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First Report of Entecavir and Tenofovir Resistance in Iraq for Chronic Hepatitis B Patients

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Abstract: The primary goal of therapy for chronic hepatitis B (CHB) is to prevent liver disease progression. In patients with drug-resistant hepatitis B virus (HBV), a combination of tenofovir disoproxil fumarate (TDF) and entecavir (ETV), which is strongest combination therapy against HBV. However, long-term tolerance data are lacking, and cost may be an issue for combination therapies. Several, well-designed, randomized controlled trials have shown that TDF monotherapy provides similar antiviral efficacy compared with the combination of TDF and ETV. Mutations in the polymerase (*Pol*) gene of hepatitis B virus (HBV) are often associated with drug resistance. The pattern of mutations varies geographically, thus giving rise to infection to HBV diversity.

This study was carried out to detect mutations in *Pol* gene of hepatitis B virus isolated from CHB Iraqi patients. Selected 20 CHB patients who's had highly viral load after treatment course (6 months) were analyzed by PCR and sequencing, also S202GCI mutation was most frequently detected 9/20 (45%) and followed by M204V/I/S (40%), L180M (35%), M250V/I/L and A181T/V (30%), T184SCGA, N236T and A194T (25%), T184ILFM (10%)**.** T184SCGA, T184ILFM, S202GCI and M250V/I/L mutations association with Entecavir resistance, A194T mutation association with Tenofovir resistance and L180M, A181T/V, M204V/I/S and N236T mutations association with multi –drug resistance.

Keywords:. *Pol* **,** CHB **,** HBV **,** Tenofovir .

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Introduction:

Hepatitis is an inflammation of the liver (1). World Health Organization was estimated in 2016; two billion people worldwide have been infected with HBV. More than 240 million are chronic carriers with serious long term complication like cirrhosis and hepatocellular carcinoma. Between 1 and 2 million of this population die annually, most of them in Asia (2).

Nucleotide analogs such as Tenofovir and Entecavir work well against both HBV and HIV. Tenofovir and Entecavir confer potent and durable HBV-DNA suppression but the best strategy in case of resistance of HBV to reverse transcriptase inhibitor Tenofovir remains unknown. New tests are being developed to study HBV resistance (3,4). Levels of Tenofovir resistance in individuals with viral failure ranged from 20% in Europe to more than 50% in sub-Saharan Africa (5,6). It is likely that 7.5-17.5% of individuals given Tenofovir plus cytosine analogue plus Entecavir will develop Tenofovir and Entecavir resistance within 1 year of treatment initiation under present practices in sub-Saharan Africa. One study has reported HBV genotypes

quasi species diversity and drug resistance mutations in antiretroviral treatment naive and treatment experienced HBV infected patients (7,8) apart from case reports of prolonged and intermittent treatment of HBV with Lamivudine and Tenofovir and development of resistant to Lamivudine and Tenofovir. This study was aimed to detect cases of resistance to Tenofovir and Entecavir in a chronic hepatitis B patients infected with Hepatitis B virus in Iraq.

Materials and Methods:

Samples:

A total of 20 blood serum samples of confirmed Chronic Hepatitis B (CHB) receiving antiviral treatment were obtained in a volume of 2 mL from Hepatology and Gastroenterology Teaching Hospital and Central Public Health Laboratory in Baghdad. Chronic Hepatitis B patients were defined as persons positive for Hepatitis B surface antigen (HBsAg) for more than six months. The serum samples obtained were retrospective samples collected for a period from February 2017 till January 2018. Patients were chosen randomly regardless of age, race, sex, and symptoms.

Viral-DNA Isolation:

HBV DNA isolation was performed using High Pure Viral Nucleic Acid Extraction Kit (Qiagen, USA) according to the manufacturer's instructions. The isolation procedure was based on spin-column method. The final elution volume of 50 µL containing viral RNA from each sample was stored at -20°C for long-term usage.

