

Quantification of hepatitis B virus (HBV) DNA and HBV genotyping simultaneously in plasma using real-time polymerase chain reaction

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ABSTRACT

Background: In Thailand, an estimated three million people are chronically infected with hepatitis B virus (HBV), mostly with genotypes C and B. Level of HBV replication as measured by quantification of HBV DNA in blood is crucial for management of chronic hepatitis B. However, HBV DNA measurement is not widely used due to limited availability and high cost of the test.

Objectives: To establish an assay for both HBV DNA load and HBV genotyping based on Fluorescence Resonance Energy Transfer (FRET) real-time polymerase chain reaction (PCR) technique and evaluate it using clinical samples.

Materials and methods: Published primers and FRET probes were used. Whole genome clones of HBV genotype B and C were used as reference. HBV DNA was extracted from clinical samples using a commercial platform (Abbott *m2000sp* system) and was then measured with the LightCycler® (Roche Diagnostics Applied Science). Results obtained with the in-house assay were compared to those obtained with COBAS® TaqMan® HBV Test, v2.0 (as a reference).

Results: We established an in-house assay with a sensitivity of 100 IU/mL of HBV DNA. Measurement of HBV DNA in 36 clinical samples showed a high correlation between HBV DNA load measured with in-house real-time PCR assay ($R=0.946$), compared to the reference assay. This assay identified HBV genotype B and genotype C based on the melting temperature peaks, 61-64 °C and 52-56 °C, respectively, and identified the presence of mixed HBV B and C genotypes if the minor population represents 10% or more of the whole population. HBV genotyping was possible in 22 of 28 samples with HBV DNA load above 2.00 log₁₀ IU/mL: 4 (18%) were genotype B and 18 (82%) were genotype C.

Conclusion: This in-house real-time PCR assay showed good performance for HBV DNA quantification in clinical samples and could distinguish B or C genotypes. It can represent an alternative to commercial assays for clinical use.

Introduction

Hepatitis B virus (HBV) infection is a major public

health problem worldwide, in particular in Asia where three-quarters of people with chronic hepatitis B infection live.¹ Individuals with chronic HBV infection are at high risk of developing severe complications such as liver fibrosis, cirrhosis and hepatocellular carcinoma. The main goal of therapy for patients with chronic HBV infection is to prevent disease progression and occurrence of complications. The

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level of HBV replication, assessed by quantification of HBV DNA in blood, is the strongest single predictive biomarker associated with disease progression and the long-term outcome of chronic HBV infection. Quantification of HBV DNA is thus crucial for appropriate management of patients with chronic HBV infection. The HBV DNA level varies during the different phase of HBV infection² and is used to define the phase of infection, make decision to start and assess the response to antiviral therapy.¹ In the 2015 World Health Organization (WHO) guidelines for the prevention, care, and treatment of persons living with chronic hepatitis B infection, quantification of HBV DNA is strongly recommended for initiation of treatment and monitoring of chronic infections.³ HBV genotype B and C are predominant in South East Asia, including in Thailand.⁴ Genotype C is associated with a high viral replication, experienced delayed HBeAg seroconversion, development of cirrhosis and hepatocellular carcinoma and non-response to interferon-based therapy.⁵⁻⁸

In Thailand, HBV infection is endemic. Currently, an estimated three million people are chronically infected predominantly with HBV genotype C or B.^{4,9} One criterion for treatment initiation in chronically HBV-infected patients recommended by the Thai Association for the Study of the Liver is a plasma HBV DNA above 2,000 IU/mL.¹⁰ In the context of elimination of viral hepatitis, it is expected that a large number of patients will need to be assessed for HBV replication level. However, HBV DNA assays currently available are commercial assays that are not affordable for many settings. We thus aimed to establish an assay which can measure HBV DNA load and HBV genotyping simultaneously based on Fluorescence Resonance Energy Transfer (FRET) real-time polymerase chain reaction (PCR) technique and evaluate it using clinical samples.

Materials and methods

Optimization of the real-time PCR assay

We used primers and FRET probes that were previous published.¹¹ The assay was based on probes hybridization. We focused on HBV genotype B and C the 2 predominant genotypes in Thailand.^{4,12} Reference strains of HBV genotype B and C were obtained after cloning whole genome of known HBV isolates.

To identify the optimal reaction conditions, we tested 6 different conditions with concentrations of primers and probes vary from 0.2 to 0.7 μ M. The final volume of PCR reaction was 20 μ L including 1X master mix, 2 mM MgCl₂ and 100 fg DNA template. PCR conditions were an initial denaturation step of 95 °C for 10 minutes, followed by 45 cycles of 95 °C for 5 seconds, 53 °C for 10 seconds, and 72 °C for 20 seconds. HBV DNA load was quantified by real-time detection of the emitted fluorescence at the annealing step of each PCR cycle (Roche LightCycler® 2.0 real time system).

