 12,309 Amplicor results generated at ARUP Laboratories during the course of routine testing. The number and proportion of samples with levels of HBV DNA which were less than detectable and within various ranges were compared. These data are presented in Table 1 and indicate that the Amplicor assay has a slightly higher percentage of samples (61.2 versus 55.9%) with results showing <4,700 (log 3.7) HBV DNA copies/ml.

Specificity. Results from clinical samples with discrepant results in the HC2 assay and 34 samples reported as having <4,700 HBV DNA copies/ml in the HC2 assay were assayed singly with the COBAS Amplicor HBV Monitor assay and in-house qualitative HBV PCR assays and are presented in Table 2. Over a 4-month period from October 1999 to February 2000, 149 samples that had Ultrasensitive HC2 results between 4,700 and ~30,000 HBV DNA copies/ml were selected for repeat testing. These samples were retested by Ultrasensitive HC2 assay when enough sample was available. Twenty-eight (18.8%) of the samples were not able to be retested due to insufficient sample volume. Of the remaining 121 samples, 89 (73.6%) samples showed results within 0.41 log of the initial results, and 32 (26.4%) samples showed results of <4,700 HBV DNA copies/ml. Among the samples in which HBV DNA was not detected upon retesting, the average initial value was 6,831 HBV DNA copies/ml, with a median initial

TABLE 1. Comparison of results using the Digene HBV Hybrid Capture 2 and COBAS Amplicor HBV Monitor assays[a]

Result category (log HBV DNA copies/ml)	COBAS Amplicor HBV Monitor[b]			Digene HBV Hybrid Capture 2[c]		
	No. of samples	%	Cumulative %	No. of samples	%	Cumulative %
<LOD[d]	6,033	49.0	49.0	6,922	55.9	55.9
2.3–3.0	687	5.6	54.6			
3.0–3.7	808	6.6	61.2			
3.7–4.0	313	2.5	63.7	455	3.7	59.5
4.0–5.0	990	8.0	71.7	1,115	9.0	68.5
5.0–6.0	602	4.9	76.6	682	5.5	74.0
6.0–7.0	571	4.6	81.3	697	5.6	79.7
7.0–8.0	573	4.7	85.9	937	7.6	87.2
8.0–8.3	261	2.1	88.0	174	1.4	88.6
>8.3	1,471	12.0	100.0	1,409	11.4	100.0

[a] Results generated at ARUP laboratories from clinical samples over similar time frames.
[b] COBAS Amplicor HBV Monitor results of <5.3 log HBV DNA copies/ml (<200,000 copies/ml) were generated by using undiluted samples; results greater than 5.3 log HBV DNA copies/ml (200,000 copies/ml) were generated with a 1:1,009 dilution of sample in NH5.
[c] Results between 3.7 and 7.8 log DNA copies/ml (4,700 and 56,000,000 copies/ml) were generated by using the Digene Ultrasensitive HBV Hybrid Capture 2 (1-ml) procedure; results found to be >7.8 log HBV DNA copies/ml (>56,000,000 copies/ml) with the ultrasensitive procedure were retested with the Digene HBV Hybrid Capture 2 Standard procedure.
[d] <LOD, less than the limit of detection. HBV DNA was not detected or was detected below the limit of detection. For the Amplicor assay, the limit of detection is 2.3 log HBV DNA copies/ml (200 copies/ml), and for the Ultrasensitive HC2 assay, the limit of detection is 3.7 log HBV DNA copies/ml (4,700 copies/ml). Results between 2.3 and 3.7 log HBV DNA copies/ml are not shown for HC2 because results are not generated in this range.

value of 6,250 HBV DNA copies/ml and a standard deviation of 1,779 HBV DNA copies/ml. Of the samples that returned results showing >4,700 HBV DNA copies/ml upon retesting, the average initial value was 9,731 HBV DNA copies/ml, with a median initial value of 8,400 HBV DNA copies/ml and a standard deviation of 4,417 HBV DNA copies/ml. Upon retesting, the average value was 10,261 HBV DNA copies/ml, with a median value of 8,600 HBV DNA copies/ml and a standard deviation of 6,062 HBV DNA copies/ml. Paired Student's *t* test comparing the initial and repeated HBV DNA results of the samples which were quantitated upon retesting indicated that the results were not statistically different ($P = 0.507$, $n = 89$). A breakdown of the initial HBV DNA level and the number and percentage of samples that repeated with or without quantitative HBV DNA results are presented in Table 3.

