Real-Time PCR Assay Using Molecular Beacon for Quantitation of Hepatitis B Virus DNA

Simon Siu-Man Sum,1 Danny Ka-Ho Wong,1 Man-Fung Yuen,1 He-Jun Yuan,2 Jian Yu,3 Ching-Lung Lai,1* David Ho,3 and Linqi Zhang3*

Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Hong Kong Special Administrative Region, and Department of Medicine, Zhongshan Hospital, Fudan University, Shanghai,† People’s Republic of China, and The Aaron Diamond AIDS Research Center, Rockefeller University, New York, New York3

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Hepatitis B virus (HBV) DNA can be detected primarily in the blood of infected patients who are positive for HBV surface antigen (HBsAg) and HBV e antigen. The detection and quantitation of HBV DNA is used extensively for monitoring disease progression and treatment efficacy of chronic HBV infection. Several commercial assays are available for accurate measurement of HBV genomic DNA, but many of them are hampered by relatively low sensitivity and limited dynamic range. The aim of this study was to develop a sensitive and accurate assay for measuring HBV genomic DNA using real-time PCR with a molecular beacon (HBV beacon assay). The performance of this assay was validated by testing serial dilutions of the two EUROHEP HBV DNA standards (ad and ay subtypes) of known concentrations. The assay showed good intra-assay (<7%) and interassay (<5%) variations for both subtypes. Its dynamic range was found to be 104 to 107 copies per reaction (1.0 × 106 to 1.0 × 109 copies ml−1). The assay was further evaluated clinically using serum samples from 175 individuals with chronic hepatitis B. The HBV DNA level measured by this assay showed good correlation with that measured by the commercially available COBAS AMPLICOR HBV Monitor test (r = 0.901; P < 0.001). The higher sensitivity and broader dynamic range of this assay compared to the existing commercial assays will provide an ideal tool for monitoring disease progression and treatment efficacy in HBV-infected patients, in particular for those with low levels of HBV viremia.

* Corresponding author. Mailing address for Ching-Lung Lai: Department of Medicine, Queen Mary Hospital, The University of Hong Kong, Pokfulam, Hong Kong. Phone: (852) 28554252. Fax: (852) 28162863. E-mail: hrlmeldc@hkucu.hku.hk. Mailing address for Linqi Zhang: The Aaron Diamond AIDS Research Center, 455 First Ave., New York, NY 10016. Phone: (212) 448-5000. Fax: (212) 725-1126. E-mail: LZhang@adarc.org.

MATERIALS AND METHODS

Preparation of M13 bacteriophage expressing HBV DNA. A fragment of HBV DNA (S gene; encoding 127 to 164 amino acids) was inserted into the genome of bacteriophage M13. This recombinant M13 phage was propagated in Escherichia coli JM101 at 37°C overnight and harvested by centrifugation. The titer of recombinant phage in the supernatant was estimated by serial 10-fold dilution, followed by counting the PFU on a plate where bacteria had grown (9).

International HBV standards. Two international HBV reference plasma de
developed by the EUROHEP Pathology Group with HBV DNA concentrations of 2.7 × 107 and 2.6 × 106 copies of serotypes ad and ay ml−1 (3) were used for standardization.

Blood samples. Blood samples were collected from 175 patients with chronic HBV infection. These patients had follow-ups in our hepatitis clinic (Queen Mary Hospital, The University of Hong Kong, Hong Kong). All were HBsAg positive for at least 6 months and were negative for antibodies to hepatitis A virus, hepatitis C virus, and hepatitis D virus.
The development of an HBV standard by using bacteriophage expressing HBV DNA. To quantify a genomic target, such as HBV genomic DNA, an accurate and reliable standard is necessary. For DNA standards, most often plasmid DNA and PCR products are the first choice, since they are easy to generate. Measuring the optical densities of plasmid DNA or PCR products can provide rough estimates of the copy numbers of a standard by dividing by the molecular weight of the plasmid or PCR products. However, this method has severe defects, largely due to the variability of optical-density-measuring instruments (spectrometers) in quantifying plasmid and PCR products, which not only varies from laboratory to laboratory but also from person to person in the same laboratory. In addition, plasmid DNA and PCR products are prone to instability, as they have been found to be sensitive to multiple rounds of freezing-thawing and incidental DNase contamination.

