Real Time-PCR HBV-DNA Analysis : Significance and First Experience in Armed Forces

Col GS Chopra, ^{SM*}, Lt Col PK Gupta⁺, Col AC Anand, ^{VSM#}, Col PP Varma, ^{VSM**}, Col V Nair, ^{VSM++},
Lt Gen Ramji Rai, ^{AVSM, VSM, PHS##}

Abstract

Background: HBV DNA quantitation is used extensively world wide for the diagnosis and monitoring of treatment of Hepatitis B virus (HBV) infection. However, it has still to be popular in India. The aim of this study was to quantitate HBV – DNA by Real time – PCR method in Hepatitis B and in immuno-compromised patients, to compare the results with HBeAg detection and to monitor the response to therapy of chronic Hepatitis B patients to antivirals.

Methods: Ninety one serum samples of Hepatitis group of patients (all HBsAg positive), 41 samples from immuno-compromised patients (all HBsAg negative) and 49 patients of Chronic Hepatitis B group (all HBsAg positive) were the subjects of this first ever study in Armed Forces. Twenty serum samples from healthy volunteers and non-hepatitis B patients served as negative controls. The amplification detection was carried out in a Rotor-Gene 2000-sequence detector.

Results : Amongst Hepatitis B group, 33 % (30/91) of the samples were positive for HBV-DNA and 26 % (24/91) of samples were positive for HBeAg. In the immuno-compromised group of patients 14.6 % (6/11) of samples were positive for HIV-DNA and 9.7 % (4/41) were positive for HBeAg. Of the Chronic Hepatitis B patients on treatment, all (100 %) were positive by HBV-DNA, whereas 29/49 (59.2 %) were positive by HBeAg before treatment. After treatment with antivirals, 06/49 (12.2 %) were positive by both tests and 11/49 (22.5 %) were positive only by HBV-DNA. 32/49 (65.3 %) patients became negative serologically after therapy.

Conclusion : HBeAg status did not necessarily reflect HBV-DNA level in the serum, as 10/91 (11 %) in the Hepatitis B group, 2/41 (4.9 %) in the immuno compromised group and 20/49 (40.8 %) patients in the Chronic Hepatitis B group were positive for HBV-DNA but negative for HBeAg. 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 quantitation and may be started in Armed Forces referral centers in the near future.

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Key Words: Real time – PCR, Chronic Hepatitis B, HBV – DNA, Antivirals.

Introduction

Hepatitis B virus (HBV) is a double stranded DNA virus of the Hepadnaviridae family that causes both acute and chronic hepatitis. According to World Health Organization estimates, 2 billion people have been infected with HBV worldwide and more than 300 million have persistent infection. Approximately 1 million people die each year worldwide from hepatocellular carcinoma or liver failure. Control of HBV infection is therefore, an important goal for public health in areas of endemicity.

The presence of Hepatitis B surface antigen (HBsAg) in serum indicates HBV infection, but does not provide information on the replicative state of the virus. Naturally occurring escape mutants of HBV with various mutations in the S gene could have no detectable HBsAg in serum [1]. Further, DNA-positive HBsAg seronegative individuals are not uncommon. Hepatitis B e antigen (HBeAg) has been considered a viral replicative marker, but the precore point mutant HBVs

cause a HBeAg negative phenotype irrespective of their status of replication [2]. These HBV-DNA positive individuals with aberrant HBV serology are infective and pose a risk especially as blood donors or health care workers. The level of HBV-DNA in serum or plasma has been shown to correlate with biochemical and histological measures of disease, and probably reflects more accurately the replicative activity of HBV. Therefore, the measurement of HBV-DNA in serum has become an important tool to identify individuals with high viral replication, to monitor patients on therapy, and to predict whether antiviral therapy will be successful. With the introduction of new antivirals like Lamivudine [(-)2'3'-dideoxy-3' thiacytidine], close monitoring of patients has become increasingly important due to the occurrence of antiviral drug resistant virus strains or the presence of flares after withdrawal from antiviral therapy [3,4].