DISCUSSION

The examination of HBV DNA levels in clinical studies has been hampered by the inability of commercially available nu-

TABLE 2. Comparison between HBV Hybrid Capture 2, COBAS Amplicor HBV Monitor, and qualitative PCR assays[a]

Sample	Assay results		
	HC2 (copies/ml)[b]	Amplicor (copies/ml)[c]	Qualitative PCR[d]
1	<4,700	114	Positive
2	<4,700	605	Positive
3	<4,700	784	Positive
4	<4,700	1,020	Positive
5	<4,700	1,460	Positive
6	<4,700	1,660	Positive
7	<4,700	1,750	Positive
8	<4,700	3,380	Positive
9	<4,700	<200	Positive
10	<4,700	80	Negative
11	<4,700	104	Negative
12	<4,700	<187	Negative
13–37	<4,700	<200	Negative

[a] Three clinical samples with discrepant HC2 results on the same specimen and 34 samples reported as <5,000 HBV DNA copies/ml in the HC2 assay were assayed singly in the following assays: COBAS Amplicor HBV Monitor, Digene HBV Hybrid Capture 2, and qualitative HBV PCR assays.
[b] Samples with HC2 results that were <4,700 HBV DNA copies/ml were considered "HBV DNA not detected."
[c] Samples with Amplicor results that were <200 HBV DNA copies/ml were considered HBV DNA not detected, although HBV DNA concentrations below this level were occasionally encountered.
[d] Qualitative PCR was considered to have a limit of detection of ~200 HBV DNA copies/ml based on replicate testing of dilution series of samples with HBV DNA concentrations determined by HC2.

TABLE 3. Comparison of results initially >4,700 HBV DNA copies/ml by the Digene Ultrasensitive HBV HC2 assay and retested with the HC2 assay[a]

Result range of initial HC2 data	Repeat HC2 result data				
	No. of samples with repeat results			% of samples with repeat results	
	Total	>4,700[b]	<4,700[c]	>4,700	<4,700
4,700–5,000	5	0	5	0	100
5,001–6,000	23	13	10	57	43
6,001–7,000	20	15	5	75	25
7,001–8,000	14	9	5	64	36
8,001–9,000	13	10	3	77	23
9,001–10,000	7	4	3	57	43
10,001–11,000	5	5	0	100	0
11,001–12,000	11	11	0	100	0
12,001–13,000	8	7	1	88	13
13,001–14,000	4	4	0	100	0
14,001–15,000	5	5	0	100	0
15,001–30,000	6	6	0	100	0

[a] From October 1999 to February 2000, 149 samples that had Ultrasensitive HC2 results between 4,700 and ~30,000 HBV DNA copies/ml were selected for repeat testing. These samples were retested by Ultrasensitive HC2 assay when enough sample was available. Twenty-eight (18.8%) of the samples were not able to be retested due to insufficient sample volume; data for the remaining 121 samples are presented.
[b] Samples with results that were >4,700 HBV DNA copies/ml repeated with results within 0.41 log HBV DNA copies/ml of the original result.
[c] Samples with results that were <4,700 HBV DNA copies/ml were considered HBV DNA not detected.

cleic acid technologies to accurately interrogate the vast range of HBV DNA concentrations encountered in patient samples. The introduction of commercial PCR methods and second-generation hybrid capture technology with improved limits of quantitation have enabled interrogation of low levels of HBV DNA. However, these tests rely on fundamentally different biochemistries, target amplification compared to signal amplification, and are therefore vulnerable to different types of analytical errors. These factors and the absence, until recently, of well-standardized materials (11, 49) contribute to a general lack of standardization of quantitative HBV DNA testing.

