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Quantitation of hepatitis B virus DNA by real-time PCR using internal amplification control and dual TaqMan MGB probes

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Abstract

Hepatitis B virus (HBV) DNA quantitation is used extensively for monitoring of antiviral treatment of HBV infection. A real-time PCR assay was developed using a TaqMan minor groove binder probe and primers corresponding to HBV pre-core region for HBV DNA quantitation. A 228 bp fragment from this genomic region of HBV was cloned and serial dilutions of plasmid DNA were used as an external DNA standard. Comparison of the real-time PCR quantitation results from 35 clinical serum samples with those obtained by COBAS Amplicor HBV DNA monitor kit (Roche Diagnostics) revealed a significant correlation ($r = 0.92$) for all the samples. The assay showed wide dynamic linear range between 2.5×10^2 and 2.5×10^{10} copies/ml serum. Sera from 25 healthy individuals tested negative indicating the high specificity of the assay. The median coefficients of variation for both intra- and inter-experimental variability were 4.9% and 10.6%, respectively, which indicated remarkable reproducibility. An internal amplification control (IC) was developed to detect the presence of PCR inhibitors in the samples to avoid false negative results. The IC had the same primer binding sites but different internal sequence and it competed with the virus-derived target. The optimum concentration of IC was found to be 100 copies/reaction. The assay was validated by testing serial dilutions of the WHO international HBV DNA standard. Since conserved regions were considered during primer and probe design, the assay should be applicable to all HBV genotypes. The real-time assay will be useful for monitoring HBV-infected patients in routine diagnostic laboratories and in clinical practice enabling analysis of a wide dynamic range of HBV DNA in a single, undiluted sample.

Keywords: HBV DNA quantitation; Real-time PCR; Internal control

1. Introduction

Hepatitis B virus (HBV) can either cause acute self-resolving hepatitis or asymptomatic chronic state or may lead to severe illness such as fulminant hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC) (Beasley, 1988). Hepatitis B is an important global problem and it is estimated that worldwide there are about 400 million people with chronic HBV infection. In India, nearly a third of the patients with acute viral hepatitis, two thirds of the cases of chronic liver disease and hepatocellular carcinoma are caused by HBV and the overall estimated carrier rate is 4.7% (Thyagarajan et al., 1996). HBV was shown to be an important contributing factor to the etiology of HCC in southern and northern India (Jayshree et al., 2003; Sarin et al., 2001). Multiple HBV genotypes (A, C and D) circulate in India (Arankalle et al., 2003; Gandhe et al., 2003; Thakur et al., 2002; Vivekanandan et al., 2004).

Interferon alfa, lamivudine or combinations of both drugs are the therapeutic options available for the management of HBV infected patients (Dienstag et al., 1995; Janssen et al., 2005; Wong et al., 1993). Quantitative and sensitive determination of viral DNA can provide indirect evidence on the level of viral replication, the degree of infectivity and changes of viral DNA during the course of infection. The efficacy of antiviral therapy is evaluated in terms of changes in the levels of HBV DNA (Mommeja-Marin et al., 2003). HBV DNA in serum can be monitored by several commercially available assays such as the Abbott solution hybridization assay, the Digene first and second generation hybrid capture assays, the Chiron quantiplex bDNA assay, the COBAS Amplicor HBA DNA monitor assay (Roche diagnostics), the VERSANT HBV DNA 1.0 assay (bDNA) (Bayer Health care diagnostics). However, these assays

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are relatively difficult to perform, take long time and expensive to restrict their use. Several real-time PCR-based HBV DNA quantitation assays have been developed in recent years using different genomic regions of HBV and different chemistries such as the TaqMan probes (Abe et al., 1999; Loeb et al., 2000; Pas et al., 2000; Weinberger et al., 2000), fluorescence resonance energy transfer (FRET)-based probes (Ho et al., 2003; Weiss et al., 2004; Stelzl et al., 2004), molecular beacon probe (Sum et al., 2004). Some assays used standards from commercial kits such as the Chiron HBV DNA Quantiplex assay and demonstrated detection range of 10 to 10^{10} copies/reaction (Jardi et al., 2001; Paraskevis et al., 2002). Paraskevis et al. (2002) also compared their HBV plasmid DNA and pooled patient sera as standards and obtained similar quantitation results with both standards. A slightly modified method of target capture with the use of an internal control for real-time PCR assay has been used by Shyamala et al. (2004) to broaden the detection range to 50 to 10^{10} IU/ ml. Increased sensitivity, detection of broad linear range of viral load and avoidance of post-PCR manipulations to minimize carryover contamination were important features of all these assays.

