Research Article

Design and Evaluation of real-time PCR for Molecular Diagnosis of Hepatitis B

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Abstract

Introduction and aim: The hepatitis B virus (HBV) was discovered in 1965. Approximately 350 million people are infected with HBV. The virus has a worldwide spread and one of the most important complications of infection is chronic hepatitis, cirrhosis, and hepatocellular carcinoma. Hepatitis can be detected by biochemical tests that determine liver function.

Methods: This study was qualitative and 30 patients with suspected hepatitis B referring to Massoud Lab in 2019 were selected. Blood samples were tested for HBsAg positive by multiplex RT-PCR with specific probes and primers as well as *β*-Actin gene as quality control. Then the obtained results were compared with the results of commercial tests.

Results: From 30 samples, 19 samples were HBsAg positive and 11 samples were HBsAg negative. Positive samples were re-examined to confirm the positive results. To compare and evaluate the quality of the results of the designed test, all of these 30 samples were analyzed using another common laboratory kit.

Conclusion: The high sensitivity, good reproducibility, and low cost make this innovative quantitative HBV real-time PCR assay especially well-suited for application to large clinical surveys.

***Keyword*:** *β*-Actin gene, HBV, Hepatitis B, Molecular Diagnosis, Real-time PCR

Introduction

Hepatitis B virus (HBV) is the best-characterized member of a small group of related viruses with a unique genome structure (Southern, 1975; Delius *et al.*, 1983). HBV infection is a major cause of chronic liver disease. It is estimated that nearly 2 billion people are infected worldwide by HBV and that more than 350 million have persistent and chronic infections (Yim and Lok, 2006). HBV carriers have a high risk of developing long-term sequelae of hepatitis B, including cirrhosis and hepatocellular carcinoma (Allice *et al.*, 2007). HBV is the prototype member of a steadily growing family of viruses called hepadnaviruses. Hepadnavir uses can be found in both mammals (orthohepadnaviruses) and birds (avihepadnaviruses) (Okamoto *et al.*, 1988; Fauquet *et al.*, 2005). The hepadnavirus

infecting humans is classified into eight genotypes today. HBV genotypes differ by at least 8% (Okamoto *et al.*, 1988) The genotypes A, B, C, D, E, F, G, and H genotypes have been detected (Naumann *et al.*, 1993; Norder *et al.*, 1994; Stuyver *et al.*, 2000; Arauz-Ruiz *et al.*, 2002; Norder *et al.*, 2004). Due to the genetic diversity of HBV, numerous subgenotypes of HBV have been described (Norder *et al.*, 2004). HBV subgenotypes differ by at least 4% (Kramvis and Kew, 2005). HBV genotypes and most subgenotypes show a distinct geographic distribution.

The global prevalence of HBV infection can be divided into three different geographic categories (Fig. 1): areas with a high prevalence (> 8%), areas with an intermediate prevalence (2 to 8%), and areas with a low prevalence (< 2%) of chronically infected persons. Areas with the highest prevalence include sub-Sahara Africa, much of Asia and the South Pacific Island region, and the Arctic/sub-Arctic region, including western Alaska, the Baffin archipelago of Canada, and all of Greenland. Intermediate areas include much of the Mediterranean region, both southern Europe and North Africa, Eastern Europe (including all of Russia), the Middle East, the Indian subcontinent, and parts of South America (McMahon, 2005).

HBV has the smallest genome among the known eukaryotic viruses and is composed of circular double-stranded DNA with a nick in the noncoding chain and a long gap with the fixed 5 '-terminal end in the coding one (Summers *et al.*, 1975). Three HBV-associated antigens are known including the surface antigen (HBsAg), core antigen (HBcAg), and antigen e (HBeAg) (Tong *et al.*, 2005).

