

# A PCR-RFLP based protocol for the detection of hepatitis B virus variants in some lamivudine-untreated chronic hepatitis B virus carriers in Pakistan

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**Abstract:** Hepatitis B virus (HBV) affects more than 350 million people worldwide and is a leading cause of morbidity and mortality in developing countries like Pakistan. Lamivudine has potential to inhibit hepatitis B virus (HBV) replication but long term lamivudine treatment results in mutations in YMDD region of HBV, making this therapy ineffective. In this study, we have optimized a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) based protocol to detect two mutations in HBV DNA polymerase gene (at codon 528 and 552) in chronic hepatitis patients, without any prior lamivudine treatment. HBV genome was extracted and tested by PCR-RFLP for detection of mutations in polymerase gene. Variations in HBV genome were not detected in enrolled patients confirming that lamivudine can be used to treat chronic Hepatitis B in these patients. Several studies have reported the natural occurrence of mutation in YMDD motif of polymerase gene in chronic hepatitis B patients, not treated with lamivudine, but these mutants were not detected in Pakistani lamivudine-untreated chronic hepatitis B patients.

**Keywords:** YMDD mutations, chronic hepatitis B, PCR-RFLP, polymerase gene, lamivudine.

## INTRODUCTION

Hepatitis B virus (HBV) affects more than 350 million people worldwide and is a leading cause of morbidity and mortality in developing countries like Pakistan (Wright, 2006; Kazmi and Khan, 2007). Use of vaccination and routine blood donor screening has largely reduced the incidence of acute hepatitis B during the last 20 years but it may still occur in adults and can result in fatal complications as cirrhosis and hepatocellular carcinoma may result due to chronic HBV infection (Chieochansin *et al.*, 2006; Hoofnagle *et al.*, 2007). HBV genome is smallest (3.2kb) among all replication competent DNA viruses, with four open reading frames encoding 7 known proteins (Dienstag *et al.*, 1999; Liaw and Chu, 2009). Among these, DNA polymerase is the focus of much attention as it is the target of many therapeutic approaches including nucleoside analogues like Lamivudine (Lisotti *et al.*, 2008). Lamivudine is used to inhibit replication of hepatitis B virus (HBV) but long-term therapy is required to achieve the target. However, tyrosine-methionine-aspartate-aspartate (YMDD) motif mutations at the active site of DNA polymerase of HBV render resistance to the infected patients against antiviral drug (Lamivudine) therapy (Liaw, 2001; Chieochansin *et al.*, 2006). 14-32% patients develop lamivudine resistance after one year of therapy (Heathcote, 2003; Hadziyannis *et al.*, 2003).

More frequent drug resistant (65% after 5 years) is reported in patients on long-term lamivudine therapy (Matsuda *et al.*, 2004). There are previous reports regarding YMDD motif mutation detection, in HBV mutants, in chronic hepatitis patients who had not received lamivudine therapy (Shin *et al.*, 2003; Ramezani *et al.*, 2008). Some cases of early emergence of mutants have been reported recently, suggesting that mutations responsible for lamivudine resistance were already present in the liver of these patients before starting antiviral therapy (Leon *et al.*, 2004).

If a drug resistant strain of HBV is detected in a patient before starting the lamivudine therapy, it may lead to selection of some other appropriate antiviral drug for therapy improving the overall patient treatment. Hence, sensitive and specific methods aimed at detecting the mutants HBV strains are essential. The aim of this study was to optimize a method for mutation detection in HBV polymerase gene by PCR-RFLP to determine the susceptibility of the use of lamivudine as anti-viral drug in chronic hepatitis patients in Pakistan.

## MATERIALS AND METHODS

### *Patients and methods*

260 chronic hepatitis B patients were tested for the presence of HBV variants having mutations in polymerase gene of virus. Apart from the known signs

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and clinical symptoms related to hepatitis B and serological screening all the samples were also confirmed for hepatitis B virus (HBV) DNA through PCR analysis using the primers specific for hepatitis B virus surface region (Data not shown). All the samples were collected after written or verbal consent from the patients visiting various diagnostic centers and pathological laboratories in different regions of Pakistan. Experimental design was approved by the Research and Ethical Committee of Bahauddin Zakariya University, Multan, Pakistan.

