Molecular Analysis of Hepatitis B Virus DNA and Mutation Status in Patients Under Lamivudine [(-) 2' 3'-dideoxy-3'-thiacytidine] Therapy

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Abstract: Quantitative analysis of HBV-DNA is extensively used worldwide for monitoring of lamivudine therapy of Hepatitis B virus (HBV) infection. We have analyzed the quantity of HBV-DNA during lamivudine therapy and investigated the relationship of lamivudine resistance to mutation type. Ninety-one hepatitis B patients were enrolled in the study where Real Time Polymerase Chain Reaction (PCR) did estimation of HBV DNA and mutation was analyzed by sequence detection via PCR. HBV-DNA was detected in the serum of 96.7% (88/91) patients with mean viral load ranging from 1×10^5 to 1×10^9. More than 80% patients responded to and 17.3% patients showed resistance to lamivudine therapy. All lamivudine resistant patients had HBV YMDD mutation of either rtL180M/M204V or rtL180M/ M204I type. PCR based analysis of HBV DNA and sequence based mutation detection can be a practically feasible approach in Bangladesh to monitor hepatitis B patients under lamivudine therapy.

Key words: Lamivudine, Hepatitis B Virus (HBV), Polymerase Chain Reaction (PCR), Mutation

INTRODUCTION

Hepatitis B virus (HBV) infection is a major health problem leading to around one million deaths annually worldwide[1]. A wide range of clinical manifestations has been established for chronic hepatitis B virus infection, from asymptomatic carriers to severe chronic liver disease, including those with cirrhosis and hepatocellular carcinoma.[2-3] 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.[4]

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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.[5]

Lamivudine [(-) 2' 3'-dideoxy–3' thiacytidine], an oral nucleoside analogue, inhibits HBV replication[6-7] and can markedly reduce serum HBV DNA levels and normalize alanine aminotransferase (ALT) levels associated with improvement in liver necroinflammatory activity,[7] but the greatest drawback with lamivudine treatment is the emergence of drug-resistant HBV mutants, the mutation of the tyrosine- methionine-aspartate-aspartate (YMDD) motif in the C domain of the HBV

DNA polymerase gene.[7-8] Close monitoring of viral titer at molecular level (e.g. HBV-DNA) and mutation detection in patient's serum has become increasingly important due to the occurrence of antiviral drug resistant virus strains. Although quantitative analysis of HBV DNA during lamivudin therapy has recently been introduced in Bangladesh, no report on the mutation status is available. The aim of this study was firstly to quantitate HBV-DNA by real time PCR method in Hepatitis B patients, secondly to compare HBV DNA mutation status of patients with lamivudine resistance and hepatitis B patients without lamivudine treatment. The mutation was analyzed by sequence detection via polymerase chain reaction (PCR).

MATERIALS AND METHODS

Patients. Ninety-one serum samples were collected from Hepatitis B group of patients, consisted of HBsAg carrier (n=10), Chronic Hepatitis B patients (n=70) and acute viral hepatitis (n=11). They were 73 men and 18 women, aged 28-63 years. Among them were 14 patients with lamivudine resistance after lamivudine treatment (100 mg/d) with mean treatment period of one year and a half; and 10 hepatitis B patients without lamivudine treatment. None of the patients was ever treated with interferons and other anti-viral drugs. Blood sampling was done at Labaid Ltd, a diagnostic and consultation center of Dhanmondi area. Informed consent of the individual patient was taken before blood collection.

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

DNA Extraction. DNA was extracted from 200 µl of serum with DNA extraction kit, (QIAGEN, Hilden, Germany). DNA was dissolved in Tris – EDTA buffer, and stored at 4ºC till used for PCR.

Quantitative Analysis of HBV DNA by Real time PCR. The HBV Rotor Gene (HBV RG) PCR kit (Sacace Biotechnologies srl. Italy), a ready to use kit of HBV DNA for real time-PCR was used. The HBV RG Master mix contained all the reagents and enzymes 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 3000 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 sequences used were - 5' - GACC ACCAAATGCCCCTAT - 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 95ºC for 10 minutes followed by 45 cycles consisting of 95ºC for 15 sec, 55ºC for 30 sec and 72ºC for 15 seconds.

The problem of carry over contamination was avoided by using pre-sterilized filtered micro tips and the tests were conducted in separate Pre PCR and post-PCR laboratory. The amplification - detection was carried out in a Rotor - Gene 3000 Sequence detector (Corbett Research). The results were considered positive if a signal was detected in Cycling A FAM, 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.

