Clinical Evaluation of the Digene Hybrid Capture II Test and the COBAS AMPLICOR Monitor Test for Determination of Hepatitis B Virus DNA Levels

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The measurement of hepatitis B virus (HBV) DNA is important for the assessment of liver disease and treatment efficacy. Most commercially available assays for the determination of HBV DNA levels have limited linear ranges. This study was performed to evaluate the clinical performance of the Digene Hybrid Capture II (Digene HC II assay) and the COBAS AMPLICOR Monitor test (COBAS-AM assay), with special emphasis on anti-HBV e antigen (HBeAg)-positive patients with low HBV DNA levels. A total of 425 Chinese patients with chronic hepatitis B were recruited. A total of 107 patients were HBeAg positive, and 318 patients were HBeAg negative. The Digene HC II assay and the COBAS-AM assay had similar intra-assay and interassay variabilities. A total of 264 patients (62.1%) had HBV DNA levels undetectable by the Digene HC II assay, and 47 patients (11.1%) had HBV DNA levels undetectable by the COBAS-AM assay ($P < 0.001$). For the 161 patients with HBV DNA levels detectable by the Digene HC II assay, the HBV DNA levels obtained by the Digene HC II assay and by the COBAS-AM assay showed an excellent correlation ($r = 0.95$; $P < 0.001$). The linear ranges of the Digene HC II assay and the COBAS-AM assay marginally overlapped. Before HBV DNA levels could be determined by the COBAS-AM assay, predilution had to be performed for 158 of 161 patients (98.1%) with HBV DNA levels detectable by the Digene HC II assay and for 10 of 264 patients (3.8%) with HBV DNA levels undetectable by the Digene HC II assay. The cost for assaying each serum sample by using different strategies was calculated. The COBAS-AM assay was more sensitive than the Digene HC II assay and more suitable for monitoring low levels of HBV viremia.

The determination of hepatitis B virus (HBV) DNA levels has been shown to be useful for assessment of the severity and prognosis of chronic hepatitis B disease as well as for prediction of the infectivity of chronic carriers of HBV surface antigen (HBsAg) (5). With the available new and potent antiviral drugs, monitoring HBV DNA levels not only will enable the evaluation of antiviral effects on HBV replication but also will be helpful in identifying the emergence of drug-resistant mutations by detecting HBV DNA breakthrough (9, 10). Recent studies have stressed the clinical importance of relatively low levels of HBV DNA. Acute exacerbation can occur in Chinese HBV e antigen (HBeAg)-negative patients with relatively low levels of HBV viremia (11). No single HBV DNA level can be defined as the cutoff value for differentiating inactive carriers from patients with HBeAg-negative chronic hepatitis (5).

Several commercially available assays for HBV DNA detection are based on hybridization assays, such as the branched-DNA (bDNA) signal amplification system (Quantiplex; Chiron Corp.) and the Digene assay (Digene Corporation, Gaithersburg, Md.). The first generation of the Digene assay (Digene Hybrid Capture System [Digene HCS assay]) has been found to be less sensitive than the bDNA assay (7). However, the second generation of the Digene assay (Digene Hybrid Cap-

ture II [Digene HC II assay]) has overcome this drawback. With the inclusion of a high-speed centrifugation step, the highly sensitive Digene HC II assay further brings the lower detection limit down to 4,700 copies/ml, but it also requires a large amount of serum (1 ml compared to the 30 μ l required for the Digene HCS assay). The bDNA assay has usually been found to overestimate HBV DNA levels compared with the Digene assay (2, 7, 8).

The AMPLICOR Monitor test (Roche Diagnostics, Branchburg, N.J.) is a competitive PCR assay. The first-generation microwell plate version of the AMPLICOR Monitor test has a linear range of 10^3 to 10^7 copies/ml. It is more sensitive than the Digene HCS assay and the bDNA assay. This first-generation AMPLICOR Monitor test, with its manual procedures and significant amplification of HBV DNA by PCR, has poor within-run and between-run reproducibilities compared with the Digene assay (6). Reproducibility has been improved in the semiautomatic COBAS AMPLICOR Monitor test (COBAS-AM assay). The COBAS-AM assay has a lower limit of detection of 3×10^2 copies/ml, but this advantage is offset by a narrow linearity range of 3×10^2 to 2×10^5 copies/ml (4). Predilution of serum with higher HBV DNA levels must be performed to extend the detection limit.