This study documents the performance attributes of the COBAS Amplicor HBV Monitor and the HBV Hybrid Capture 2 tests and examines their performance using shared replicate samples. We find that Deming regression comparing the Amplicor assay to the HC2 assay shows poor agreement between the methods, with the large standard error of the estimate (SEE = 0.710) indicating that a sample tested by both assays can have results that differ by 2 logs 5% of the time. The upper limit of linearity of the Amplicor assay, using undiluted samples, was found to be approximately 200,000 HBV DNA copies/ml, which agrees with the manufacturer's package insert, but this range can be expanded through the use of dilution of high-titer samples. In addition, we find that the Amplicor assay has lower within-run variance than the Ultrasensitive HC2 assay when paired samples detected in both assays are compared. The observed greater variation in HC2 results could be attributable to technical issues such as loss or partial removal of the pellet during decanting or could be due to an inherent increase in variation near the limit of detection of the HC2 assay.

A previous study indicated that the dilution level for a sample tested by the COBAS Amplicor HBV Monitor assay could be determined by the serology profile (46), but this is not a practical solution for all laboratories. It was noted in our study that when the quantitation standard OD is weak but a strong target OD is observed in a reaction in which an undiluted sample is tested by the Amplicor assay, it is likely that the sample is greater than 200,000,000 HBV DNA copies/ml. Using this criterion, it may be possible to reduce the number of dilutions necessary to quantitate samples greater than 200,000,000 HBV DNA copies/ml. By using both protocols for the HC2 assay and diluting high-titer samples for the Amplicor assay, these assays will have similar dynamic ranges, although the Amplicor assay is more sensitive and requires much less sample volume to achieve greater sensitivity.

The most striking differences between the Amplicor and HC2 assays were noted near the limit of detection of the HC2 assay. Of 149 samples that were quantitated as less than 30,000 HBV DNA copies/ml with the Ultrasensitive HBV HC2 assay, a large proportion of them, 28 (18.8%), were not able to be retested due to insufficient sample volume, and 32 (26.5%) of the remaining samples were not quantified upon retesting with the HC2 assay. In addition, six samples selected for correlation studies were found to have HBV DNA levels not detectable by the Amplicor or qualitative PCR assay, suggesting that the HBV DNA results generated using the HC2 assay were false-positive results. A separate evaluation of 37 samples with <4,700 HBV DNA copies/ml by HC2 found 22% (8 of 37) of samples positive by both Amplicor and in-house qualitative PCR, suggesting that the Amplicor assay is more sensitive and specific than the Ultrasensitive HC2 method and that the samples tested positive by Amplicor are likely true results as evidenced by the corroborating qualitative HBV PCR data which target a different HBV gene. Analysis of results near the lower limit of the AMR of the HC2 assay suggests that specificity of results in this area of the AMR of the HC2 assay is suspect and as such warrants the inclusion of a grey zone or an increase in the claimed lower limit of the AMR. In addition, the inability to repeat tests of nearly 20% of samples in these experiments due to insufficient sample volume also highlights the shortcomings of the Ultrasensitive HC2 assay, which requires a 1-ml sample input volume, making the possibility of repeat testing in a grey zone difficult for samples that have limited volume. While the apparent lack of specificity near the limit of detection of the HC2 assay appears to account for the discrepancies noted, there are other potential concerns that are appropriate to contemplate for all quantitative HBV DNA assays. The potential for contamination is always present for assays measuring HBV DNA, especially with nucleic acid amplification technologies in addition to specimen stability concerns, technical error, and general assay specificity. Similar systematic evaluations of other molecularly based HBV DNA assays, including the Amplicor assay, have not been performed, but data from the HC2 assay suggest that such studies would be useful.

All HC2 testing was performed as specified by the package insert instructions, which state that incorrect technique may lead to false-positive results; however, the lack of an internal control in the HC2 assay makes it difficult to determine if the inconsistent results experienced are due to an error of technique or lack of specificity in the Ultrasensitive HC2 assay near the lower limit of detection. These problems are addressed by the Amplicor assay through the enhanced specificity of PCR combined with the addition of an internal control early in the extraction procedure. In addition, the Amplicor assay requires 1/10 of the volume required for the Ultrasensitive HC2 assay, making the option of repeat testing available for almost all samples received for clinical testing.

