Evaluation of a Rapid One-step Real-time PCR Method as a High-throughput Screening for Quantification of Hepatitis B Virus DNA in a Resource-limited Setting

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ABSTRACT

Virological monitoring is the best predictor for the management of chronic hepatitis B virus (HBV) infections. Consequently, it is important to use the most efficient, rapid and cost-effective testing systems for HBV DNA quantification. The present study compared the performance characteristics of a one-step HBV polymerase chain reaction (PCR) vs the two-step HBV PCR method for quantification of HBV DNA from clinical samples. A total of 100 samples consisting of 85 randomly selected samples from patients with chronic hepatitis B (CHB) and 15 samples from apparently healthy individuals were enrolled in this study. Of the 85 CHB clinical samples tested, HBV DNA was detected from 81% samples by one-step PCR method with median HBV DNA viral load (VL) of $7.50 \times 10^3$ IU/ml. In contrast, 72% samples were detected by the two-step PCR system with median HBV DNA of $3.71 \times 10^3$ IU/ml. The one-step method showed strong linear correlation with two-step PCR method ($r = 0.89; p < 0.0001$). Both methods showed good agreement at Bland-Altman plot, with a mean difference of 0.61 log_{10} IU/ml and limits of agreement of $-1.82$ to $3.03$ log_{10} IU/ml. The intra-assay and interassay coefficients of variation (CV%) of plasma samples ($4.7 \log_{10}$ IU/ml) for the one-step PCR method ranged between 0.33 to 0.59 and 0.28 to 0.48 respectively, thus demonstrating a high level of concordance between the two methods. Moreover, elimination of the DNA extraction step in the one-step PCR kit allowed time-efficient and significant labor and cost savings for the quantification of HBV DNA in a resource limited setting.

Keywords: HBV DNA viral load, One-step PCR, Two-step PCR, Resource limited settings.


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Conflict of interest: None

INTRODUCTION

Viral hepatitis is the commonest liver disease in Bangladesh. Globally, over 350 million people are infected with hepatitis B virus (HBV), and around 1 million die annually due to the consequences of this infection.1 Bangladesh belongs to the intermediate prevalence region for HBV infection where carrier rates of varies from 7.5 to 10%.2 Studies from Bangladesh show that HBV is responsible for 31.25% cases of acute hepatitis, 76.3% cases of chronic hepatitis, 61.15% cases of cirrhosis of liver and 33.3% cases of hepatocellular carcinoma (HCC).3-6

Currently, available target amplification assays, such as the polymerase chain reaction (PCR), have a much lower limit of detection (as low as 100 copies/ml) and is becoming more widely available worldwide. They are preferable for the initial evaluation of patients and more importantly, for virological monitoring of both treated and untreated patients. Therefore, HBV DNA seems to be the best predictor in the management of HBV infection.7-9

As more HBV DNA quantitative assays become available, it is important to use an accurate, highly sensitive, real-time PCR having standardized target amplification technology for HBV DNA detection.10 In order to ensure comparability between the assays, HBV DNA levels should be universally reported in IU/ml that have been calibrated with the World Health Organization (WHO) international standard for HBV DNA.11 Simultaneously, the assay employed should equally quantify all HBV genotypes. As there are assay-to-assay
Mg

conserved sequence of HBV-DNA, specific probe, dNTPs,
38 µl of HBV PCR mixture (a pair of primer to target
mixture was added. Each reaction mixture contained
10 minutes at room temperature, and 40 µl of reaction
tubes. Five microliter of test specimen along
HBV-DNA from plasma specimen within 0.2 ml PCR
containing tubes and plasma was separated and stored in
multiple aliquots at –20°C until time of testing. All plasma
samples were tested and quantified for HBV DNA using
both the HBV PCR kits of different testing technologies;
one-step (DNA extraction and amplification in single PCR
tube) HBV PCR kit (Sansure, China) and the two-step
manual DNA extraction followed by DNA amplification) PCR method for quantification
of HBV DNA. Performance characteristics, including
analytical sensitivity, precision and reproducibility, were
also studied.

MATERIALS AND METHODS

The study included a total of 100 blood samples consis-
ting of 85 randomly selected samples from chronic HBV
infected patients who were referred from several health
centers to the Department of Virology, Bangabandhu
Sheikh Mujib Medical University (BSMMU), Dhaka,
Bangladesh, for HBV DNA quantitative testing. Patients
were recruited after taking their verbal informed consent.

