Quantitation of Hepatitis B Virus DNA in Plasma Using a Sensitive Cost-Effective “In-House” Real-Time PCR Assay

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Abstract

Background: Sensitive nucleic acid testing for the detection and accurate quantitation of hepatitis B virus (HBV) is necessary to reduce transmission through blood and blood products and for monitoring patients on antiviral therapy. The aim of this study is to standardize an “in-house” real-time HBV polymerase chain reaction (PCR) for accurate quantitation and screening of HBV. Materials and Methods: The “in-house” real-time assay was compared with a commercial assay using 30 chronically infected individuals and 70 blood donors who are negative for hepatitis B surface antigen, hepatitis C virus (HCV) antibody and human immunodeficiency virus (HIV) antibody. Further, 30 HBV-genotyped samples were tested to evaluate the “in-house” assay’s capacity to detect genotypes prevalent among individuals attending this tertiary care hospital. Results: The lower limit of detection of this “in-house” HBV real-time PCR was assessed against the WHO international standard and found to be 50 IU/mL. The interassay and intra-assay coefficient of variation (CV) of this “in-house” assay ranged from 1.4% to 9.4% and 0.0% to 2.3%, respectively. Virus loads as estimated with this “in-house” HBV real-time assay correlated well with the commercial artus HBV RG PCR assay (r = 0.95, P < 0.0001). Conclusion: This assay can be used for the detection and accurate quantitation of HBV viral loads in plasma samples. This assay can be employed for the screening of blood donations and can potentially be adapted to a multiplex format for simultaneous detection of HBV, HIV and HCV to reduce the cost of testing in blood banks.

Key words: Hepatitis B virus quantitation, real-time polymerase chain reaction

Introduction

Despite the wide availability of good hepatitis B surface antigen (HBsAg)-based detection systems for hepatitis B virus (HBV), there is evidence that transmission of HBV through blood and blood products occurs during the serological window period and more importantly during the later stages of infection due to occult hepatitis B infection. Individuals negative for HBsAg but positive for HBV DNA in blood or tissues with or without the presence of HBV antibodies are categorized as occult HBV infection. In a recent study done from 2001 to 2006 in 93 Italian transfusion centers screening 34,05,497 units, a positivity of 57.8 per million donations with a viral load range of 6 to 3,340 IU/mL was reported (Velati et al., Impact of nucleic acid testing for hepatitis B virus, hepatitis C virus, and human immunodeficiency virus on the safety of blood supply in Italy: a 6-year survey, 2008-in press). Occult HBV infection was detected in 13-71% of liver tissues and in 5-55% of serum from chronic liver disease patients negative for HBsAg and hepatitis C virus antibody (HCV-Ab). Individuals with occult HBV infection have been shown to be infectious to immunocompromised individuals, especially from organ donors. HBV DNA detection in blood banks can therefore reduce the transfusion risk from individuals with such occult HBV infection or variants of HBV, which may escape detection by the existing serological screening methods.

Materials and Methods

Clinical samples

This study included patients with chronic HBV infection who were referred from the departments of Gastroenterology, Nephrology and Haematology to the clinical virology department for purposes of blood collection. All patients were recruited after a verbal consent in addition to a general consent that was obtained for all investigations as part of our routine patient management in this hospital. Blood was collected in tubes containing 0.5% ethylene diamine tetraacetic acid. Plasma was separated from whole blood collected from 30 individuals with chronic HBV infection after centrifugation at 1500 rpm for 10 minutes at 4°C and stored in multiple aliquots at −60°C until time of testing. All the chronically HBV infected individuals were screened for HBsAg (DiaSorin S.p.A., Italy) and HBeAg (DiaSorin S.p.A., Italy). In addition, blood samples were also collected from 70 donors at the Ida Scudder blood bank in this tertiary care centre. All donors were negative for antibody to human immunodeficiency viruses (HIVs) 1 and 2, HBsAg and HCV-Ab. The blood donor samples were not screened for anti-HBcAg IgG or IgM as this is not included in the donor screening in this hospital.
blood bank. Blood was collected from the first 10 donors reporting for the day (Monday through Friday, except on holidays) after completion of the donor’s questionnaire and physical examination. Samples were coded and the identity blinded to the person performing the assay.