An important next solution for the measurement of HBV DNA levels in clinical samples may be in the form of real-time PCR assays calibrated to the first HBV DNA World Health Organization international standard 97/746 (11, 49), which could potentially allow for a extended dynamic range in a single, sensitive, standardized PCR test requiring minimal sample input volume. These assays have only recently become available in the form of commercial kits, and evaluations of these kits have not been presented in the literature, but studies of in-house-developed real-time PCR assays suggest linear ranges of 8 logs or greater (2, 15, 30, 44, 45, 55). In addition, 96-well formats, as used in many of the real-time PCR systems, offer potential for increased automation using liquid handling devices for nucleic acid extraction and PCR setup with the additional safeguard against contamination through use of a sealed PCR plate. As clinical trials are completed for new therapies using HBV DNA laboratory tests referenced against international standard material, it is likely that a more clear relationship between HBV DNA levels, disease states, drug efficacy, and clinical outcomes will become apparent.

ACKNOWLEDGMENTS

This study was in compliance with human subject research regulations and was approved by the University of Utah Institutional Review Board.

Special thanks to the Molecular Hepatitis/Retrovirus laboratory at ARUP for performing the testing on the Digene Hybrid Capture 2 assays.

COBAS Amplicor HBV Monitor kits were supplied by Roche Molecular Systems, Pleasanton, Calif. The cost of reagents for the Digene Hybrid Capture 2 testing was paid for by Roche Molecular Systems.

