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Simultaneous detection of Hepatitis B virus and Hepatitis C virus in human plasma using Taq-man chemistry

Naresh Kumar Y[1], Rahmathulla S[1], Madhavi C[1], Ramachandra V V[1], Vishnupriya S[2], Mohammed Aejaz Habeeb[1], Khaja M N[1].

[1]Center for Liver Research and Diagnostics, Deccan College of Medical Sciences and Allied Hospitals, Kanchanbagh, Hyderabad-500058, Andhra Pradesh, India.

[2]Department of Genetics, Osmania University, Tarnaka, Hyderabad-500007, Andhra Pradesh, India.

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Corresponding author

Naresh Kumar Y
Center for Liver Research and Diagnostics,
Deccan College of Medical Sciences and Allied Hospitals,
Kanchanbagh, Hyderabad-500058,
Andhra Pradesh, India.
Phone: +91 40 24342954.
Fax: +91 40 24342954.
Email: naresh_id@rediffmail.com

Abstract

Designing a rapid, reliable and sensitive assay, for detection of hepatitis B virus and Hepatitis C virus variants by real-time PCR, is challenging at best. A recent approach for quantifying the viral load using the sensitive fluorescence principle, was used in this study. A total of 350 samples were collected from outpatient unit, CLRD. Complete Human HBV DNA and HCV sequences were obtained from the National Centre for Biotechnology Information (NCBI); primers and probes were designed and synthesized from core, surface and x region of Hepatitis B and UTR region of HCV. Real-time based detection was done, using standard kit and in-house generated standards and RT-PCR protocols. A standard curve was generated by using the Smart Cycler II software and serial dilution 102 to 108 of cloned viral regions, the calibration curve was linear in a range from 102 to108 cp/ml for both HBV and HCV, with R2 value of 0.999 and 0.995. Out of 100 predetermined HCV negative samples, 02 samples were found positive with in-house developed RT-PCR assay, the positivity of this sample was confirmed by sequencing the amplified product. Low cost of this assay procedure and précised sample volume will permit the assay to be implemented for routine screening of Hepatitis B and C virus mono-infection and co-infection using Real Time PCR , Nucleic acid Chip technology and Fluorescent End Point detection systems. 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 HBV and HCV virus monitor.

Key words: Hepatitis B virus, Hepatitis C virus, Nucleic acid test, Real-time PCR

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The hepatitis B virus (HBV) and hepatitis C virus (HCV) often cause persistent infection, leading to chronic hepatitis, cirrhosis and hepatocellular carcinoma[1,2]. Given the burden of these diseases and the current potential for therapy, there is a persuasive call for diagnosis of active HBV and HCV infection. A variety of HBV and HCV markers have been used to detect HBV and HCV infection. Nucleic acid tests, such as PCR-based[3-7] assays are a promising approach, to diagnose and

monitor the treatment efficacy in highly endemic infectious viral diseases that include HBV and HCV.

HBV has a circular genome of approximately 3,200 base pairs and is divided into eight different genotypes (A to H) based on entire genome sequence divergence of greater than 8%[8,9]. In addition, a number of common mutations have been described in HBV strains, and recombinant genomes have been reported[10-13]. These include variability within the basic core promoter, precore/core region, and the pre-S1 gene, which shows great heterogeneity[14-17].

There is increasing evidence that measuring the level of HBV DNA in serum is useful in monitoring the efficacy of antiviral therapy[17], detecting the occurrence of drug resistant mutants and relapse after discontinuing antiviral therapy. The real-time PCR method based on the Taq-man chemistry uses a dual-labeled fluorescent probe containing a reporter dye that is quenched by a second fluorescent dye[18]. 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[19]. However, due to the great heterogeneity in HBV and HCV genomic sequences, designing primer and probe sets to detect and quantify all genotypes by real-time PCR is challenging at best. The aim of the present study is to develop a newer, rapid and sensitive assay for simultaneous detection of Hepatitis B Virus and Hepatitis C virus in patients.

Materials and methods

Study samples and controls

A total of 350 samples consisting of HBV positive (n=100), HBV, HCV, MTB and HIV negative (n=100), HCV positive (n=100), HIV positive (n=25) and *Mycobacterium tuberculosis* positive (n=25) were collected from subjects attending outpatient unit at Centre for Liver Research and Diagnostics. HBV, HIV and HCV infection was screened using enzyme linked immune-sorbent 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)]. MTB samples were screened using PCR for 6110 region. 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), HCV positive samples were confirmed by HCV Real-TM Quant SC (Sacace Biotechnologies Italy).