To determine the limit of detection of the assay, 10-fold serial dilutions of HBV genotype B and C standard controls starting from 8 log₁₀ IU/mL were mixed in ratio 1:1 and tested as described above.

Evaluation of in-house assay using clinical samples

Clinical samples were obtained from the remaining EDTA plasma samples of HBV-infected individuals who received an HBV DNA load test using the COBAS® AmpliPrep/COBAS® TaqMan® HBV test v2.0 (Roche molecular diagnostics) at the Clinical Microbiology Laboratory Service Unit (CMSU), Associated Medical Sciences-Clinical Service Center (AMS-CSC). To ensure that the clinical samples tested in this study cover the range of all HBV DNA levels, we selected 4-5 plasma samples at each log₁₀ of HBV DNA level.

HBV DNA was extracted from patient's plasma using a commercial automatic DNA extractor (m2000sp Instrument, Abbott laboratories, USA) and the level of HBV DNA was then measured using the in-house real-time PCR on the Roche LightCycler® 2.0 real time system (Roche Diagnostics Applied Science). Results were compared to those obtained from COBAS® TaqMan® HBV Test version 2.0 as reference (Roche diagnostic GmbH, Mannheim, Germany).

HBV genotyping using the in-house real-time PCR assay

After the PCR was completed, HBV genotype B and C were identified by analyzing the melting curve and melting temperature of each genotype. To determine the ability of the assay to detect mix infection of genotype B and C, we analyzed the melting curve of various proportions of HBV standard genotype B and C with the final concentration at 5 log₁₀ IU/mL. HBV DNA quantification was performed using Roche LightCycler® 2.0 real time system and melting temperature peaks were identified in the melting curve.

To verify the genotypes identified by the melting temperature peaks, all samples with detectable HBV DNA were genotyped using a single tube semi-nested multiplex PCR technique which amplified products were visualized with gel electrophoresis.¹³ If the results between the 2 techniques were discrepant, sample's amplicons were then sequenced using primers targeting viral surface and polymerase genes, and the BigDye Terminator Mix V.1.1 (Applied Biosystems).¹² The sequences were aligned using the Bioedit software. HBV genotype was then identified by phylogenetic analysis using clustalW software.

Statistical analysis

Correlation analysis and Bland-Altman plot were generated to compare HBV DNA quantification using the two techniques. The Cohen's Kappa statistical test was used to compare the concordance of the two methods for HBV genotyping. All data analyses were performed using STATA™ version 10.1 software (Statacorp, College Station, TX).

Results

Development of real-time PCR assay for measuring of HBV DNA load and HBV genotyping

We tested 6 experimental conditions to identify the optimal concentrations of primers and probes. The condition corresponding to the concentration of 0.5 μ M for each primer and 0.2 μ M for each probe provided the best result with a high fluorescent signal (Cycle Threshold for

detection of HBV genotype B and C were 25.38 and 27.55, respectively) and lesser amount of reagents and was thus selected for the subsequent analyses. The sensitivity (limit

of detection) of this assay for HBV DNA detection was 100 IU/mL of HBV DNA (Table 1).

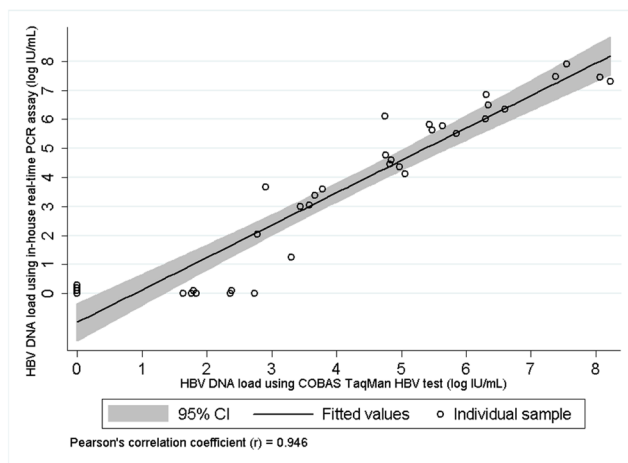
Table 1 Cycle threshold values derived from amplification plots of various concentrations of mixed standard HBV genotype B and C controls (ratio 1:1).