Polymerase (*Pol*) gene PCR Amplification:

Approximately 2.5 kb length of polymerase (*Pol*) gene of HBV was amplified using 2 sets of published oligonucleotides (9). Oligonucleotides used in this study are listed in Table (1) and A fragment of 902bp was amplified in the first round PCR using sense *Pol*3-F and antisense *Pol*3-R A second round of PCR using of oligonucleotides(*Pol*4-F, *Pol*4-R) was detect length of PCR product (609), amplification reactions were carried out in a 96-well Thermal Cycler (Bio Rad, USA). The first round of PCR was undertaken for 35 cycles (94°C for 1 min, 60°C for 1 min, and 72°C for 1min) followed by an extension reaction at 72°C for 7 min. The second round PCR was performed for 30 cycles (94°C for 1 min, 56°C for 1 min, and 72°C for 1 min) followed by extension at 72°C for 7 min. First round PCR reaction was composed of 12.5 uLof Go Taq Green Master Mix, 1.0 uL of each oligonucleotides(10 pmol), 5.5 uL sterile dH2O, and 5 uL of extracted HBV DNA. The second round PCR reaction was composed of the same reagent concentrations for each of oligonucleotides, except that only 2 uL of the first round PCR product was used as template. According to submitted sequence of *pol* gene of HBV (EU594413.1), four primers had been designed covering whole gene using Primer3 software.

Table (1): Sequences of *Pol* gene primers.

Primer Name	Sequence (5'-3')	Product size (bp)
*Pol*3-F	GCTCAAGGAACCTCTATGTATC	902
*Pol*3-R	GCACGGGACGTAAACAAA	
*Pol*4-F	CTGTTGTCCTATCCCGCAAATA	609
*Pol*4-R	ACTCCACAGTAGCTCCAAATTC	

PCR Purification and Sequencing:

A 10µL aliquot of each PCR reaction from the second round PCR was analyzed on 1 % agarose by gel electrophoresis and viewed under UV illumination. The agarose was pre-stained with Ethidium Bromide. The corresponding amplicons were extracted from the agarose gel and purified using Gel Extraction Kit (Qiagen, USA) according to the manufacturer's instruction. Final elution contained 35 uL of purified PCR amplicons from which 5µL was reanalyzed on 1% agarose gel to confirm that the purification step was performed precisely. For PCR product, 5µl was directly loaded to well at 70v/Amp for 85 min. Detection about product under U.V light.

Sequencing of PCR products:

PCR product were send for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using software and alignment with original Sequence by NCBI (blast).

Results:

PCR Amplification of *Pol* Gene:

Pol gene amplification was observed in all 20/35 Hepatitis B serum samples. The remaining 15 sera showed negative amplification. The Reverse Transcriptase *Pol* region was amplified fragment by fragment with accurate amplicon sizes as shown in Figure (1) and Figure (2).

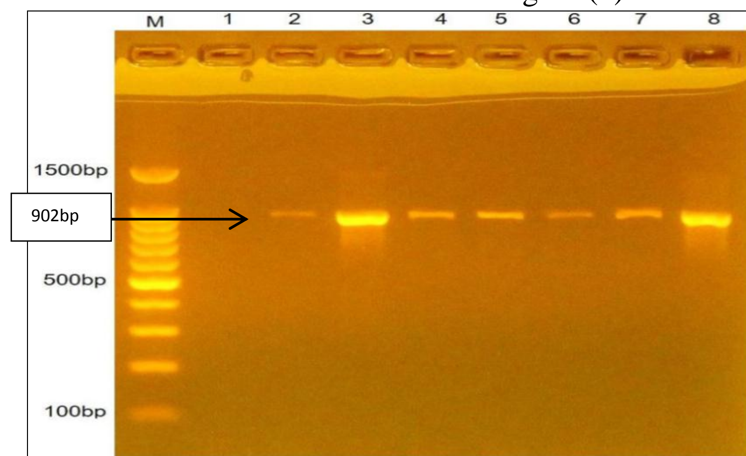

Figure (1): PCR product of *Pol*3 gene in HBV electrophoresed on 1% agarose gel electrophoresis stained with Ethidium Bromide 70vol/hr showing 902 bp bands. Marker used in this gel is (100 pb).

