Real Time PCR for Viral Load Quantification of Hepatitis Virus B in Côte d’Ivoire, West Africa

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ABSTRACT

Approximately 350 million people are chronically infected with Hepatitis B virus (HBV) worldwide. Detection and quantification of circulating HBV in plasma or serum play an important role in diagnosing and in monitoring of HBV infection as well as assessing response to therapy. Several in-house quantitative assays for HBV-DNA have been developed, based mostly on real-time PCR methodology, showing a remarkable sensitivity and a wide broad linear range for quantification. In Côte d’Ivoire, serological test were used as gold standard for the detection of Hepatitis B surface antigen (HBsAg) and the viral load was not determine or unavailable for all patients. The objective of this study was to evaluate the performance of real time PCR for quantification of viral load of HBV in clinical samples from Côte d’Ivoire. 80 clinical serum samples were tested by real time PCR assay to determine the viral load for HBV. The sensitivity and the specificity were determined by the use of HBV-DNA positive and non-specific DNA negative controls. 66.25 % (53/80) samples resulted positive for real time PCR for HBV-DNA. 50% (40/80) of the clinical samples have showed low titer, while 16.25% (13/80) of samples were detected with high titer and 33.75 % (27/80) were undetectable and negative. The real time PCR showed lower viral load of 5 IU/ml corresponding to 14 copies/ml in clinical samples. The molecular test was specific and reproducible for clinical tested samples. No specific amplification of other DNA pathogens was found positive for real time HBV PCR. This study was the first application of molecular quantification for viral load by using real time PCR method for HBV in Côte d’Ivoire, West Africa.

Keyswoeds: Hepatitis B Virus; real time PCR; quantification; viral load, West Africa

INTRODUCTION

Hepatitis B virus (HBV) is a well-known agent of acute and chronic hepatitis, with an estimated 350 million chronic carriers around the world (WHO 2002). HBV exhibits genetic variability which gives rise to the well-recognized subtypes and genotypes of the virus. Worldwide HBV has been classified into eight genotypes (A-H) on the basis of the entire genomic sequence (Okamoto et al., 1988; Norder et al., 1994; Stuyver et al., 2000; Arauz-Ruiz et al., 2002). HBV genotypes have distinct geographical distributions (Magnus and Norder 1995; Nordor et al 1994). Genotypes A and D occur frequently in Africa and Europe (Lindh et al., 1997; Baptista et al., 2003), while genotypes B and C are prevalent in Asia (Okamoto et al., 1988). Genotype E is almost entirely restricted to Africa, and F is found preferentially in Central and South America (Norder et al., 1993; Doumbia et al., 2013).

HBV is one of the most viral infections in Côte d’Ivoire and the prevalence was estimated by 10-25% for global population. Previous studies indicated that HBV genotype E was predominant in Côte d’Ivoire (Doumbia et al., 2013).
Chronic HBV infection increases the risk for liver cirrhosis and hepatocellular carcinoma. In Africa, the detection of hepatitis infection was based on the serology tests that cannot identify all strains because of the genomic variations.

Detection and quantification of circulating HBV in plasma or serum play an important role in diagnosing and in monitoring HBV infection as well as assessing the response to therapy (Pawlotsky. 2002; Berger et al., 2001). Basis on HBV-DNA viral load, the evolution of the disease can be predicted for the treatment efficacy, the emergence of resistance and the detection of occult hepatitis. Owing to its high sensitivity, specificity, and broad dynamic range, quantitative real-time PCR has become increasingly important in the diagnostic laboratory and has been used for HBV detection, genotyping, and quantification. This study was to evaluate the performance of molecular real time to detect and to quantify HBV viral load in clinical samples.

**METHODOLOGY**

**Clinical Samples**

Eighty serum samples tested positive or negative previously in serological tests anti-Hbs/anti-Hbe by CNR Hepatitis of Institut Pasteur Côte d’Ivoire were used. All patients were tested for HBsAg or anti-HBe/anti-Hbs and 35 samples were previous positive for serological tests. Epidemiological data of the patients were collected for the Hepatitis project in CNR Hepatitis (Data not shown). Informed written consent was obtained from all patients. The serum samples were collected in 2011-2012 from HBV-infected patients and stored at –20°C after serological analysis.