5 ml of the peripheral blood from 260 HBV chronically infected patients was drawn. These patients had hepatitis signs, were untreated with any antiviral drug including Lamivudine and were hepatitis B surface antigen (HBsAg) seropositive. HBV DNA was extracted by phenol-chloroform method as described by Kao *et al.* (2000).

### PCR-RFLP

DNA fragments containing polymerase gene were amplified for the detection of mutations at 528 and 552 codons, that are associated with lamivudine resistance, analyzed further by restriction fragment length polymorphism. Both positive and negative controls were added to increase the authenticity of the reaction. Primer pair HBV409 (5'CATCCTGCTGCTATGCCTCATCT3') and HBSR1 (5'GACCCATAACATCCAATGACATAGCCCATG3') were used for first round PCR. During the second round PCR, F1 (5'CACTGTTTGGCTTTCAGTCAT3') and B2 (5'GTTCAAATGTATACCCAAAG3') amplified the region for subsequent analysis for examining presence or absence of methionine in YMDD motif at codon 552 while F3 (5'GTGGGCCTCAGTCCGTTTCTC3') and B2 amplified the region that was subjected to the detection of leucine at codon 528 in polymerase.

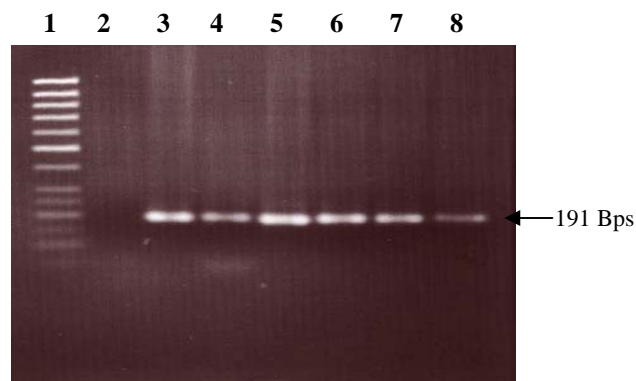
Reaction mixture was 50 µl for first and second round PCR carried out in 0.2 ml tubes. For first round PCR, 10<sup>nl</sup> of extracted HBV DNA while for second round PCR, first round PCR product (1µl) was used as template while the final concentrations of the PCR reagents for each type of PCR were: 1X PCR buffer (10 mM Tris HCl, pH 8.8 at 25°C, 50 mM KCl, and 0.08% Nonidet P-40), 2.0 mM MgCl<sub>2</sub>, 0.15 mM dNTPs (0.2 mM for second round PCR), 50 pM each of forward and reverse primers and 1.5U of Taq DNA polymerase per reaction. Thermocycler conditions for amplification were one cycle 94°C for 5 min, 32 cycles of 94°C for 45 sec (30 sec for second round PCR), 50°C for 45 sec (55°C for second round PCR) and 72°C for 60 sec, and one cycle of 72°C for 7 min (10 min for second round PCR) followed by a hold at 4°C. 8 µl of the PCR products was analyzed by electrophoresis on 2% agarose gel. A set of restriction enzymes *Nde* I and *Hin* I was used to detect mutations at 552 and 528 codons of the polymerase respectively. A 5µl aliquot of each PCR product was digested with *Hin* I

(2.5U) or *Nde* I (5U) at 37°C for overnight. The resulting DNA fragments were resolved by electrophoresis on 7% non-denaturing polyacrylamide gel.

### RESULTS

260 patients, in total, were enrolled in this study; 152 of them were male (59%) while 108 (41%) patients were female. Patients were divided into 3 age groups: group A contained 36 patients in the range of 15-30 years; 98 were enrolled in group B with age ranged between 31-45 years and group C included the remaining 126 patients in age range of 46-55 years. Samples were positive for hepatitis B surface antigen (HBsAg) for more than six months, indicating that all of them were chronic hepatitis B patients. Furthermore all of them were also found positive for the presence of hepatitis B e antigen (HBeAg).