Plasmid control template and standard curve preparation

The pGEM-T easy vector (Promega make) was used to clone the part of Human HBV X-gene and UTR regions of HCV. The recombinant plasmid was propagated in *Escherichia coli* and purified. The purified plasmid DNA was quantified at 260nm 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.

Taq-man primer and probe designing

Complete Human HBV DNA sequences (n=944) and HCV sequences (n=346) were selected from the NCBI nucleotide database, excluding incomplete and redundant sequences. Those sequences lacking clear genotype information were genotyped by using NCBI's viral genotyping tool. The sequences were aligned using K-Align software application tool from European Molecular Biology Information (EMBI) and a consensus nucleotide sequence was generated by Jalview application tool from EMBI. The consensus sequence was imported and based on this sequence, data primer and probe sets were designed from most conserved sites, including Surface, Core and X regions of HBV genome and UTR region of HCV (Table 1).

Nucleic acid isolation and real-time quantification

HBV DNA and HCV RNA were isolated using the silica based technology as mentioned by Boom *et al*[17] from plasma samples. The PCR was then performed on a Smart Cycler 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 Ex-Taq (Takara) and 1.5 µl of primers and probe. The amplification was performed as follows: initial hot start denaturation at 50°C for 20 min 95°C for 15 min, followed by 10 cycles of denaturation at 95°C for 10 sec, annealing and extension at 46°C for 40 sec and followed by 38 cycles of denaturation at 95°C for 05 sec, annealing and extension at 60°C for 40 sec. 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 Smart Cycler II analysis software version 2.0 following the manufacturer's instructions (Cepheid).

Results

The standard calibration curve was generated by using the Smart Cycler II software and serial dilution 10^2 to 10^8. The calibration curve was linear in a range from 10^2 to10^8 copies/ml (Figs. 1 & 2), with R2 value of 0.999. Reproducibility as measured by dual

Table 1. Primers and probes

Primer & 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
Sense Primer	5’ - CTCAATGCCTGGAGATTTGG – 3'	UTR
Anti Sense Primer	5' -TTGGGGTTTAGGAAGTGTGC – 3'	UTR
Probe	5' – CY55 – GGGTCTCGTAGACCGTGCAACA – BHQ2 – 3'	UTR
Sense Primer	5’ - CCGGTGAGTACACCTGGAAT – 3'	UTR
Anti Sense Primer	5' - CAAGCACCCTATCAGGCAGT – 3'	UTR
Probe	5' – CY55 – GCTAGCCGAGTAGTGTTGGGTCGCG – BHQ2 – 3'	UTR

Table 2. Controls and result interpretation.

Control	Stage for control	FAM Channel	Cy3 Channel	Interpretation
Negative Control	RNA/DNA isolation	Negative	Negative	Valid Result
HCV/HBV positive Control	RNA/DNA isolation	Positive	Positive	Valid result
TE-buffer	Amplification	Negative	Negative	Valid Result
HCV/HBV positive control	Amplification	Positive	Positive	Valid result

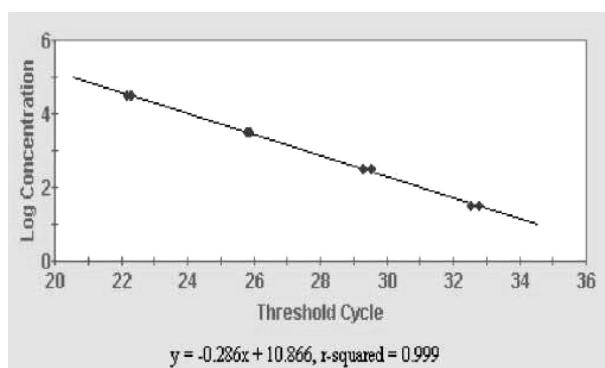

Fig 1. Standard curve originated using serial dilutions (HBV control).