**Viral DNA Purification**

Viral DNA purification was performed using the Nuclisens magnetic extraction protocol (Biomerieux, France). Briefly, 500 µl serum were added to 1 ml of lysis buffer. The volume of 40 µl of silica was added to the solution and incubated 10 min at room temperature. The solution was centrifuged 1 min, 6000 g and the pellet was washing with different washed buffers by magnetic stirring in Minimag (Biomerieux, France). In the end, 50 µl DNA were eluted and stored at –20°C or used immediately in molecular test. The positive DNA control was obtained from a positive anti-Hbs and anti-Hbe serum. Negative clinical samples were used for the DNA purification to evaluate the presence of the inhibitors and the specificity of the molecular test (Table 1).

**Quantitative Real Time PCR**

The molecular test was based on the genotype specific target of HBV surface region (Paraskevis et al., 2010). The assay was carried out into a 7500 Applied Biosystems. The reaction mixture contained 1X Go-Taq Flexi buffer (Promega, Germany), 1.25 mM MgCl₂, Rox dye (Invitrogen, California), 1Unit Go-Taq DNA polymerase, 0.25mM dNTPs (Promega, Germany), 0.375 µM each primer (hbv 305, hbv 460), 0.25 µM Probe (Paraskevis et al., 2010) labeled with carboxyfluorescein (FAM) at the 5’end and with N,N,N,N-tetramethylrhodamine (TAMRA) at the 3’end. The final reaction volume was 20 µl containing 5 µl of extracted viral DNA and 15 µl of PCR-mix. Negative controls were performed with 5µl of sterile RNase free-water. The amplification profile was as follows; initial denaturation: 95°C for 10 min; followed by 40 cycles of amplification at 95°C for 15 sec, 55°C for 30 sec. Fluorescence of FAM liberated from the probe by TaqMan was

<table>
<thead>
<tr>
<th>Samples</th>
<th>Pathogens</th>
<th>Sample type</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Neisseria meningitis</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>2</td>
<td>Mycobacterium ulcerans</td>
<td>Biopsy</td>
</tr>
<tr>
<td>3</td>
<td>Mycobacterium tuberculosis</td>
<td>Cerebrospinal fluid</td>
</tr>
<tr>
<td>4</td>
<td>Pseudomonas aeruginosa</td>
<td>Culture</td>
</tr>
<tr>
<td>5</td>
<td>Human immunodeficiency virus</td>
<td>Plasma</td>
</tr>
<tr>
<td>6</td>
<td>Plasmodium falciparum</td>
<td>Dry Blood Spot</td>
</tr>
</tbody>
</table>

**Table 1. Pathogens tested in the specificity test of real time PCR for HBV**
measured to determine the amplification threshold cycle (Ct), which was the first cycle at which fluorescent emission was 10-fold higher than the standard deviation of the mean baseline emission.

Analytical Sensitivity and Specificity

The analytical sensitivity of the real-time PCR assay was determined by the use of a low-titer dilution series in elution buffer of an HBV genotype E specimen from clinical sample tested positive by serological analysis. HBV-DNA was serial diluted to concentrations of 500,000, 50,000, 5,000, 500, 50 and 5 IU/ml, corresponding to 14.10^6 to 14 copies/ml, respectively. Each sample was tested in duplicate in quantification test. The threshold cycles (Ct) were set manually after PCR was completed to generate the standard curve; the unknowns were then calculated based on the standard curve. 5 µl DNA of several pathogens were for the molecular test for the specificity of the detection (Table 1). The use of others DNA is important because the co-infection and the presence of inhibitors in several clinical samples. The DNA extraction was performed after pre-treatment using the protocol of magnetic extraction performed by Biomerieux. 5 µl of extracted DNA from each serum were tested for the performance of the assay for clinical samples. The inhibitory test performed for all negative samples by 1/10 dilution of DNA.