REFERENCES

1. **Aspinall, S., A. D. Steele, I. Peenze, and M. J. Mphahlele.** 1995. Detection and quantitation of hepatitis B virus DNA: comparison of two commercial hybridization assays with polymerase chain reaction. J. Viral Hepatitis **2:**107–1411.
2. **Brechtbuehl, K., S. A. Whalley, G. M. Dusheiko, and N. A. Saunders.** 2001. A rapid real-time quantitative polymerase chain reaction for hepatitis B virus. J. Virol. Methods **93:**105–113.
3. **Butterworth, L. A., S. L. Prior, P. J. Buda, J. L. Faoagali, and W. G. Cooksley.** 1996. Comparison of four methods for quantitative measurement of hepatitis B viral DNA. J. Hepatol. **24:**686–691.
4. **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.
5. **de Man, R. A., P. Marcellin, F. Habal, P. Desmond, T. Wright, T. Rose, R. Jurewicz, and C. Young.** 2000. A randomized, placebo-controlled study to evaluate the efficacy of 12-month famciclovir treatment in patients with chronic hepatitis B e antigen-positive hepatitis B. Hepatology **32:**413–417.
6. **DiDomenico, N., H. Link, R. Knobel, T. Caratsch, W. Weschler, Z. G. Loewy, and M. Rosenstraus.** 1996. COBAS AMPLICOR: fully automated RNA and DNA amplification and detection system for routine diagnostic PCR. Clin. Chem. **42:**1915–1923.
7. **Dienstag, J. L., R. P. Perrillo, E. R. Schiff, M. Bartholomew, C. Vicary, and M. Rubin.** 1995. A preliminary trial of lamivudine for chronic hepatitis B infection. N. Engl. J. Med. **333:**1657–1661.
8. **Di Marco, V., O. Lo Iacono, C. Camma, A. Vaccaro, M. Giunta, G. Martorana, P. Fuschi, P. L. Almasio, and A. Craxi.** 1999. The long-term course of chronic hepatitis B. Hepatology **30:**257–264.
9. **Evans, A. A., A. P. O'Connell, J. C. Pugh, W. S. Mason, F. M. Shen, G. C. Chen, W. Y. Lin, A. Dia, S. M'Boup, B. Drame, and W. T. London.** 1998. Geographic variation in viral load among hepatitis B carriers with differing risks of hepatocellular carcinoma. Cancer Epidemiol. Biomarkers Prevent. **7:**559–565.
10. **Fei, G. Z., S. P. Sylvan, G. B. Yao, and U. B. Hellstrom.** 1999. Quantitative monitoring of serum hepatitis B virus DNA and blood lymphocyte subsets during combined prednisolone and interferon-alpha therapy in patients with chronic hepatitis B. J. Viral Hepatitis **6:**219–227.
11. **Heermann, K. H., W. H. Gerlich, M. Chudy, S. Schaefer, R. Thomssen, and the Eurohep Pathobiology Group.** 1999. Quantitative detection of hepatitis B virus DNA in two international reference plasma preparations. J. Clin. Microbiol. **37:**68–73.
12. **Hennig, H., I. Puchta, J. Luhm, P. Schlenke, S. Goerg, and H. Kirchner.** 2002. Frequency and load of hepatitis B virus DNA in first-time blood donors with antibodies to hepatitis B core antigen. Blood **100:**2637–2641.
13. **Hilleman, M. R.** 2001. Overview of the pathogenesis, prophylaxis and therapeusis of viral hepatitis B, with focus on reduction to practical applications. Vaccine **19:**1837–1848.
14. **Ho, S. K., T. M. Chan, I. K. Cheng, and K. N. Lai.** 1999. Comparison of the second-generation digene hybrid capture assay with the branched-DNA assay for measurement of hepatitis B virus DNA in serum. J. Clin. Microbiol. **37:**2461–2465.
15. **Ho, S. K., W. C. Yam, E. T. Leung, L. P. Wong, J. K. Leung, K. N. Lai, and T. M. Chan.** 2003. Rapid quantification of hepatitis B virus DNA by real-time PCR using fluorescent hybridization probes. J. Med. Microbiol. **52:**397–402.
16. **Hwang, S.-J., R.-H. Lu, M. L. Wood, Y.-J. Wang, F.-Y. Chang, and S.-D. Lee.** 1999. Comparison of the nucleic acid-based crosslinking hybridization assay and the branched DNA signal amplification assay in the quantitative measurement of serum hepatitis B virus DNA. J. Clin. Lab. Anal. **13:**296–300.
17. **Janssen, H. L., G. Gerken, V. Carreño, P. Marcellin, N. V. Naoumov, A. Craxi, H. Ring-Larsen, G. Kitis, J. van Hattum, R. A. de Vries, P. P. Michielsen, F. J. ten Kate, W. C. Hop, R. A. Heijtink, P. Honkoop, S. W. Schalm, et al.** 1999. Interferon alfa for chronic hepatitis B infection: increased efficacy of prolonged treatment. Hepatology **30:**238–243.
18. **Kao, J. H., M. Wood, P. J. Chen, M. Y. Lai, and D. S. Chen.** 1999. Comparison of two methods for quantification of hepatitis B viral DNA. J. Gastroenterol. Hepatol. **14:**423–426.
19. **Kessler, H. H., K. Pierer, E. Dragon, H. Lackner, B. Santner, D. Stunzner, E. Stelzl, B. Waitzl, and E. Marth.** 1998. Evaluation of a new assay for HBV DNA quantitation in patients with chronic hepatitis B. Clin. Diagn. Virol. **9:**37–43.
20. **Kessler, H. H., S. Preininger, E. Stelzl, E. Daghofer, B. I. Santner, E. Marth, H. Lackner, and R. E. Stauber.** 2000. Identification of different states of hepatitis B virus infection with a quantitative PCR assay. Clin. Diagn. Lab. Immunol. **7:**298–300.