The aim of the present work was the development of highly sensitive genomic assays that employ easily prepared and stable DNA-containing internal and external standards.

The advantage of using M13 phage as the standard in a PCR-based assay is that no DNA extraction is required, since the single-stranded circular form of DNA is automatically released into the PCR mixture once it is heated to 95°C. Also, it has been demonstrated by limiting-dilution PCR (10) that the correlation between the number of PFU and the DNA copy number is 100%. Therefore, in this HBV beacon assay, the HBV DNA concentration in the standard was reflected by the concentration of the recombinant M13 phage measured by PFU.

In addition, we further tested the stability of the M13 bacteriophage standard by incubating the recombinant phage at 4, 25, and 37°C, respectively, for 2, 4, 6, 8, 12, and 24 h. After incubations at different temperatures and for different durations, the HBV beacon assay was carried out for each of the incubated M13 bacteriophage standards. The results showed that standards incubated at both 4 and 25°C were stable for all durations and standards incubated at 37°C were stable for 6 h. More importantly, since the HBV fragment containing recombinant genomic DNA is packaged inside the capsid protein of the M13 bacteriophage, it is also relatively resistant to the actions of DNase.

Sensitivity and linear range of the HBV beacon assay. The sensitivity of the HBV beacon assay was determined by quantifying the HBV genomic DNA of known concentration (EUROHEP HBV reference plasma). The lower limit of detection of the HBV beacon assay was found to be 100 copies/ml. A linear relationship ($r^2 = 0.999$) was obtained between the threshold and the log$_{10}$ concentration of the genomic HBV DNA.

Accuracy of HBV beacon assay. The accuracy of the HBV beacon assay was determined by testing EUROHEP standards from a single extraction in 10 separate runs, both undiluted and in sequential ninefold dilutions. The result curve was almost linear against the theoretical value ($r = 0.999; P < 0.001$) (Fig. 1).

Comparison of HBV beacon assay with COBAS AMPLICOR HBV Monitor test. The quantitation values of the HBV beacon assay were compared with those of the COBAS AMPLICOR HBV Monitor test. Among the 175 samples, the HBV beacon assay identified 128 (73%) and the COBAS AMPLICOR HBV Monitor test identified 119 (68%) positive samples. Of the 56 samples which were undetectable by the COBAS Amplicor HBV Monitor test (Roche Diagnostics, Branchburg, N.J.) is a target amplification assay. The assay was performed according to the instructions of the manufacturer.

In order to compare the HBV beacon assay with a commercially available assay, all 175 blood samples were tested with the COBAS Amplicor HBV Monitor test (lower limit of detection, 200 DNA copies/ml). All viral DNA was extracted from 200 μl of serum using the QIAamp DNA Blood Mini kit (QIAGEN GmbH, Hilden, Germany) following the manufacturer’s instructions. DNA was eluted from the QIAamp spin column with 70 μl of DNase-free water, which served as the template for the HBV beacon assay. This DNA preparation had a concentration factor of 2.86 and a yield of 83% ± 3% compared with the original amount of DNA in the sample.