For the detection of YMDD motif mutations, in the first round PCR, a DNA fragment of 498 bps was amplified. The low visibility of the band on agarose gel due to low viral load necessitated a second round PCR using first round PCR product as a template. In second round PCR, 191 bps DNA fragment (fig. 1) was amplified by F3 and B2 primers for the subsequent RFLP analysis for mutation detection at 528 codon. Like-wise another second round PCR through F1 and B2 primers resulted in the amplification of 119 bps DNA fragment (fig. 2). This fragment was subjected to restriction analysis for the detection of the mutation at 552 codon.

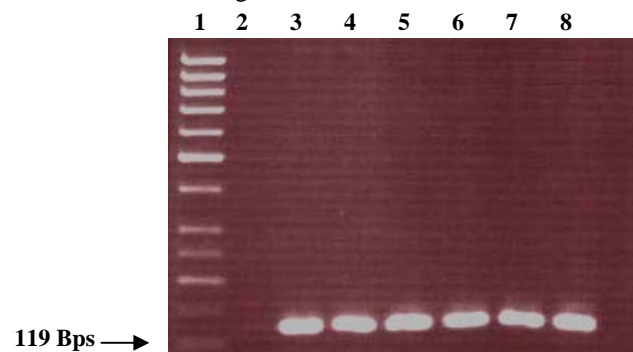


**Fig. 1:** Analysis of second round PCR amplified product of 528 codon region of polymerase gene on 2% agarose gel.

Lane 1: 50bp DNA ladder (Fermentas, UK)  
Lane 2: Negative control  
Lane 3: Known positive sample control  
Lane 4-8: Amplified DNA products (191bp)

To detect C/T to A base change at nucleotide 669, responsible for methionine instead of leucine at 528 codon (L<sup>528</sup> to M<sup>528</sup>), the F3B2 PCR product of 191 bps was digested with *Hin* III. PAGE analysis showed that for all the samples the fragment remains uncut showing the

presence of leucine at 528 codon. This wild type pattern showed the absence of mutation that can be responsible for HBV resistance against Lamivudine.



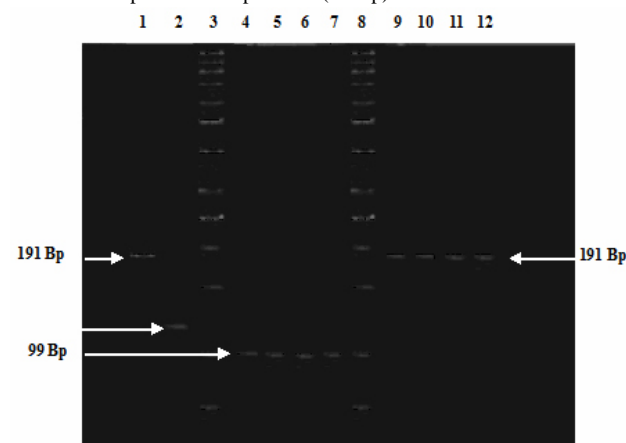
**Fig. 2:** Analysis of second round PCR amplified product of 552 codon region of polymerase gene on 2% agarose gel.

Lane 1: 50bp DNA ladder (Fermentas, UK)

Lane 2: Negative control

Lane 3: Known positive sample control

Lane 4-8: Amplified DNA products (119bp)



**Fig. 3:** Gel image of RFLP assay for detection of HBV variants at codon 528 and 552 in YMDD nucleotide sequence after *Hin*III and *Nde*I enzyme digestion respectively.

