Real Time PCR as a Diagnostic Tool for HBV Infection in Iraq

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ABSTRACT :

BACKGROUND:

HBV infection is a worldwide infectious disease that acutely infects 2 billion yearly, thus finding a precise, accurate and sensitive diagnostic test for this infection is highly advisable. Real Time PCR has been introduced to achieve that mission.

OBJECTIVE:

Confirm the role of Real Time PCR as a precise tool for diagnosis of HBV infection in Iraq.

PATIENTS AND METHODS:

In this study, 40 patients of HBV with HBs Antigen positive serological test and 20 individuals with HBs Antigen negative were selected to be the material of the study and they were tested by Real Time PCR to estimate the exact amount of HBV genome in their blood.

RESULTS:

All the cases with HBs Antigen positive have had viral load with different values and no case with HBs Antigen negative have been found to have any viral element.

CONCLUSION:

Real Time PCR is useful and precise tool for the diagnosis and monitoring of patients with HBV infection and further studies are required to find a useful classification of the viral load and a correlation need to be found between viral load and the serological panel and especially HBe Antigen and HBe Antibody.

***KEY WORDS*:** real time pcr, hbv, diagnosis

INTRODUCTION:

Hepatitis can be caused by toxins, drugs, too much alcohol, or a variety of viruses. Viruses that infect the liver are called hepatitis viruses. Each virus is unique and is identified by a letter of the alphabet, in the order of its discovery. Hepatitis B infection can cause either a short-term (acute) infection or a long-term or lifelong (chronic) infection.

Hepatitis B (HBV for short) is a virus that lives in human blood and bodily fluids (semen, vaginal secretions, etc.). HBV makes more copies of itself by infecting the liver (this could be reflected by increasing amount of circulating viral genome). When the patient is infected with chronic hepatitis B over a long period of time HBV may damage the liver to the point that it cannot perform many of the important jobs that it must do to keep you healthy [(1)].

Worldwide an estimated 2 billion persons have been infected and 400-800 million are chronically infected with HBV. Complications from HBV are the 10th leading cause of death worldwide. HBV is responsible for up to 70% of all cases of liver cancer worldwide. In 2007, the U.S. Centers for Disease Control and Prevention (CDC) estimated that 43,000 Americans were newly infected with HBV. It is also estimated that between 2 and 3 million Americans have chronic HBV infection [(2)].

Hepatitis B virus DNA (HBV DNA) carries the genetic blueprint of the virus. How many HBV DNA particles or "units" are found in a blood sample indicates how rapidly the virus is reproducing in the liver.

Diagnostics for chronic hepatitis B have evolved from the simple detection of HBsAg through the complex antibody response against individual viral proteins and to the detection and quantification of viral DNA. Implementation of increasingly sensitive methods of HBV DNA quantification has greatly aided the diagnosis and management of disease.

To measure HBV DNA, also called "viral load," a laboratory measures how many HBV DNA units are found in a milliliter of blood. This result is written in international units per milliliter or IU/mL. High levels of HBV DNA, which can range from thousands up to millions, indicate a high rate

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of HBV replication. Low or undetectable levels – less than 2,000 IU/mL – indicate an "inactive" infection [3].

The aim of this study is to estimate the usefulness of Real Time PCR as a sensitive and specific tool to diagnose active hepatitis B in Iraqi Patients.

Viral load tests that quantify HBV in peripheral blood (serum or plasma fractions) are currently the most useful and most widely used. Assessment of HBV DNA in plasma or serum should therefore be performed along with other tests to establish this diagnosis.

Practice guidelines for the management of chronic hepatitis B have been published by a number of professional societies [4,5]. These consensus documents recommend the quantification of HBV DNA in the initial evaluation of chronic hepatitis B and during management, particularly in the decision to initiate treatment and in therapeutic monitoring [6].

Viral load measurement plays a significant role during therapy, as most guidelines propose that suppression of HBV replication is a major therapeutic goal. Measurement of viral load at 3- to 6-month intervals during treatment has been recommended [7]. Monitoring intervals can also be guided by specific treatment, with shorter intervals (every 3 months) for lamivudine and longer intervals (at least every 6 months) for other nucleoside/nucleotide reverse transcriptase inhibitors due to the more rapid emergence of resistance during lamivudine therapy [8].

PATIENTS, METHODS AND MATERIALS:

Forty patients who have been identified to have positive HBs Ag serological test and 20 individuals who were negative for test and been considered as controls were been selected to be the raw material of this study.