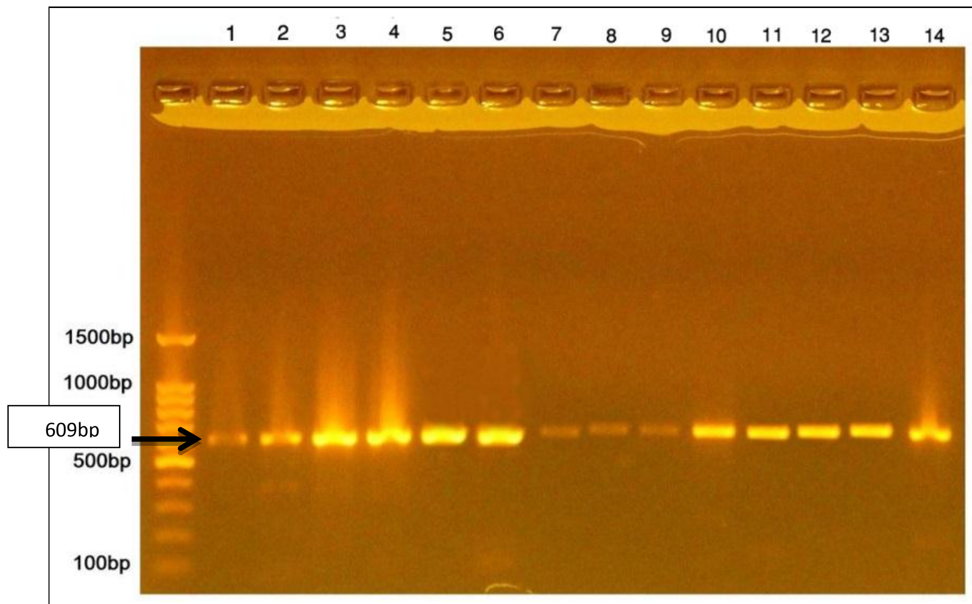

Figure (2): PCR product of *Pol*4 gene in HBV electrophoresed on 1% agarose gel electrophoresis stained with Ethidium Bromide 70vol/hr showing 609 bp bands. Marker used in this gel is (100 pb).

Pol Gene Sequences of Hepatitis B:

Alignment of forward sequences of each fragment of *Pol* gene against the reference strain produced a full length of 1500 bp of *Pol* gene. The results were received by email then analyzed using genious software.

Case Report of Mutations in *Pol* Gene of HepatitisB:

Analysis of *Pol* gene sequence revealed that genome of twelve patients contained mutations that caused drug resistance. The mutations were listed in Table 2. Based on genome analysis, Most patient possessed mutation rtL80V/I, which caused resistance to lamivudine (LAM). The disease of those patients, were diagnosed as chronic hepatitis B and were on lamivudine treatment about 6 months, then Adefovir (ADV) or/and Entacavir (ETV) were added as an antiviral therapy. The patient was followed up till January 2018 and HBV DNA was still detected.

Table (2): List of *Pol* gene mutations associated with drug resistance

Mutations	Patient Id (Sequence)	%	Association With Drug Resistance
L180M	7/20	35	Lamivudine, Entecavir & Clevudine resistance
A181T/V	6/20	30	Adefovir ,Tenofovir & Telbivudine resistance
M204V/I/S	8/20	40	Lamivudine, Entecavir, Clevudine& Telbivudine resistance
N236T	5/20	25	Adefovir & Tenofovir resistance
T184SCGA	5/20	25	Entecavir resistance
T184ILFM	2/20	10	Entecavir resistance
A194T	5/20	25	Tenofovir resistance
S202GCI	9/20	45	Entecavir resistance
M250V/I/L	6/20	30	Entecavir resistance

Patients were found to have mutations L180M, M204I, T184A, and rtM250V, which associated with resistance to lamivudine, entecavir, clevudine and telbivudine (multidrug resistance) were diagnosed as decompensated liver cirrhosis.

The patients also had multiple mutations in HBV *Pol* gene (RT region), which were S202I, N236T, A181T/V, A194T and M250L. These mutations were known to cause resistance towards entecavir and tenofovir (10).

The HBV genomic variations in *Pol* gene regions have clinical Importance according on genotype. The mutations in reverse transcriptase region have been reported to have association with drug resistance in patients(11). In Brazil, in a recently published study (Gomes *et al*., 2015) with samples from various regions of Brazil, the resistance rate of mutations associated with nucleos(t)ide analogues (NA) was found to be 1.6% in naive patients. Expanding the prevalence for the Northern and North-eastern regions, the prevalence of resistance mutations to NA was found to be 2.6%.One of the resistance mutations found in the North-eastern Region was rtA194T, which can be associated with resistance to TDF (12).

Conclusion:

This is a first report from Iraq of occurrence of Tenofovir, Entecavir and multi-drug mutations L180M, N236T, T184SCGA, T184ILFM, S202GCI, A181T/V, A194T and M204 V/I mutations which could lead to prediction of effectiveness of antiviral therapy as well as severity of the disease. Therefore, it is of importance to evaluate antiviral therapy by surveillance of the significant sites of mutations. Early detection of HBV drug resistance is crucial for clinicians to decide on the choice of antiviral treatment and further management of CHB patients.

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