Mutation Analysis in Lamivudin Resistant Samples. The serum HBV DNA mutation of patients with lamivudine resistance was analyzed by sequence detection via polymerase chain reaction (PCR). Primers used for PCR and sequencing are as follows: Upstream primer: 5' CTCCAATCACTCACCA AC 3'; downstream primer: 5' GGGTTTAAATGT ATACCCA 3'; sequencing primer: 5' GTAATTCCC

ATCCC 3'. All the primers above were synthesized by Maxim Biotech (USA). The amplification reaction contained 1 µl each of 25 µmol/L specific primers, 1 µl 10 mmol/L dNTP mixture (dATP, dGTP, dCTP, dTTP), 4 µl 25 mmol $MgCl_2$, 2.5 U *Taq* DNA polymerase (Promaga) and 5 µl 10 × PCR buffer solution. The total volume was brought to 50 µl using ddH_2O. The PCR amplifications were performed in a PTC-200 peltier thermal cycler (MJ Research, USA) under the following conditions: After an initial denaturation for 5 min at 94°C, samples were subjected to 35 cycles of amplification (94°C 45 s, 55°C 45 s, 72°C 1 min), followed by a final extension of 5 min at 72°C. The purification of PCR products was performed by QIAquick PCR purification kit according to manufacture's instructions (QIAGEN, USA). Sequence analysis of the PCR products was performed by DYEnamicTM ET dye terminator cycle sequencing Kit (Amersham Bioscience, USA) in a MegaBACETM 500 according to manufacture's instructions; sequence analysis software was used to analyze the results. Analysis of HBV-DNA mutations was done at the Department of Pathology, University of Pittsburgh, Pittsburgh, PA-15213; USA

RESULTS AND DISCUSSION

Measurement of HBV DNA by Real Time PCR. 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 96.7% (88/91) patients of Hepatitis B group with mean viral load ranging from 1×10^5 to 1×10^9). In HbsAg carrier group, 80% (8/10) was found positive for HBV-DNA with viral load ranging from 1.7×10^5 to 1.5×10^8; in chronic hepatitis B 100% (70/70) with viral load ranging from 1.8×10^6 to 1.96×10^9; and Acute Viral Hepatitis 90.9% (10/11) (Table 1). The twelve samples, retested after one week for confirming the reproducibility of the results, showed similar positive or negative results as shown earlier. HBV - DNA was not found to be positive amongst any of the negative controls. Quantitative analysis of HBV-DNA is very useful for treatment follow-up and to see the response to antiviral in Hepatitis B patients. In the present study, 82.7% (67/81) patients recovered after antiviral therapy as detected by a decrease in HBV DNA concentration to less than 500 copies/ml. 17.3% (14/81) patients showed resistance to lamivudine therapy HBV-DNA concentration either increased or remained same (Table 2).

Table 1. HBV-DNA status of Hepatitis B patients

Disease	HBV-DNA		
	Positive	Negative	Viral Load (Copies/ml)
HbsAg Carrier (n=10)	8	2	1.7×10^5 - 1.5×10^8
Chronic Hepatitis B (n=70)	70	0	1.8×10^6 - 1.96×10^9
Acute Viral Hepatitis (n=11)	10	1	1.0×10^5 - 1.2×10^7

Table 2. Hepatitis B Patient's Response to Lamivudine Therapy

Lamivudive Therapy	HBV-DNA Concentration (Copies/ml serum)	
	Before Treatment	After Treatment
Patients Responded to Lamivudine Therapy (n=67)	1.7×10^5 - 1.96×10^9	< 500
Patients Not Responded to Lamivudine Therapy (n=14)	1.55×10^5 - 1.6×10^9	1.8×10^6 - 1.0×10^{10}
Patients Not Treated with Lamivudine (n=10)	1.0×10^5 - 1.9×10^5	

The quantitative real time PCR assay for the detection of HBV-DNA is a highly sensitive method. 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[9]. Our study shows that the real time PCR assay for HBV-DNA using HBV Rotor Gene PCR kit is specific and is reproducible for the detection and quantitation of HBV-DNA in clinical serum samples.

Mutation Analysis of Lamivudine Resistant HBV-DNA. Fourteen patients with lamivudine resistance and 10 hepatitis B patients without lamivudine treatment were studied in this work. The serum HBV DNA mutation was analyzed by sequence detection via polymerase chain reaction (PCR). Our results indicated that all lamivudine resistance patients had HBV YMDD mutation. Among them, 8 patients (8/14; 57.14%) had rtL180M/M204V mutation, 5 patients (5/14; 35.71%) had rtL180M/ M204I mutation. Mutation status of one resistant patient could not be classified and 10 hepatitis patients without lamivudine treatment had no mutations (Table 3).

Table 3. Types of Mutations in Lamivudine Resistant HBV DNA

Group	Type	n
Patients with Lamivudine Treatment	rtL180M/M204V	08
	rtL180M/M204I	05
	No Classification	01
Patients with Lamivudine Treatment	No Mutation	10

Chronic hepatitis B virus (HBV) can be treated with a nucleoside analogue, lamivudine (2', 3'-dideoxy- 3'-thiacytidine). In the short term, substantial inhibition of HBV replication can be achieved. However, resistance to lamivudine emerges in approximately 14% of patients after 1 year, rising to 43% after 3 years. Resistance is associated with mutations in the highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motif (codons 203-206 of the reverse transcriptase (rt)), which is part of the catalytic site of the HBV polymerase.[10-14] The L180M and M204V mutations act synergistically to increase resistance to lamivudine.[10]

CONCLUSION

Analysis of HBV DNA by real time PCR and sequence based mutations detection may be helpful to evaluate Hepatitis B patients taking lamivudine therapy.

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