All subjects have been stratified against age, sex, history of contact with infected patient, geographical distribution, and history of blood transfusion or chronic renal disease seeking for the primary cause of infection. The subjects have been obtained from the teaching hospital of gastroenterology and hepatology and some of them from Al Razy specialized private laboratory while the control subjects have been obtained from another patients with different complaints and been checked for HBs Ag (all were negative for test) . All the samples were operated at Al Razy specialized laboratory from January – March /2011 with Smart Cycler II® Real Time PCR instrument *(Cephied Company).*

Viral genome (HBV DNA) has been extracted and purified using a ready- to- use kit for HBV DNA extraction (Ribo Column®) (Lot. No. 07D11B737) *(Sacace Biotechnologies)*. In brief, patient's plasma has been used and lysis buffer was used to lyse the viral particles, then subsequent steps of purification, washing and elution to produce a purified genomic material ready to be utilized in Real Time PCR. No Known inhibitor of Real Time PCR was used (e.g. Heparinized sample have been excluded from the study).

Internal Control (HBV IC) with known concentration (Specific for each lot) has been used to monitor the amplification and exclude any loss of genomic DNA or PCR inhibition, thus enabling to calculate precisely the HBV viral load. HBV IC 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.

Real Time PCR has been done using HBV Real-TM Quant. ® Kit *(Sacace Biotechnologies).*

A total volume of 25 µL has been utilized for reaction with a 12.5 µL from the reaction Mix (provided by the kit) and 12.5 µL from the extracted viral genome.

With each run, 6 standards with identified concentrations (provided in the data card) have been used to quantify the concentration of the viral genome within the sample. From these 6, 3 standards for the HBV DNA (QS1 HBV, QS2 HBV, and QS3 HBV) and the other 3 standards for the internal control (QS1 IC, QS2 IC, and QS3 IC). These standards are used by the system to create a standard curve on which the calculation of the HBV DNA depends.

Protocol of Real Time PCR has been specified according to the manufacturers instructions 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.

RESULTS AND DISCUSSION:

The study has been designated to test 40 patients and 20 individuals as control cases. All the cases and the controls have been distributed in

accordance to different factors in addition to the molecular study.

In regard to the **sex**, 35 of the cases were male and 5 females while in control individuals they were 16 and 4 respectively.

The mean age of all cases was 38 years and they are distributed in different geographical regions.

All the cases were positive for the serological test (HBs Ag +) and all of them were diagnosed for the first time (Baseline Viral Load) while in controls, 2 of the individuals were HBs Ag + (this could be due to previous exposure).

Results of the tests have determined using the software which is specific for that test. Briefly,

there is an equation which determines the exact level of the viral DNA:

HBV DNA /IC DNA x coefficient = copies HBV DNA /ml.

The coefficient is specific for each lot and reported in the HBV TM Quant Data Card provided in the kit. Furthermore, the concentrations of the HBV DNA are determined in IU/ml as the software uses a specific formulation to calculate the HBV DNA in IU/ml. The conversion factor varies from 1-6 according to the system and kit been used. In our study we calculate the conversion factor to be 1.69 (~ 1.7).

HBV Real-TM Quant is linear from 2 x 10^2 to 1 x 10^8 copies/ml. Test results greater than 100.000.000 copies/ml are above the upper limit of quantitation of the test and should be reported as "greater than 100.000.000 copies/ml".

HBV DNA has been extracted successfully from all the cases and the results were variable.

Viral load has been obtained for all of the participants and their results have been classified according to the level of viral load as shown in (Table 1) with the maximum number of cases have got a viral load below 10,000 IU/ml (about 50% of the cases)

Table 1: Distribution of cases according to their viral loads

No. of Patients (%)	Category (IU/ml)
8 (20%)	≥ 1,000,000
4 (10 %)	100,000 – 1,000,000
8 (20%)	10,000 -100,000
20 (50%)	< 10,000

The amount of viral genome has been obtained by detecting a visible increase in fluorescence as the cycles progressing during the reaction (Growth Curve) as illustrated in Figure 1 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 .

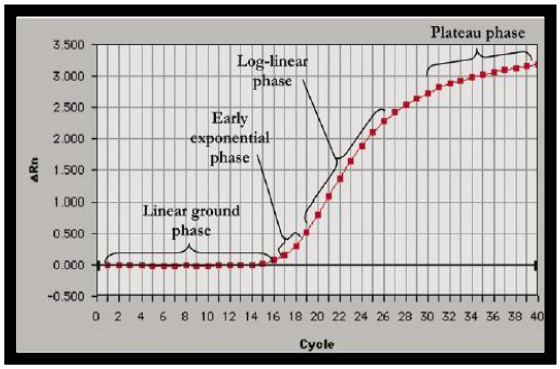

Figure 1: Showing the typical Real Time growth curve with its phases in which any fluorescence (identified as Y axis) detected above a certain level (named as threshold) as the reaction cycles progress (identified as X axis) would be regarded as (Positive) and those who fail to cross the threshold would be regarded as (Negative).

