ISSN: 2279 - 0594

Available Online at www.jbpr.in

Journal of Biomedical and Pharmaceutical Research 2 (4) 2013, 75-80

RESEARCH ARTICLE

QUANTIFICATION OF HBV DNA AND ITS IMPACT ON VIRAL LOAD MONITORING

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Received 20 July 2013; Revised 30 July 2013; Accepted 05 August 2013

ABSTRACT

HBV is a causative agent for chronic hepatitis, and can cause liver cirrhosis and hepatocellular carcinoma. Chronic carriers are at high risk of long term complications of infection, including chronic hepatitis, cirrhosis and hepatocellular carcinoma. Study includes the ease, reliability and sensitivity of HBV DNA quantification by RT PCR and its clinical significance in viral load monitoring.

Keywords: Carcinoma, Real Time PCR, Quantification, TaqMan Probe, Amplification.

INTRODUCTION:

Hepatitis B virus (HBV) is known to be highly infective and associated with long-term morbidity and mortality due to complications like cirrhosis, portal hypertension and hepatocellular carcinoma. HBV is a DNA virus classified in the virus family Hepadnaviridae. Humans are the only known natural host. HBV enters the liver via the bloodstream, and replication occurs only in liver tissue. The intact, infectious virus is 42–47 nm in diameter and circulates in the blood in concentrations as high as 108 virions per ml. The inner core of the virus contains hepatitis B core antigen, hepatitis B e antigen (1-4). Present study was carried out to Quantitate HBV DNA in plasma/serum and its relevance to patients.

MATERIALS AND METHOD:

In this study, a total eight blood samples were used. They were also obtained after getting written consent from patients at SMI hospital. The patients which were found positive in HBsAg Card assay were referred to nucleic acid amplification test for the quantitation of Hepatitis B Virus (HBV) DNA in human serum or plasma (5,6). Extraction of nucleic acid for HBV DNA quantitation through High Pure System nucleic acid extraction kit (Roche) The COBAS® TaqMan® HBV Test processes plasma and serum specimens and isolates HBV DNA through a generic manual specimen preparation based on nucleic acid binding to glass fibers. The HBV virus particles are lysed by incubation at an elevated temperature with a protease and chaotropic lysis/binding buffer that releases nucleic acids and protects the released HBV DNA from DNases in plasma and serum. A known number of HBV Quantitation Standard DNA molecules are introduced into each specimen along with the lysis reagent. Subsequently, isopropanol is added to the lysis mixture that is then centrifuged through a column with a glass fiber filter insert. During centrifugation, the HBV DNA and HBV Quantitation Standard DNA are bound to the surface of the glass fiber filter. Unbound substances, such as salts, proteins and other cellular impurities, are removed by centrifugation. The adsorbed nucleic acids are washed and eluted with an aqueous solution. The disposables allow for a parallel processing of 12 specimens or multiples thereof. The processed specimen, containing HBV DNA and HBV Quantitation Standard DNA, is added to the amplification/detection mixture. The HBV target DNA and the HBV Quantitation Standard DNA are then amplified and detected on the COBAS® TaqMan® 48 Analyzer using the amplification and detection reagents provided in the Test kit (7-10).

Selective Amplification:

Selective amplification of target nucleic acid from the specimen is achieved in the COBAS® TaqMan® HBV Test by the use of AmpErase (uracil-N-glycosylase) enzyme deoxyuridine triphosphate (dUTP). The AmpErase enzyme recognizes and catalyzes the destruction of DNA strands containing deoxyuridine26, but not DNA containing deoxythymidine. Deoxyuridine is not present in naturally occurring DNA, but is always present in amplicon due to the use of deoxyuridine triphosphate as one of the dNTPs in the Master Mix reagent; therefore, only amplicon contains deoxyuridine. Deoxyuridine renders contaminating amplicon susceptible to destruction by the AmpErase enzyme prior to amplification of the target DNA. Also any nonspecific product formed after initial activation of the Master Mix by manganese is destroyed by the AmpErase enzyme, thus improving sensitivity and

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specificity. The AmpErase enzyme, which is included in the Master Mix reagent, catalyzes the cleavage of deoxyuridine-containing DNA at the deoxyuridine residues by opening the deoxyribose chain at the C1-position. When heated in the first thermal cycling step the amplicon DNA chain breaks at the position of the deoxyuridine, thereby rendering the DNA nonamplifiable. The AmpErase enzyme is inactive at temperaturerocesses to allow the accurate quantitation of HBV DNA in each specimen.

