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Drug-Resistance Associated Mutations in Polymerase (*Pol*) Gene of Hepatitis B Virus Isolated from Iraqi Chronic Hepatitis B patients (CHB)

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Abstract
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 patients Iraqi**.** Selected 20 CHB patients who's had highly viral load after treatment course (6 months) were analyzed by PCR and sequencing, showed in sequence also **rtL80V/I** mutation was most frequently detected 12/20(60%) and followed by **rtS202GCI** (45%), **rtM204V/I/S** (40%), **rtL180M** (35%), **rtM250V/I/L** (30%), **rtT184SCGA** and **rtA194T** (25%), **rtT184ILFM** (10%), **rtV173L** and **rtN236T (5%)** and no show **rtA181T/V** mutation**.** Most of CHB patients included within age groups from (20–70) years and male to female ratio was (1.33:1)

INTRODUCTION:

HBV is the most common blood borne viral infection worldwide; that is capable of causing hepatitis by attacking the liver leading to acute (transient) and chronic infection. Acute infection may cause serious illness and 0.5% terminates with fatal, fulminate hepatitis. While chronic infection has serious consequences, about twenty five percent terminate to cirrhosis and hepatocellular carcinoma (HCC) (2).
World Health Organization 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, and over 4 million are acute carriers (3).Thirty percent of world's population has had contact with or are carriers of the hepatitis virus (4,5). Between 1 and 2 million of this population die annually, most of them in Asia (6).
HBV is transmitted by perinatal, percutaneous, and sexual exposure, as well as by close person-to-person contact presumably by open cuts and sores, especially among children in hyper endemic areas (1). Due to high mutational rates in the HBV-DNA, the virus could be classified into several genotypes with a standard cut-off of 8% genomic divergence The most common mutation genotype in Iraq is genotype D(2). High serum hepatitis B virus (HBV) DNA levels are an independent risk factor for disease progression to cirrhosis and hepatocellular carcinoma (HCC) in patients with chronic hepatitis B (CHB) (7).
By contrast, reducing HBV DNA concentrations to very low or undetectable levels through long-term nucleos(t)ide analogue (NUC) therapy is associated with reduced risk of mortality and/or HCC (8). Over the past two decades, treatment of CHB has greatly improved with the availability of NUCs, including lamivudine (LAM), adefovir (ADV), entecavir (ETV), telbivudine, and tenofovir, which target particular sites of viral polymerases (9,10). Oral antiviral agents have been developed in the last two decades for the treatment of chronic hepatitis B (CHB). However, antiviral resistance remains an important challenge for long-term CHB therapy. All of the clinically available oral antiviral agents are nucleoside or nucleotide analogues that target the activity of viral reverse transcriptase (RT), and all are reported to have resistant mutations. Since the hepatitis B virus (HBV) RT, like other viral polymerases, lacks proofreading activity, the emergence of drug-resistance occurs readily under selective pressure from the administration of antiviral agents. The molecular diagnosis of drug-resistant HBV is based on sequence variations, and current diagnostic methods include sequencing, restriction fragment polymorphism analysis, and hybridization (11).

3. MATERIALS AND METHODS:

Samples:

A total number of 80 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 (HBs Ag) 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 -20ؠC for long-term usage.

Polymerase (P) Gene PCR Amplification:

Approximately 2.5 kb length of polymerase (P) gene of HBV was amplified using 2 sets of published oligonucleotides (6). oligonucleotides used in this study are listed in Table (3.1) and A fragment of 902bp was amplified in the first round PCR using sense Pol3-F and antisense Pol3-R A second round of PCR using of oligonucleotides(Pol4-F, Pol4-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 uLof 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.

Post PCR Purification and Sequencing:

A 10 uL 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 5 uL was reanalyzed on 1% agarose gel to confirm that the purification step was performed precisely.

Table (3.1) sequences of primers that use to detection *POL* gene.

Primer Name	Sequences (5'-3')	Size	Length of PCR product (bp)
Pol3-F	GCTCAAGGAACCTCTATGTATC	22mer	902
Pol3-R	GCACGGGACGTAAACAAA	18mer	
Pol4-F	CTGTTGTCCTATCCCGCAAATA	22mer	609
Pol4-R	ACTCCACAGTAGCTCCAAATTC	22mer	

Casting of the horizontal agarose gel:
The agarose solution was poured into the gel tray after both the edges were sealed with cellophane tapes and the agarose was allowed to solidify at room temperature for 30 minutes. The comb was carefully removed and the gel was placed in the gel tray. The tray was filled with 1X TAE-electrophoresis buffer until the buffer reached 3-5 mm over the surface of the gel.

