

Original Article

A multi-center clinical study comparing Sansure Magb and CAP/CTM HBV tests in the quantitative detection of HBV DNAXiaoyu Fu¹, Deming Tan¹, Xiaoguang Dou², Jinjun Chen³, Juan Wu¹¹ Key Laboratory of Viral Hepatitis, Department of Infectious Disease; Xiangya Hospital, Central South University, Changsha, China² Department of Infectious Disease, Shengjing Hospital, China Medical University, Shenyang, China³ Department of Infectious Disease, Nanfang Hospital, Southern Medical University, Guangzhou, China**Abstract**

Introduction: As the most reliable means of diagnosing hepatitis (HBV) infection and predicting the prognosis of HBV-related chronic liver disease, the COBAS AmpliPrep/COBAS TaqMan real-time polymerase chain reaction (PCR) (CAP/CTM) assay provides a highly sensitive and accurate method for quantifying HBV DNA. However, the high cost of the COBAS reagents is prohibitive in many developing countries. Thus, we compared the Sansure magnetic bead (Magb) assay, a novel technology developed by a Chinese company, with the CAP/CTM assay. **Methodology:** The reproducibility and sensitivity of the Sansure Magb assay were first validated using HBV DNA reference samples. Next, the quantitative results for the two assays using 635 blood samples collected from chronic hepatitis B patients and 10 healthy controls were compared.

Results: The Sansure Magb assay showed high reproducibility and was at least as sensitive and specific as the CAP/CTM assay. Among the patient samples, 407 tested positive by both methods, with 386 (94.84%) showing quantitative differences of less than 1 log unit and 21 (5.16%) showing quantitative differences of between 1 and 2 log units. The results from the assays were closely correlated. Bland-Altman plot analysis showed that only 6.6% of the data points fell outside the 95% limits of agreement, which suggests that the differences between methods are clinically acceptable.

Conclusions: This study demonstrates that the Sansure Magb assay is highly sensitive and reproducible. Based on its reduced cost, the Sansure Magb assay may be more applicable than the CAP/CTM assay for HBV diagnosis in developing countries such as China.

Key words: COBAS AmpliPrep/COBAS TaqMan real-time PCR assay; magnetic bead assay; HBV DNA quantification.

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Introduction

Hepatitis B virus (HBV) infection is a major global public health problem. According to the World Health Organization (WHO), about two billion people worldwide have or have had HBV infection, and about 360 million have chronic HBV-related liver diseases, including cirrhosis and hepatocellular carcinoma. HBV is estimated to cause 600,000 deaths per year worldwide [1]. Hepatitis B is endemic in Asia and Africa [2]. The results of national hepatitis epidemiological surveys in China show that approximately 93 million people are chronically infected with HBV, 20 million of whom have chronic hepatitis B [3].

The HBV DNA level is the most direct and reliable marker of HBV replication activity and infectivity. Studies have revealed that baseline HBV DNA levels are closely associated with the incidence of cirrhosis [4] and exhibit a clear dose-response relationship with the

incidence of hepatocellular carcinoma [5]. Additionally, during antiviral treatment for chronic HBV infection, HBV DNA levels are important indicators of antiviral efficacy and disease prognosis. Dynamic monitoring of HBV DNA levels is crucial for planning treatment, determining the need for combination therapy, scheduling follow-up assessment, evaluating long-term efficacy, deciding when to withdraw treatment, and determining whether resistance has developed [6,7]. Though various assays have been established to diagnose and monitor the course and outcome of chronic liver disease caused by HBV infection, direct detection and quantification of serum HBV DNA is considered the most reliable means of diagnosing HBV infection, particularly in the case of certain genetic variations in HBV [8].

Currently, there are two major types of methods that can be used for quantitative detection of HBV DNA. One is based on polymerase chain reaction (PCR)

