A latest and promising approach for prediction of viral load in hepatitis B virus infected patients

Naresh Yalamanchili, Rahamathullah Syed, Madhavi Chandra, Vishnupriya Satti[1], Ramachandra Rao, Aejaz Habeeb Mohammed, Khaja Mohammed Nanne

Department of Gastroenterology, Centre for Liver Research and Diagnostics, Owaisi Hospital and Research Centre, Kanchanbagh, Hyderabad, [1]Department of Genetics, Osmania University, Tarnaka, Hyderabad, India

INTRODUCTION: Designing a rapid, reliable and sensitive assay for detection of hepatitis B virus (HBV) variants by real-time PCR is challenging at best. A recent approach for quantifying the viral load using a sensitive fluorescent principle was brushed in this study.

MATERIALS AND METHODS: A total of 250 samples were collected from the outpatient unit, CLRD. Complete Human HBVDNA sequences ($n = 944$) were selected from the National Centre for Biotechnology Information (NCBI), primers and probes were designed and synthesized from the core, surface, and x region. Real-time based quantification was carried out using a standard kit and in-house generated standards and RT-PCR protocols.

RESULTS AND DISCUSSION: The standard calibration curve was generated by using serial dilution 10^2 to 10^8. The calibration curve was linear in a range from 10^2 to 10^8 copies/ml, with an R^2 value of 0.999. Reproducibility as measured by dual testing of triplicates of serum samples was acceptable, with coefficients of variation at 6.5%, 7.5%, and 10.5%. Our results showed that amplification performance was good in the case of the x-region-based design (98%). Out of 100 negative samples screened by enzyme linked immunosorbent assay and the standard RT-PCR kit, one sample was detected as positive with the in-house developed RT-PCR assay, the positivity of the sample was confirmed by sequencing the amplified product, NCBI accession EU684022.

CONCLUSION: This assay is reproducible showing limited inter- and intra-assay variability. We demonstrate that the results of our assay correlated well with the standard kit for the HBV viral load monitor.

Introduction

Human hepatitis B virus (HBV) is the major epidemiological agent chronically infecting more than 350 million people worldwide. The infection can cause acute and chronic liver disease, including cirrhosis and hepatocellular carcinoma (HCC).[1,2] A significant proportion of chronic infections terminate in HCC, leading to more than one million deaths annually.[3,4]

HBV has a circular genome of approximately 3200 base pairs and is divided into eight different genotypes (A to H) based on entire genome sequence divergence of greater than 8%.[5,6] In addition, a number of common mutations have been described in HBV strains and recombinant genomes have been reported.[7-10]These include variability within the basic core promoter, precore/core region, and the pre-S1 gene, which shows great heterogeneity.[11-13]

There is increasing evidence that measuring the level of HBV Deoxyribonucleic acid (DNA) in serum is useful in monitoring the efficacy of antiviral therapy,[3,14] detecting the occurrence of drug-resistant mutants and relapse after discontinuing antiviral therapy.[4] The real-time Polymerase Chain Reaction (PCR) method based on the TaqMan chemistry uses a dual-labeled fluorescent probe containing a reporter dye that is quenched by a

Address for correspondence: Mr. Naresh Yalamanchili, Centre for Liver Research and Diagnostics, Owaisi Hospital and Research Centre, Kanchanbagh, Hyderabad, India. E-mail: naresh_id@rediffmail.com

second fluorescent dye.[15] In real-time PCR, there is a direct relationship between the starting template copy number and the number of cycles needed to measure a positive signal from the reporter dye.[16] However, due to the great heterogeneity in HBV genomic sequences, designing primer and probe sets to detect and quantify all HBV genotypes by real-time PCR is challenging at best. The aim of this study is to develop a newer, rapid, and sensitive assay to detect and quantify HBV in patients.