Another 15 samples from apparently healthy individuals;
doctors, residents and laboratory personnel's from the
Department of Virology after assessing their HBsAg and
anti-HBc (total) sero-negative status. Briefly, 4 ml of blood
was collected in EDTA (ethylenediaminetetraacetic acid)
tubes and plasma was separated and stored in

Two-step PCR method: The HBV-DNA extraction
procedure was performed with the INSTANT Virus DNA
Kit (AJ Roboscreen GmbH, Germany) according to the
manufacturer's instructions. Briefly, 200 µl plasma was
added into the tube containing 200 µl lysis solution along
with 25 µl of proteinase K, mixed vigorously and incubated
at 50°C for 15 minutes in a heating thermal block. Then,
400 µl of binding solution was added, passed through
spin filter in a 2.0 ml receiver tube, and 500 µl of washing
solution was added. After centrifuging at 12000 rpm
for 1 minute, 650 µl of washing solution was added into
new receiver tubes and centrifuged again. Then, 60 µl
prewarmed elution buffer was added to yield extracted
DNA which was stored at –20°C until DNA quantification.
The extracted HBV DNA was amplified with RoboGene®
HBV DNA Quantification Kit (AJ Roboscreen GmbH,
Germany) according to the manufacturer's instructions. Probes and primers of this kit were specific for a
subsequence of the HBV-S gene encoding HBsAg which
which can amplify all eight HBV genotypes (A-H) with equal
efficiency. Amplification of HBV DNA and standards
were detected by the probes labeled with FAM/Green
channel and internal control was detected in VIC/
Yellow channel. ROX was added as passive fluorescence
dye to eliminate nonspecific amplification. HBV DNA
amplification was performed with the ABI 7300 real-time
PCR System using 5 µl of extracted DNA in a 25 µl of
reaction mixture, containing 9.6 µl of PCR grade water,
2.5 µl of 10× PCR buffer, 5 µl of HBV/IC specific primer,
probe and dNTP's and 0.4 µl of Taq polymerase (5 U/µl).

Thermal cycling conditions used were: 95°C for 4 minutes
followed by 45 cycles at 57°C for 1 minute, 95°C for
30 seconds and 45°C for 30 seconds with total run time of

One-step PCR method: This method utilized 5 µl of
nucleic acid lysis buffer to allow rapid lysis and release of
HBV-DNA from plasma specimen within 0.2 ml PCR
reaction tubes. Five microliter of test specimen along
with 4 quantitative references (provided by the National
Institute for Food and Drug Control, China) were added
to the respective PCR reaction tubes, incubated for
10 minutes at room temperature, and 40 µl of reaction
mixture was added. Each reaction mixture contained
38 µl of HBV PCR mixture (a pair of primer to target
conserved sequence of HBV-DNA, specific probe, dNTPs,
Mg²⁺ , buffer solution), 2 µl of enzyme mixture (Hot start
Taq enzyme, UNG enzyme) and 0.2 µl of positive internal
control (cloning plasmid without HBV target sequence).

HBV-DNA and the four quantitative references were
detected in FAM channel (reporter: FAM, Quencher:
None) and internal control was detected in VIC channel
(reporter: VIC, Quencher: None). ROX was added as
passive fluorescence dye to eliminate variations among
different tubes and achieve more accurate quantification.

HBV DNA quantification was performed using the
following thermal cycling conditions: 50°C for 2 minutes,
94°C for 5 minutes followed by 45 cycle at 94°C for
15 seconds, 57°C for 30 seconds with total run time about
2 hours with the ABI 7300 real-time PCR system. Results
were saved automatically upon completion of the
reactions. Evaluation of precision and reproducibility of
the one-step HBV PCR was performed with four plasma
samples of different concentrations that were run in
duplicates for two consecutive days considering two-step
HBV PCR as a set method.

Evaluation of one-step HBV PCR method: The HBV-DNA extraction
efficiency varies from kit to kit and
test to test, and the extraction process is not only time
consuming but also tend to increase the risk of carry-
over contamination. Accordingly, direct PCR without
DNA extraction for quantitative use has been reported
in several studies. In this study, we analyzed the
performance characteristics and comparability of two
HBV DNA methods based on different technologies: a
one-step (DNA extraction and amplification in single PCR
tube) with a two-step (manual DNA extraction followed
by DNA amplification) PCR method for quantification
of HBV DNA. Performance characteristics, including
analytical sensitivity, precision and reproducibility, were
also studied.
approximately 3 hours. The pre-extracted standards were calibrated using a WHO calibrated reference HBV DNA preparation obtained from the German Federal Agency for Sera and Vaccines (PEI). Sample results were accepted only when the internal control was amplified. HBV DNA concentration was expressed in IU/ml.