PCR set up for HBV DNA Quantitation:

HBV DNA Quantitation is carried out in **COBAS® TaqMan® 48 Analyzer,** which is Real Time PCR instrument from Roche. The Quantitation of HBV DNA is done by FDA approved test which is already incorporated with this instrument. Since it is FDA approved test therefore it is completely closed system and cycling conditions are not mentioned.

RESULTS:

In this study a total of six human blood samples were collected in serum separation tube (SST) and the resultant serum was further subjected for DNA extraction by high pure nucleic acid extraction system utilizing silica columns. Master mix was prepared according to samples for Real time PCR set up. Quantitation was done utilizing amplification parameters provided by manufacturer (Roche) using Amplilink software version 3.3.

Out of six specimens five got quantified for HBV DNA along with quantitation standard (QS) and their titer values ranges from 1.30E+2 to 4.61E+2 IU/ml.

The COBAS® TaqMan® 48 Analyzer automatically determines the HBV DNA titre of the specimen or control based upon the Cycle Threshold (Ct) values for the HBV DNA and HBV Quantitation Standard DNA and the lot-specific calibration coefficients. **The HBV DNA titer is expressed in International Units (IU)/mL.** The conversion factor between HBV copies/mL and HBV International Units/mL is 5.82 copies/ IU using the WHO HBV International Standard for NAT testing 97/746.

Table 5: Titre values in IU/ml for HBV DNA in the human serum samples and controls.

Sr. No.	Analyte for HBV DNA quantification	Amplification status for Target and QS	Cycle Threshold (Ct values) w.r.t. cycle no.		Titer values of HBV DNA in (IU)/ml	No. of HBV DNA copies per ml
			QS	Target		
01	Sample 1	Detected & quantified	29.2	30.1	2.25E+3 or 2.25×10^3	1.3×10^4
02	Sample 2	Detected & quantified	28.2	33.4	1.30E+2 or 1.30×10^2	7.57×10^2
03	Sample 3	Detected & quantified	28.8	32.6	5.22E+2 or 5.22×10^2	3.03×10^3
04	Sample 4	Not detected, QS detected	29.6	-	-	-
05	Sample 5	Detected & quantified	28.6	32.2	3.23E+2 or 3.23×10^2	1.88×10^2
06	Sample 6	Detected & quantified	28.8	29.2	4.61E+4 or 4.61×10^4	2.68×10^5
07	Negative Control	Target not detected but QS amplified	28.6	-	-	-
08	High Positive Control	Detected & quantified	28.6	32.2	3.23E + or 3.23×10^2	18.8×10^3

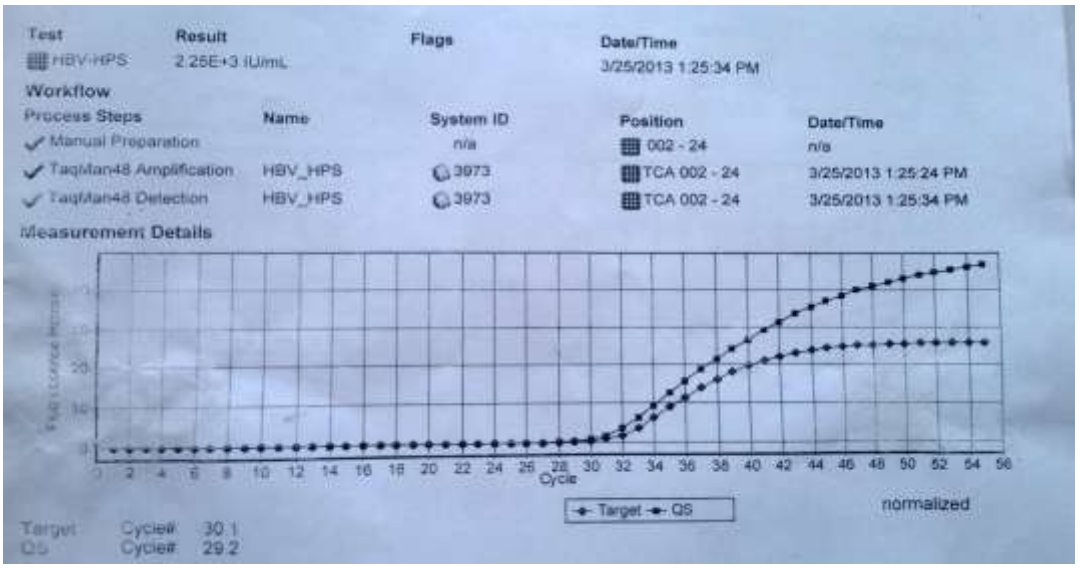

Figure 1: Showing titer value of HBV DNA in a human serum and a graph of amplification of QS and target (HBV DNA