DNA loading:
PCR products were loaded directly. For PCR product, 5µl was directly loaded to well. Electrical power was turned on at 100v/mAmp for 85 min. DNA moves from Cathode to plus Anode poles. The Ethidium bromide stained bands in gel were visualized using Gel imaging system.

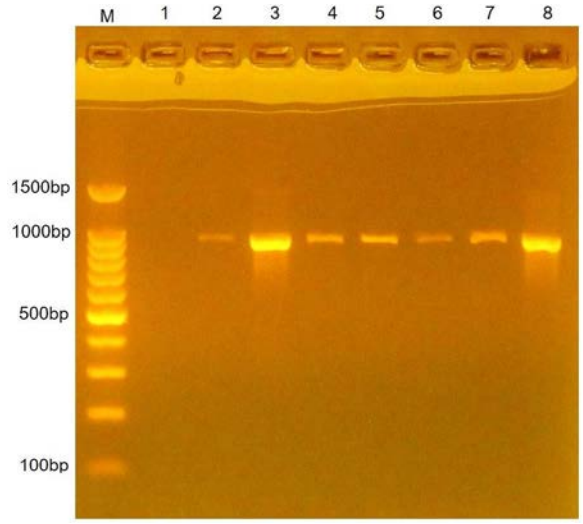
. Standard Sequencing:
PCR product were send for Sanger sequencing using ABI3730XL, automated DNA sequencer, by Macrogen Corporation – Korea. The results were received by email then analyzed using genious software.

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 (4.1) and Figure (4.2).

***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 P Gene of HepatitisB
Analysis of P gene sequence revealed that genome of twelve patients contained mutations that caused drug resistance. The mutations found are as listed in Table 3.Based on genome analysis, Most patient possessed mutation rtL80V/I, which was responsible to cause resistance to lamivudine (LAM). The disease of those patients, (20-64) years-old male almost, 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.



Figure(4.1): Results of the *Pol*3 gene of HBV were fractionated presence of on 1% agarose gel electrophoresis stained with Eth.Br. Lane1:100bp DNA marker

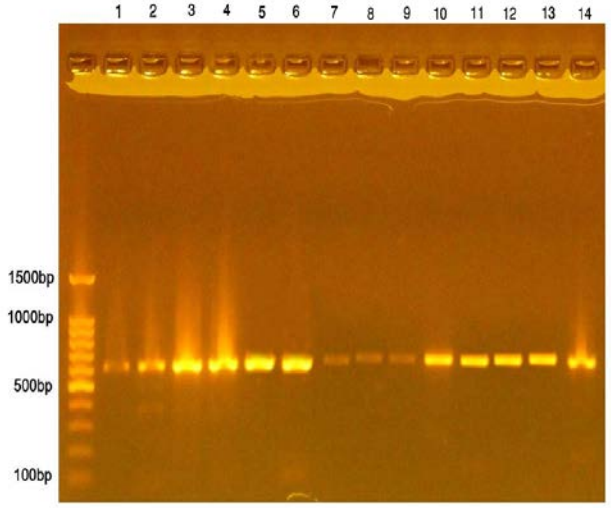


Figure (4.2): Results of the *Pol*4 gene of HBV were fractionated presence of on 1% agarose gel electrophoresis stained with Eth.Br. Lane1:100bp DNA marker

Patients were found to have mutations rtL80V/L, rtV173L, rt L180M, rtM204I, rtT184A, and rtM250V, which were associated with resistance to lamivudine, entecavir, emitricitabine, famciclovir, and telbivudine (multidrug resistance) were diagnosed as decompensated liver cirrhosis. The patients were treated with tenofovir and no history of treatment failure found. The patients were not followed up thereafter.
The patients also had multiple mutations in HBV *Pol* gene (RT region), which were S202I, N236T, and M250L. These mutations were known to cause resistance towards entecavir and adefovir.
Also analysis of the HBV *Pol* gene sequence of patients showed presence of two significant mutations which were L180M and M204I, indicating possible multi-drug resistance to lamivudine, emtricitabine, famciclovir, and telbivudine. The patients were presented with liver cirrhosis secondary to chronic HBeAg positive.
The patient initially started on lamivudine for a period of two years, then switched to tenofovir due to failure of viral suppression. It was also stated that no resistant test was available in hospital. HBV DNA was still detected, however the viral load had remarkably descended from approximately 1,000,000 IU/mL

(just before switching to tenofovir) to <200IU/mL. The patient was reported thereafter to have no more symptoms or signs of liver decomposition. Based on sequence analysis, patients showed the presence of L180V, M204I, and V173L mutations in *Pol* gene sequence of HBV DNA. These mutations were associated with resistance to lamivudine, emtricitabine, telbivudine, and entecavir.

Table (4.1): List of P Gene Mutations Found in Patients in This Study and Association with Drug Resistance.