Quantitative assays of the HBV-DNA are used

*Senior Advisor (Pathology and Immunology), **Senior Advisor (Medicine and Nephrology), ++Senior Advisor, (Medicine and Haematology), Army Hospital (R and R), Delhi Cantt., +Reader, Department of Blood Transfusion, #Professor and Head, Department of Medicine, Armed Forces Medical College, Pune, ##Director General Medical Services (Army), Army Head Quarters, New Delhi.

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extensively to quantify the level of HBV-DNA in serum samples [5,6,7]. Various commercially available test kits for HBV-DNA provide conflicting quantitative results, with considerable difference[8].

During the past few years, real time PCR assays have been developed to overcome the problems of conventional PCR assays [9,10]. In the present study, a sensitive real time PCR assay was used and validated for the detection and quantitation of HBV-DNA in certain clinical situations. The detection occurred via the fluorescent labeling of oligonucleotide probes that bind specifically to the PCR amplicate. Detection of the fluorescence intensity during the course of real time PCR enables verification as well as quantification of the product. The aim of the study was firstly to quantitate HBV-DNA by real time PCR method in Hepatitis B and immuno-compromised patients, secondly to compare the results of HBV-DNA estimation with HBeAg detection and lastly to serologically monitor the response to therapy of chronic Hepatitis B patients on antivirals.

Material and Methods

Patients: Ninety one serum samples were collected from Hepatitis B group of patients, 41 samples collected from immunocompromised patients and 49 samples collected from chronic Hepatitis B patients. The Hepatitis B group of patients consisted of HBsAg carrier (n=20), Chronic Hepatitis B patients (n=67) and acute viral hepatitis (n=4) (Table 1). These patients were all initially positive for HBsAg. The immunocompromised group consisted of Renal transplant patients (n=24), HIV positive patients (n=3), CRF patients (n=4) and Haematological malignancies (n=10). None of these patients were HBsAg positive. The Chronic Hepatitis B group of patients were all Chronic Hepatitis B patients and were all HBsAg positives.

Controls : 20 serum samples from healthy volunteers and Non-Hepatitis B patients and negative for Hepatitis B-seromarkers served as negative controls for the study.

DNA extraction : DNA was extracted from 200 µl of blood with DNA extraction kit, (QIAGEN, Hilder, Germany). DNA was dissolved in Tris – EDTA buffer, and stored at 4°C till used for PCR.

Real time PCR: The HBV Rotor Gene (HBV RG) PCR kit, a ready to use kit of HBV DNA for real time-PCR was used. The HBV RG Master contained all the reagents and enzymes (with the exception of Mg^{2+}) for the specific amplification of a 110 bp sequence of the HBV genome and for the direct detection of the PCR product in cycling A FAM of the Rotor Gene 2000 instrument. In order to detect potential PCR inhibition in the absence of a HBV PCR product, the kit also contained an inhibition control to monitor the amplification efficiency of the system.

The primer sequence used were – 5' – GACCACCAATGCCCTAT – 3' (forward primer) and 5' – CCRAGAYYGAGATCTTCTGCGAC – 3' (reverse primer), where 'R' and 'Y' are the spacers used in the primer sequence. The amplification was performed in a 50µl reaction mixture

containing: 30 µl of mixture of HBV RG Master mix (Buffer, dNTP, Primer, Probe and enzymes) and HBV RG Inhibition control mixed with 20 µl of DNA template to each reaction. The Real-time PCR cycling parameters consisted of denaturation at 95°C for 10 minutes followed by 45 cycles consisting of 95°C for 15 sec, 55°C for 30 sec and 72°C for 15 seconds.