In addition, 10 plasma samples each from HIV-1 and HCV RNA-positive chronically infected individuals were also tested. Samples with simulated dual infection containing HBV DNA and HIV-1 or HCV RNA (n = 5) and simulated triple infection containing HBV DNA, HIV-1 RNA and HCV RNA (n = 5) were also tested. Two hundred microlitres each of HBV DNA, HIV-1 RNA and HCV RNA positive samples with viral load ranging from 10^6 to 10^8 IU/mL for HBV DNA, 10^6 to 10^4 IU/mL for HIV-1 and HCV RNA were taken and mixed at different combinations to simulate dual or triple infection. For simulated dual infection samples, 200µl of negative plasma was added to make up the volume to 600µl of samples.

"In-house" HBV PCR assay

Nucleic acid extraction

HBV DNA was extracted from plasma samples using the QIAamp MinElute virus spin kit (Qiagen GmbH, Hilden, Germany). A sample volume of 200 µL was used for the extraction as per manufacturer's instruction. Nucleic acid was eluted in 60 µL elution buffer provided by the manufacturer.

Amplification

The probe for the detection of HBV DNA was labeled with reporter dye (ROX) at the 5’ end and with black hole quencher as the non-fluorescent quencher at the 3’ end (Operon Biotechnologies GmbH, Cologne, Germany). The primer and probe sequences are shown in Table 1. Primer and probe sequences were kindly provided by Prof. Richard Tedder (University College London, London, UK). The specificity of the primers and probe were checked using the BLAST search. This search indicated that the primer and probe set had the capacity to detect all HBV genotypes (A-H). Amplification was performed using 10 µL of extract in a 25 µl volume containing 12.5 µL of 2X QuantiTect Multiplex RT-PCR NoROX buffer and 10 pm each of HBV primers (HBV TAQ 1, HBV TAQ 2) and 10 pm of HBV probe (HBV TAQ PR). The reaction mixture was amplified using the following thermal cycling conditions: 50°C for 30 min followed by 95°C for 15 min and 45 cycles at 95°C for 45 s and 60°C for 75 s. Amplification and detection of HBV DNA was performed using the Rotor-Gene™ 3000. Standard curves were generated using the in-built software (Rotor-Gene version 6.0) in the Rotor-Gene 3000. The assay described here is adapted from the previously published work.[6]

Artus HBV RG PCR (commercial) assay

Nucleic acid extraction

HBV DNA isolation was also performed using the QIAamp Blood mini kit as per the manufacturer’s instructions. A sample volume of 200µL was used for extraction. Elution was performed using 50 µL of AVE buffer (provided by the manufacturer).

Amplification

This assay amplified a 134 bp region of the HBV genome that was detected by the cycling A.FAM channel. This assay uses an internal control that was detected by the cycling A.JOE channel. All the five standards provided by the manufacturer were pre-extracted. Amplification and detection of HBV DNA was performed using the Rotor-Gene 3000 as per the manufacturer’s instructions. Standard curves were generated using the in-built software (Rotor-Gene version 6.0). A sample result is accepted only when the internal control is amplified.

HBV genotyping

This “in-house” real-time PCR was further evaluated for its capacity to detect the various genotypes prevalent among individuals seen in this tertiary care centre. HBV genotyping was performed using PCR-RFLP as standardized in this laboratory.[7]

HBV “in-house” standard

The “in-house” standard was made from a clinical HBV DNA-positive sample. This was vortexed, aliquoted and stored at −60°C. This standard was run in parallel with the WHO international standard for HBV DNA 97/746 from the National Institute for Biological Standards and Control (NIBSC, UK) to assess the HBV viral load. This “in-house” standard was further validated by testing it in triplicate in three different assays.

Results

Calibration of the “in-house” standard against the WHO

### Table 1: Primers and probes used for hepatitis B virus “in-house” real-time assay

<table>
<thead>
<tr>
<th>Primer/probe names</th>
<th>Primer sequences</th>
<th>Nucleotide position</th>
<th>Product size (base pair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HBV TAQ 1</td>
<td>GTG TCT GCG GCG TTT TAT CA</td>
<td>379-398</td>
<td>98</td>
</tr>
<tr>
<td>HBV TAQ 2</td>
<td>GAC AAA CGG GCA ACA TAC CTT</td>
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<td></td>
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<tr>
<td>HBV TAQ PR</td>
<td>ROX-CCT CTT CAT CCT GCT GCT</td>
<td>403-430</td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATG CCT CAT C-BHQ</td>
<td></td>
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</table>
The viral load of the “in-house” standard as assessed using the WHO standard was estimated to be $10^8$ IU/mL. Ten-fold dilutions of this HBV standard were made down to 10 IU/mL using normal human plasma and dilutions from $10^6$ IU/mL to 10 IU/mL were tested in all the runs to generate the standard curve.