RESULTS

Analytical Sensitivity and Specificity

The analytical sensitivity observed for real time PCR was 5 IU/ml or 14 copies/ml. An excellent correlation between the target template concentration and the threshold cycle (Ct) was observed (Table 2). The slope and y intercept are 3.25 and 41.41, respectively, and the R^2 value is 0.996. The assay has showed negative results for all pathogens (Table 1) for real time of HBV and the good specificity of the assay (Table 2).

Performance of the Real Time PCR for Clinical Samples

The performance of the assay was tested on 80 samples, 35 samples were previous positive in serological tests (Data not shown). 66.25 % (5/80) serum samples resulted positive for real time PCR for HBV (Table 3).

For the significance of viral load for clinical monitoring, we have presented three titer groups. Among the 80 samples tested for viral load of HBV, 50 % (40/80) of samples have showed low titer by the Ct>30, while only 16.25 % (13/80) of samples were detected with high viral load by Ct<30 (Table 3). The high viral load detected for clinical was higher than 5,000 IU/ml corresponding for 1400 copies/ml. The corresponding of clinical samples result in log copies/ml ranged from 4 to 1 that indicated the sensitivity of the quantitative assay. 33.75% (27/80) of the tested serum samples resulted negative

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Table 2. Clinical linear dynamic range of real time HBV PCR

<table>
<thead>
<tr>
<th>DNA dilution (log)</th>
<th>Threshold cycle (Ct)</th>
<th>Predicted Viral load (IU/ml)</th>
<th>copies/ml</th>
<th>Titer group</th>
</tr>
</thead>
<tbody>
<tr>
<td>10^0</td>
<td>22</td>
<td>500,000</td>
<td>1,400,000</td>
<td>High</td>
</tr>
<tr>
<td>10^-1</td>
<td>25</td>
<td>50,000</td>
<td>140,000</td>
<td></td>
</tr>
<tr>
<td>10^-2</td>
<td>28</td>
<td>5,000</td>
<td>14,000</td>
<td></td>
</tr>
<tr>
<td>10^-3</td>
<td>31</td>
<td>500</td>
<td>1,400</td>
<td>Low</td>
</tr>
<tr>
<td>10^-4</td>
<td>34</td>
<td>50</td>
<td>140</td>
<td></td>
</tr>
<tr>
<td>10^-5</td>
<td>37</td>
<td>5</td>
<td>14</td>
<td></td>
</tr>
<tr>
<td>10^-6</td>
<td>nd</td>
<td>nd</td>
<td>nd</td>
<td>undetectable</td>
</tr>
</tbody>
</table>

nd. not detected
and showed Ct>37 or undetectable amplification signal (Table 3).

DISCUSSION

Molecular quantification of HBV viral load is very important for monitoring response of the therapy and for detection of occult hepatitis (Welzel et al., 2006). In Côte d’Ivoire, the accessibility of viral DNA load is limited because high costs of the tests or the unavailability of molecular tests in national care hospitals. In the current study, we described the first application of a sensitive real time TaqMan PCR in clinical samples for HBV. Although measuring serum HBV-DNA is the gold standard for monitoring viral load, it is relatively expensive and not yet readily available in some areas. The development of quantitative method for viral load contributes to the accessibility by low cost of the diagnosis in tropical countries. Côte d’Ivoire is a country seriously affected by chronic HBV infection and the prevalence is 5-20% of the general population and the predominance of genotype E was recently confirmed in West Africa (Doumbia et al., 2013; Kei et al., 2005).

The new assay showed improved sensitivity of 5 IU/mL corresponding of 14 copies/ml. These results are similar to commercial available TaqMan assays and to previous studies (Weiss et al., 2004 Katsoulidou et al., 2007). The detection limit of the assay is low as previous assay by 16 to 22 IU/ml (Paraskevis et al., 2010). We used the linear regression for viral load quantification and the setting 1IU/ml = 2.8 copies/ml. The DNA extraction magnetic method for all clinical sera can explain the high sensitivity of the real time PCR. Threshold cycles have been found lower in some samples by using other DNA extraction method as phenol chloroform protocol (Data not shown).

