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Original Article

Hepatitis B Virus (HBV) DNA Quantification by Real Time PCR

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ABSTRACT

Hepatitis B virus (HBV) is an important cause of human chronic liver diseases and is a major public health problem**.** Quantification and detection of Hepatitis B virus (HBV) DNA plays a significant role in diagnosing and monitoring infection related to HBV as well as assessing therapeutic response. The great variability among HBV genotypes and the enormous range of clinical HBV DNA levels present challenges for PCR-based amplification techniques.For the identification of the HBV Viral load, firstly isolated the DNA of HBV by using silica column method and further processed in Real Time PCR .A sensitive and reproducible real-time PCR assay based on Taq Man technology was developed for the detection and quantitation of hepatitis B virus (HBV) DNA in serum, and compared with an "in-house" qualitative PCR assay. Recent advances in antiviral therapy, based on the development of new and more powerful nucleotide analogues, have dramatically improved chronic hepatitis B management, including the prevention of allograft reinfection in those patients undergoing liver transplantation for HBV related disease. Total 51 specimens were collected from the patients who has been positive for (HBsAG) serological test.28 cases were high viral load, 10 have <10 IU/ml viral load and **13** specimens target DNA was not detected.

Keywords: Real Time PCR, Hepatocellular Carcinoma, TaqMan Probes, DNA Amplification.

1. INTRODUCTION

Hepatitis B is an infectious disease caused by the hepatitis B virus (HBV) [3]. The virus lives in human blood and bodily fluids (semen, vaginal secretions, etc.). Hepatitis B infection can cause either a short-term (acute) infection or a long-term or lifelong (chronic) infection. It may take 30 to 180 days for symptoms to begin [3]. Chronically infected carriers have a high risk of developing liver damage and hepatocellular

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carcinoma (HCC) and liver cancer is the commonest cause of death. Hepatitis B virus (HBV) is a partially double stranded DNA containing virus of Hepadna viridae family [3]. Hepatitis B virus (HBV), a 3.2 kb *Orthohepadnavirus*, is a well-known agent of acute and chronic hepatitis, with an estimated 350 million chronic carriers around the world[1,2].How many HBV DNA particles or "units" are found in a blood sample indicates how rapidly the virus is reproducing in the liver. It enters into the liver by blood circulation. Transmission of hepatitis B virus results from exposure to infectious blood or body fluids containing the virus. It is 50 to 100 times more infectious than HIV [4]. Without intervention, a mother who is positive for HBsAg has a 20% risk of passing the infection to her offspring at the time of birth. This risk is as high as 90% if the mother is also positive for HBeAg. HBV can be transmitted between family members within households, possibly by contact of nonintact skin or mucous membrane with secretions or saliva containing HBV [5].The infection has been preventable by vaccination since 1982 [3,7]. Vaccination is recommended by the World Health Organization in the first day of life if possible [3]. The firstdose is generally recommended within a day of birth [9]. Vaccination at birth is recommended for all infants of HBV infected mothers [10]. A combination of hepatitis B immune globulin and an acceleratedcourse of HBV vaccine prevents HBV transmission around the time of birth in 86% to 99% of cases[11]. Liver transplantation is sometimes used for cirrhosis[3].

The tests, called assays, for detection of hepatitis B virus infection involve serum orblood tests that detect either viral antigens (proteins produced by the virus) or antibodies produced by the host. Interpretation of these assays is complex [8]. Detection of serological markers is the mainstay of diagnosis of HBV infection and the most reliable marker of HBV carriage is HBV surface antigen (HBsAg) in serum. The minority of chronic HBV carriers in whom HBeAg can be detected have a particularly high risk of progressive liver disease and end stage liver failure [6]. The monitoring of hepatitis B virus DNA in serum is as important as serological markers in predicting the clinical outcome of infection.

Recent advances include real-time target amplification methods for detecting and quantifying viral genomes and next-generation sequencing (NGS) techniques. Viral load tests that quantify HBV in peripheral blood (serum or plasma fractions) are currently the most useful and most widely used. Viral load measurement plays a significant role during therapy, as most guidelines propose that suppression of HBV replication is a major therapeutic goal. The quantitative PCR (qPCR) assay is based on the specific amplification of HBV DNA using primers targeted to the S-gene and detection in real-time with SYBR Green dye.

2. MATERIAL AND METHODOLOGY

Analization of Hepatitis B from the patients who have been positive for HBsAg Serological test. Test is intended for the use as an aid in the management of patients with chronic HBV infection undergoing antiviral Therapy. Clinical specimens such as serum or plasma were collected from the patients who have been positive for serological test and transported at 4°C in virus transport medium. Viral genome (HBV DNA) has been extracted and purified from patient's plasma by using silica column method to produce a purified genomic material and ready to be utilized in Real Time PCR. The HBV viral load was monitored utilizing COBAS TaqMan 48 Real Time PCR from Roche . This technique uses thermostable recombinant enzyme DNA polymerase (ZO5) for reverse transcription and as well as PCR amplification.

Real-time quantitative PCR for HBV DNA

PCR uses a number of temperatures and a thermostable DNA polymerase to create double-stranded DNA amplicons. The reaction was carried out using a commercial SYBRGreenreaction mix. Amplification of HBV DNA & HBV quantitation standard DNA is measured independently at different wavelengths. Emission intensity of individual reporter dye effectively increases in each cycle that allows independent identification of HBV DNA & HBV quantitation standard DNA. HBV IC(Internal Control) is recombinant DNA containing-structure which carried through all steps of analysis from extraction to amplification. The extracted DNA need to be used in PCR at the same day of extraction. Protocol of Real Time PCR has been specified as follow: stage 1: 95 C° for 900 seconds and in stage 2 : a 2 temperature cycle with 95C° for 20 seconds and 60 C° for 40 seconds has been repeated for 42 times. Visualization of the results will start immediately after starting the reaction (therefore, it's a Real Time PCR). The dyes which have been used are Cy3 for the sample and FAM for the internal control. The signals from these dyes are calculated and presented as numbers.