**Sensitivity (lower limit of detection)**

Log dilutions of the WHO international standard for HBV DNA 97/746 for HBV DNA were performed to assess the analytical sensitivity of this “in-house” HBV real-time assay. The lower limit of detection of this assay as assessed by the second WHO international standard for HBV DNA 97/746 was 50 IU/mL.

**Specificity**

Seventy blood donor samples that were negative for HBsAg were negative by this “in-house” assay. Ten samples each that were positive for HIV-1 and HCV RNA were also negative in this HBV real-time assay. HBV DNA in five samples each of the simulated dual infection (HBV with HCV or HIV-1) and triple infection (HCV, HBV and HIV-1) were also correctly identified.

**Reproducibility**

The intra-assay reproducibility of this “in-house” HBV real-time PCR was calculated with triplicate testing of three plasma samples with different concentrations of HBV DNA load (low, medium and high) in the same run. The intra-assay CV ranged from 0.0% to 2.3% [Table 2] and the interassay CV ranged from 1.4% to 9.4% [Table 3]. The intra-assay and interassay CV for this assay using actual copy numbers ranged from 1.2 to 19.7 and 5.3 to 22.7, respectively.

**Correlation between artus HBV RG PCR real-time assay**

The viral loads of the 30 patients as per this “in-house” HBV real-time PCR assay was compared with the artus HBV RG PCR assay and the correlation coefficient was 0.95 ($P < 0.0001$, 95% CI for $r$ 0.90-0.98) [Figure 1]. The Bland-Altman plot showing the comparison of the two assays is shown in [Figure 2]. The HBV viral DNA loads used for comparison ranged from 341 IU/mL to $7.6 \times 10^7$ IU/mL. Among the 30 samples compared, two samples gave identical results while 25 samples gave a higher viral load (log difference in viral loads ranged from 0.1 to 2.3 Log IU/mL) and three samples gave lower viral loads (log difference in viral loads ranged from 0.1 to 0.3 Log IU/mL) compared with the artus HBV RG PCR real-time assay.

The serological profile of the thirty patients samples are given in Table 4. All the samples were HBsAg positive. The correlation coefficient for HBeAg positive and negative samples compared between the artus and the in-house assay were 0.91 and 0.96 respectively.

**Detecting HBV genotypes prevalent in this region**

The HBV genotypes tested include HBV genotype A ($n = 6$), D ($n = 19$) and C ($n = 5$). These “in-house” HBV assays detected all the tested genotypes prevalent in this region.

**Discussion**

We describe an “in-house” real-time PCR that quantitates plasma HBV DNA in infected individuals with good accuracy. This assay has excellent analytical sensitivity with a good dynamic range for detection of HBV DNA.

For quantitation of HBV DNA there are a variety of commercial assays available that are routinely used in diagnostic laboratories. Most commercial quantitative assays have a limited dynamic range. Hence, clinical samples may need to be diluted and retested to get the exact viral load. This “in-house” HBV real-time PCR assay exhibits a wide dynamic range of approximately $10^8$ IU/mL.

### Table 2: Intra-assay variation of HBV viral DNA load in the “in-house” HBV real-time assay

<table>
<thead>
<tr>
<th>Run no.</th>
<th>Sample ID</th>
<th>Replicates</th>
<th>Mean log (IU/mL)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>I</td>
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<td>7.1</td>
<td>7.0</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>5.0</td>
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<td>5.0</td>
<td>5.0</td>
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<tr>
<td></td>
<td>3</td>
<td>2.9</td>
<td>3.0</td>
<td>3.0</td>
<td>3.0</td>
</tr>
<tr>
<td>II</td>
<td>1</td>
<td>6.9</td>
<td>6.8</td>
<td>6.8</td>
<td>6.8</td>
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<tr>
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<td>3.5</td>
<td>3.4</td>
</tr>
<tr>
<td>III</td>
<td>1</td>
<td>6.9</td>
<td>6.9</td>
<td>6.9</td>
<td>6.9</td>
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<tr>
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<td>3.6</td>
<td>3.5</td>
<td>3.6</td>
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</tr>
</tbody>
</table>

All viral load values are shown as log units (IU/mL)
and hence samples do not have to be diluted and retested. The lower detection limit of this assay is 50 IU/mL. Given these results, the assay may be adapted to a pool-testing format for large-scale screening as in blood banks and transfusion centres.