Concentration of standard HBV controls (IU/mL)	Cycle threshold of 1 st run	Cycle threshold of 2 nd run	Mean cycle threshold
100,000,000 (8 Log ₁₀)	15.63	15.78	15.71
10,000,000 (7 Log ₁₀)	20.83	20.93	20.88
1,000,000 (6 Log ₁₀)	24.70	24.25	24.48
100,000 (5 Log ₁₀)	27.72	27.65	27.69
10,000 (4 Log ₁₀)	31.15	31.98	31.57
1,000 (3 Log ₁₀)	36.02	35.63	35.83
100 (2 Log ₁₀)	38.23	38.45	38.34
10 (1 Log ₁₀)	Undetectable	Undetectable	Undetectable
1 (0 Log ₁₀)	Undetectable	Undetectable	Undetectable
0.1 (-1 Log ₁₀)	Undetectable	Undetectable	Undetectable
0.01 (-2 Log ₁₀)	Undetectable	Undetectable	Undetectable

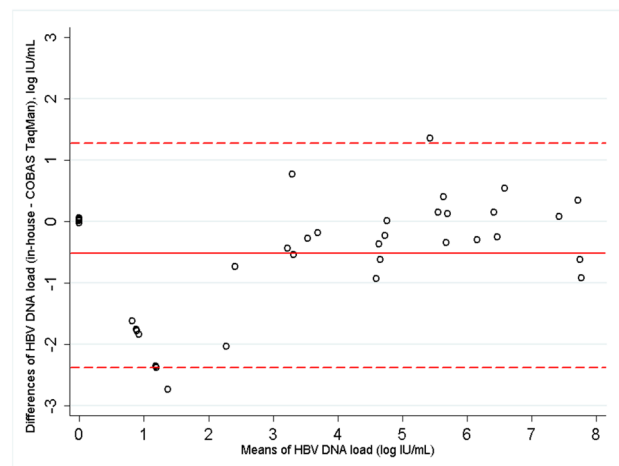
Evaluation of in-house assay for HBV quantification and genotyping

Thirty-six clinical samples were tested for HBV DNA quantification with both in-house real-time PCR assay and the COBAS® TaqMan® HBV Test v2.0 (reference assay). There was a high correlation between the two assays (Figure 1) and an excellent linear relationship (Pearson's correlation coefficient, $r=0.946$) (Figure 1A). Bland-Altman analysis showed the mean difference (in-house HBV DNA

assay – COBAS® TaqMan® HBV Test) was $-0.54 \log_{10}$ IU/mL with standard deviation (SD) of $0.95 \log_{10}$ IU/mL (Figure 1B). The in-house HBV DNA PCR assay could not quantify 7 samples with HBV DNA level ranged from 1.63 – $2.74 \log_{10}$ IU/mL measured by COBAS® TaqMan® HBV test. If only samples with HBV DNA above $3 \log_{10}$ IU/mL measuring by the reference method were considered for Bland-Altman analysis ($n=23$), the mean difference was improved to $-0.22 \log_{10}$ IU/mL with SD of $0.64 \log_{10}$ IU/mL.



(A)



(B)

Figure 1 Log₁₀-transformed HBV DNA level results of 36 samples with both Roche COBAS® TaqMan® HBV test and in-house real-time HBV DNA assays. (A) Correlation between plasma HBV DNA levels measured with the Roche assay and the in-house HBV real-time assay. The line indicates the best fit of the data to a linear regression. (B) Bland-Altman plot of HBV DNA obtained with two assays. Solid line represents the mean difference and the upper and lower dashed lines represent ± 1.96 standard deviation.

Detection of mixed genotype infection

Standard HBV genotype B showed a range of melting temperature peaks around 61-64 °C and standard genotype C showed a range around 52-56 °C (Figure 2). We assessed the melting temperature peaks of mixture of various ratio of standard genotype B and C. The results indicated mixed genotype infection could be detected with a sensitivity of 10%, i.e. viral population representing 10% or more of the whole population (Table 2).

Using the in-house assay, HBV genotype could be clearly identified in 22 of 28 samples tested (79%), 4 (18%)

were genotype B and 18 (82%) were genotype C. By a single tube semi-nested multiplex PCR technique, 15 of these 22 samples were successfully verified HBV genotype: 2 samples with genotype B and 13 samples with genotype C (Table 3). The concordance of the two methods for HBV genotyping was considered to be fair (Kappa=0.41, 95%CI: 0.13-0.68). Of the 6 samples with discrepant results between the 2 techniques, analysis of the HBV sequences showed that 2 samples were genotype B, 4 were C, which were corresponding to the results obtained from the technique based on real-time PCR.