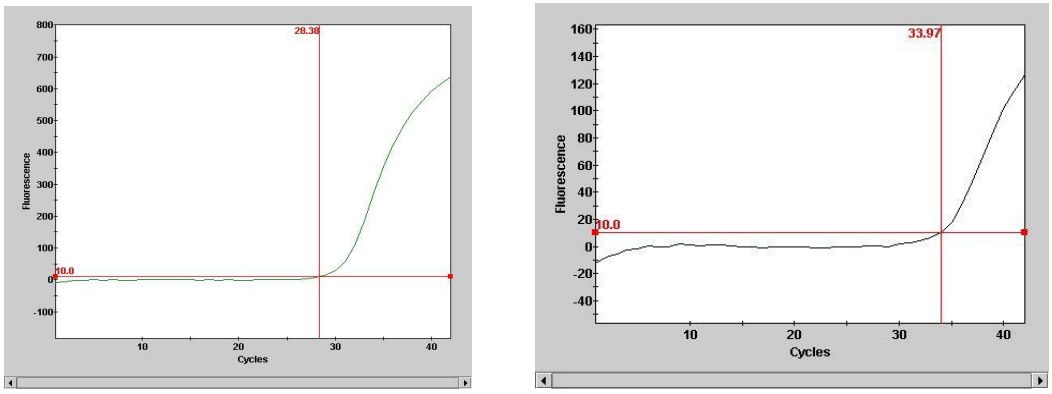

Figure 2: Showing two positive cases with two different (Ct)s (28.3 and 33.9) which indicates different levels of viral genome .

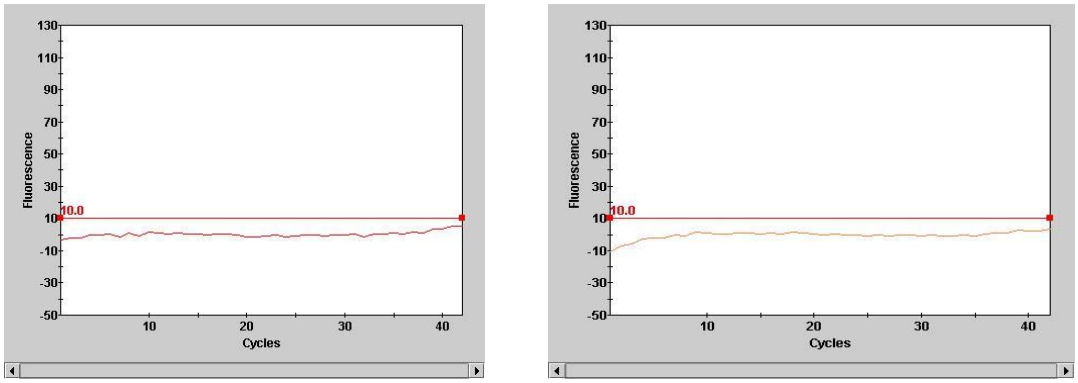

Figure 3: two examples of negative samples, the fluorescence failed to cross the threshold because of the low level (or absence) of viral genome (both are control).

Threshold level needs to be specified appropriately as not to be too high (which causes late detection beyond the exponential phase) or to be too low (which may cause the background to prematurely crosses the threshold). For all of our samples and controls, the threshold was to be stabilized at 10 Fluorescence Unit (FU).

In consistent with results of our study, many studies have recommended using Real Time PCR as a diagnostic mean in clinical virology [9].

The significance of detecting viral load in patients with hepatitis B seems to assist in risk estimation of hepatocellular carcinoma and liver cirrhosis. Beginning with a value of 10^4 copies/ ml the risk of cirrhosis and HCC is significantly increasing proportionately with increasing virus load. This close correlation is clearly still present even after adjustment for all other risk factors. For this reason a new clinical threshold level of 10^4 copies/ml (corresponding to approx. 2x 10^3 IU/ ml) has been included in the German and European guidelines as a decision-making criteria [10, 5]. It is also used in the US guidelines [11].

Further applications of PCR in HBV need to be applied and discussed more deeply as the use of PCR in HBV genotyping in Iraq as this topic seems to play a role in the monitoring and follow up of patients during the course of treatment. HBV genotyping by PCR have been discussed in many studies with a high significance [12].

CONCLUSION:

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. More precise classification of the viral load might be helpful to start and monitor treatment and to differentiate those who are inactive carrier from chronic hepatitis B.

Further estimation of serological panel (especially HBe Ag) might clarify some hints about the replication of the virus at time of diagnosis.

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