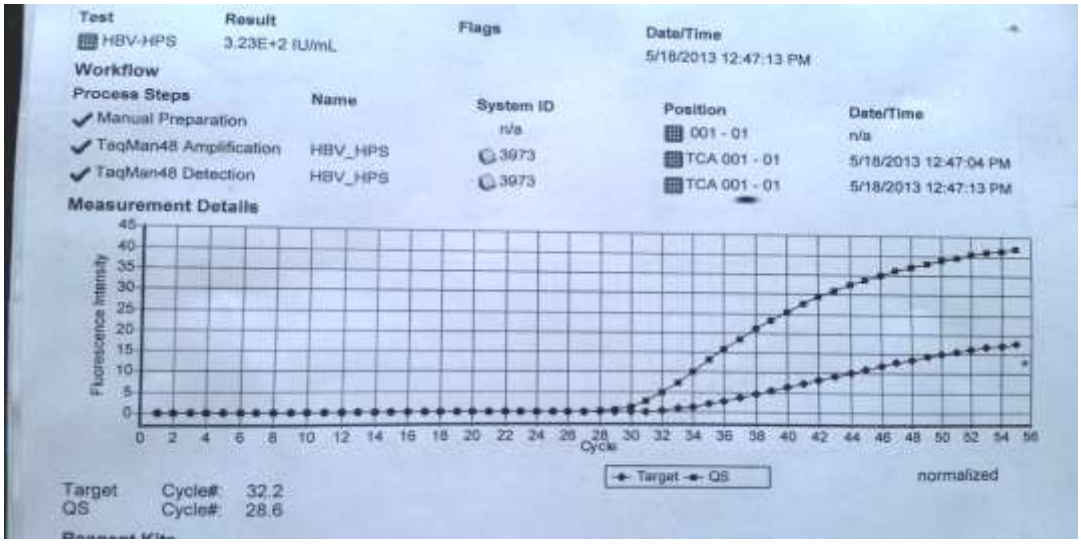

Figure 2: Showing titer value of HBV DNA in a human serum and a graph of amplification of QS and target (HBV DNA).

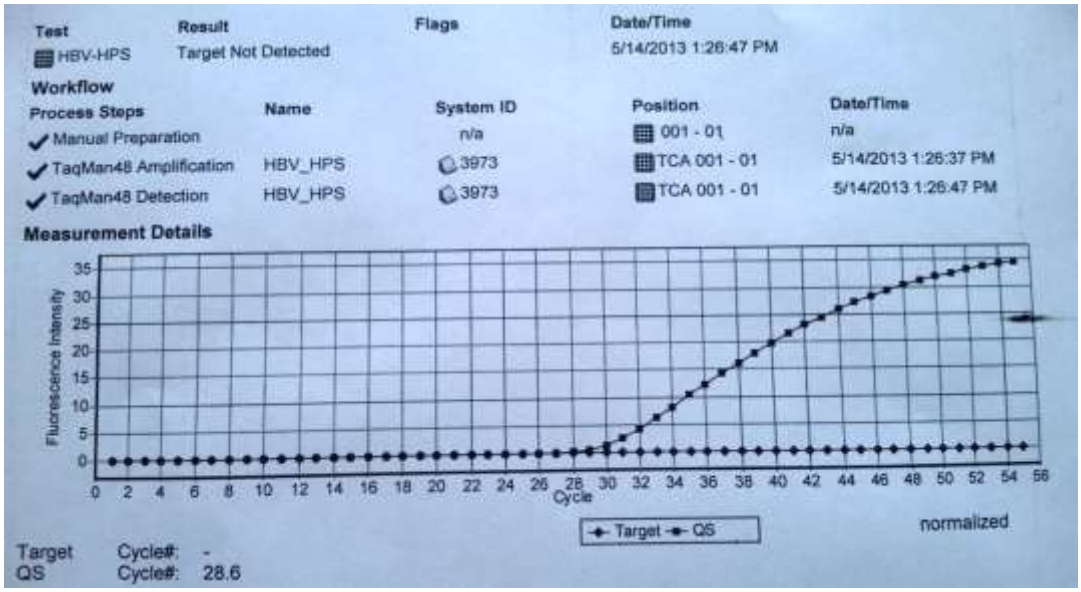

Figure 3: Negative control showing a graph with QS amplified and target was not detected.