techniques, such as real-time fluorescence quantitative PCR (FQ-PCR). The other is based on nucleic acid hybridization, such as the dot blot and liquid hybridization methods. Real-time PCR techniques have the advantage of being sensitive, specific, precise, reproducible, automated, and rapid. Among commercially available assays for HBV DNA quantification, the COBAS Ampliprep/COBAS Taqman (CAP/CTM) platform developed by Roche Molecular System (Pleasanton, USA) is frequently used [9]. The CAP/CTM assay uses a dual-labeled hybridization probe targeting the precore and core regions of HBV DNA, paired with automated extraction based on the high DNA affinity of silica gel-covered magnetic beads. Due to its superior specificity, high sensitivity, accuracy, and reproducibility, this method has been approved by the United States Food and Drug Administration and has become the international gold standard for quantitative detection of HBV DNA [10]. However, the CAP/CTM HBV DNA assay is expensive and only compatible with the CAP/CTM system produced by Roche. This poses a financial challenge for developing countries, including China. An affordable alternative method of HBV DNA quantification that does not sacrifice sensitivity or accuracy would have a considerable impact in these areas. Currently, the China Food and Drug Administration (CFDA) has approved several real-time FQ-PCR reagents for HBV DNA detection, but the differences between these methods and the internationally recognized CAP/CTM HBV DNA assay remain to be determined.

In this study, we assessed the Sansure magnetic bead (Magb) assay, which was designed to target a conserved segment of the S-gene, as a method for HBV DNA quantification. The Sansure Magb assay was developed by Sansure Biotech. (Changsha, China) and has a comparatively low cost. We evaluated the precision and reproducibility of the Sansure Magb assay and also systematically compared it to the CAP/CTM assay for HBV DNA quantification.

Methodology

Reproducibility, sensitivity, and specificity testing

To assess the reproducibility of the Sansure Magb assay (Sansure Biotech. Changsha, China), two different reference products were used to conduct imprecision intra- and inter-assays on high- and low-concentration HBV samples. Three different batches of HBV assay kits were tested on the same ABI 7300 system, and each sample test was repeated 10 times. The coefficient of variation (CV%) of the logarithmic concentration was used to evaluate the precision of the kit.

To compare the sensitivity of the Sansure Magb and CAP/CTM assays, a series of eight twofold dilutions of the low-concentration HBV reference sample were made. The dilutions were assayed in triplicate with the Sansure Magb assay and the CAP/CTM assay.

To assess the specificity of the Sansure Magb assay, 10 samples from healthy (HBV-negative) patients were assessed in duplicate by both the Sansure Magb and the CAP/CTM assays.

Samples

A total of 635 plasma and serum samples were collected from outpatients and inpatients with chronic hepatitis B treated in the Department of Infectious Diseases, Xiangya Hospital of Central South University; the Department of Infectious Diseases, Shengjing Hospital of China Medical University; and the Division of Infectious Diseases, Nanfang Hospital of Southern Medical University. The study samples were left over from samples collected for diagnosis or monitoring. All patients had been diagnosed positive for hepatitis B surface antigen (HBsAg) at least six months prior to sample collection. HBsAg and hepatitis B e antigen (HBeAg) detection was performed using an automatic chemiluminescence immunoassay analyzer (Architect, Abbott Laboratories, Green Oaks, USA). All samples were collected after the patients had fasted for at least eight hours. Serum and plasma was isolated within two hours of blood collection and then stored at -70°C . All samples tested negative for HCV as assessed by the third-generation hepatitis C virus antibody (anti-HCV) enzyme immunoassay test (Abbott Laboratories). The study subjects waived informed consent. The test results were used only as evaluation of the reagents, and study results were not made available to the patients, nor were they used as a basis for clinical diagnosis or treatment. Informed consent was obtained from each patient and no patient's private information was disclosed. An additional 10 samples from healthy donors were obtained from a local blood bank and were provided without personal information.

COBAS Ampliprep/COBAS Taqman Assay

The AmpliPrep/COBAS TaqMan system (Roche Molecular System) was used for the CAP/CTM HBV DNA assay. The manufacturer's instructions were strictly followed. The lower limit of detection (LOD) of this assay was reported to be 12 IU/mL, and the upper limit was reported to be 1.1×10^8 IU/mL.