Linear dynamic ranges: The linear dynamic ranges of the one-step HBV DNA quantification kit was $1 \times 10^2$ to $1 \times 10^{11}$ IU/ml, whereas it was $5 \times 10^2$ to $5 \times 10^9$ IU/ml for two-step HBV DNA quantification kit.

Quality assessment of the laboratory work: All the tests were performed maintaining proper specimen collection, separation, processing, and storage conditions as per laboratory criteria. Any cross contamination during the work were strictly controlled and retested when detected.

STATISTICAL ANALYSIS

Sample results were log transformed for analysis. Spearman’s correlation coefficient and linear regression analysis were performed to measure overall correlation between assays. Bland-Altman plots were used for analysis of agreement between the assays. All statistical analyses were performed with the SPSS 19.0 software package for Windows. A two-tailed p-value less than 0.05 was considered as statistically significant.

RESULTS

Of the 85 samples from CHB patients, the one-step PCR system detected 69 (81%) samples with HBV DNA level between $3.14 \times 10^2$ IU/ml and $4.67 \times 10^8$ IU/ml (median, $7.50 \times 10^3$ IU/ml), whereas, the two-step PCR system detected 61 (72%) samples with VL ranging from $1.02 \times 10^2$ to $3.13 \times 10^9$ IU/ml (median, $3.71 \times 10^3$ IU/ml). Of the total detected samples, 80% were detected by both one-step and two-step PCR systems, and were quantitated at various level with mean differences in quantification of 0.61 log$_{10}$ IU/ml (one-step and two-step). All 15 apparently healthy individuals tested for HBV DNA by both the PCR kits had undetected VL. The features of both assays are described in Table 1. The intra-assay coefficients of variation (CV%) ranged from 0.33 to 0.59, while the inter-assay (CV%) ranged from 0.28 to 0.48 for the one-step PCR method (Table 2).

Comparison of one-step and two-step PCR methods showed strong linear correlation between the assays ($r = 0.89, 0.97; p < 0.0001$) (Graph 1). These two PCR methods also showed good agreement at Bland-Altman plot, with mean difference of 0.61 log$_{10}$ IU/ml (one-step and two-step) and limits of agreement of $-1.82$ to $3.03$ log$_{10}$ IU/ml (Graph 2).

Table 1: Features of one-step and two-step PCR methods

<table>
<thead>
<tr>
<th>Features</th>
<th>One-step PCR</th>
<th>Two-step PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Kit description</td>
<td>DNA extraction and amplification in single PCR tube</td>
<td>Manual DNA extraction followed by DNA amplification</td>
</tr>
<tr>
<td>Lowest detection limit (IU/ml)</td>
<td>$1 \times 10^2$</td>
<td>$5 \times 10^2$</td>
</tr>
<tr>
<td>Number of detected samples (n, %)</td>
<td>69 (81.2%)</td>
<td>61 (71.8%)</td>
</tr>
<tr>
<td>Median VL (IU/ml)</td>
<td>$7.50 \times 10^3$</td>
<td>$3.71 \times 10^3$</td>
</tr>
<tr>
<td>Category of VL (IU/ml)</td>
<td>$&lt;10^2$</td>
<td>$16 (18.8%)$</td>
</tr>
<tr>
<td></td>
<td>101-10000</td>
<td>$24 (28.2%)$</td>
</tr>
<tr>
<td></td>
<td>10001-999999</td>
<td>$22 (25.9%)$</td>
</tr>
<tr>
<td></td>
<td>$&gt;10^6$</td>
<td>$15 (17.6%)$</td>
</tr>
<tr>
<td></td>
<td>$30 (35.3%)$</td>
<td>$24 (28.2%)$</td>
</tr>
<tr>
<td>PCR technology</td>
<td>Hot start Taq enzyme, UNG enzyme</td>
<td>Taq polymerase</td>
</tr>
<tr>
<td>Log linear relationship</td>
<td>Slope -3.44</td>
<td>-3.23</td>
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<tr>
<td></td>
<td>Intercept 46.83</td>
<td>42.31</td>
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<tr>
<td></td>
<td>Amplification efficiency (R$^2$)</td>
<td>0.99</td>
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<tr>
<td>Methods performance</td>
<td>DNA extraction No</td>
<td>Yes</td>
</tr>
<tr>
<td>Run time (hours)</td>
<td>1:35</td>
<td>2:47</td>
</tr>
</tbody>
</table>