The problem of carry over contamination was avoided by using pre-sterilized filtered microtips and the tests were conducted in separate Pre PCR and PCR laboratory. The amplification – detection was carried out in a Rotor – Gene 2000 Sequence detector (Corbett Research). The results were considered positive if a signal was detected in Cycling A FAM (Fig. 1), and negative if no signal was detected in Cycling A FAM, but however a signal from the Inhibition control was present in Cycling A JOE. If no signal was detected in either Cycling A FAM or Cycling A JOE no conclusions could be made and it was concluded that PCR was inhibited. Quantitation of the HBV-DNA was done in all cases and the result was considered significant and positive if viral load was more than 1×10^5 viral copies/ml. HBeAg was done by ELISA method using a commercial kit (M.B.S., Galeno, Milano, Italy)

Reproducibility of results: Twelve serum samples, 7 positive and 5 negative samples, were retested after one week of the initial tests, to see the reproducibility of the results of HBV-DNA quantitation.

Monitoring of patients after antiviral therapy:

Forty-nine patients of Chronic Hepatitis B group of patients were treated with Lamivudine or Interferon or combination of both for a minimum duration of six months. Pre treatment and post treatment monitoring of serological markers HBV-DNA and HBeAg were done in all cases.

Results (Tables 1-4)

Of the ninety-one patients of Hepatitis B group, real time-PCR detected HBV-DNA in 33% (30/91) of samples. HBeAg was positive in 26% (24/91) of samples. Of these 20 patients were positive by both the tests, whereas 57 patients were negative by both the tests. Ten patients were positive by real time-PCR for HBV-DNA but were negative for HBeAg. In the immunocompromised group of patients, real time-PCR detected HBV-DNA in 14.6%(6/41) of samples, whereas

Fig. 1 : Quantitation data for Cycling AFAM/Sybr. Amplification fluorescent plot of various samples and standards

HBeAg was positive in 9.7%(4/41) of samples. Of these four patients were positive by both tests and two patients were positive by real time-PCR method but were negative for HBeAg. 35 patients were negative by both the tests.

In the chronic Hepatitis B group, real time PCR detected HBV-DNA in all 49 cases (100%), whereas HBeAg was positive in 29/49 (59.2%) patients. 29 patients were positive by both the tests. After 6 months of antiviral therapy, 6/49 (12.2%) were positive by both HBeAg and HBV-DNA and 11/49 (22.5%) were positive only by HBV-DNA. 32/49(65.3%) patients became sero-negative after treatment. The HBV load in these patients varied between 112291 to 180561410776 viral copies/ml. The mean HBV-DNA levels in the positive chronic Hepatitis B subgroup of patients was significantly higher, as compared to other subgroups.

Total of 181 patients of different subgroups were analyzed in the present study. Of these, 53 patients were positive by both HBV-DNA and HBeAg. 32 patients were positive by HBV DNA only, 4 patients by HBeAg and 92 patients were negative by both tests. The sensitivity and negative predictive value of HBeAg for detection of HBV was 62.4% and 74.2% respectively. HBV – DNA was not found to be positive amongst any of the negative controls. The twelve samples, retested after one week for confirming the reproducibility of the results, showed similar positive or negative results as shown earlier.

Discussion

The quantitative real time PCR assay for the detection of HBV-DNA is a highly sensitive method. Furthermore, the assay is less laborious than competitive PCR [9]. The entire process can be completed within a single day, including extraction of DNA. 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. 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 (Fig 2) [11].

The common problems with PCR were the lack of standardized controls, poor reproducibility and contamination due to carry over amplicons. These problems were not faced with the real time PCR method used in this study. It has further advantage in that this assay is highly reproducible with low inter and intra-experimental variabilities. 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 [10,12]. The test is also more rapid than

Fig. 2 : Standard curve with various concentrations

Table 3

Comparison of positivity of test results by the two methods

	Positive by both tests	Positive by HBV DNA only	Positive by HBeAg only
1. Hepatitis B group (n=91)			
HBsAg carriers (n=20)	3	4	1
Chr Hepatitis B (n=67)	16	5	3
Viral Hepatitis (n=4)	1	1	-
2. Immuno compromised group (n=41)			
Renal transplant (n=24)	1	1	-
HIV I (n=3)	2	-	-
CRF (n=4)	-	-	-
Haematological malignancies (n=10)	1	1	-
3. Chronic Hepatitis group (n=49)			
Chronic Hepatitis B	29	29	-