Materials and Methods

Study samples and controls

A total of 250 samples consisting of HBV positive (*n* = 100), HBV, HCV and HIV negative (*n* = 100), HCV positive (*n* = 25) and HIV positive (*n* = 25) were collected from subjects attending the outpatient unit at Centre for Liver Research and Diagnostics. HBV, HIV, and HCV infections were screened using enzyme linked immunosorbent assay [ELISA-Pathozyme HBsAg (M/S Omega Diagnostics Limited U.K.), Ortho HCV 3.0 ELISA test system with enhanced SAVe, Ortho HIV-1/HIV-2 Ab Capture ELISA Test System (M/S Ortho Clinical Diagnostics USA)]. HBV positive samples were further screened for HBeAg using ELISA and the HBV DNA levels were determined by HBV Real-TM Quant SC (Sacace Biotechnologies, Italy).

Real-time PCR quantification of hepatitis B virus using SmartCycler II

The serum HBV DNA was extracted with a SACACE Ribo-Sorb kit, and real-time PCR was then performed on a SmartCycler II instrument (Cepheidmake) using a SACACE HBV monitor kit. The amplification was performed as follows: initial hot start denaturation at 95°C for 900 s, followed by 42 cycles of denaturation at 95 °C for 20 s, annealing and extension at 60 °C for 40 s. The fluorescence was measured at the annealing stage of each cycle. The quantification of sample was carried out using the internal control co-efficient value and the fluorescent values of both sample and internal control.

Plasmid control template and standard curve preparation

The pGEM-T easy vector (Promega make) was used

to clone the part of Human HBVX-gene. The recombinant plasmid was propagated in *Escherichia coli* and purified. The purified plasmid DNA was quantified at 260 nm using a spectrophotometer. The quantified plasmid DNA was used to derive a standard curve using dilution series 10^2, 10^4, 10^6 and 10^8.

TaqMan primer and probe designing

Complete Human HBV DNA sequences (*n* = 944) were selected from the National Centre for Biotechnology Information (NCBI) nucleotide database, excluded in complete and redundant sequences. Those sequences lacking clear genotype information were genotyped by using NCBI's viral genotyping tool. The sequences were aligned using the K-Align software application tool from European Molecular Biology Information (EMBI) and a consensus nucleotide sequence was generated by the Jalview application tool from EMBI. The consensus sequence was imported into a word file and based on this sequence primer and probe sets were designed from most conserved sites including surface, core, and x regions of HBV genome [Table 1].

DNA isolation and real-time quantification of hepatitis B virus

HBV DNA was isolated using the silica-based technology as mentioned by Boom *et al.*[17] from plasma samples. The PCR was then performed on a SmartCycler II instrument (Cepheid) using primers and probes described in Table 1. The PCR was run in a total volume of 25 µl containing 12.5 µl of template, 11 µl of Premix

Table 1: Designed primer probe sets

Primer and probe.	Nucleotide sequence	Region
Sense primer	5′-TACTGTTGTTAGACGACGA-3′	Core
Anti-sense primer	5′-TAACATTGAGATTCCCGAGAT-3′	Core
Probe	5′-FAM-AAGAACTCCCTCGCCTCGCA–TAMRA-3′	Core
Sense primer	5′-TTCCTCTTCATCCTGCTGCT-3′	Surface
Anti-sense primer	5′-TTAGAGGACAAACGGGCAAC-3′	Surface
Probe	5′-FAM-TGCCTCATCTTCTTGTTGGTT-TAMRA-3′	Surface
Sense primer	5′-ACTCCCCGTCTGTGCCTTCT-3′	x
Anti-sense primer	5′-GATCTGGTGGGCGTTCAC-3′	x
Probe	5′–FAM-CCGGACCGTGTGCACTTCGCTT-TAMRA-3′	x

Ex-Taq (Takara), and 1.5 µl of primers and probe. The amplification was performed as follows: initial hot start denaturation at 95 °C for 10 s, followed by 45 cycles of denaturation at 95 °C for 5 s ,annealing and extension at 60 °C for 20 s. Real-time monitoring was achieved by measuring the fluorescence at the end of the annealing phase for each cycle. The quantitative analyses were conducted by using SmartCycler II analysis software version 2.0 following the manufacturer's instructions (Cepheid).