Lane 1: Uncut DNA fragment for 528 codon (191 bp)

Lane 2: Uncut DNA fragment for 552 codon (119 bp)

Lane 3 & 8: 50 bp ladder (Fermentas, UK)

Lane 4 to 7: Wild type pattern for 552 codon (99 bp & 20 bp) (20 bp band is not visible)

Lane 9 to 12: Wild type pattern for 528 codon (191 bp)

For detecting YMDD mutation, the F1B2 PCR product of 119 bps was digested with *Nde*I to examine base change of A to G at nucleotide 741 for methionine to valine (YM<sup>552</sup>DD to YV<sup>552</sup>DD). In this case again only the wild type pattern was seen for all the samples since the restriction analysis generated two bands of 99 bps and 20 bps (fig. 3). In few samples, for the first instance after digestion a mixture of bands equal to size of uncut (119 bps) representative of mutation either of M<sup>552</sup> to V<sup>552</sup> or a base change of G to T at nucleotide 743 for methionine to isoleucine (YM<sup>552</sup>DD to YI<sup>552</sup>DD) as well as digested fragments (99 bps and 20 bps) was observed. In order to

confirm it the F1B2 PCR product was digested with *Hin*III and then with more amount of *Nde*I but this showed only the fragment representative for wild type pattern for YMDD region. This confirmed that all the samples from chronic hepatitis B patients were free from HBV mutant strains.

## DISCUSSION

Hepatitis B virus (HBV) is a blood-borne pathogen and a member of the hepadnavirus family. Replication of HBV takes place from intermediate RNA molecule, called pre-genomic RNA, via reverse transcriptase enzyme. The largest part of HBV genome encodes viral polymerase that contains both RNA-dependent DNA polymerase (reverse transcriptase) as well as DNA-dependent DNA polymerase activity (Allen *et al.*, 1999). Lamivudine is among the many current therapeutic approaches for treating HBV infection that focus on inhibition of viral replication by inactivating the DNA polymerase (Ramezani *et al.*, 2008).

Lamivudine can be used for the short-term treatment of chronic HBV as it inhibits the DNA replication of HBV. However, during long-term treatments Lamivudine resistant strains has been detected (Chieochansin *et al.*, 2006). Mutant viral genomes are frequently found in HBV as it employs reverse transcription to replicate its genome. It is likely that HBV mutations, conferring resistance to lamivudine, may pre-exist under the conditions of high levels of viral replication. Pre-existing polymerase gene mutants have been documented in some lamivudine-naïve asymptomatic HBV carriers or chronic hepatitis B patients (Kobayashi *et al.*, 2001; Kirishima *et al.*, 2002; Lee *et al.*, 2006). These mutations, especially in YMDD region, renders patients treatment ineffective. Therefore, the development of a detection method and monitoring of polymerase gene mutations is important in the lamivudine treatment of chronic hepatitis B patients when the mutant virus is still little friction in the total HBV population (Bowden *et al.*, 2003; Pichkovskaia *et al.*, 2004). In the present study, high level of HBV DNA was isolated from patient blood samples and HBsAg was present but all viral genome were without a pre-existing YMDD mutation. So, lamivudine can be used in these patients as an antiviral drug against HBV.

Our findings are in agreement with some previous studies (Kirishima *et al.*, 2002; Zhang *et al.*, 2003; Ramezani *et al.*, 2008). Absence of HBV mutant strains may be explained by experimental conditions such as the number of enrolled patients and the detection method used to identify the polymerase gene, especially YMDD motif, mutants and epidemiological and geographic variations as YMDD mutants have significantly low replication ability and it is generally considered that the naturally occurring YMDD mutants are only a very small proportion of the total HBV, and very sensitive investigative tools are

required to detect them (Heo *et al.*, 2004; Ramezani *et al.*, 2008). Direct sequencing is a conventional method for screening DNA mutations but it can only detect major HBV populations (Hong *et al.*, 2004). In addition to that it requires sophisticated instrument and is unaffordable for majority of the population of developing country like Pakistan. The problem could be solved by developing and establishing relatively cost effective and sensitive method like PCR-RFLP.

In conclusion, we have optimized a sensitive and specific PCR-RFLP method using mutant-specific primers to detect the HBV polymerase gene mutations. This assay might prove to be an effective tool for monitoring the viral genome in chronic hepatitis B patients in order to make decision regarding the use of lamivudine as anti-viral therapy as the use of lamivudine against mutant HBV will be a waste of time and money.

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