The widely held picture of the HBV genome is of a circular DNA molecule that has about 3,200 nucleotides but is only partly double-stranded. One strand (the long, or L, strand) is complete but has a nick at a fixed position. The complementary strand (the short, or S, strand), however, is incomplete, extending for a variable distance from a fixed 5' terminus, which is some 250 nucleotides 5' to the nick in the L strand. It is generally believed that the S strand is at least 50% of the length of the genome but that after that point it is quite variable in length (Robinson *et al.*, 1974).

The diagnosis of HBV reactivation was based on HBsAg and hepatitis B surface antibody (antiHBs) antibody titers. Early reports, described 2 separate clinical scenarios during immunosuppressive therapy: (1) HBsAg-positive patients experiencing an increase in serum HBsAg titer and (2) HBsAg-negative/anti-HBs–positive patients showing anti-HBs decline associated with the reappearance of HBsAg (seroreversion) (Wands *et al.*, 1975; Tien *et al.*, 2018).

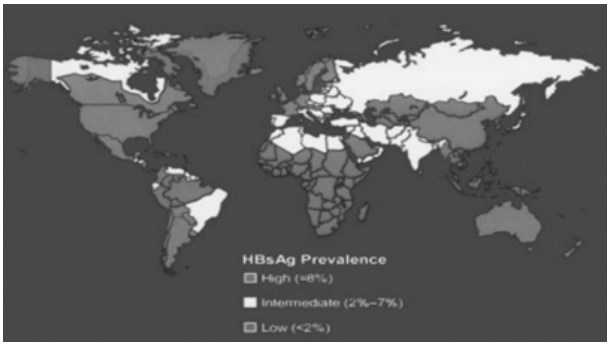

Figure 1. Geographic distribution of chronic hepatitis B virus (HBV) infection. (*From the Centers for Disease Control and Prevention*).

With the availability of quantitative HBV DNA assays, HBV reactivation could be tracked by the temporal relationship of the rise in HBV DNA titers with hepatitis and chemotherapy administration (Lok *et al.*, 1991; Yeo *et al.*, 2000). Other definitions have been used, such as "the occurrence of hepatitis during or immediately after cytotoxic chemotherapy, accompanied either by an increase in HBV-DNA levels of 10-fold, or an absolute increase that exceeds 9log10 copies/mL, in the absence of other systemic infections (Yeo *et al.*, 2000). Thus, other than close monitoring before, during, and after chemotherapy, the sensitivity of the HBV DNA assays is crucial. (Pawlotsky, 2002). Chronic infection with the hepatitis B virus (HBV) is a major risk factor for development of hepatocellular carcinoma (HCC). The

pathogenesis of cancer in HBV infection has been extensively analyzed, and multiple factors appear to play a role (Bréchot, 2004).

Over 95% of acutely infected adults completely and spontaneously recover from the infection, while most neonatally transmitted infections become persistent (Ganem and Prince, 2004). Chronic HBV infection often progresses to the development of life-threatening complications such as cirrhosis and hepatocellular carcinoma (HCC) (Bréchot, 2004). On a worldwide basis, over 350 million people are chronically infected by HBV and about 1 million of them die each year from the complications of chronic infection. As many of these patients do not have a sustained response to currently available therapies (nucleoside analogs and/or interferon) (Ganem and Prince, 2004), it is very important to improve our understanding of HBV pathogenesis if we are to develop better treatments. The experimental approaches examining HBV pathogenesis have been difficult because the host range of HBV is limited to man and chimpanzees, and because in vitro systems for the propagation of HBV do not exist (Guidotti *et al.*, 1996). Many methods have been described for the quantitative analysis of nucleic acid sequences (Thomas and Mathews, 1980; Lamontagne *et al.*, 2016). Researchers have developed several methods of quantitative PCR and RT-PCR. One approach measures PCR product quantity in the log phase of the reaction before the plateau (Rutledge and Stewart, 2008). This method requires that each sample has equal input amounts of nucleic acid and that each sample under analysis amplifies with identical efficiency up to the point of quantitative analysis. A gene sequence (contained in all samples at relatively constant quantities, such as *β*-actin can be used for sample amplification efficiency normalization. Another method, quantitative competitive (QC)-PCR, has been developed and is used widely for PCR quantitation. QC-PCR relies on the inclusion of an internal control competitor in each reaction (García-Cañas *et al.*, 2004; Zaker Bostanabad *et al.*, 2020). The efficiency of each reaction is normalized to the internal competitor.

