

# The incidence of Drug- Resistant Mutants in Patients With Chronic HBV Infection After Lamivudine Treatment

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**Background:** Hepatitis B virus (HBV) is replicated through reverse transcription of polymerase gene. Lamivudine can postpone the clinical progression in Hepatitis B infected patients, but the long-term monotherapy causes the emerge of YMDD (tyrosine-methionine-aspartate-aspartate), LLAQ (leucine-leucine-alanine-glutamine) motifs and increases the Alanine aminotransferase (ALT) and HBV DNA levels.

**Objectives:** The aim of this study was to investigate rtL180M and rtM204V mutations in polymerase gene of HBV in infected patients after Lamivudine therapy.

**Patients and Methods:** Fifty sera samples collected from patients who referred to Blood Transfusion Center in Tehran were studied. The samples were divided into two groups; treated and untreated based on antiviral treatment status. From 50 patients, 34% were males and 66% were females, aged between 18 and 80 years. All of samples were tested for anti hepatitis B e antibody (anti-HBe), hepatitis B e antigen (HBeAg), hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (anti-HBc) by enzyme-linked immunosorbent assay (ELISA) method. The ALT levels were measured using a commercial kit. Then HBV-DNA was extracted from serum samples using a commercial kit and a fragment of the P gene was amplified by nested PCR. Also, HBV viral load was detected by Real-Time PCR. HBV genotypes and polymerase gene mutations were determined by direct sequencing of the polymerase gene of HBV. Phylogenetic tree was constructed using neighbor-joining (NJ) method.

**Results:** About 6% (3 of 50) of samples were HBeAg positive and 94% (47 of 50) of patients were HBeAg negative by ELISA method. The patients' ALT level was between 16 and 95 IU/L with the mean of 37.58 IU/L. Also, 48% (24 of 50) of samples had <104 IU/mL viral load, 52% (26 of 50) of them had >104 IU/mL viral load. About 10% (5 of 50) of samples was treated with Lamivudine with specific substitution of amino acid located at codons 80, 180 and 204. In addition, phylogenetic tree showed that genotype D of HBV was dominant among Iranian HBV infected patients

**Conclusions:** This study showed that the presence of mutation at codons rtL80V, rtL180M and rtM204V in A, B and C domains is associated with higher viral load and resistance to Lamivudine (3TC) respectively.

**Keywords:** YMDD Mutant; Polymerase Gene; Hepatitis B Virus; Lamivudine

## 1. Background

Hepatitis B infection has three states, acute, chronic and asymptomatic carrier. Hepatitis B virus (HBV) infection ranks as the tenth leading cause of death (1). HBV strains are divided into 8 genotypes (A-H), based upon an inter-group divergence of at least 8 percent in the complete nucleotide sequence (2), with almost similar clinical manifestations of all these genotypes (3). Also HBV is divided into 9 serologic groups based on HBV surface antigen (HBs Ag) (4, 5).

Chronic hepatitis B (CHB) infection causes developing cirrhosis, hepatic decompensation and hepatocellular carcinoma (1, 6). This disease is common in Asia and Africa whereas is not common in Western countries (1). HBV

has high mutational rate due to the presence of active replication and high number of viral copy production as well as lack of proofreading activity in HBV polymerase (6).

HBV genotypes do not spread with the same incidence in the world (7, 8). Genotype D has the most prevalence in Mediterranean region, India, Southern Europe, North and South Africa (9). Previous investigations showed that predominance serotype D was found in Iran (10, 11). The length of HBV genome is 3200 base pairs (bp) encoding four known genes, called C, X, P and S (12). The P gene encodes a polypeptide (90 kD), with four different activities; the terminal protein (TP) acts as a primer for synthesis negative DNA strand, the RT enzyme uses RNA as a tem-

### Implication for health policy/practice/research/medical education:

In order to reduce of liver damage in the group of chronic hepatitis B patients with Lamivudine therapy that causes the mutations in P gene of HBV and no response to treatment.

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plate for negative DNA strand transcript, RNase-H decomposes RNA template, and DNA polymerase enzyme synthesizes positive DNA strand from negative DNA strand (13).

