

## Detection of Hepatitis B Virus DNA by Real-Time PCR in Chronic Hepatitis B Patients, Ilam, Iran

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**Abstract:** Fifty-eight sera of chronic Hepatitis B patients were subjected during the period of March 2009 to April 2010 in Ilam cities in west of Iran. Sera assayed by real-time PCR and ELISA methods. Twenty serum samples from healthy volunteers and Non-Hepatitis B patients and negative for Hepatitis B seromarkers served as negative controls for the study. Among fifty-eight sera, ELISA showed fifty-five (94.8%) of the samples were positive for HBsAg and three (5.2%) showed negative results. While real-time PCR specified fifty-eight (100%) positive results in chronic Hepatitis B patients. HBsAg status did not necessarily reflect HBV-DNA level in the serum, as 5.2% of chronic Hepatitis B patients were positive for HBVDNA but negative for HBsAg. HBV-DNA was not found to be positive amongst any of the negative controls. Real time - PCR is a sensitive and reproducible assay for HBV-DNA quantization.

**Key words:** Real time - PCR • Chronic Hepatitis B • Ilam • Iran

### INTRODUCTION

Originally known as serum hepatitis, hepatitis B has only been recognized as such since World War II and has caused current epidemics in parts of Asia and Africa. Hepatitis B is recognized as endemic in China and various other parts of Asia. Over one-third of the world's population has been or is actively infected by hepatitis B virus HBV [1].

The hepatitis B virus is a member of the Hepadnavirus family. It consists of a proteinaceous core particle containing the viral genome in the form of double stranded DNA with single stranded regions and an outer lipid-based envelope with embedded proteins. The envelope proteins are involved in viral binding and release into susceptible cells. The inner capsid relocates the DNA genome to the cell's nucleus where viral mRNAs are transcribed. Three subgenomic transcripts encoding the envelope proteins are made, along with a poorly understood transcript encoding the X protein, whose function is still under debate [2].

A fourth pre-genomic RNA is transcribed, which is exported to the cytosol and translates the viral polymerase and core proteins. Polymerase and pre-

genomic RNA are encapsidated in assembling core particles, where reverse transcription of the pre-genomic RNA to genomic DNA occurs by the polymerase protein. The mature core particle then exits the cell via normal secretory pathways, acquiring an envelope along the way. Hepatitis B is one of a few known non-retroviral viruses which employ reverse transcription as part of its replication process.

The study was focused to quantities HBV - DNA by Real time - PCR method in chronic Hepatitis B patients, to compare the results with HBsAg detection by ELISA.

### MATERIALS AND METHODS

**Samples:** Fifty-eight sera of chronic Hepatitis B patients were subjected during the period March 2009 to April 2010. All sera were obtained of patients referred to Ebnesina laboratory in Ilam city in the west of Iran. All sera were stored at -20°C until use.

**Serological ELISA Assay:** Serum HBsAg and HBsAb, were measured by commercially available enzyme immunoassays according to the manufacturer's instructions.

**DNA Extraction:** For the isolation of HBV DNA from plasma, the QIAamp DNA mini kit (QIAGEN, Hilden, Germany) was used, following manufacturer's instructions. DNA was extracted from 200 $\mu$ L plasma with 1 $\mu$ L of the internal control (1X IPC DNA; Applied Biosystems, Foster City, CA, USA) and eluted in 50 $\mu$ L buffer.

**Real Time PCR Assay:** Amplification was performed in a 50 $\mu$ L reaction mixture containing 1X TaqMan Universal PCR Master Mix, 20 $\mu$ M of each primer and probe and 5 $\mu$ L of extracted DNA. All samples were performed in duplicate. As an internal control, we used the TaqMan Exogenous Internal Positive Control Reagents kit with 1X IPC Mix (primers and TaqMan probe labeled with VIC) and 1X IPC DNA. Absolute quantification of HBV DNA was performed with CFX96 Real Time (Bio Rad-USA). Amplification starts with an incubation at 50°C for two min for uracil N'-glycosylase inactivate possible contaminating amplicons, followed by 10 min at 95°C that activates AmpliTaq Gold Polymerase and inactivates uracil N'-glycosylase. PCR cycling program consisted of 45 two-step cycles of 15 s at 95°C and 60 s at 60°C. The method used in this test is real-time PCR of the precore/core region of the HBV genome. It has a linear range from  $10^2$  to  $5 \times 10^9$  IU/mL.

## RESULTS

Among fifty-eight sera, ELISA showed fifty-five (94.8%) of the samples were positive for HBsAg and three (5.2%) negative results, while real-time PCR specified fifty-eight (100%) positive results in chronic Hepatitis B patients.

We first validated the HBV plasmid standard curve of the real-time PCR detection assay using the VQC panel, containing both HBV DNA-negative control and well-characterized samples ranging from  $4.27 \times 10^7$  to 700 copies per ml. The  $\log_{10}$  theoretical HBV DNA concentrations were compared to the  $\log_{10}$  HBV DNA concentrations determined by the real-time PCR detection assay, which were calculated through interpolation of the Ct values from the standard curve. Regression analysis showed that the slope approached 1.0 (95% confidence interval, 0.83 to 1.04) and the  $y$  intercept approached 0 (95% confidence interval, 20.30 to 0.64). The VQC sample with a viral HBV copy number of 975/ml still generated a detectable signal in the real-time PCR detection assay, while the sample with 700 copies/ml could not be detected in the format used.

The detection limit of this assay was 100 DNA copy per reaction. A linear standard curve was obtained between 100 and  $10^9$  DNA copies per reaction ( $r > 0.990$ ).

## DISCUSSION

HBV viral load measuring is a very important tool for monitoring HBV infected patients. There is an increasing trend for the use of combination of nucleotide analogs, which lead to a fast dynamic of viral load. It is expected that monitoring the HBV viral load together with the detection of resistance related mutations in the HBV genome will be able to provide clinicians with important guidelines for the HBV treatment.

The most direct and reliable measurement of viral replication is HBV DNA quantification that can replace other indirect methods to assess the efficacy of antiviral therapy used to treat HBV infected patients, such as serologic markers or measurement of liver enzyme function. Histology is adequate to evaluate the efficacy of a treatment, however, lacks the ability to discriminate rapidly any difference at multiple time points in clinical trials, comparing active drugs or combination therapy. Monitoring the HBV viral load can predict the evolution to cirrhosis and hepatocellular carcinoma [3], as well as a rapid and sustained response to treatment, as a predictive factor for a favorable treatment outcome. It can also provide an early detection of treatment failure that may be related to poor adherence to therapy or selection of a resistant virus. The likelihood of resistance to nucleos(t)ide analogues is very low when HBV-DNA level is undetectable during therapy and increases proportionally to the HBV-DNA level [4].

An assay with a lower limit of detection of  $10^3$  IU/mL may be sufficient to monitor. Our results have shown that we could detect samples with viral load below 1000 IU/mL. For all of these reasons, real-time PCR quantification assays are strongly recommended over other technologies, especially in clinical trials, because they are very sensitive and have a broad dynamic range of quantification [5].

HBV DNA was detected in a 55 of sera with HBsAb and sera with no HBV serological marker. This shows that determining HBV replication only by detection of HBV marker is not enough and real-time PCR should be used simultaneously.

In conclusion, HBsAg status did not necessarily reflect HBV-DNA level in the serum, as 5.2% of chronic Hepatitis B patients were positive for HBVDNA but negative for HBsAg. HBV-DNA was not found to be

positive amongst any of the negative controls. Real time - PCR is a sensitive and reproducible assay for HBV-DNA quantization.

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