Clinically, HBV-DNA titers vary greatly, from levels as high as 1010 copies/ml during acute HBV infection (Whalley et al., 2001), to very low levels in HBe antigen-negative chronic carriers and in patients undergoing antiviral therapy, and in those with occult HBV infection (20).

Low viral load samples were detected in occult hepatitis infection and were determined negative in serological tests. In this study, 43.75% of samples detected previous positive for serological tests, were detected positive with 66.25% in real time PCR. The use of DNA of several clinical pathogens samples for the test specificity is important because the evidence of inhibitors and co-infection with others blood pathogens in tropical countries. The predominance of the genotype E in Côte d’Ivoire explains the use of genotype E as positive control for molecular tests (Doumbia et al., 2013). The specific primers in this study cover the HBV genotype E circulating in the country and the region of the HBV surface region was amplified for this assay (Paraskevis et al., 2010). However the genetic diversity among HBV genotype E strains was very low compared to the other genotypes (Kei et al., 2005). 33.75% of samples were found negative for viral quantification; because the storage of samples was not optimal those have been frozen several times after serological tests. Specific advantages of this assay include the following:

(i) good clinical sensitivity (5 IU/ml);
(ii) excellent clinical specificity (100%);
(iii) broad linear dynamic range (5-5.10^5 IU/ml);
(iv) low sample volume requirement (100-500 µl);
(v) significantly lower cost than commercial assays.

The quality of the molecular assay can depend of several factors in tropical context, the storage and the transport of the sera, the extraction protocol, the qualification of technical workers to avoid false-positive or cross-contamination and the integration in external quality control network (Saldanha et al., 2001). Our results demonstrated the good performance for specificity and sensitivity of real time HBV to detect clinical samples and confirm previous studies for the circulating of HBV genotype E in West Africa (Doumbia et al., 2013; Kei et al., 2005). In this study, we identified the samples in three groups of

Table 3. Viral load detection of clinical samples by real time PCR

<table>
<thead>
<tr>
<th>Samples Testing Results</th>
<th>Low titer (Ct&lt;30)</th>
<th>High titer (Ct&gt;30)</th>
<th>Detectable titer (Ct≤37)</th>
</tr>
</thead>
<tbody>
<tr>
<td>positive</td>
<td>40 (50%)</td>
<td>13 (16.25%)</td>
<td>53 (66.25%)</td>
</tr>
<tr>
<td>negative</td>
<td>40 (50%)</td>
<td>67 (83.75%)</td>
<td>27 (33.75%)</td>
</tr>
<tr>
<td>Total</td>
<td>80 (100%)</td>
<td>80 (100%)</td>
<td>80 (100%)</td>
</tr>
</tbody>
</table>
viral load: high, low and undetectable, but no correlation between epidemiological data, serological and HBV-DNA titer was possible because missing data. Future studies will investigate the seroprevalence of co-infection for HBV and Hepatitis C virus in Côte d'Ivoire that included all epidemiological data and compare different available methods.

These characteristics make the novel real-time HBV-PCR assay described herein particularly well suited for clinical and epidemiological studies for which good sensitivity and genotype inclusivity are mandatory, and the low sample volume and low cost are advantageous in tropical countries. The first application of viral load quantification for HBV in Côte d'Ivoire can ameliorate the availability of quantitative test in HBV for the monitoring for response of therapy in the public care health system.

CONCLUSION

Although measuring serum HBV-DNA is the gold standard for monitoring viral load, it is relatively expensive and not yet readily available in some areas. This is the first application by quantification of viral load for HBV in real time PCR in Côte d'Ivoire and this can ameliorate the detection of HBV by usually the gold serological standard method for all patients. The detection limit and the sensitivity of the assay was very high and can been implanted for clinical samples for the National Health Program for Hepatitis B Virus.

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