The correlations between the Digene HC II assay and the COBAS-AM assay have been evaluated only in one study, by Poljak et al. (8), for 252 Slovenian patients. Patients with chronic hepatitis B have a wide range of HBV DNA levels (0 to 10^{11} copies/ml). However, the linear ranges of both the

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Digene HC II assay and the COBAS-AM assay are relatively narrow, being within 3 log₁₀ copies/ml. Repeat testing of serum samples will increase the cost of the determination of HBV DNA levels. How to economically select them in clinical studies has not been well delineated.

The aims of this study were (i) to determine the clinical values of the Digene HC II assay and the COBAS-AM assay for detecting HBV viremia in Chinese patients with chronic hepatitis B and (ii) to explore a viable strategy of combining the Digene HC II assay and the COBAS-AM assay to extend the range of determination of HBV DNA levels in clinical settings.

MATERIALS AND METHODS

Patients. A total of 425 patients with chronic hepatitis B were recruited from the Hepatitis Clinic of Queen Mary Hospital, Hong Kong. All had been HBsAg positive for at least 6 months and were negative for antibodies to hepatitis A virus, hepatitis C virus, and hepatitis D virus. None of the patients had a history of drug abuse or heavy drinking. One serum sample was taken from each patient for determination of the HBV DNA level. Liver function tests at the time of serum collection were also performed. Serum HBsAg, HBeAg, and antibody to HBeAg were tested by microparticle enzyme immunoassays (Abbott Laboratories, North Chicago, Ill.). The patients were chosen first to provide a wide range of HBV DNA levels and second to include a substantial proportion of patients who were positive for antibody to HBeAg and therefore more likely to have low levels of HBV DNA.

Methodology. (i) Digene HC II assay. The Digene HC II assay was performed by following the manufacturer's instructions. In brief, 30 µl of each serum sample, triplicate samples of calibrators 1 and 2 (containing 0 and 1.42×10^5 copies of HBV DNA/ml, respectively), duplicate samples of calibrators 3, 4, and 5 (containing 2.83×10^7 , 5.66×10^8 , and 1.70×10^9 copies of HBV DNA/ml, respectively), and one replicate of two positive controls were added to a 96-well microplate containing 30 µl of denaturation reagent. After the microplate was shaken at 1,100 rpm for 1 min and incubated at $65^\circ\text{C} \pm 2^\circ\text{C}$ for 30 min to lyse HBV, 30 µl of a probe mixture containing RNA probes specific for both the ad and the ay strains of HBV was added to each well and incubated at $65^\circ\text{C} \pm 2^\circ\text{C}$ for 60 min. Then, 75 µl of the solution containing the RNA-DNA hybrid was transferred from each hybridization microplate well to the corresponding well of the capture microplate. The capture microplate was shaken on a rotary shaker at $1,100 \pm 100$ rpm for 60 min. After the HBV RNA-DNA hybrid was captured onto a solid phase coated with antibodies specific to the RNA-DNA hybrid, the liquid from the capture microplate was removed and mixed with 75 µl of detection reagent 1, containing alkaline phosphatase-labeled antibodies specific to the RNA-DNA hybrid. This mixture was incubated at 20 to 25°C for 30 min. Multiple anti-RNA-DNA hybrid antibodies would bind to each captured hybrid, and these antibodies would in turn be conjugated to several alkaline phosphatase molecules. After six washes with wash buffer (provided by manufacturer), 75 µl of detection reagent 2 (chemiluminescent substrate) was applied. After 15 min of incubation, the emitted light was read in a DML 2000 Luminometer.

The validation of the test was based on the variance of the results for the calibrators. HBV DNA levels were calculated by comparing the results for the samples against the standard curve obtained from the results for calibrators 2 to 5. HBV DNA levels were reported in copies per milliliter. The linear range of the Digene HC II assay was 1.42×10^5 to 1.70×10^9 copies/ml.