The amplification was performed in a 50-μl reaction mixture containing 1× TaqMan buffer A (Applied Biosystems, Branchburg, N.J.), 25 mM MgCl$_2$, 2.5 mM deoxynucleoside triphosphate, 20 pmol of molecular beacon/μl (5'-FAM-C GTGCGACTGCGCTCTGCTTCTCTTCGAGGG-DABCYL-3', where FAM represents the fluorophore 6-carboxyfluorescein and DABCYL represents a quenching chromophore, 4-dimethylaminophenylazobenzoic acid), 20 pmol of the forward (sense) primer LQZ105/μl (5'-TCGCTGTGAATGTTCTGGCGGCGGTTTAT-3'), 20 pmol of the reverse (anti-sense) primer LQZ106/μl (5'-TAG AGGACAAAACGGGCAACATACC-3'), and 1.00 U of AmpliTaq Gold DNA polymerase. The HBV beacon assay was performed using the PRISM 7000 sequence detection system (Applied Biosystems). The PCR program consisted of an initial denaturation (95°C) for 10 min, followed by 55 amplification cycles (94°C for 15 s and 60°C for 30 s). For each run, a standard curve was created in a 7-log-unit range by 1:10 serial dilutions of the HBV DNA phage standard, and all samples were run in duplicate.

Statistical analysis. Pearson’s correlation coefficient was used to assess the strength of the linear correlation between the log-transformed values of the HBV beacon assay and the COBAS Amplicor HBV Monitor test using SPSS software (version 10.0).
HBV beacon assay) was identified in 11 (19.6%) samples by the HBV beacon assay. The median HBV DNA level of these samples was 1,860 copies/ml, and the range was from 146 to 5,890 copies/ml.

The correlation plot of the log10 HBV DNA copies/ml determined by the COBAS AMPLICOR HBV Monitor test and the HBV beacon assay is shown in Fig. 2. The correlation coefficient found between the two assays with HBV DNA levels above both detection limits was 0.901 (P < 0.001), using the following equation: log_{10} (HBV DNA by COBAS AMPLICOR HBV Monitor test) = 0.901 \times \log_{10} (HBV DNA by HBV beacon assay) = 2.795.

**DISCUSSION**

The quantitation of the HBV DNA level is very useful in monitoring the progression of the disease and the efficacy of treatment in chronic HBV infection (8). As the clinical significance of low HBV DNA levels in chronic hepatitis B patients is increasingly recognized (5, 2), an assay which can detect a lower level of HBV DNA becomes an important tool for such research.

Compared with commercially available assays, the HBV beacon assay has the highest sensitivity (10 HBV DNA copies/reaction, which is equivalent to 100 HBV DNA copies/ml of serum). Also, the linear range of HBV quantitation for most commercial assays is 3 to 4 log_{10} units, while the HBV beacon assay has a linear range of 7 log units.

The HBV beacon assay uses the M13 phage as an accurate and reliable standard. The M13 phage is easy to generate and quantify. Furthermore, it is easy to maintain and transfer due to its resistance to DNase treatment and temperature changes. Importantly, it is precise because no DNA extraction is needed, since a single circular form of DNA is released automatically once the PCR mixture is heated to 95°C.

The two EUROHEP reference standards have been used for the standardization of HBV DNA test kits and also for quality control trials (6, 2). We used the reference standards to validate the HBV beacon assay, and our results show the linearity over 8 log units, while the coefficient of variation was low for both the ad and ay subtypes. According to our results in the evaluation of clinical samples, the specificity of the assay was high. The results of the assay showed high correlation with those of the COBAS AMPLICOR HBV Monitor test. The HBV beacon assay was also more sensitive, since it could detect HBV DNA levels as low as 100 copies/ml. For the 11 samples which were detectable only by the HBV beacon assay, 2 were below the detection limit of the COBAS AMPLICOR HBV Monitor test; the others cannot be explained but are probably due to variations in the operational conditions of the assays. Further studies are required to verify the tests, particularly for samples with very low levels of HBV DNA.

The HBV beacon assay could handle 96 samples for each run, while the COBAS AMPLICOR HBV Monitor test could handle only 21 samples.

In conclusion, the HBV beacon assay allows sensitive and accurate quantitative measurement of serum HBV DNA over a wide range of 7 log units. The M13 bacteriophage as a standard is stable in different temperatures and in the presence of DNase. Therefore, this assay can be reliably used for clinical studies and research on viral loads in patients with chronic hepatitis B.

**REFERENCES**