Results

The standard calibration curve was generated by using the SmartCycler II software and serial dilution 10^2 to 10^8. The calibration curve was linear in a range from 10^2 to 10^8 copies/ml [Figure 1], with an R^2 value of 0.999. Reproducibility as measured by dual testing of triplicates of three serum samples was acceptable, with coefficients of variation at 6.5%, 7.5%, and 10.5%. Good correlation was found between the current study procedure and the standard SACACE HBV monitor test, with all 110 samples included in the study.

To test the performance of our primers and probes in real-time PCR, we used serial diluted sets of known positives of HBV. Our results showed that amplification performance was good in the case of primer and probe set designed from the x region [Figure 2]. Therefore, we constructed a plasmid with x region using the p-GEMT easy vector for being used as a control for deriving the standard curve.
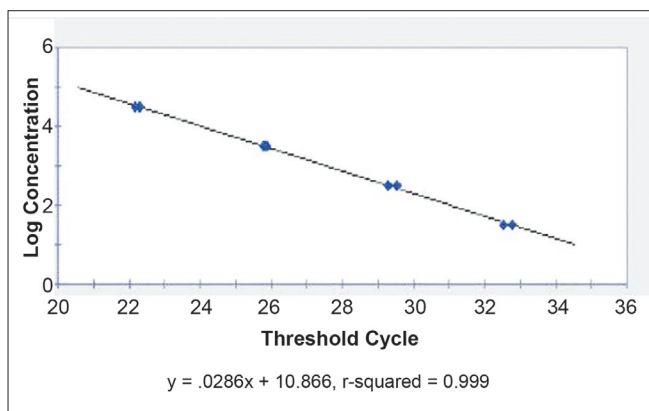
To develop a low cost method and cover all genotypes and serotypes of human HBV, we designed a set of primers and probes in this study. The total cost for analyzing one sample is in and around 250 INR (Indian rupee).

The precision of our in-house real-time assay was determined by assessing the intra- and interassay co-efficient of variation (CV). For interassay variability 10 samples included 8 positive HBV samples with determined viral load and 2 negative samples were screened triplicate in the same assay, which has given a mean CV value of 0.084. Hundred and ten samples previously tested by a SACACE HBV monitor test was tested using our in-house real-time PCR assay.
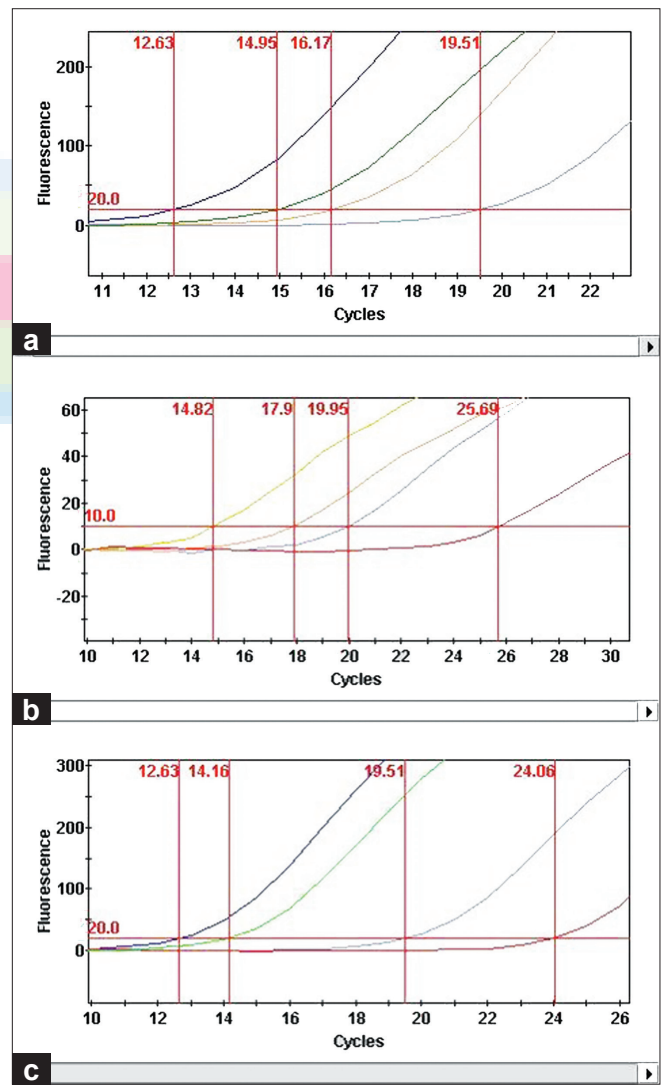