(ii) COBAS-AM assay. The COBAS-AM assay is a competitive PCR assay based on the coamplification of target HBV DNA and quantitation standard (QS) DNA. The QS DNA was a linearized plasmid which differed from the target HBV DNA only in a 21-mer internal sequence. The 21-mer internal sequences allowed differential detection of the QS and the target HBV by specific probes. The QS DNA was added at a fixed known copy number to each specimen.

The conserved precore and core regions of HBV were amplified with primer HBV-104D and biotinylated primer HBV-104UB, provided by the manufacturer. After an amplicon of 104 bp was hybridized with a magnetic ball coated with probes for HBV DNA or QS DNA, avidin-horseradish peroxidase conjugates were added to bind the biotin-labeled amplicon. A substrate containing hydrogen peroxide and 3,3',5,5'-tetramethylbenzidine was used to form a colored complex, which was measured by using a COBAS AMPLICOR Analyzer at a wavelength of 660 nm. HBV DNA levels were calculated from the ratio of the total absor-

bance of target HBV DNA to that of QS DNA and the input number of QS DNA.

For the COBAS-AM assay, pre-PCR sample preparation was performed according to the instructions of the manufacturer. Then, the remaining steps were finished automatically by using the COBAS AMPLICOR Analyzer.

The linear range of the COBAS-AM assay was 3×10^2 to 2×10^5 copies of HBV DNA/ml. For a specimen with an HBV DNA level higher than 2×10^5 copies/ml, serial 1:10 dilution of the specimen is recommended. In this study, serum specimens with HBV DNA levels of 0.14×10^6 to 100×10^6 copies/ml in the Digene HC II assay were prediluted $1:10^3$ and serum specimens with HBV DNA levels higher than 100×10^6 copies/ml in the Digene HC II assay were prediluted $1:10^5$ in dilution buffer (Basematrix; Roche, Branchburg, N.J.) before the HBV DNA levels were retested with the COBAS-AM assay.

Statistical analyses. The Statistical Program for Social Sciences (SPSS 11.0 for Windows; SPSS Inc., Chicago, Ill.) was used for all statistical analyses. The Student *t* test was used to compare the difference between the means. Regression analysis and correlation were used to define the relationships between continuous variables. The chi-square test with the Yates correction factor or the Fisher exact test was applied for categorical variables. *P* values of less than 0.05 were used to indicate statistical significance.

RESULTS

Demographics. The male/female ratio of the patients recruited was 294:131, and the median age was 38.5 years (range, 15.3 to 81.6 years). A total of 107 patients were HBeAg positive, and 318 patients were HBeAg negative.

Precision of the Digene HC II assay. In five runs of the Digene HC II assay, the mean intra-assay coefficients of variation (CVs) of calibrators 1 and 2 were 9.8 and 9.0%, respectively. Three samples were tested with the Digene HC II assay on three different days by the same operator. The interassay CV was 28.6%.

Precision of the COBAS-AM assay. Three samples were tested in three replicates in the same run of the COBAS-AM assay. The intra-assay CV was 10.8%. Five samples were retested with the COBAS-AM assay on three different days by the same operator. The interassay CV was 22.3%.

Clinical evaluation of the Digene HC II assay and the COBAS-AM assay. A total of 264 patients (62.1%) had HBV DNA levels undetectable by the Digene HC II assay. A total of 47 patients (11.1%) had HBV DNA levels undetectable by the COBAS-AM assay ($P < 0.001$).

For the 161 samples with HBV DNA levels detectable by the Digene HC II assay, the HBV DNA levels were remeasured by the COBAS-AM assay with predilution. The results obtained with the COBAS-AM assay and the Digene HC II assay were significantly correlated ($r = 0.95$; $P < 0.001$) (Fig. 1), with the following equation: $\log_{10}(\text{HBV DNA determined by the COBAS-AM assay}) = 0.394 + 0.995 \log_{10}(\text{HBV DNA determined by the Digene HC II assay})$.

The slope obtained by a least-square fit to a line was 0.995; this was quite close to 1.0.