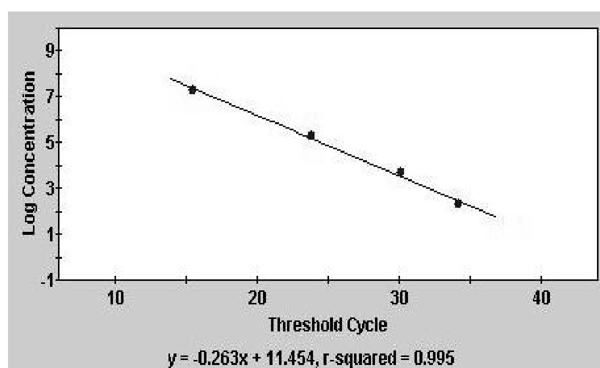

Fig 2. Standard curve originated using serial dilutions (HCV control).

testing of triplicates of three serum samples was acceptable. Good correlation was found between current study procedure and standard SACACE HBV and HCV monitor test, with all 350 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 and HCV. Our results showed that amplification performance was good in case of primer and probe set designed from x region

of HBV and UTR region of HCV. So, we constructed a plasmid with x region and UTR regions using p-GEMT easy vector for being used as a control for deriving standard curve.

To develop low cost method and cover all genotypes and serotypes of human HBV and HCV, we designed a set of primers and probes in this study. The total cost for analyzing one sample is approx. 400 Indian rupees (≈ $ 8).

The precision of our in-house real-time assay was determined by assessing the intra and inter assay co-efficient of variation (CV). For inter assay variability 18 samples including 08 positive HBV samples, 08 positive samples of HCV with determined viral load, and 02 negative samples, were screened triplicate in the same assay, which has given a mean CV 0.084. Two ten samples previously tested by a SACACE HBV and HCV monitor test were tested using our in-house real-time PCR assay. Correlation between the two assays was good, with an R^2 value of 0.99.

The results are interpreted by the device software through the presence of crossing of fluorescence curve with the threshold line. HCV cDNA is detected on the Cy3 (Cyanine 3) channel, HBV DNA on the FAM (6 carboxy fluorescein) channel. Results are accepted as relevant if positive and negative controls of amplification along with negative and positive controls of extraction are passed (Table 2).

The sample is considered to be positive for HCV if, in the channel Cy3 the value of Ct is lower than 33. The sample is considered to be positive for HBV if, in the channel FAM the value of Ct is lower than 33.The sample is considered to be negative if, in both the channels FAM and Cy3 the Ct value is not determined (the fluorescence curve does not cross the threshold line) and the Ct value is higher than 33.

Analytical specificity of the primers and probes was validated with 90 negative samples. They did not generate any signal with the HCV/HBV primers and probes. The specificity of the HCV/HBV real-time Taq-man PCR assay was 100%. Analytical sensitivity of the assay is HCV RNA – 10 copies / ml and HBV DNA – 10 copies / ml. The detection was carried out on the control standard and its dilutions by negative plasma starting from a sample volume of 1 ml. The potential cross-reactivity of the HCV/HBV was tested for, also against the HIV and Tuberculosis control. Cross-reactivity was not observed with any of these pathogens.

Out of four oligo sets designed for HBV detection by RT-PCR, X gene set showed highest specificity and sensitivity (98%), followed by surface (74%) and core (70%), whereas in case of HCV UTR derives set is found promising.

Discussion

We have established and evaluated a sensitive and cost effective in-house adaptable real-time PCR based assay for effective simultaneous detection of HBV and HCV in blood samples with a lower limit of detection of 10 copies / ml. This assay is reproducible showing limited inter and intra assay variability and good amplification efficacy in different genotypes and serotypes. We demonstrate that the results of our assay correlated well with the standard kit available in market for HBV and HCV viral load prediction.

We also made the entire procedure low cost and rapid, which will be helpful in detecting HBV and HCV in patient's blood accurately and efficaciously. The sensitivity of Taq-man procedure for quantifying viral DNA has already been proved by various studies[19,21,22], however they are being limited by certain aspects like limited samples, & designing of primer and probes sets from regions of un-conserved nature.

In this study we made use of the huge sequence database from NCBI site and analyzed the different regions of HBV and HCV for predicting the most conserved regions using latest bioinformatics 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 non clear genotype information; genotypes of these sequences were predicted by using the online NCBI viral genotyping tool. Various HBV genotypes undergo rapid mutagenesis because their reverse-transcription (RT) lacks proof-reading functions[23,24] and also because during the therapy there are certain regions being modified specifically as per the drug used[17] : so there is a need for a reliable quantification tool for predicting the viral load in a specific and sensitive manner. Toward that end, in this study we eliminated the drug inducible mutation regions and variable regions, for selecting the conserved portion of both HBV and HCV viral genomes.

Conclusion

Taq-man chemistry application in the present study can be made applicable in certain commercial fluorescent quantification instruments available in the market (Gene machine™ -Bioron), at lowest price, in replacement of high-cost real-time PCR.

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Conflict of interest: None

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