DISCUSSION:

HBV has eight genotypes (A-H) due to variation in nucleotide sequences greater than 8%. Various publications are found that the clinical course and outcome of antiviral therapy dependent on the genotype of the infecting HBV strains. After that real time come in field of molecular diagnostic of epidemic diseases (11). The Roche Real Time PCR accurately identifies the genotype in one step reaction by means of primer specificity. It provides a useful tool for rapid detection and would make large scale longitudinal HBV related studies simple & feasible. TaqMan hepatitis B virus (HBV) analyte specific reagent is designed for the quantification of HBV DNA in serum or plasma. Analytical sensitivity and precision were assessed with commercially available HBV standards, while clinical serum specimens from HBsAg commercially available HBV standards, while clinical serum specimens from HBsAg seropositive patient and healthy blood donor were used to determine clinical sensitivity, specificity and correlation with other commercially available assays. Analytical studies yielded a limit of detection of 2.4 IU/ml, with good linearity and correlation with expanded HBV DNA titre over a range. Clinical sensitivity and specificity of the assay combined with automated sample processing are both 100%. TaqMan HBV providing sensitive and accurate quantification of HBV DNA levels over a range of 8 logs 10 IU/ml. Hepatitis B virus (HBV) infection continues to be a leading cause of chronic liver disease, with more than 350 million people chronically infected worldwide. Chronic HBV carriers are at increased risk for the development of cirrhosis and hepatocellular carcinoma. The direct detection of HBV DNA in serum or plasma has become an important tool in diagnosis of chronic HBV infection. Furthermore, serum HBV DNA level may be an important prognostic indicator as well as an important marker for measuring therapeutic response and to the development of resistance to antiviral agents, a variety of commercially available HBV DNA assays reporting in standardized units with improved sensitivity and wide dynamic ranges are currently available.

The COBAS TaqMan HBV test is one of the commercially available Real Time PCR assay designed for the quantitative detection of HBV DNA in human serum and plasma. The benefits of nucleic acid amplification and detection using Real Time PCR include substantial reduction in labor, decreased test turnaround time, and reduced potential for contamination with exogenous DNA. The disadvantages of technically demanding manual sample preparation methods are also numerous. Use of automated sample processing in clinical diagnostic laboratories provides a labor-saving approach to reducing the number of failed sample preparations, while potentially limiting the occurrence of specimen to specimen contamination during processing. This approach may also reduce laboratory space requirements and decreased dependence on laboratory technologists. Nucleic acid extraction from biologic specimens is technically demanding, and reliable PCR result depends on it. The amount of intra hepatic HBV DNA was significantly lower in occult HBV infection than in overt disease. It is known that maintained high levels of HBV DNA are associated with progressive liver disease. Serum DNA levels are a prognostic factor, and contribute to define the phase of chronic hepatitis B (CHB) infection, the treatment indication, and allow an assessment of the efficacy of antiviral therapy. High levels of HBV DNA are an independent risk factor for cirrhosis and hepatocellular carcinoma HCC in Asia. 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. The major polymerase mutations that have been reported to be selected during antiviral therapy with either lamivudine or famciclovir are presented. Antiviral resistance to lamivudine has been mapped to the YMDD motif in the HBV polymerase gene. With the new nomenclature, the major mutations in the reverse transcriptase selected during lamivudine therapy are designated rtM2041/V and rtL180M .The latter B-domain mutations have been associated with adefovir or entecavir resistance.The conserved catalytic domains A-E coincide with the ‘a determinant; of the S gene in the overlapping reading frame. The changes to the S gene selected during antiviral therapy are also listed. Changes in the S gene that have been associated clinically with hepatitis B immunoglobulin (HBIG) breakthrough and vaccine failure is within the “a”determinant located at codon sG145R.The S gene encodes for a B-cell epitope at codons 124-148 and a T-cell epitope at codons 28-51. The majority of reported change in the S gene are located in the “a” determinant. However, not all these changes are associated with vaccine/HBIG escape. Mutations that have been tested in functional antigen-antibody binding studies to confirm this phenotype .changes in the S gene may alter the overlapping polymerase gene. Mutation selected in the S gene after nucleoside analog therapy. There is the potential that some changes selected during antiviral therapy may alter the antigenicity or the S gene. The polymerase mutation at rtV173L plus rtM204V selected during lamivudine treatment result in change to the S gene at codons sE164D and sI195M. In antigen-antibody binding studies, this S-gene mutant had reduced binding affinities compared with wildtype virus. Virus encoding these