To further estimate the agreement of the COBAS-AM assay and the Digene HC II assay, the mean HBV DNA levels obtained with both assays were plotted against their differences. According to Bland and Altman (1), the limits of agreement are defined as the mean of differences plus or minus two times the standard deviation. If the differences are normally distributed (Gaussian), 95% of the differences will lie between these limits (1). As shown in Fig. 2A, there was good agreement between the assays for HBV DNA levels in the lower range of the Digene HC II assay ($<10^6$ copies/ml in the

FIG. 1. Correlation between HBV DNA levels determined by the COBAS-AM assay and the Digene HC II assay. The dotted line is the line of equality.

COBAS-AM assay). The mean difference was close to zero (0.077), and 95% of the difference was between -0.86 and 1.02 after logarithmic transformation. However, for sera with HBV DNA levels in the median range of the Digene HC II assay (10^6 to 10^9 copies/ml in the COBAS-AM assay), the mean difference was 0.34 , and the limits of agreement were -0.52 and 1.0 orders of magnitude (Fig. 2B). In the higher range of the Digene HC II assay, the mean difference between the assays was 0.61 , and the limits of agreement were 0.13 and 1.09 orders of magnitude (Fig. 2C).

The linear ranges of the Digene HC II assay and the COBAS-AM assay marginally overlapped. The upper detection limit of the COBAS-AM assay was 2×10^5 copies/ml. A total of 158 of 161 patients (98.1%) with HBV DNA levels detectable and 10 of 264 patients (3.8%) with HBV DNA levels undetectable by the Digene HC II assay had HBV DNA levels higher than the detection limit of the COBAS-AM assay. Predilution had to be performed for these samples before HBV DNA levels could be measured by the COBAS-AM assay. For the remaining 3 of 161 patients (1.9%) with HBV DNA levels detectable and 207 of 264 patients (78.4%) with HBV DNA levels undetectable by the Digene HC II assay, HBV DNA levels were detectable by the COBAS-AM assay without predilution.

Cost evaluation of the Digene HC II assay and the COBAS-AM assay. The price of the COBAS-AM assay was about three times higher than that of the Digene HC II assay per reaction (\$83 versus \$26 [U.S. dollars]). In order to determine the optimal strategy for the measurement of HBV DNA levels in clinical settings, we further analyzed the chance of requiring retesting by the COBAS-AM assay when the Digene HC II assay was used (strategy A) and the chance of requiring predilution of serum samples when the COBAS-AM assay was used (strategy B). A third option was to use the Digene HC II assay for all HBeAg-positive patients and the COBAS-AM assay for all HBeAg-negative patients (strategy C).

For HBeAg-positive patients, 14 of 107 samples (13.1%) had HBV DNA levels undetectable by the Digene HC II assay and

FIG. 2. Difference from the mean for HBV DNA levels determined by the COBAS-AM assay and the low range, median range, and high range of the Digene HC II assay. The mean of the difference is indicated by the solid line. The mean and two standard deviations (2SD) is indicated by the dotted line.

required retesting by the COBAS-AM assay. A total of 95 of 107 samples (88.9%) required predilution when the COBAS-AM assay was used.

For HBeAg-negative patients, 250 of 318 samples (78.6%) had HBV DNA levels undetectable by the Digene HC II assay and required retesting by the COBAS-AM assay. A total of 73 of 318 samples (23.0%) required predilution when the COBAS-AM assay was used.

For HBeAg-negative patients, HBV DNA levels were moderately correlated with serum alanine transaminase (ALT) levels ($r = 0.27$; $P < 0.001$) (Fig. 3). Table 1 shows the percentages and odds ratios of predilution required when different ALT levels were used as cutoff values. The average costs per sample of the three strategies are shown in Table 2.

DISCUSSION

This study confirmed that the COBAS-AM assay was more sensitive (by $\sim 3 \log_{10}$ copies/ml) for the determination of HBV DNA levels than the Digene HC II assay. The HBV DNA levels obtained by the Digene HC II assay and the COBAS-AM assay were significantly correlated ($r = 0.95$; $P < 0.001$). This correlation was much better than the correlation between the first generations of both assays ($r = 0.6$; $P < 0.001$), as reported by Pawlotsky et al. (6), indicating that the

FIG. 3. HBV DNA levels in HBeAg-negative patients with different ALT levels. Error bars indicate standard deviations.

semiautomatic COBAS-AM assay significantly improves the sensitivity and precision of the AMPLICOR Monitor test.