Methods

Samples: For validation of assay as well as for sensitivity and specificity testing different panels of clinical samples and material were used in this study:

1) The probes and primers were checked for any cross-reactivity against other non-Hepatitis genomes that may co-exist in clinical samples. It was tested experimentally using a panel of viruses commonly found, to determine the specificity of the multiplex PCR.

2) For further evaluation of real-time PCR assay and to determine diagnostic sensitivity, we used clinical samples (n=30) received in the laboratory for routine testing of HBV in three months (from January 2018 to March 2018).

Samples were from the existing clinical samples bank stored at -80 °C, kept in the laboratory. All the samples had a unique identification number and had been recorded in such a manner that subjects cannot be identified, directly or through identifiers. All positive PCR products obtained in the testing using the PCR assay were confirmed by sequencing using rotor gene 6000.

Nucleic acid isolation: HBV DNA was isolated from 100 µl of serum using the High Pure Viral Nucleic Acid kit (DNA-TECHNOLOGY, Russia). Viral nucleic acids were eluted from the filter column with 50 µl of nuclease-free double distilled water and stored at -80 °C until further use.

Primer design for HBV: A set of primer and probe reported by Garson et al., (2005) was aligned with all the sequences representing eight HBV genotypes (A–H) (Accession no. genotype A: AP007263.1, genotype B: AB602818.1, genotype C: AB644286.1, genotype D: FJ692536.2, genotype E: AP007262.1, genotype F: AF288628.1, genotype G: AP007264.1, genotype H: AB516395.1). Further total of six degenerated

nucleotides were added to make it suitable to amplify all eight HBV genotypes. All eight HBV genotypes which we studied are presented in Table 1. The probe is tagged with 5' Fam as a reporter and 3'BHQ as a quencher at 3' end.

Table 1. Features of designed primer for HBV

Sequence ID	Oligonucleotide sequence 5'–3'	Length	Product size
HBV Fwd	GTGTCTGCGGCG TTTTATCA	20	98
HBV Rvs	GACAMACGGGCA ACATACCTT	21	
HBV Probe	[5' Fam] CCTCTKCATCCK GCTGCTATGCCT YMWC[3'BHQ]	28	

Primer design for Human *β*-actin: An alignment of primers and probe with the nucleotide sequence of *β*-actin, using MEGA is shown in Table 2. The designed probe is tagged with HEX as reporter and quenched 3'BHQ. Complementarity of self-designed primers and probes was tested experimentally by testing primer sets against human total nucleic acid under both reverse transcription (RT) and/or real time PCR conditions. The designed primers and probes were synthesized by integrated DNA technology (IDT) with HPLC purification.

Table 2. Features of designed primers for *β*-actin

Sequence ID	Oligonucleotide sequence 5'–3'	Length	Product size
β-Actin Fwd	ACCGAGCGCGGC TACAG	17	60
β-Actin Rvs	CTTAATGTCACG CACGATTTCC	22	
β-Actin Probe	[HEX]TTCACCAC CACGGCCGAGC[B HQ1	19	

Designing of primers and probes: The self-designed primers and probes targeting HBV were checked bioinformatically, with all the genomes submitted to NCBI and they were found suitable to amplify all the types and subtypes of HBV. They were blasted using the 'NCBI Blast' program with human genome and genomes of other commonly found viruses, available on NCBI database and found no complementarity with any of the above. The primers and probes were tested with more than 4500 whole genomes of HBV submitted to NCBI and it showed that primers and probes were suitable to amplify all the types and subtypes of HBV. This set of primers and probes also did not show any complementarity with the human genome or any other virus genome, as confirmed by the NCBI Blast program. The primers and probe sequences for HBV and *β*-actin are mentioned in Table 1 and Table 2.