21. **Kessler, H. H., E. Stelzl, E. Daghofer, B. I. Santner, E. Marth, H. Lackner, and R. E. Stauber.** 2000. Semiautomated quantification of hepatitis B virus DNA in a routine diagnostic laboratory. Clin. Diagn. Lab. Immunol. **7:**853–855.
22. **Krajden, M., J. Minor, L. Cork, and L. Comanor.** 1998. Multi-measurement method comparison of three commercial hepatitis B virus DNA quantification assays. J. Viral Hepatitis **5:**415–422.
23. **Kuhns, M. C., A. L. McNamara, R. P. Perrillo, C. M. Cabal, and C. R. Campbel.** 1989. Quantitation of hepatitis B viral DNA by solution hybridization: comparison with DNA polymerase and hepatitis B e antigen during antiviral therapy. J. Med. Virol. **27:**274–281.
24. **Lai, C. L., R. N. Chien, N. W. Leung, T. T. Chang, R. Guan, D. I. Tai, K. Y. Ng, P. C. Wu, J. C. Dent, J. Barber, S. L. Stephenson, D. F. Gray, et al.** 1998. A one-year trial of lamivudine for chronic hepatitis B. N. Engl. J. Med. **339:**61–68.
25. **Lai, C. L., M. Rosmawati, J. Lao, H. Van Vlierberghe, F. H. Anderson, N. Thomas, and D. Dehertogh.** 2002. Entecavir is superior to lamivudine in reducing hepatitis B virus DNA in patients with chronic hepatitis B infection. Gastroenterology **123:**1831–1838.
26. **Lau, G. K., M. Tsiang, J. Hou, S. Yuen, W. F. Carman, L. Zhang, C. S. Gibbs, and S. Lam.** 2000. Combination therapy with lamivudine and famciclovir for chronic hepatitis B-infected Chinese patients: a viral dynamics study. Hepatology **32:**394–399.
27. **Leung, N. W. Y., C.-L. Lai, T.-T. Chang, R. Guan, C.-M. Lee, K.-Y. Ng, S.-G. Lim, P.-C. Wu, J. C. Dent, S. Edmundson, L. D. Condreay, and R.-N. Chien.** 2001. Extended lamivudine treatment in patients with chronic hepatitis B enhances hepatitis B e antigen seroconversion rates: results after 3 years of therapy. Hepatology **33:**1527–1532.
28. **Lewin, S. R., R. M. Ribeiro, T. Walters, G. K. Lau, S. Bowden, S. Locarnini, and A. S. Perelson.** 2001. Analysis of hepatitis B viral load decline under potent therapy: complex decay profiles observed. Hepatology **34:**1012–1020.
29. **Liu, C. J., P. J. Chen, M. Y. Lai, J. H. Kao, and D. S. Chen.** 2001. Hepatitis B virus variants in patients receiving lamivudine treatment with breakthrough hepatitis evaluated by serial viral loads and full-length viral sequences. Hepatology **34:**583–589.
30. **Loeb, K., K. 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.
31. **Lok, A. S., E. J. Heathcote, and J. H. Hoofnagle.** 2001. Management of hepatitis B: 2000—summary of a workshop. Gastroenterology **120:**1828–1853.
32. **Lok, A. S., and B. J. McMahon.** 2001. Chronic hepatitis B. Hepatology **34:**1225–1241.
33. **Mahoney, F. J.** 1999. Update on diagnosis, management, and prevention of hepatitis B virus infection. Clin. Microbiol. Rev. **12:**351–366.
34. **Makris, A., L. Zignego, and S. J. Hadziyannis.** 1991. Measurement of hepatitis B viral DNA in serum by solution hybridization and comparison with the dot-blot hybridization technique. Hepatogastroenterology **38:**53–55.
35. **Marin, I. J., M. Poljak, K. Seme, J. Meglic-Volkar, M. Maticic, G. Lesnicar, and V. Brinovec.** 2001. Comparative evaluation of semiautomated COBAS AMPLICOR hepatitis B virus (HBV) MONITOR test and manual microwell plate-based AMPLICOR HBV MONITOR test. J. Clin. Microbiol. **39:**758–761.
36. **Michalak, T. I., C. Pasquinelli, S. Guilhot, and F. V. Chisari.** 1994. Hepatitis B virus persistence after recovery from acute viral hepatitis. J. Clin. Investig. **93:**230–239.
37. **Mommeja-Marin, H., E. Mondou, M. R. Blum, and F. Rousseau.** 2003. Serum HBV DNA as a marker of efficacy during therapy for chronic HBV infection: analysis and review of the literature. Hepatology **37:**1309–1319.
38. **Mutimer, D., D. Dowling, P. Cane, D. Ratcliffe, H. Tang, K. O'Donnell, J. Shaw, E. Elias, and D. Pillay.** 2000. Additive antiviral effects of lamivudine and alpha-interferon in chronic hepatitis B infection. Antivir. Ther. **5:**273–277.
39. **Nagata, I., G. Colucci, G. V. Gregorio, P. Cheeseman, R. Williams, G. Mieli-Vergani, and D. Vergani.** 1999. The role of HBV DNA quantitative PCR in monitoring the response to interferon treatment in chronic hepatitis B virus infection. J. Hepatol. **30:**965–969.
40. **Niesters, H. G., M. Krajden, L. Cork, M. de Medina, M. Hill, E. Fries, and A. D. Osterhaus.** 2000. A multicenter study evaluation of the Digene Hybrid Capture II signal amplification technique for detection of hepatitis B virus DNA in serum samples and testing of EUROHEP standards. J. Clin. Microbiol. **38:**2150–2155.
41. **Noborg, U., A. Gusdal, P. Horal, and M. Lindh.** 2000. Levels of viraemia in subjects with serological markers of past or chronic hepatitis B virus infection. Scand. J. Infect. Dis. **32:**249–252.
42. **Noborg, U., A. Gusdal, E. K. Pisa, A. Hedrum, and M. Lindh.** 1999. Auto-