Table 2: Precision and reproducibility of one-step PCR method

<table>
<thead>
<tr>
<th>Expected VL (log$_{10}$ IU/ml)</th>
<th>Interassay variations (log$_{10}$ IU/ml)</th>
<th>Intra-assay variations (log$_{10}$ IU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Run 1</td>
<td>Run 2</td>
</tr>
<tr>
<td>4</td>
<td>4.53</td>
<td>4.32</td>
</tr>
<tr>
<td>5</td>
<td>5.62</td>
<td>5.31</td>
</tr>
<tr>
<td>6</td>
<td>6.61</td>
<td>6.44</td>
</tr>
<tr>
<td>7</td>
<td>7.67</td>
<td>7.41</td>
</tr>
</tbody>
</table>
DISCUSSION

Real-time PCR techniques have greatly improved the ability to quantify wide ranges of HBV DNA concentrations in patients with CHB infection and are the method of choice recommended by the American Association for the Study of Liver Diseases (AASLD).\(^\text{16}\) In this study, we verified the performance specifications of a European conformity (CE) and FDA-approved in vitro diagnostics (IVD)-licensed one-step HBV PCR method (Sansure, China) with CE-marked IVD-licensed two-step HBV PCR method (AJ Roboscreen GmbH, Germany) for quantification of clinical samples for HBV DNA. A variety of commercial assays with different dynamic detection ranges are available for quantitation of HBV DNA in diagnostic laboratories. In order to compare results of viral load generated from different manufacturers, it is essential to have validated, internationally acceptable standards.\(^\text{17}\)

The one-step assay demonstrated excellent analytical sensitivity with a good dynamic range for detection of HBV DNA. Results of VL from the one-step PCR were compared with the two-step PCR validated with the WHO standard of HBV. Furthermore, one-step PCR method showed excellent correlation \(r = 0.89, p < 0.0001\) of the expected HBV DNA values with the two-step PCR.

Precision and reproducibility analysis is a basic requirement of a good quantitation assay, and the one-step PCR exhibited very low interassay and intra-assay variation in our study. The one-step PCR showed a maximum intra-assay variation of 0.59% at a \(10^4\) IU/ml concentration and the assay was linear and reproducible between 4 and \(7 \log_{10}\) IU/ml. The type of samples used for accuracy of the results may also influence the determined performance characteristics of the assay. Therefore, fine tuning of this assay would make it a highly satisfactory HBV VL detection kit.

The main reason for difference between the two methods may be due to the difference in sample volumes, which were 200 µl for two-step and only 5 µl for one-step PCR. Besides, the final elution volume of 60 µl used in the two-step PCR made a substantial difference in the obtained DNA concentration, which was not required for one-step. The effect of sample volume on the sensitivity of HBV detection have been shown in various studies.\(^\text{18,19}\)

Although, the one-step and two-step PCR methods employed different principles for sample processing and targeted different regions for amplification, the results for clinical samples correlated remarkably well. Moreover, avoiding the regular extraction procedure in the one-step HBV PCR limited the use of consumables plastic materials along with other necessary reagents to a great extent. This in turns greatly reduced the cost of the entire one-step PCR assay by nearly half the amount. The mean differences in quantification between assays were 0.61 \(\log_{10}\) IU/ml and showed an adequate level of agreement. Therefore, both the PCR kits can be interchangeably used for therapeutic monitoring of CHB patients.

Our study demonstrated a high level of concordance between the one-step PCR utilizing direct plasma real-time PCR with the classical two-step real-time PCR for HBV DNA quantification. Eliminating the DNA extraction in the one-step PCR allowed considerable work and cost savings. In conclusion, the direct plasma one-step real-time PCR is a simple, economical, time-efficient and accurate method for quantification of plasma HBV DNA. Therefore, it may be used for high-throughput screening and evaluation of prognosis of CHB patients in resource-limited countries like Bangladesh.

The one-step PCR positive and two-step PCR negative or the vice versa results could not be analyzed due to...
variations in sample volume. Furthermore, precision and reproducibility were not vigorously checked due to budget and kit constrains.

REFERENCES