The purpose of HBV infected patient treatment is to suppress virus replication, long-lasting maintenance of undetectable levels of serum HBV DNA and prevention of liver damage (14). Nowadays Antiviral agents are oral nucleoside analogues such as Lamivudine (LAM), Entecavir (ETV), Telbivudine (TBV), Adefovir (ADV) and Tenofovir (TDF) that are used for chronic hepatitis B management. These antiviral agents inhibit HBV reverse transcriptase and also control HBV replication, enhancing hepatitis B e antigen (HBeAg) seroconversion and reducing liver damages (15, 16). The best antiviral agent is determined by its ability to act as a competitive inhibitor of the HBV polymerase relative to that of the natural substrate (15). Long term use of nucleotide/nucleoside analogues can lead to viral resistance.

Lamivudine (B-L, 2,3-dioxy-3-thiacytidine) has the highest rate of antiviral resistance and Entecavir has the least rate of mutation resistance (15). Mutations that cause resistance to nucleoside/nucleotide analogues mainly involve polymerase gene and this gene contains 7 functional domains (A - G) and these mutations are located essentially in domain A through E (14). Factors such as age, gender, and disease status had no effect on the incidence of Lamivudine-resistant mutations (17). Due to drug resistance mutations, recent studies suggest the need for a combinational antiviral therapy (18).

## 2. Objectives

In this study the presence of rtL180M and rtM204V mutations in the HBV polymerase gene was investigated using the sequencing method among hepatitis B infected patients after lamivudine treatment.

## 3. Patients and Methods

### 3.1. Patients

Fifty sera from patients, who referred to Blood Transfusion Center in Tehran, were collected in 2011. A total of 50 patients were included in this study, there were 17 (34%) male and 33 (66%) female aged between 18 and 80 years (mean 40.6). Based on the antiviral therapy procedure, patients divided into two groups; 1) HBV infected Patients (27 cases that treated with lamivudine (100 mg/dL) with a mean treatment period of 18 months and 2) HBV infected patients (23 cases) not treated with lamivudine. Also, patients who were not treated with other antiviral drugs excluded from this study. All of sera samples were stored at -70°C for further study. The questionnaires were completed by patients finally.

### 3.2. ELISA Method

All patients were tested for serologic factors including hepatitis B surface antigen (HBsAg), hepatitis B e antigen (HBeAg), hepatitis B e antibody (anti-HBe) and hepatitis B core antibody (anti-HBc) by enzyme-linked immunosorbent assay (ELISA) method using DIA.PRO kit All serological tests were performed as instructed by the manufacturers. Also the levels of ALT were measured using PARS AZMUN kit, Iran.

### 3.3. Real Time PCR

HBV DNA was extracted from serum samples using the QIAamp DNA mini kit (QIAGEN, Germany) according to manufacturer instructions. 200 µL serum samples were used and eluted into 50 µL of buffer. The HBV DNA levels were detected by Real Time PCR using Artus HBV LC PCR kit (Artus GmbH, Hamburg, Germany).

### 3.4. Nested PCR

The nested polymerase chain reaction assay (PCR) was carried out on extracted HBV DNA by specific primers (10). Briefly, The target polymerase gene was amplified in a 50 µL reaction consisting of 5 µL 10×PCR buffer with 1.5 mM MgCl<sub>2</sub>, 1 µL 10 mM dNTPs, 1 µL of outer primer 10 pmol/µL, 0.5 µL AmpliTaq Gold DNA Polymerase (5U/µL), and 36.5 µL nuclease free water. The thermal profile used in Techne thermocycler consisted of 94°C for 5 minutes, followed by 30 cycles at 94°C for 45 seconds, 63°C for 45 seconds, 72°C for 45 seconds, followed by a final extension at 72°C for 10 minutes and a final hold program at 4°C (10).

The outer primers were F1 (sense, nt 56-76, 5'-CCTGCTG-GTGGCTCCAGTTC-3') and R1 (antisense, nt 1395-1416, 5'-CGTCCCGCG (AC) AGGATCCAGTT-3') (10). In second round of Nested-PCR, 48 µL master mix containing the primer F2 (sense, nt 298-320, 5'-CYTGGCCWAAATTCGAGTCCC-3') and R2 (antisense, nt 997-1019, 5'-GCAAANCCCCAAAAGAC-CACAAT-3') and 2 µL first round PCR products were used under the same conditions. The cycling profile for the second round PCR to produce amplicon, consisted of 94°C for 5 minutes, followed by 30 cycles at 94°C for 45 seconds, 56°C for 45 seconds, 72°C for 45 seconds, followed by a final extension at 72°C for 10 minutes and a final hold program at 4°C.