The present study demonstrated that the HBV DNA levels obtained by the Digene HC II assay were lower than those obtained by the COBAS-AM assay. The difference might have been due to the different methods and standards used in the two assays. Because the scatter of differences did not widen with higher HBV DNA levels, these findings suggested that the predilution procedure for the COBAS-AM assay probably did not introduce significant variance. Another possible explanation could be sample preparation. The COBAS-AM assay has a polyethylene glycol precipitation step, whereas there is no polyethylene glycol precipitation or proteinase K digestion step in the Digene HC II assay. The presence of too many proteins might affect the performance of the Digene HC II assay, e.g., the formation or capture of the RNA-DNA hybrid in the Digene HC II assay. It has been found that the Digene HC II assay detects HBV DNA levels 0.25 log₁₀ copies/ml lower than the Digene HCS assay (3). A proteinase K digestion step is included in the latter assay (3).

The linear ranges of the two assays marginally overlapped. There was no recommended effective strategy concerning sample predilution for the COBAS-AM assay. This study demonstrated that the results obtained with the Digene HC II assay served as a reliable reference for determining predilution to be done for the COBAS-AM assay. The error percentage was

TABLE 2. Cost evaluation of Digene HC II and COBAS-AM assays

Patients	Strategy ^a	Estimated price per sample (U.S. dollars)
All	A	79.5
	B	115.8
	C	86.0
HBeAg positive	A	38.4
	B	156.7
HBeAg negative	A	93.3
	B	102.1
HBeAg negative with elevated ALT levels	A	76.2
	B	125.0
HBeAg negative with normal ALT levels	A	99.2
	B	94.2

^a In strategy A, the Digene HC II assay is used first, and then the COBAS-AM assay is used for sera with HBV DNA levels undetectable by the Digene HC II assay. In strategy B, the COBAS-AM assay is used first, and then sera with HBV DNA levels higher than 2×10^5 copies/ml are remeasured with a predilution step. In strategy C, the Digene HC II assay is used for all HBeAg-positive patients, and then the COBAS-AM assay is used for HBeAg-negative patients.

3.1% (13 of 425). However, screening of all clinical samples with the Digene HC II assay before the COBAS-AM assay was too expensive and laborious.

By estimating the average cost of determining HBV DNA levels per sample, we found that measuring HBV DNA levels directly with the Digene HC II assay and then retesting samples with HBV DNA levels undetectable by the Digene HC II assay with the COBAS-AM assay was much more cost-effective for HBeAg-positive patients and HBeAg-negative patients with elevated serum ALT levels than was the strategy of measuring HBV DNA levels directly with the COBAS-AM assay. For HBeAg-negative patients with normal ALT levels, measuring HBV DNA levels directly with the COBAS-AM assay was slightly more cost-effective and therefore was a more suitable selection. The third option, that of using the Digene HC II assay for all HBeAg-positive patients and the COBAS-AM assay for all HBeAg-negative patients, is also a viable alternative.

In conclusion, the COBAS-AM assay was much more sensitive than the Digene HC II assay and was suitable for the determination of low levels of HBV viremia. However, serial dilution would be required for samples containing HBV DNA levels higher than 2×10^5 copies/ml.

TABLE 1. Percentages and odds ratios for HBeAg-negative patients with HBV DNA levels higher than the higher limit of detection of the COBAS-AM assay

ALT level (U/L) ^a	Proportion (%) of patients with HBV DNA levels higher than 2×10^5 copies/ml	P value	Odds ratio (95% confidence interval)
<0.5 ULN vs ≥ 0.5 ULN	7/87 (8.0%) vs 67/231 (29.0%)	0.001	4.7 (2.1–10.6)
<1.0 ULN vs ≥ 1.0 ULN	32/237 (13.5%) vs 42/81 (51.9%)	<0.001	6.9 (3.9–12.2)
<1.5 ULN vs ≥ 1.5 ULN	50/278 (18.0%) vs 24/40 (60.0%)	<0.001	6.84 (3.4–13.8)
<2.0 ULN vs ≥ 2.0 ULN	58/295 (19.7%) vs 16/23 (69.6%)	<0.001	9.3 (3.7–23.8)

^a ULN, upper limit of normal.

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