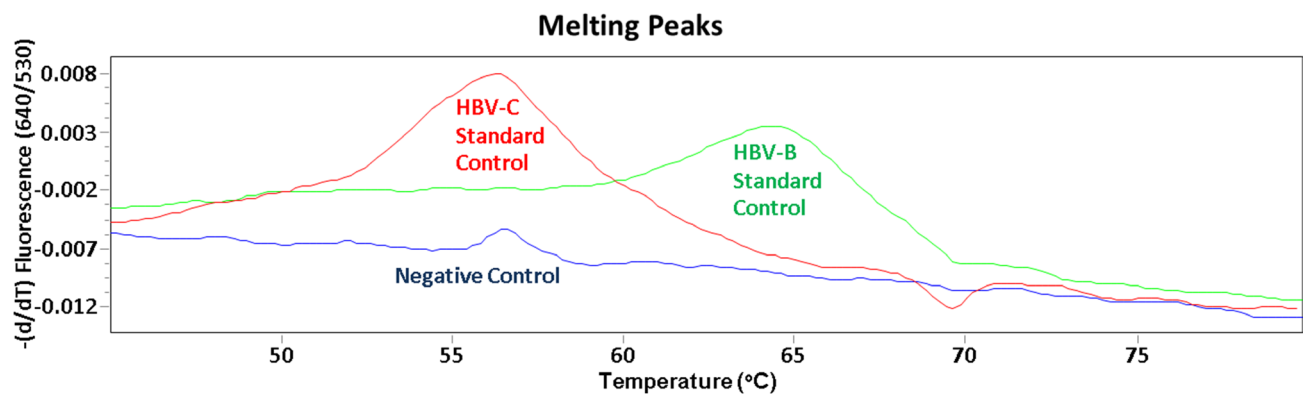


Figure 2 Melting peaks of HBV standard genotype B and C. Plotted as the derivative of fluorescence versus temperature.

Table 2 Melting temperature peaks according to HBV standard genotype B:C ratio.

Genotype B: Genotype C ratio	Melting temperature peak 1	Melting temperature peak 2
100:0	None	62.59
95:5	None	61.86
90:10	52.99	61.53
80:20	53.46	61.26
50:50	54.37	62.53
20:80	55.28	62.35
10:90	55.88	62.86
5:95	55.15	None
0:100	55.57	None
Control	None	None

Table 3 Comparison of HBV genotyping methods: in-house real-time PCR versus single tube semi-nested multiplex PCR.

Genotyping		in-house real-time PCR		
		B	C	Total
single tube semi-nested multiplex PCR	B	2	-	2
	C	-	13	13
	B and C	1	1	2
	Indeterminate	1	3	4
	Not done	-	1	1
	Total	4	18	22

Discussion

We established and evaluated a FRET PCR assay using clinical samples from HBV-infected patients in Thailand. This assay can be used to simultaneously quantify HBV DNA and also identify genotype B or C infecting the patient. This assay displayed a high sensitivity and very good correlation with the COBAS® TaqMan® assay. It was possible to detect a minor virus population representing 10% or more of the whole population.

We evaluated our assay against the COBAS® TaqMan® HBV Test v2.0. Comparison of HBV DNA results showed a high correlation of HBV DNA load between the 2 assays with an excellent linear relationship. However, the correlation was lower for HBV DNA level below 3 log₁₀ IU/mL. This is probably due to the low amount of DNA extract input (10 µL) used in this assay as compared to the input (50 µL) used in the reference assay. However, this 3 log₁₀ IU/mL threshold may not be relevant for clinical use since several guidelines, including the Thai guidelines, for HBV management do not recommend treatment for persons with normal ALT levels and low levels of HBV replication (<2,000 IU/mL).^{3,10}

Furthermore, our assay was able to accurately identify HBV genotypes as compared to the single tube semi-nested multiplex PCR technique in 71.4% (15 of 21). However, our assay could also identify HBV genotype in 6 samples that could not be clearly identified with the single tube semi-nested multiplex PCR technique. This discrepancy may be due to the high sensitivity of in-house assay based on real-time PCR while the single tube semi-nested multiplex PCR technique is a conventional technique and use gel electrophoresis for detection of PCR products. The whole process (viral isolation, quantification, and genotyping) of this assay takes about 4 hours and its cost is estimated to be 2-3 times cheaper than the commercial HBV DNA quantification assays.

However, this study has some limitations which include: 1) the lack of DNA internal control for extraction step, however the results of HBV DNA highly correlated between the 2 assays, and 2) the low number of clinical samples tested. Additional testing is probably needed to confirm and validate the performance of this assay.

In conclusion, we have established a real-time PCR assay for simultaneous quantification of HBV DNA and HBV genotyping in plasma that can be used in Thailand and in the Asia Pacific region where HBV genotype B and C are circulating. This assay may represent a cheaper alternative to current commercial assays for the quantification of HBV DNA and contribute to appropriate management of patients with chronic HBV infection.

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Conflict of interest:

The authors declare that they have no conflict of interest.

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