Sansure Magb assay

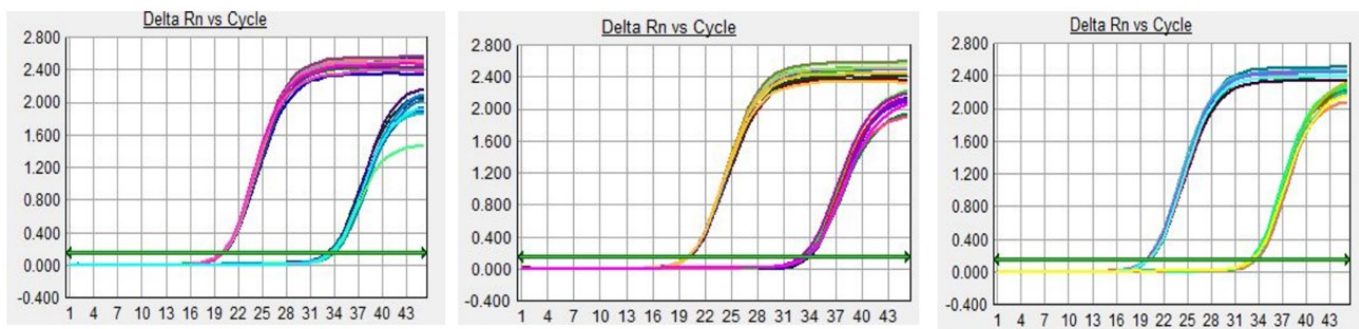
A Sansure Magb reagent kit (Sansure Biotech, Xiangya, China) and ABI 7500 Real-Time PCR System (Applied Biosystems, Carlsbad, USA) were used for the Sansure Magb assay. The magnetic bead method was used to extract HBV DNA from serum samples. A pair of specific primers was designed to target a conserved region of HBV DNA, and a specific fluorescent probe (TaqMan probe, with the 5'-end labeled with the fluorescein FAM), together with the PCR reaction solution, were subjected to real-time quantitative PCR analysis using a fluorescence quantitative PCR analyzer. The instructions of the Sansure Magb reagent kit were strictly followed. The protocol was as follows. A batch of 1.5 mL sterile centrifuge tubes were labeled as negative control, positive control, quantitative reference materials A–D, and test samples. Then, 300 μ L of DNA extraction solution 1 was added to each tube, and 200 μ L of the corresponding sample was added to each tube. The tubes were vortexed for 10 seconds and centrifuged. Next, 100 μ L of DNA extraction solution 2 mix was added to each tube. The tubes were vortexed for 10 seconds and allowed to sit at room temperature for 10 minutes. After centrifugation, the tubes were placed on a magnetic separator. After 3 minutes, the solution was slowly aspirated (avoiding the brown material adsorbed on the tube wall). Then, 600 μ L of DNA extraction solution 3 and 200 μ L of DNA extraction solution 4 were added to each tube. The tubes were vortexed for 5 seconds. After centrifugation, the tubes were again placed on a magnetic separator. After about 3 minutes, the supernatant was divided into two layers, and the liquid was slowly aspirated from the bottom of the tube and discarded. After another minute, the residual liquid at the bottom of the tube was aspirated and discarded. All

tubes were then transferred to a centrifuge tube rack; 50 μ L of PCR mix was added to each tube, and the brown residue on the tube wall after the PCR mix elution was aspirated and collected. This was repeated several times until the elution was complete. All brown solutions collected were transferred to a 0.2 mL PCR reaction tube. The lid was closed, and the tube was moved to the amplification area, where it was subjected to detection using an ABI 7500 Fluorescence Real-Time PCR System. The PCR conditions were as follows: 50°C for 2 minutes for UNG enzyme reaction; 95°C for 2 minutes for Taq enzyme activation; 45 cycles of 95°C for 15 seconds and 58°C for 30 seconds; and then 25°C for 10 seconds. The LOD of the Sansure Magb assay, indicated as an HBV DNA level that can be detected over 95% of the time, was 10 IU/mL, and the upper limit reached 8.7×10^8 IU/mL.

Statistical methods

Data are expressed as mean \pm standard deviation. SPSS version 16.0 was used for all data analysis, and $p < 0.05$ was considered statistically significant. Pearson's correlation analysis was performed to determine the linear relationship between quantitative variables. A Bland-Altman plot (difference plot) was constructed to further analyze the agreement between the two assays. In this plot, the x-axis shows the mean value measured using the two methods, and the y-axis shows the difference between the values measured using the two methods (in log units).

Figure 1. Real-time PCR results from three different experiments.



Exp. 1

Exp. 2

Exp. 3

SYBR Green fluorescence charts are shown for three different Sansure Magb experiments in which 10 high-concentration and 10 low-concentration replicates per experiment were amplified by real-time PCR. The high concentration samples uniformly reached the threshold (green line) at an earlier cycle than that of the low concentration samples.

Results

Reproducibility testing for the Sansure Magb assay

Reference samples with high and low HBV DNA concentrations were used to determine whether the Sansure Magb Assay yields data that are reproducible. Ten replicates of each sample were assayed, and the experiment was performed three times with different batches of Sansure Magb kits. The amplification charts show that the high and low samples were easily distinguishable (Figure 1).