TaqMan Probe assay for HBV

The HBV COBAS TaqMan, was introduced by Roche Molecular Diagnostics, is a real-time PCR assay reported to equally amplify HBV genotypes. TaqMan probe consists of oligonucleotide sequence labeled with reporter and quencher dye. The TaqMan probes are designed in such a way, which contains complimentary sequence for target. The reporter region of the TaqMan probe binds. to the 5' end of the target. When these dual labeled fluorescent probes are present in bound state the quencher region emits the fluorescence which is absorbed by the reporter region. This type of emission of fluorescence of quencher region and absorbance based on the 'forster type energy transfer effect' (FRET).

Cycle Threshold (CT) ValueDuring Real Time PCR

The amount of viral genome has been obtained by detecting a visible increase in fluorescence as the cycles progressing during the reaction. The Cycle at which the fluorescence

crosses a certain level (called Threshold) will be named as (Cycle Threshold) (Ct) which depends on the starting amount of the viral genome therefore, the software of the Real Time PCR utilizes that Ct (of standard samples) to create a standard curve to calculate precisely the viral load of unknown samples. The threshold cycle is inversely proportional to the original relative expression level of the gene of interest.

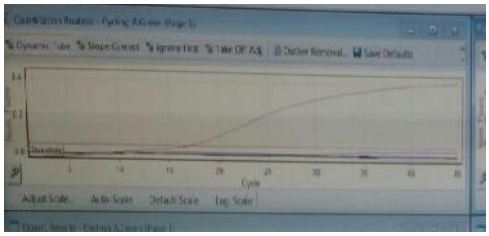

Fig 1: Graph showing cycle threshold (green channel) which indicate viral genome

3. RESULTS

Total **51**specimens were collected from the patient who has been positive for (HBsAg) serological test. The specimens were collected from Shri Mahant Indiresh Hospital and further they were processed and their DNA was isolated by silica column method. After DNA extraction to check presence or quantity of HBV DNA the specimens were processed in Real Time PCR. Viral load were obtained from maximum number of patients and their results have been classified according to the level of viral load. Out of the total **51** clinical sample processed in the 1 month of this study, **28** cases were high viral load, **10** have <10 IU/ml viral load and **13** specimens target DNA was not detected. According to this study Male were mostly effected as compared to Female.

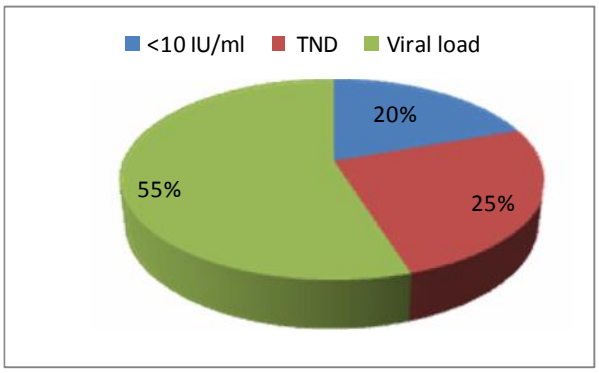

Fig 2: Pie chart for Results interpretation

Significance of real time PCR in molecular diagnosis

It is a very important tool in the Molecular Lab which used for the diagnostics of various diseases such as viral, Bacterial infections and also used for the detection of the high viral loads. It also gives the diagnostics results within 2 hours which is also very workable for the Emergency admitted patients. It gives the introduction of novel technology platforms to the medical and Biomedical Science.

4. DISCUSSION AND CONCLUSION

Earlier molecular biology tools can be used to detect and quantify viral genomes, sequence them, assign them to a phylogenic clade or subclade (genotype or subtype), and identify clinically relevant nucleotide or amino acid substitutions, such as those associated with resistance to antiviral drugs. Recent advances include real-time target amplification methods for detecting and quantifying viral genomes and next-generation sequencing (NGS) techniques. Real Time PCR has been proved to be a useful tool for accurate estimation of the viral amount in the patients blood and this was reflected by its sensitivity for detection. The quantitative real time PCR assay for the detection of HBV-DNA is a highly sensitive method. Advances in the molecular diagnosis of drug resistance using highly sensitive methodologies such as DNA Amplification by PCR can further detect upcoming viral resistance at an early stage when the variant represents only a minor fraction of the total viral population. Such new tools are especially relevant for patients at high risk for disease progression or acute exacerbation. Furthermore, the assay is less laborious than competitive PCR. The real time PCR assay was performed by a single step, requiring a single tube, a single enzyme and a single set of primers with a target specific fluorogenic probe. The development and validation of a real-time polymerase chain reaction (PCR) method based on SYBR-Green for, measuring HBV DNA in serum and plasma. Post PCR data analysis of the real time PCR could be performed by using a computer based data system using standard controls and extrapolating the results by using a standard curve. The dynamic ranges of real time PCR assays usually range from 10^2 – 10^8 genome equivalents per litre (geq/l) of HBV-DNA in serum with a good linearity. The test is also more rapid than the conventional PCR assays (1.5 vs.5 hrs).TaqMan HBV providing sensitive and accurate quantification of HBV DNA levels over a range of 8 logs 10 IU/ml.

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