Then horizontal gel electrophoresis on 1% agarose gel in TAE 1x buffer at 85 V/h was carried out using ladder specific, a single 721 bp band was showed after the second round of Nested-PCR. The polymerase gene was amplified by PCR in a 100µL volume and then was purified with high pure PCR product purification kit (Roche, Germany).

### 3.5. HBV DNA Sequencing

PCR product was sequenced with dideoxy method using PCR primer (Takapozist Co., Iran). The polymerase gene was analyzed using CEQ 8000 analysis software. Using the CLUSTAL W 1.8 software package, the nucleotide-sequences of HBV strains were aligned with a refer-

ence panel of sequences representative of each subtype retrieved from the Gen Bank database. Phylogenetic tree was drawn using the neighbor joining method. The amino acid sequence encoded by the nucleotide sequence was also analyzed to assess possible drug mutations. Data were analyzed with SPSS, version 16 ( $P < 0.05$ ).

#### 4. Results

A 6% (3 cases) of samples were HBeAg positive and 94% (47 cases) of patients were HBeAg negative employed ELISA method. All patients were positive for HBV DNA by PCR assay that 48% (24 cases) of samples had a viral load of  $< 10^4$  IU/mL and 52% (26 cases) of them had viral load  $> 10^4$  IU/mL. The mean of HBV DNA level was  $2 \times 10^7$  IU/mL. About 54% (27 of 50 cases) of samples had treatment history with antiviral therapy using Lamivudine and 46% (23 cases) of them had no treatment history. A 33.3% (1 case)

of HBeAg positive/HBeAb negative patients had the viral load less than  $10^4$  IU/mL and 66.6% (2 cases) of them had the viral load more than  $10^4$  IU/mL; none of them were treated. Also 49% (23 from 47 cases) of HBeAg negative/HBeAb positive patients had a viral load less than  $10^4$  IU/mL and 51% (24 cases from 47) of them had a viral load more than  $10^4$  IU/mL that these patients had treatment history (Tables 1, 2).

The patients' ALT levels were between 16 and 95 IU/L with the mean of 37.58 IU/L. ALT levels in 18.5% (5 of 27 cases) and 4% (1 of 23 cases) of treated and untreated patients were high, respectively. The PCR products were sequenced in the region of the HBV polymerase gene encoding the YMDD motif. Only 10% (5 cases) of patients that were taken antiviral therapy had specific substitution of amino acid; L80V, L180M and M204V. These patients were HBeAg negative and had viral load of  $> 10^4$  IU/mL.

**Table 1.** Frequency of HBeAg/HBeAb among Patients With Different Viral Load

Viral Load	$< 10^4$ IU/mL, No. (%)	$> 10^4$ IU/mL, No. (%)	Total, No. (%)
HBeAg Positive/Anti-HBe Negative	1 (2)	2 (4)	3 (6)
HBeAg Negative/Anti-HBe Positive	23 (46)	24 (48)	47 (94)
Total	24 (48)	26 (52)	50 (100)

**Table 2.** Frequency of HBeAg/HBeAb in Treated and Untreated Patients With Different Viral Load

Viral Load	$< 10^4$ IU/mL, No. (%)		$> 10^4$ IU/mL, No. (%)		Total, No. (%)
	Treated	Untreated	Treated	Untreated	
HBeAg Positive / Anti-HBe Negative	–	1 (2)	–	2 (4)	3 (6)
HBeAg Negative / Anti-HBe Positive	20 (40)	3 (6)	7 (14)	17 (34)	47 (94)
Total	20 (40)	4 (8)	7 (14)	19 (38)	50 (100)

Also, phylogenetic analysis results revealed that all HBVs infected the Iranian HBV patients were genotype D. The studied sequences from this study were compared with GenBank sequences in a BLAST search, the results of blast showed that 75% of isolates from this study were similar to Iranian isolates (accession No. JN040770). Also, 20% and 5% of sequences were similar to isolates from other countries (Syria, accession No. JN257205 and Turkey, accession No. JF754628, respectively).