In order to compare the viral load results generated from different laboratories, it is essential to have validated, internationally acceptable standards. We have therefore used the WHO standard of HBV to calibrate an internal standard that was used to quantitate the viral load of the samples, which was expressed in IU/mL. Using this “in-house” standard, the viral load of the samples was compared with a commercial real-time assay. The “in-house” HBV real-time assay showed excellent correlation ($r = 0.95$, $P < 0.0001$) with the commercial assay.

In this study, two different methods of extraction were used for the “in-house” real-time assay and the artus commercial assay. The QIAamp MinElute virus spin kit used for extraction of HBV DNA in the “in-house” real-time PCR assay was used to improve the sensitivity of the assay and with a view to use this extraction method for both RNA and DNA extraction. A small difference in efficiency may exist between both extraction methods used.

This “in-house” assay exhibited very low interassay and intra-assay variation, which is a basic requirement of a good quantitation assay. Further, fine tuning of this assay would make it highly satisfactory as a screening assay.

This assay uses primer and probe from the conserved region of the surface gene of HBV. Recent studies have shown the surface gene PCR-based assays to be efficient and sensitive compared with the core and ‘X’ regions of HBV. Another study compared the COBAS AMPLICOR monitor with an “in-house” real-time PCR amplifying the surface gene. This study reported that the COBAS AMPLICOR monitor assay failed to recognize increasing levels of viraemia in individuals on treatment due to core mutations that develop during treatment.

This assay, which uses the manual nucleic acid extraction protocol and the real-time Taqman chemistry, is very sensitive and can potentially be used to detect occult HBV infection. The total assay duration is 5.5-6 hours, including the extraction protocol. The assay has only a single round of amplification with a better sensitivity compared with conventional nested PCR. The assay is easy to perform and can be used for high-throughput screening. Further, because this is a non-nested PCR, cross contamination, which is inherent to all conventional nested PCR, could be avoided. This assay was able to detect all the HBV genotypes prevalent in this region. Since this assay does not have an internal control, it cannot confirm

### Table 3: Interassay variation of HBV viral DNA load in the “in-house” HBV real-time assay

<table>
<thead>
<tr>
<th>Sample ID</th>
<th>Run No.</th>
<th>Mean log (IU/mL)</th>
<th>SD</th>
<th>CV (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>7.0</td>
<td>6.8</td>
<td>6.9</td>
<td>1.4</td>
</tr>
<tr>
<td>2</td>
<td>5.5</td>
<td>5.3</td>
<td>5.3</td>
<td>0.25</td>
</tr>
<tr>
<td>3</td>
<td>3.0</td>
<td>3.4</td>
<td>3.3</td>
<td>0.31</td>
</tr>
</tbody>
</table>

All values are shown in log units (IU/mL).

### Table 4: Serological profile of the samples included in this study

<table>
<thead>
<tr>
<th></th>
<th>HBsAg</th>
<th>HBeAg</th>
</tr>
</thead>
<tbody>
<tr>
<td>POS</td>
<td>30</td>
<td>16</td>
</tr>
<tr>
<td>NEG</td>
<td>0</td>
<td>14</td>
</tr>
</tbody>
</table>

and hence samples do not have to be diluted and retested. The lower detection limit of this assay is 50 IU/mL. Given these results, the assay may be adapted to a pool-testing format for large-scale screening as in blood banks and transfusion centres.

### Figure 1: Correlation of plasma hepatitis B virus (HBV) viral DNA loads between artus HBV RG polymerase chain reaction (PCR) assay and “in-house” HBV real-time assay ($n = 30$)

### Figure 2: Bland-Altman plot depicting the correlation of artus hepatitis B virus (HBV) RG polymerase chain reaction (PCR) assay with the “in-house” HBV PCR assay ($n = 30$). The graph displays a scatter diagram of the differences plotted against the averages of the two measurements. Horizontal lines are drawn at the mean difference (bold line) and at the mean difference plus and minus 1.96 times the standard deviation of the differences.
a true negative sample, which is similar to conventional PCR. This “in-house” assay is considerably inexpensive compared with the commercial quantitative HBV real-time PCR assays currently available. A rough estimate of the cost of this “in-house” assay amounts to less than 50% of the commercial real-time assay.

In summary, this “in-house” real-time HBV quantitative assay could have wide applications in diagnostic laboratories and blood banks.

Acknowledgement

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References


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