Sensitivity of HBeAg = 62.4%; Negative predictive value of HBeAg = 74.2%

Table 4

Serological response of chronic Hepatitis B patients on antiviral therapy (n=49)

Serological marker	Became negative	Remained positive
HBeAg (n=29)	23	6
HBV-DNA (n=49)	32 (65.3%)	17 (34.7%)

Table 1

Quantitative analysis of HBV DNA by RT-PCR and HBeAg by ELISA in Hepatitis B infection (n=91)

Disease	HBV-DNA		HBeAg	
	Positive	Negative	Positive	Negative
HBsAg carriers (n=20)	7	13	4	16
Chr. Hepatitis B (n=67)	21	46	19	48
Viral Hepatitis (n=4)	2	2	1	3
Total	30 (33%)	61	24 (26%)	67

Table 2

Quantitative analysis of HBV DNA by RT-PCR and HBeAg by ELISA in immuno compromised patients (n=41)

Disease	HBV-DNA		HBeAg	
	Positive	Negative	Positive	Negative
Renal transplant (n=24)	2	22	1	23
HIV I (n=3)	2	1	2	1
CRF (n=4)	0	4	0	4
Haematological malignancies (n=10)	2	8	1	9
Total	6 (14.6%)	35	4 (9.7%)	37

the conventional PCR assays (1.5 vs.5 hrs).

Contrary to common opinion, the disappearance of HBsAg from the serum, the development of anti-HBs antibodies and normalization of liver function may not reflect complete virological recovery from acute HBV infection. HBV-DNA, and possibly HBV virions, can be present in the serum and the viral genome can persist in a transcriptionally active form in blood for over 5 years after clinical and serological recovery from acute infection [13]. Our data also in a limited number of patients suggests that the HBV-DNAemia prevalence may be high among HBsAg negative patients. We also analyzed HBV-DNA in immunocompromised patients and it was found to be positive in 14.6% of patients, suggesting that these groups of patients require close follow up and monitoring for HBV.

The present data indicate that the HBeAg status does not necessarily reflect the HBV-DNA level in the serum, which is in agreement with previous study [14]. Measurement of serum HBV-DNA concentrations in Hepatitis B patients facilitates prediction of hepatic inflammatory activity in Hepatitis B. In the present study HBV-DNA was detected in the serum of 33% (30/91) patients of Hepatitis B group, 14.6% (6/41) of immuno compromised group and 100% (49/49) of the chronic Hepatitis B group. Whereas, HBeAg was positive in 26% (24/91), 14.6% (6/41) and 59.2% (29/49) patients respectively in the above groups. So, the real time PCR method of detection of HBV – DNA is more sensitive in immuno-compromised hosts, as the traditional method of detection of HBeAg can miss some cases of Hepatitis B infection. The patients who are positive for HBV-DNA but negative for HBeAg respond poorly to treatment. In the viral hepatitis subgroup, out of four patients studied only two patients were positive by HBV-DNA, which may be due favorable treatment response leading to drastic reduction in viral loads.

HBV-DNA estimation is very useful for follow-up and to see the response to antivirals in chronic Hepatitis B patients. In the present study, 65.3% (32/49) patients recovered after antiviral therapy. Only 12.2% (6/49) were positive by both the tests, whereas 22.5% (11/49) were positive only by HBV-DNA.

Our study shows that the real time PCR assay for HBV-DNA using Real Art HBV Rotor Gene PCR kit is specific and is reproducible for the detection and quantitation of HBV-DNA in clinical serum samples and on monitoring of patients of Chronic Hepatitis B on antiviral therapy. The assay may be especially useful for detection of small subgroup of patients who are HBeAg negative but carry the HBV virion. It is especially useful for early diagnosis in immuno compromised patients who present with altered liver

function test but negative for HBsAg / HBeAg markers. So these patients can be given anti-viral therapy at the appropriate time. Real time – PCR method can be further useful for follow up of these patients and to see the response to the treatment given, hence can be started in referral centers of the Armed Forces in near future.

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