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The Lamivudine/ Entacavir Resistance Mutations Among Treatment Naïve Chronic HBV Infected Patients Mashhad, Iran

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ABSTRACT

Background and Aim: Despite significant progress in Hepatitis B Virus (HBV) treatment, the emergence of drug resistance mutations is a main challenging health threat. The data is lacking regarding circulation mutant strains in northeastern Iran; therefore, the present study was conducted to investigate HBV reverse transcriptase (RT) inhibitors drug resistance mutations in a group of treatment naïve patients in Mashhad, Iran.

Materials and Methods: In this study, 25 patients were included. The genomic DNA was extracted from serum samples, and the *RT* gene of HBV was amplified using specific primers. The PCR products were then subjected to gel electrophoresis and were next sent for sequencing. Finally, the sequences were analyzed using the HBVseq database, mutation list analysis software supported by Stanford University (https://hivdb.stanford.edu).

Results: The mean age of the patients was 42.7±16.5. Among the patients, 56% were men. Among 23 cases (92%), no resistance mutation was observed, while 2 cases showed mutations outside the YMDD motif of viral reverse transcriptase causing Lamivudine or Entecavir resistance. The detected mutations included: rt T184A, S202I, S202H, V180I, I169L, and V173L. All sequenced samples were identified as genotype D.

Conclusion: Lamivudine/Entecavir resistant variants are circulating in a minority of treatment naïve patients, which may indicate transmission of mutated stains to these patients or may be due to prolonged replication of the virus. This finding might be considered an alarm for increasing circulating mutant variants.

Keywords: Hepatitis B Virus (HBV), Lamivudine, Resistance, *RT* gene

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1. Introduction

Chronic hepatitis B (CHB) infection is one of the most common liver diseases, affecting about 300 million people worldwide **(1)**. Clinically, HBV Infection may cause acute hepatitis and fulminant hepatitis, and chronic infection may lead to cirrhosis, hepatic insufficiency, and hepatocellular carcinoma **(2)**. Data are lacking regarding precise estimation of HBV infection among the Iranian population. One meta-analysis reported high seroprevalence of 30.9% (95% CI: 27.88–33.92) among IV drug abusers; and a prevalence of about 1% among the general population **(3)**. However, it seems logical that such estimation needs to be confirmed by additional epidemiological data. To cope with the virus and the development of therapeutic approaches, the viral replication cycle should be precisely noticed. The genome of HBV is a partially double-stranded relaxed circular dsDNA (cccDNA), approximately 3200 bp size. After infection,

the virus enters the host's liver cells, thereafter, the rcDNA genome is released into the nucleus, where the rcDNA forms the closed covalent circular DNA (cccDNA) configuration **(4)**. The cccDNA serves as a stable mini chromosome and encodes all viral RNA transcripts **(5)**, among which pgRNA (pregenome RNA) forms relaxed circular DNA (rcDNA) through the function of the reverse transcription (RT) enzyme of HBV **(6)**. Therefore, replication of HBV is completely dependent on the activity of the viral RT enzyme. The enzyme is error-prone, and misincorporation of nucleotides in the growing DNA strand leads to the emergence of mutated progeny viruses **(7)**.

In recent decades, several anti-HBV drugs have been developed and approved, including conventional alpha Interferon, pegylated alpha Interferon and Nucleoside/Nucleotides analogues (NAs). Three classes of NAs are now available: i) L-Nucleoside analogues, including Lamivudine, Telbivudine, and Emtricitabine, ii) deoxyguanosine analogue (Entecavir, ETV), and iii) acyclic nucleoside phosphonate analogues such as Adefovir and Tenofovir **(8)**. These drugs are incorporated into the growing DNA during the DNA synthesis step, leading to chain termination and thereby inhibiting viral replication **(9)**.

Resistances to NAs result from amino acid (aa) changes in HBV RT. The resistance mutations have been defined as primary and secondary drug resistance mutations. Eight main amino acid changes are related to primary drug resistance, including rtI169T, rtA181T/V, rtT184S/C/G/A, rtA194T, rtS202G/C/I, rtM204V/I, rtN236T, and rtM250V, where rt indicates reverse transcriptase. The secondary resistances are related to the following three codon changes: rtL80I, rtV173L, and rt180M **(10)**. Primary mutations reduce sensitivity to an antiviral agent by replacing amino acids, while secondary mutations result from compensatory mechanisms to regenerate replication defects caused by primary drug resistance and are generally associated with a low level of drug resistance **(11)**.