Optimization of RT PCR singleplex assay: Optimization of Real-time PCR was performed for singleplex detection of HBV using the Rotor Gene system (England). Concentrations of primers, probes, and Mg2þ were optimized in a final reaction volume of 25 µl to obtain maximum dRn and minimal threshold cycle (CT). The reaction mix contained 10 µl buffer, 0.5 µl of HBV forward and reverse primer and 0.35 µl *β*-actin forward and reverse primer and 0.25 µl probe HBV and 0.2 µl probe *β*-actin and 7.8 µl H_2O (DNAse and RNAse free). Finally, 5 µl extracted total nucleic acid was added to 17.5 µl of prepared master mix making it a final volume of 25 µl. The primers and probes were diluted in a working concentration of 10 pmol/µl. Samples were amplified using the following conditions: preincubation at 10 min at 45 °C, followed by incubation at 10 min at 95 °C, then 45 cycles of denaturation at 95 °C for 15 s, annealing, and extension at 20 S at 54.8 °C, and 15 S at 72 °C, respectively and finally 30 S at 40 °C for cooling. After amplification, a CT value was assigned to each sample. Fluorescent signals were recorded during each annealing step of the amplification cycle. The

human β-actin was to be amplified in all the samples as it is abundant so the primer and probe concentration was reduced to a level that there should not be any effect on the amplification of both the viruses. Amplification reactions were performed with a real time thermocycler (Rotor Gene) program consisting of a preincubation step of 45 °C for 10 min, then a denaturation step of 10 min at 95 °C followed by 45 cycles of denaturation at 95 °C for 15 S, annealing for 20 S at 55 °C and extension at 72 °C for 15 S. The fluorescence was acquired at the time of annealing. All the three channels for FAM, HEX and BHQ1 were selected. All the primers and probes were designed in such a way that there was not any primer dimer or cross dimer formation resulting in unwanted fluorescence.

Results

In this study, serum samples were obtained from 30 patients and after DNA extraction, real-time PCR test kit was performed to evaluate the efficiency and function of designed probes and primers. According to Table 3, all results are in accordance with the results of commercial diagnostic kits and the diagnostic test is quite significant.

No	Commercial Kit	Singleplex RT-PCR
Sample 1	Detected	Detected
Sample 2	Detected	Detected
Sample 3	Not detected	Not detected
Sample 4	Detected	Detected
Sample 5	Detected	Detected
Sample 6	Detected	Detected
Sample 7	Detected	Detected
Sample 8	Detected	Detected
Sample 9	Detected	Detected
Sample 10	Not detected	Not detected
Sample 11	Not detected	Not detected
Sample 12	Detected	Detected
Sample 13	Detected	Detected
Sample 14	Detected	Detected
Sample 15	Detected	Detected
Sample 16	Detected	Detected
Sample 17	Not detected	Not detected
Sample 18	Detected	Detected
Sample 19	Not detected	Not detected
Sample 20	Not detected	Not detected
Sample 21	Not detected	Not detected
Sample 22	Not detected	Not detected
Sample 23	Not detected	Not detected
Sample 24	Not detected	Not detected
Sample 25	Not detected	Not detected
Sample 26	Detected	Detected
Sample 27	Detected	Detected
Sample 28	Detected	Detected
Sample 29	Not detected	Not detected
Sample 30	Not detected	Not detected
Control positive	Detected	Detected
Control Negative	Not detected	Not detected

Table 3. Comparing results of Commercial Kit and Singleplex RT-PCR

Conclusion

The high sensitivity, good reproducibility, and low cost make this innovative quantitative HBV real-time PCR assay especially well-suited for application to large clinical surveys.

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