mutate only have the ability to behave as a vaccine/HBIG escape. The risk of hepatitis B virus infection through transfusion has been reduced subsequently with the introduction of hepatitis B surface antigen (HBsAg) screening in blood donors. Generally, HBV infection is diagnosed by the detection of HBsAg in the serum or plasma of an individual. Detection of HBsAg in blood is a diagnostic marker for infection with HBV and in the blood banks screening for HBsAg is carried out routinely to detect HBV infection. Occult HBV infection is defined as the presence of HBV DNA in blood or liver tissues in patients negative for HBsAg but who may or may not be positive for HBV antibodies. It is possible that, donors with occult HBV infection, who lack detectable HBsAg might have exposure to HBV infection indicated by positive anti-HBc positive for antibodies against HBV core antigen and HBV DNA, are a potential source of HBV infection. Low levels of viraemia have been shown to continue long after clinical recovery from acute, self-limiting HBV infection. HBV is also transmitte long after clinical recovery from acute, self-limiting HBV infection. HBV is also transmitted very frequently when liver is transplanted from HBsAg negative, anti-HBc positive blood donors which shows that liver harbours infectious HBV in some persons negative for HBsAg but positive for anti-HBc. However, some HBsAg negative individuals with positive anti-HBc and/or positive for antibodies against HBsAg (anti-HBs) continue to be positive for HBV DNA. Due to limitations in current blood screening practices in developing countries, donation by such individuals is a potential source of HBV transmission to the recipients.

There is higher risk of HBV DNA detection in persons who lacked anti-HBs compared to those with detectable anti-HBs levels. The infectivity of anti-HBs-positive, HBV DNA-positive blood components is low, with only 10 per cent transmission of HBV infection8. All blood donors with occult HBV infection may not transmit the disease in blood recipients. Factors, such as viral load in the blood component and immune status of patient, may play a role in viral transmission.

Routine anti-HBc screening of individual blood donations and nucleic acid amplification testing (NAT) by pooling of sera is done in some countries to exclude these donations. In India, detection of HBV infection among blood donors is carried out by HBsAg screening while detection of anti-HBc is rarely done. Screening blood donors for anti-HBc is not mandatory in India; and blood reactive for anti-HBc would normally be transfused to patients. Recommendations for India include not transfusing blood with high titre anti-HBc, although the titre is not defined. Therefore, there is an urgent need to clarify the prevalence of viraemia among HBsAg negative, anti-HBc positive cases and to evaluate the infectivity of blood components from such donors because anti-HBc screening is not mandatory in many countries including India. Hence, the aim of this study was to evaluate the presence of anti-HBc amongst the first time blood donors from Delhi and to determine the presence or absence of HBV DNA in the serum samples from HBsAg negative, anti-HBc positive blood donors by PCR method to assess the magnitude of occult HBV infection in these subjects(12-14).

CONCLUSION:

Serological markers are key elements in diagnosing acute hepatitis B virus (HBV) infection and determining its possible evolution towards chronicity. Once treatment of chronic HBV is initiated with approved anti-hepadnaviral agents, such as lamivudine, interferon-alpha, or adefovir dipivoxil, the measurement of HBV DNA in serum can not only help monitor treatment efficacy but also indicates breakthrough infection should drug resistance emerge. Should chronic carriage ensue, those persons who are unable to resolve HBV infection enter into a low replication phase of infection marked by the seroconversion of HBeAg to anti-HBe. This change occurs in about 10% (5%-20%) of chronic adult carriers per year. The inactive carrier state is marked by continued HBsAg positivity, in contrast to a drop in HBV DNA levels to less than 10^5 copies/mL. In addition, ALT levels and anti-HBc IgM decline and normalize; liver histology shows a significant reduction in necro-inflammation. 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.

ACKNOWLEDGMENT:

The authors are grateful to Honorable Chairman, Shri Guru Ram Rai Education Mission for his kind support and guidance.

Conflict of Interest: None

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