Among NAs, Lamivudine acts as an RT inhibitor in both HIV and HBV infections, though the dosage for HBV therapy is higher than for HIV. Despite remarkable improvement in HBV infection treatment with Lamivudine, the drug is associated with high resistance levels **(12)**. The main domain of creation resistance mutations is the YMDD motif (rtY203-rtD206), located in the C domain of HBV polymerase. In this regard, substituting Methionine for Valine, Isoleucine, and rarely Serine at 204 renders mutated motifs: YVDD rtM204V, YIDD rtM204I, and YSDD rtM204S. These structural changes cause less drug affinity to the viral polymerase, where the drug exerts its inhibitory effect **(13)**.

Drug resistance mutations have important effects on infection control, considering the prevalence of HBV infection **(14, 15)**. It is important to monitor HBV drug resistance mutations among these patients. Therefore, the present study was performed to investigate RT resistance mutations among treatment-naïve patients in Mashhad, Iran.

2. Materials and Methods

Study Group

The present study was conducted on the serum samples of 25 HBV-positive treatment naïve patients referred to the central health center of Mashhad. The demographic characteristics of the participants are shown in Table 1. Sera were taken from the patients and kept frozen until DNA extraction was used.

DNA Extraction and PCR

Based on the manufacturer's instructions, DNA was extracted from serum samples using DynaBio™ Viral Nucleic Acid (DNA/RNA) Extraction Mini Kit (Takapouzist, Tehran, Iran). Next, the RT gene of HBV was amplified by polymerase chain reaction (PCR) using previously described specific primers; forward: UP4: 5′-TTC CTG CTG GTG GCT CCA GTT C-3′ and reverse: DOWN2: 5′-TTC CGC AGT ATG GAT CGG CAG -3′ **(16)**.

The PCR reaction was performed in a total volume of 25 µL, as shown in Table 1. The thermal program consisted of 40 cycles of denaturation (95°C, 5 sec), annealing (63°C, 20 s), and extension (72°C, 60 s). The PCR products were then subjected to gel electrophoresis on 1.5% agarose gel and were sent for sequencing (Topaz gene company, Karaj, Iran).

Sequencing and Data analysis

Sequencing was performed based on the Sanger method, and the results were visualized using Chromas software. Sequences were then subjected to BLAST at the NCBI website (https://blast.ncbi.nlm.nih.gov) to ensure sequence homology with the HBV RT gene. After that, the sequences were analyzed using Stanford University's HBV drug resistance database (HBVseq). The database analyzes submitted HBV RT sequences and determines the genotype and provides data regarding known drug resistance mutations. The database is online available at https://hivdb.stanford.edu.

Table 1. PCR reaction components

Materials	Volume (µL)
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
Master mix (2 X)	12
DNA ~ (200-400 ng)	variable (1-2)
H_2O (deionized water)	up to 25

Table 2. Demographic characteristics of the HBV-positive patients included in the study

		Patients (N)	Percentage (%)
Age Range	Less than 20 yr	2	8
	40-20 yr	9	36
	More than 40 yr	14	56
Gender	Men	14	56
	Women	11	44

3. Results

The mean age of the patients was 42.7±16.5, among whom 56% were men. The patients' age and gender characteristics are presented in Table 2.

As demonstrated in Figure 1, a 1225bp band corresponding to the amplified HBV RT gene was observed on gel electrophoresis.

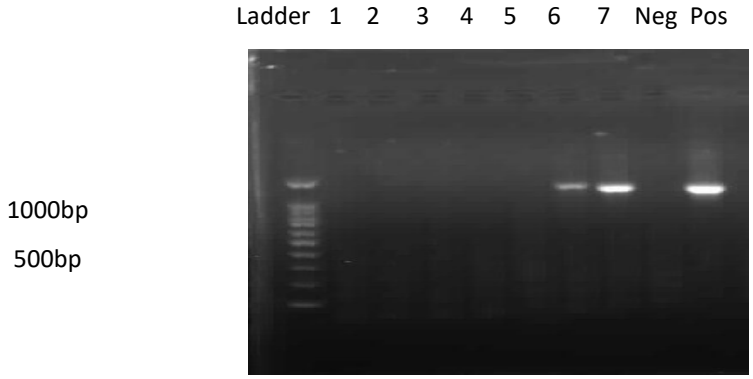

Figure 1. 1225bp band corresponding to amplified RT gene with PCR

Based on sequence analysis, all patients were defined as group D genotype. In two patients, primary and secondary resistance mutations to Lamivudine and/or Entecavir were observed. The detected mutations are listed in Table 3.