Figure 2: Quantification data: amplification fluorescence plot for various samples using core (a), surface (b) and x (c) oligo sets

$y = .0286x + 10.866$, r-squared = 0.999

Figure 1: Standard curve originated using serial dilutions

Correlation between the two assays was good, with an R^2 value of 0.99.

Out of 100 negative samples screened by the ELISA and standard RT-PCR kit, one sample was detected as positive with the in-house developed RT-PCR assay, the positivity of the sample was confirmed by sequencing the amplified product, NCBI accession EU684022. To avoid the possibility of contamination, this sample was simultaneously processed twice using both standard and in-house assays. Out of four oligo sets designed for HBV detection by RT-PCR, the X gene set showed highest specificity and sensitivity (98%) followed by the surface (74%) and core (70%).

Discussion

We have established and evaluated a sensitive and cost-effective in-house adaptable real-time PCR based assay for effective quantification of Human HBV virus in blood samples with a lower limit of detection of 50 copies/ml. This assay is reproducible showing limited inter- and intra-assay variability and good amplification efficacy in different HBV genotypes and serotypes. We demonstrate that the results of our assay correlated well with the standard kit available in a market for HBV viral load prediction.

We also made the entire quantitative procedure in a low cost and rapid manner, which will be helpful in detecting HBV in patient's blood in less time and accurate manner. The sensitivity of the TaqMan procedure for quantifying viral DNA has already been proved by various studies;[2,3,16,18,19] however, they are being limited by certain aspects such as limited samples, designing of primer, and probes set from regions of unconserved nature.

In this study, we made use of huge sequence database from the NCBI site and analyzed the different regions of HBV for predicting the most conserved regions using latest software applications. Different genotypes and serotypes were analyzed for targeting the conserved segments from entire viral genome. In this study, we have considered the sequences of nonclear genotype information; genotypes of these sequences were predicted by using the online NCBI viral genotyping tool.

Various HBV genotypes undergo rapid mutagenesis because of their reverse-transcription (RT) lacks proof-reading functions[20,21] and also during the therapy there are certain regions being modified specifically as per the drug used,[4,14] so there is a need for reliable quantification tool for predicting the viral load in a specific and sensitive manner. However, in this study we eliminated the drug inducible mutation regions and variable regions for selecting the conserved portion of viral genome.

TaqMan chemistry application in this study can be made applicable in certain commercial fluorescent quantification instruments available in a market at a least price in replacement with high cost real-time PCR. This makes the assay more cost-effective for quantifying the viral load.