To assess the intra-assay variability, the means, standard deviations, and coefficients of variation (CV) for the threshold Ct values and logarithmic concentrations of the ten samples within each individual experiment were calculated. According to the data, the CV values ranged between 0.35% and 3.46% (Table 1, Exp. 1, Exp. 2, and Exp. 3 intra-assay variability). These results suggest that the concentration determined by the Sansure Magb experiments is highly reproducible.

To further determine the inter-assay variability, the means, standard deviations, and coefficients of variation (CV) for the threshold Ct's and logarithmic

concentrations of all 30 samples combined (10 from each experiment) were calculated. The CV values ranged between 0.56% and 3.65%, which is similar, but slightly higher than, the intra-assay range (0.35% to 3.46%) (Table 1, Exp. 1–3 inter-assay variability). Collectively, the data suggest that both the intra-assay and inter-assay variabilities for the Sansure Magb assay are low, which indicates that the assay is highly reproducible.

Comparison of the sensitivity and specificity of the Sansure Magb and CAP/CTM assays for HBV DNA quantification

The Sansure Magb assay is reported by the manufacturer to have a slightly more favorable range of detection than that of the CAP/CTM assay (Sansure Magb: 10 IU/mL - 8.7×10^8 IU/mL; CAP/CTM: 12 IU/mL - 1.1×10^8 IU/ml). However, the LOD for the two assays have not been directly compared. To compare the sensitivity of the Sansure Magb and CAP/CTM assays, a series of eight twofold dilutions of the low-concentration reference sample were first prepared, and the concentrations were measured in triplicate by each

Table 1. Statistical analysis of intra- and inter-assay precision of experiments 1–3.

| | Value assessed | High-concentration HBV sample | | Low-concentration HBV sample | |
|-------------------------|----------------|-------------------------------|---------------------------|------------------------------|---------------------------|
| | | Ct | Logarithmic concentration | Ct | Logarithmic concentration |
| Exp. 1 | Mean | 19.98 | 6.50 | 33.40 | 2.30 |
| Intra-assay variability | STDEV | 0.0917 | 0.0281 | 0.2302 | 0.0728 |
| | CV | 0.46% | 0.43% | 0.69% | 3.16% |
| Exp. 2 | Mean | 19.97 | 6.50 | 33.33 | 2.33 |
| Intra-assay variability | STDEV | 0.0753 | 0.0227 | 0.2593 | 0.0805 |
| | CV | 0.38% | 0.35% | 0.78% | 3.46% |
| Exp. 3 | Mean | 19.94 | 6.52 | 33.08 | 2.41 |
| Intra-assay variability | STDEV | 0.1664 | 0.0527 | 0.2338 | 0.0732 |
| | CV | 0.83% | 0.81% | 0.71% | 3.04% |
| Exp. 1–3 | Mean | 19.96 | 6.51 | 33.27 | 2.34 |
| Inter-assay variability | STDEV | 0.1153 | 0.0363 | 0.2728 | 0.0856 |
| | CV | 0.58% | 0.56% | 0.82% | 3.65% |

STDEV: standard deviation; CV: coefficient of variation.

Table 2. Comparison of the Sansure Magb and CAP/CTM assays for the detection of limiting dilutions of HBV DNA

| Sample dilution | Log value, Sansure Magb assay (mean ± SD) | CV%, Sansure Magb assay | Log value, CAP/CTM assay (mean ± SD) | CV%, CAP/CTM assay |
|-----------------|---|-------------------------|--------------------------------------|--------------------|
| Undiluted | 3.855 ± 0.022 | 1.69% | 3.854 ± 0.018 | 1.40% |
| 2X dilution | 3.558 ± 0.020 | 1.88% | 3.554 ± 0.018 | 1.54% |
| 4X dilution | 3.283 ± 0.024 | 2.16% | 3.288 ± 0.026 | 2.33% |
| 8X dilution | 2.942 ± 0.024 | 2.46% | 2.919 ± 0.025 | 2.55% |
| 16X dilution | 2.665 ± 0.021 | 2.42% | 2.679 ± 0.016 | 1.80% |
| 32X dilution | 2.347 ± 0.020 | 2.58% | 2.361 ± 0.019 | 2.37% |
| 64X dilution | 2.104 ± 0.019 | 2.74% | 2.027 ± 0.025 | 3.63% |
| 128X dilution | 1.675 ± 0.023 | 4.14% | 1.729 ± 0.023 | 4.07% |
| 256X dilution | 1.395 ± 0.020 | 4.40% | 1.493 ± 0.023 | 4.58% |

Sansure Magb: Sansure magnetic bead assay; CAP/CTM: COBAS AmpliPrep/COBAS TaqMan real-time PCR; CV: coefficient of variation.

method. Results showed that all the eight dilutions could be detected by both the Sansure Magb assay and the CAP/CTM assay (Table 2). These findings suggest that the Sansure Magb assay is at least as sensitive as the CAP/CTM assay.