#### 5. Discussion

Polymerase gene is responsible for reverse transcriptase in HBV but it does not have proof reading activity which causes these sort of mutations. By pressure created by the antiviral agents, the selected viruses had replicative advantage in presence of antiviral agents (15). Previous studies showed that using of Lamivudine may reduce serum HBV DNA levels and normal ALT levels in patients (19), however, long term use of Lamivudine cause emerging YMDD mutant and increasing in ALT and HBV DNA levels. Then, this is emphasis that all patients with high levels of viral load, after long term use of Lamivudine,

should be examined for the presence of drug resistance in polymerase gene (20).

One of the factors that increase the risk of drug resistance is ALT, a high level of ALT and aspartate aminotransferase (AST) enzymes in the samples can be a sign of liver damage (12). The other factor is the incomplete suppression of viral replication (12). Detection of HBeAg is an indication of active replication in HBV (21). Also the anti-HBe antibody usually is a sign for inactivation of viral replication, or a response to treatment (21). Detection of HBV-DNA level is a criterion for determining the state of infection, the risk of progression toward cirrhosis and HCC, identification of patients who need antiviral therapy, and identifying emergence of drug resistance. In our study, despite the positivity of anti-HBe, 12.2% (6 cases) of patients had high ALT levels with the mean of 76 IU/L and the mean viral load was  $5 \times 10^6$  IU/mL.

The results of sequencing showed that 10% (5 cases) of treated patients had rtL180M / M204V mutations due to amino acid substitution from Leucine to methionine located in LLAQ motif (rtL180M) which were converted to LMAQ motif, also in YMDD motif (rtM204 V) due to the

amino acid substitution from methionine to valine that were converted to YVDD motif and also, these patients had the amino acid substitution rtL80V in A domain. All these patients were received Lamivudine for a period of 18 months. The statistical results showed that there is a significant relationship between liver enzyme and viral load in patients with rtL180M / M204V mutations. These results were in agreement with a study on Japanese patients treated with Lamivudine that showing rtL80V (domain A) and rtL180M in conjunction with the M204V/I changes that conferred Lamivudine resistance. these doubles and triplicates changes are associated with higher viral loads (12).

Ghandehari et al. in 2011 showed that the rate of YMDD motif mutant in chronic hepatitis B virus infected patients who treated with lamivudine, was approximately 14% (22). another study in 2008, reported a 31% rate YMDD mutation in 107 patients in North China (23). A study in 2005 showed that 41.3% of HBV infected patients had rtL180M / M204V mutation and also patients undergo the treatment with other medications than Lamivudine had shown no mutation, also this study indicated that rtM204V mutation is always combined with rtL180M and the occurrence rate of this kind of combinational mutation was 100% (23).

In our study mutation in polymerase gene was not observed in the all patients who have not received prior Lamivudine therapy. An investigation in Iranian population showed that patients without Lamivudine therapy had no rtL80M / M204V mutations (24). Also, another study on infected blood donors with HBV showed that Lamivudine resistance mutations (rtL180M / rtM204V) strains are not detected in Iranian blood donors (10). Sequence analysis of our study showed similar results with previous study, that genotype D of HBV was dominant among Iranian patients (10). Also, all HBV infected patients had the same amino acids including I92L, P110S, T119N, N122I, I123F, Y125H, D132N, K150Q, S224A, I225V, N249H, S257C, W258Y, T260S, E264D, V267I, L268Q, L270I, Q272E.

Also, sequence analysis showed that, 75% of the nucleotide sequence of HBV isolates had similarity with HBV isolates from Iranian patients and 25% had similarity with the nucleotide sequence of HBV isolates from other countries (Syria and Turkey), therefore the presence of these circulating isolates from Iranian patients may be the result of traveling to such countries.

In conclusion, HBV mutations which play an important role in Lamivudine resistance, usually located at polymerase gene, including rtM204V in C domain and rtL180M in B domain and L80V in A domain and triple mutations in this gene are associated with higher viral loads and level of ALT. Therefore evaluation of these sites of polymerase gene is necessary for detecting Lamivudine resistance in HBV patients treated with Lamivudine.

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## Authors' Contribution

None declared.

## Financial Disclosure

The authors declare that they have no conflict of interest.

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