References

1. Liu WC, Mizokami M, Buti M, Lindh M, Young KC, Sun KT, *et al*. Simultaneous quantification and genotyping of hepatitis B virus for genotypes A to G by real-time PCR and two-step melting curve analysis. J Clin Microbiol 2006;44:4491-7.
2. Qian WP, Tan YQ, Chen Y, Peng Y, Li Z, Lu GX,*et al*. Rapid quantification of semen hepatitis B virus DNA by real-time polymerase chain reaction. World J Gastroenterol 2005;11:5385-9.
3. Lu YQ, Han JX, Qi P, Xu W, Zu YH, Zhu B. Rapid quantification of hepatitis B virus DNA by real-time PCR using efficient TaqMan probe and extraction of virus DNA. World J Gastroenterol 2006;12:7365-70.
4. Gaeta GB, Stornaiuolo G.Therapy of chronic hepatitis B: Focus on telbivudine. Dig Liver Dis2007;39 Suppl 3: S372-8.
5. Thippavazzula R, Mogili C, Chandra M, Khaja MN, Habeeb MA, Habibullah CM. Prevalent HBV genotypes and subtypes in a South Indian population. J Clin Virol 2006;37:58-64.
6. Sakurai M, Sugauchi F, Tsai N, Suzuki S, Hasegawa I, Fujiwara K,*et al*. Genotype and phylogenetic characterization of hepatitis B virus among multi-ethnic cohort in Hawaii. World J Gastroenterol 2004;10:2218-22.
7. Lai ME, Melis A, Mazzoleni AP, Farci P, Balestrieri A.Sequence analysis of hepatitis B virus genome of a new mutant of ayw subtype isolated in Sardinia. Nucleic Acids Res1991;19:5078.
8. Sugauchi F, Mizokami M, Orito E, Ohno T, Kato H, Suzuki S,*et al*. A novel variant genotype C of hepatitis B virus identified in isolates from Australian Aborigines: Complete genome sequence and phylogenetic relatedness. J Gen Virol 2001;82:883-92.
9. Kurbanov F, Tanaka Y, Fujiwara K, Sugauchi F, Mbanya D, Zekeng L,*et al*. A new subtype (subgenotype) Ac (A3) of hepatitis B virus and recombination between genotypes A and E in Cameroon. J Gen Virol 2005;86:2047-56.

10. Stuyver L, De Gendt S, Van Geyt C, Zoulim F, Fried M, Schinazi RF,*et al*. A new genotype of hepatitis B virus: Complete genome and phylogenetic relatedness. J Gen Virol 2000;81:67-74.
11. Sugauchi F, Kumada H, Acharya SA, Shrestha SM, Gamutan MT, Khan M,*et al*. Epidemiological and sequence differences between two subtypes (Ae and Aa) of hepatitis B virus genotype A. J Gen Virol 2004;85:811-20.
12. Laperche S, Thibault V, Bouchardeau F, Alain S, Castelain S, Gassin M,*et al*. Expertise of laboratories in viral load quantification, genotyping, and precore mutant determination for hepatitis B virus in a multicenter study. J Clin Microbiol 2006;44:3600-7.
13. Behzad-Behbahani A, Mafi-Nejad A, Tabei SZ, Lankarani KB, Torab A, Moaddeb A. Anti-HBc and HBV-DNA detection in blood donors negative for hepatitis B virus surface antigen in reducing risk of transfusion associated HBV infection. Indian J Med Res 2006;123:37-42.
14. Lai CL, Gane E, Liaw YF, Hsu CW, Thongsawat S, Wang Y, *et al*. Telbivudine versus lamivudine in patients with chronic hepatitis B. N Engl J Med 2007;357:2576-88.
15. Holland PM, Abramson RD, Watson R, Gelfand DH. Detection of specific polymerase chain reaction product by utilizing the 5'----3' exonuclease activity of ThermusaquaticusDNA polymerase. Proc Natl Acad Sci U S A1991;88:7276-80.
16. Welzel TM, Miley WJ, Parks TL, Goedert JJ, Whitby D, Ortiz-Conde BA. Real-time PCR assay for detection and quantification of hepatitis B virus genotypes A to G. J Clin Microbiol 2006;44:3325-33.
17. Boom R, Sol CJ, Heijtink R, Wertheim-van Dillen PM, van der Noordaa J. Rapid purification of hepatitis B virus DNA from serum. J Clin Microbiol 1991;29:1804-11.
18. Chopra GS, Gupta PK, Anand AC, Varma PP, Nair V, Ramji R. Real time-PCR HBV-DNA analysis: Significance and first experience in armed forces. Medical Journal Armed Forces India 2005;61:234-7.
19. Abe A, Inoue K, Tanaka T, Kato J, Kajiyama N, Kawaguchi R,*et al*. Quantitation of hepatitis B virus genomic DNA by real-time detection PCR. J Clin Microbiol 1999;37:2899-903.
20. Seeger C, Mason WS.Hepatitis B virus biology. Microbiol Mol Biol Rev 2000;64:51-68.
21. Lu X, Block T. Study of the early steps of the hepatitis B virus life cycle. Int J Med Sci 2004;1:21-33.