To further determine whether the Sansure Magb is comparable to the CAP/CTM assay for quantifying HBV DNA in patient samples, 635 samples were collected from patients with hepatitis B. Out of these samples, 407 (64%) tested positive by both methods, 152 (24%) tested negative by both methods, 63 (10%) were positive only by the Sansure Magb assay, and 13 (2%) were positive only by the CAP/CTM assay (Table 3). Among the samples that were positive by both methods, 386 (94.84%) showed quantitative differences of less than 1 log unit, and 21 (5.16%) showed quantitative differences of between 1 and 2 log units. These results suggest that the two methods yield highly similar results, but that the Sansure Magb assay may, in fact, have a higher sensitivity.

To further assess the specificity of the two methods, HBV DNA levels in 10 healthy controls were measured. All the controls tested negative by both methods, suggesting that both the Sansure Magb and the CAP/CTM are specific (data not shown). Collectively, the data suggest that the Sansure Magb assay is at least as sensitive and specific as the CAP/CTM assay.

Assessment of the correlation between the two assays

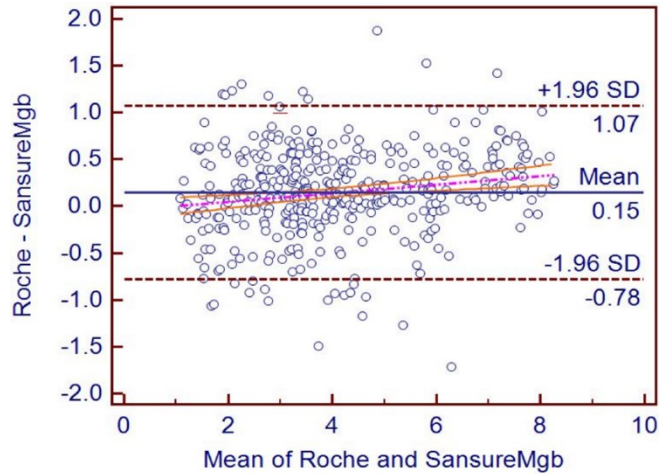
Pearson’s correlation analysis was performed on the 407 samples that tested positive using both methods. The results showed a correlation coefficient of $r = 0.933$ ($p < 0.05$) (data not shown). The regression equation was as follows:

$$\text{Value Sansure} = 0.053 + 1.005 \text{ Value Roche}$$

These results suggest that the HBV DNA levels quantified using Sansure Magb assay closely correlated with those found using the Roche CAP/CTM assay.

To further analyze the agreement between two different assays, a Bland-Altman plot (difference plot) was constructed. As shown in Figure 2, for the 407

Figure 2. Bland-Altman plot of the Sansure Magb assay and the Roche CAP/CTM assay.



The x-axis shows the mean value measured using the two methods, and the y-axis shows the difference between the values measured using the two methods (in log unit). Dotted red lines show the 95% limits.

samples found to be positive using both methods, the mean difference of the two sets data was 0.15 log units (the middle solid horizontal line), with a standard deviation of 0.47. The 95% limits of agreement (upper and lower limits denoted by the upper and lower horizontal solid lines, respectively) was $0.15 \pm 1.96 \times 0.47$, i.e., (-0.78, 1.07). Only 6.6% (27/407) of the data points were outside the 95% limits of agreement. For the data points that fell within the 95% limits of agreement, the maximum difference between the values measured using the Sansure Magb assay and the Roche CAP/CTM assay was 1.07 log units. The magnitude of this difference is considered clinically acceptable. These results confirm that the two methods show satisfactory agreement.