A sensitive quantitative competitive real-time PCR assay was developed for HBV DNA quantitation with TaqMan chemistry (Heid et al., 1996), by using the TaqMan minor groove binder fluorescent probes on ABI PRISM 7000 Sequence Detection System. The assay was standardized using a synthetic HBV DNA standard and serially diluted serum samples from hepatitis B patients. The efficacy of the assay was evaluated on 35 HBV DNA positive serum samples and sera from 25 healthy individuals and the results were compared with those obtained with the COBAS Amplicor HBV monitor test (Roche diagnostics). An internal amplification control (IC) was developed to detect the presence of PCR inhibitors in the samples to avoid false negative results. This internal control had the same primer sites but different internal sequence and it competed with the virus-derived target. The amplification of the IC could be distinguished easily from that of the virus by the use of a probe with a different fluorophore label. The sensitivity of the real-time PCR assay was estimated further by testing serial dilutions of the WHO international standard for HBV NAT assays (97/746).

2. Materials and methods

2.1. Serum samples

HBsAg and HBV DNA positive serum samples from chronic hepatitis B patients ($n = 35$) collected earlier and stored at −20 °C were used. Sera from 25 healthy individuals negative for HBV markers were used as negative controls. The same sets of samples were tested with commercially available COBAS Amplicor HBV monitor kit (Roche diagnostics).

The WHO international standard for HBV DNA NAT assays, 97/746, 5×10^5 IU per vial (obtained from National Institute for Biological Standards and Control, U.K.) was reconstituted in 0.5 ml sterile water, diluted serially 10-fold in human serum negative for HBV. Viral concentrations of 1×10^6, 1×10^5, 1×10^4, 1×10^3, 1×10^2 IU/ ml were processed for DNA isolation by a spin column method. The five samples containing 2×10^4, 2×10^3, 2×10^2, 2×10^1 and 2 IU/5 μl each were tested in triplicate in three different experiments and quantitated against the nine-point standard curve (10^9 to 10^1 copies/reaction) obtained with the HBV plasmid standard.

2.2. Isolation of DNA from the serum samples

DNA was purified from 200 μl serum using DNAzol (Invitrogen, Carlsbad, USA) as per the manufacturer's instructions and the DNA pellet was resuspended in 50 μl water. Alternatively, DNA was purified from 200 μl serum by using a spin column method (QIAamp Blood DNA kit, QIAgen, Hilden Germany). The DNA was eluted from the spin column in a volume of 50 μl water. DNA obtained by both methods was stored at −20 °C until used for PCR testing.

2.3. Oligonucleotide primers and Taqman MGB probes

2.3.1. HBV specific primers

Forward primer HBVF1 (nt 1774–1797) 5′-TAGGAGGCTGTAGGCATAAATTGG-3′, reverse primer HBVR1 (nt 1882–1864) 5′-GCACAGCTTGGAGGCTTGT-3′ which amplify 109 bp fragment from pre-core region were designed. For that 60 full-genome sequences, representative of all eight genotypes (A–H) were aligned. Similarly 118 pre-core/core region sequences (done in our lab for pre-core mutant analysis) were aligned and conserved regions were selected to locate the primers and probe using primer-express software (PE Biosystems, Foster, CA). The primers were tested by the BLAST algorithm to ensure that they would only amplify HBV DNA. A TaqMan MGB probe, HBVP1 (nt 1825–1840) 5′-TCACCTCTGCCTAATC-3′ was selected similarly. The probe had reporter dye FAM (6-carboxy-fluorescein) attached to the 5′-end and a non-fluorescent quencher (NFQ) and minor groove binder (MGB) attached to the 3′-end. The probe for competitor, HBVQCP2: 5′-TTC TCC GGC CGC TTG-3′ was labeled with another fluorophore, NED at the 5′-end, NFQ and MGB at 3′-end.

2.4. Construction of HBV standard and competitor DNA template

To obtain a construct for use as an external standard (pHBVEXT) for the quantitation of HBV, a 228 bp fragment encompassing the HBV pre-core region of HBV genome was PCR amplified from a serum sample from a HBV chronic carrier (HBV genotype D) and TA-cloned into pGEM-T Easy vector (Promega, Madison, USA) according to the manufacturer's instructions.

A competitor construct (pHBVQC) was obtained by PCR amplification with a set of composite primers, forward primer: 5′-**TAGGAGGCTGTAGGCATAAATTGG**TGAACAAGATGGATTGCA-3′ and the reverse primer: 5′-**GCACAG-**