Table 3. Resistance mutations in studied patients

Resistance mutation	Patients (N)	Percentage (%)
None	23	92
180I, 202H*	1	4
169L, 173L, 184A, 202I	1	4
Total	25	100

*Substituted amino acid has been mentioned in the text

4. Discussion

The present study provides data regarding circulating drug resistance mutations in our treatment of naïve HBV-infected patients. The mutations reported in the present study consist of primary and secondary drug resistance mutations. The mutations: rtS202H, I169L, T184A, and S202I confer primary resistance to Entecavir (ETV). The secondary resistance mutation V180I is responsible for Entecavir/Lamivudine cross-resistance. In contrast, secondary mutation V173L causes resistance to Lamivudine. Multiple resistance mutations were observed in two patients; both were over the age of 65 yrs. The observed mutations were all outside the YMDD motif (rtY203-rtD206). Spontaneous accumulation of drug resistance mutations in the elderly has been previously reported in treatment-naïve patients due to prolonged continuous replication of the virus **(17-19)**.

Antiviral drugs are used in combination regimens to ensure precise inhibition of viral replication. However, still minor mutant variants continue to replicate under the selection pressure of antivirals. These variants will become the dominant strain in the patient.

Several viral and host factors are responsible for drug resistance mutations. First, the mutations occur spontaneously due to the high replication rate of HBV. Secondly, the RT enzyme is error-prone and lacks proofreading to correct nucleotide misincorporations **(20)**, and third, there is inadequate treatment and person-to-person transmission of mutant variants **(21)**. In this regard, it has been shown that the emergence and selection of mutations inside the YMDD motif of HBV polymerase occur rapidly during inadequate Lamivudine treatment **(22, 23)**. However, mutations outside YMDD-motif generally emerge due to long-term accumulation of spontaneous mismatches **(24)**.

Several studies have reported different resistance mutations in Iran, though this field still lacks data. Similar to our finding, in one study in South Khorasan in 2016, mutation rtV173L was observed in two cases (3.2%), a compensatory mutation conferring Lamivudine resistance **(25)**. The other mutations reported in this study have been previously reported in other countries **(21, 26-28)**. We did not observe other reported mutations in Iran, including Q149K, L122F, N118D/T, L157M, H124Y, rtA181T, rtA181S, rtM204I, rtA181T, rtN236, and rtA181V**(25, 29, 30)**.

In the present study, all patients were genotype D, consistent with other studies reporting genotype D as the dominant genotype in Iran **(31-34)**. In a broader view, genotype D is predominant in the Middle East and Mediterranean countries. HBV genotypes play a critical role in therapeutic management as well as disease prognosis **(35)**. According to recent studies, genotype D is associated with more severe diseases in Asia and may predict the occurrence of hepatocellular carcinoma in young patients **(36)**. Genotypes other than genotype D have been reported in two studies in the Kermanshah and Khuzestan provinces of Iran **(37, 38)**.

To express some limitations of this study, a larger sample size could result in complete information, and studies with more included samples are recommended. Studies among patients receiving anti-HBV treatment also could provide data on this group. Nevertheless, this study is one of the first reports on drug resistance in Mashhad. It is also noteworthy that some detected mutations have not previously been reported in most Iranian studies.

It should be considered that sequencing is not routinely performed in cases of HBV treatment failure; therefore, mutation detection investigations can help to explain the cause of treatment failures. Such research should be performed in defined intervals to follow the emergence of new circulating mutations among the infected population.

5. Conclusion

Primary and secondary Lamivudine/Entecavir resistance mutations are circulating in a minority of treatment naïve patients. This finding might indicate transmission of mutated stains to these patients or prolonged virus replication. Mutations in the *RT* gene of HBV polymerase may rapidly emerge among patients with short-term inadequate Lamivudine therapy and may be transmitted to other individuals. Some mutations were not previously reported in most Iranian studies; this might be an alarm for the increasing circulation of mutant variants in the population.

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Ethical Approval

The investigation was performed in accordance with the Helsinki declaration on human research. The study proposal was reviewed and approved by the ethical committee of Mashhad University of Medical

Sciences; ethical code no: IR.MUMS.MEDICAL.REC. 1397.671.

Authors' Contribution

Author ES did the main experimental work and wrote the manuscript with MY. AAS helped in sampling and provided patient samples. MD helped in proposal design and data interpretation with MY. ST and AM helped with technical issues and experimental work. MY was the principal investigator, supervised the study and wrote the manuscript with ES.

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Conflict of Interest

No conflict of interest to declare.

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