Discussion

The CAP/CTM assay is currently the standard for HBV DNA determination. However, the cost of this assay may be prohibitive, especially in countries where hepatitis is common. In an effort to facilitate the application of a less expensive assay for HBV DNA detection, we tested the Sansure Magb assay. Initial

Table 3. Comparison of HBV DNA positivity results for the Sansure Magb and CAP/CTM assays

| HBV DNA results (n = 635) | | CAP/CTM assay | | |
|------------------------------|----------|--------------------------------------|---------------------------------------|-----------|
| | | Positive 407 (64%) | | Negative |
| Sansure Magb assay | Positive | 386 (94.84%) | 21 (5.16%) | 63 (10%) |
| | Negative | Quantitative difference < 1 log unit | Quantitative difference < 2 log units | 152 (24%) |
| | | 13 (2%) | | |

CAP/CTM: COBAS AmpliPrep/COBAS TaqMan real-time PCR.

assessment of 10 replicate high- and low-concentration samples in three independent experiments demonstrated that the assay has acceptable intra- and inter-assay variability. The CV values were less than 5%, suggesting that the Sansure Magb assay possesses good reproducibility.

We also performed experiments to directly compare the sensitivity and specificity of the Sansure Magb and CAP/CTM assays. Limiting dilution assays demonstrated that the sensitivity for the Sansure Magb assay is at least as great as for the CAP/CTM assay. Furthermore, using 635 samples from chronic hepatitis B patients, we identified 407 samples as positive by both assays. An additional 63 samples tested positive by the Sansure Magb assay only, while 13 samples tested positive by the CAP/CTM assay, which suggests that the Sansure Magb assay may, in fact, be more sensitive. Overall, the Sansure Magb assay and Roche CAP/CTM assay have similar LODs and linear ranges with respect to the quantification of HBV DNA, and the former is slightly better (LOD lower, linear range larger) than the latter, which could explain why the Sansure Magb assay was able to detect more positives. Additional experimentation using healthy control samples demonstrated specificity for the Sansure Magb assay. Systematic comparison of the quantification results of 635 blood samples of chronic hepatitis B patients demonstrated that the two sets of data correlate highly. Furthermore, Bland-Altman plot analysis showed that the maximum difference in the HBV DNA level among the 407 samples that tested positive using both methods was 1.07 log units, which is clinically acceptable.

The Sansure Magb was carefully designed for optimal detection of HBV DNA. The S-gene, which is targeted in this assay, encodes the surface antigen (HBsAg). Though the clinical significance of S-gene mutants remains to be further investigated [11], the variability of this gene represents a challenge for the sensitivity of immunologic and molecular-based assays. Therefore, the magnetic bead assay was designed to specifically target a conserved region of the S-gene.

HBV viruses are categorized as eight genotypes (A–H), which have a distinct geographical distribution. Differences in genotypes affect disease severity, course, likelihood of complications, and response to treatment and possibly vaccination [12]. The most common HBV genotypes in China are B and C, though genotypes A and D can also be found [13]. Consistent with the epidemiological statistics in China, the Sansure Magb assay was established for use primarily with genotypes B and C, and the genotype test results fully met the quality requirements of the standard product [14].

Magnetic beads are generally used as labels in diagnostic immunoassays. Usually, the magnetic beads are stable over time and are not affected by reagent chemistry or photo-bleaching, which makes them an ideal carrier in the Sansure Magb assay. The magnetic background in a bio-molecular sample is usually insignificant. Furthermore, sample turbidity or staining has no impact on magnetic properties, and magnetic beads can be manipulated remotely by magnetism. Nucleic acids that are attached to magnetic beads can be extracted with high sensitivity, which eliminates the need for heating or centrifugation and saves time. Furthermore, the assay shows strong anti-interference ability and is not affected by high blood fat or other variables between specimens. The ability to use ROX dye as an internal control also helps to eliminate variations among different samples while improving accuracy in quantitation. These features may have contributed to the low levels of variability of the Magb assay.

Conclusions

Overall, our results show that the Sansure Magb assay can be used as an affordable alternative to the Roche CAP/CTM assay for HBV DNA testing without sacrificing sensitivity or accuracy. This is of particular significance in developing countries, where the high cost of the Roche CAP/CTM assay can pose a serious obstacle to the widespread application of HBV DNA quantitative detection and impede the efficient and reliable diagnosis of HBV infection and determination of prognosis of chronic HBV-related liver diseases.

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Corresponding author

Deming Tan, Key Laboratory of Viral Hepatitis, Department of Infectious Disease; Xiangya Hospital, Central South University, Xiangya Road 87, Changsha, China
 Phone.: +86-731-8432-7221; Fax: +86-731-8432-7281.
 Email: dmt3008@163.com

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