mated quantitative analysis of hepatitis B virus DNA by using the Cobas Amplicor HBV monitor test. J. Clin. Microbiol. **37:**2793–2797.
43. **Nolte, F. S.** 1998. Branched DNA signal amplification for direct quantitation of nucleic acid sequences in clinical specimens. Adv. Clin. Chem. **33:**201–235.
44. **Paraskevis, D., C. Haida, N. Tassopoulos, M. Raptopoulou, D. Tsantoulas, H. Papachristou, V. Sypsa, and A. Hatzakis.** 2002. Development and assessment of a novel real-time PCR assay for quantitation of HBV DNA. J. Virol. Methods **103:**201–212.
45. **Pas, S. D., E. Fries, R. A. De Man, A. D. Osterhaus, and H. G. 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.
46. **Poljak, M., I. J. Marin, K. Seme, V. Brinovec, M. Maticic, J. Meglic-Volkar, G. Lesnicar, and A. Vince.** 2001. Second-generation hybrid capture test and Amplicor monitor test generate highly correlated hepatitis B virus DNA levels. J. Virol. Methods **97:**165–169.
47. **Roche, B., C. Feray, M. Gigou, A. M. Roque-Afonso, J. L. Arulnaden, V. Delvart, E. Dussaix, C. Guettier, H. Bismuth, and D. Samuel.** 2003. HBV DNA persistence 10 years after liver transplantation despite successful anti-HBS passive immunoprophylaxis. Hepatology **38:**86–95.
48. **Roth, W. K., S. Buhr, C. Drosten, and E. Seifried.** 2000. NAT and viral safety in blood transfusion. Vox Sang **78**(Suppl. 2)**:**257–259.
49. **Saldanha, J., W. Gerlich, N. Lelie, P. Dawson, K. Heermann, and A. Heath.** 2001. An international collaborative study to establish a World Health Organization international standard for hepatitis B virus DNA nucleic acid amplification techniques. Vox Sang. **80:**63–71.
50. **Tsiang, M., J. F. Rooney, J. J. Toole, and C. S. Gibbs.** 1999. Biphasic clearance kinetics of hepatitis B virus from patients during adefovir dipivoxil therapy. Hepatology **29:**1863–1869.
51. **Weusten, J. J., H. A. van Drimmelen, and P. N. Lelie.** 2002. Mathematic modeling of the risk of HBV, HCV, and HIV transmission by window-phase donations not detected by NAT. Transfusion **42:**537–548.
52. **Wolters, L. M., B. E. Hansen, H. G. Niesters, S. Zeuzem, S. W. Schalm, and R. A. de Man.** 2002. Viral dynamics in chronic hepatitis B patients during lamivudine therapy. Liver **22:**121–126.
53. **Yotsuyanagi, H., K. Yasuda, S. Iino, K. Moriya, Y. Shintani, H. Fujie, T. Tsutsumi, S. Kimura, and K. Koike.** 1998. Persistent viremia after recovery from self-limited acute hepatitis B. Hepatology **27:**1377–1382.
54. **Yuen, M. F., E. Sablon, C. K. Hui, H. J. Yuan, H. Decraemer, and C. L. Lai.** 2001. Factors associated with hepatitis B virus DNA breakthrough in patients receiving prolonged lamivudine therapy. Hepatology **34:**785–791.
55. **Zanella, I., A. Rossini, D. Domenighini, A. Albertini, and E. Cariani.** 2002. Quantitative analysis of hepatitis B virus DNA by real-lime amplification. Eur. J. Clin. Microbiol. Infect. Dis. **21:**22–26.
56. **Zollner, B., P. Schafer, H. H. Feucht, M. Schroter, J. Petersen, and R. Laufs.** 2001. Correlation of hepatitis B virus load with loss of e antigen and emerging drug-resistant variants during lamivudine therapy. J. Med. Virol. **65:**659–663.