Mutations	Patient Id (Sequence)	%	Association With Drug Resistance
rtL80V/I	12/20	60	Lamivudine resistance
rtV173L	1/20	5	Lamivudine resistance
rtL180M	7/20	35	Lamivudine, Entecavir & Clevudine resistance
rtA181T/V	0/20	0	Adefovir ,Tenofovir & Telbivudine resistance
rtM204V/I/S	8/20	40	Lamivudine, Entecavir, Clevudine& Telbivudine resistance
rtN236T	1/20	5	Adefovir & Tenofovir resistance
rtT184SCGA	5/20	25	Entecavir resistance
rtT184ILFM	2/20	10	Entecavir resistance
rtA194T	5/20	25	Tenofovir resistance
rtS202GCI	9/20	45	Entecavir resistance
rtM250V/I/L	6/20	30	Entecavir resistance

DISCUSSION:

Genotype D had a worldwide distribution but most prevalent in the Mediterranean and Western Asian countries. In our study, it has been found that genotype D (100%) was dominant when analysis sequence. This finding differs from another study (12). Our study result was comparable with many previous reports on the geographical distribution of HBV genotypes(13, 14). The HBV isolated from this patient contained a 31-nucleotide deletion at the location which was also the beginning of PreS1open reading frame. This deletion has been reported exclusively for in Genotype D of Hepatitis B and non-human primate isolates (15). Based on ethnicity.

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.

For antiviral therapy, six nucleoside/nucleotide analogues (lamivudine, adefovir, entecavir, telbivudine, Clevudine and tenofovir) are approved at the present by European Association for the Study of Liver. Lamivudine resistance mutants were reported to harbor M240V/I in the YMDD motif of the polymerase gene (16). Adefovir resistance was caused by N236T and /or A181V amino acid substitution (17), whereas entecavir resistance resulted from HBV reverse transcriptase substitutions at positions T184, S202, or M250L which emerge in the presence of lamivudine resistance substitutions M204I/V and L180M (18). Resistance to telbivudine has been associated with M204I mutation (19). Emtricitabine resistance was often accompanied with lamuvudine resistance because of L180M, V173L, and M204I mutations (20). The results of this study have demonstrated drug resistant HBV mutations in twenty patients. All patients were male more than female with the age ranging from 20-76 years. Based on the genome analysis, HBV isolated from 12/20 patients (60%) contained mutations associated with lamivudine resistance in combination with either of emtricitabine, telbivudine, or adefovir. Another study has also reported high frequency of lamivudine resistance in up to 70% of HBV patients who were treated 5 years with lamivudine, 29% after 5 years with adefovir, 20% after 2 years with telbivudine, and 1% after 5 years with entecavir (21). The mutations found in the four patients were L180M/V, M204I, A181T, and V173L. Among these, L180M/V and M204I were frequently observed. Some study reported that in most cases, the M204V/I mutation was not present alone but linked with a leucine to methionine exchange at position 180 (L180M) (22). Similarly, 8/20and7/20 patients contained both M204I and L180M/V respectively. Mutations associated with entecavir and adefovir resistance were the next frequently observed in two patients each (40%).

It was observed that the HBV isolates with lamivudine resistant mutations were all from patients who were on lamivudine treatment at some points. Interestingly, two patients have completely recovered after switching of antiviral therapy from lamivudine to tenofovir. This indicates the efficacy of tenofovir as treatment for HBV patients. This finding is supported by Fung et al. (2012) (23) stating that tenofovir has shown high barrier to resistance, produced high rates of viral suppression, and showed no evidence of resistance through six years for previously untreated chronic hepatitis B patients. Similar finding was also demonstrated whereby regression of cirrhosis during treatment was observed with tenofovir in chronic hepatitis B patients in a year follow-up (24). The remaining 8 patients did not recover in sequencing analysis.

One patients had HBV mutant associated with adefovir resistanc in this patient of which adefovir treatment as second-line treatment with lamivudine was confirmed. Genomic analysis revealed that HBV from this patient also had lamivudine resistant mutation. This finding supports earlier studies (25) that demonstrated adefovir resistance occurred more frequently in second line treatment of lamivudine-resistant patients than in naive patients. Therefore administration of adefovir in patients with history of lamivudine resistance should be abandoned. The other patient's treatment history was unclear, but there was a possibility that this patient has also received adefovir treatment.

Analysis on symptoms of 12 patients with HBV Pol gene mutants revealed that the disease of three patients were diagnosed as liver cirrhosis and the remaining two as chronic hepatitis B and drug induced hepatitis, respectively.

All three patients with liver cirrhosis contained HBV mutant L180M in combination with M204I. However, to conclude if there is any association of hepatitis B disease symptoms with the presence of drug resistant mutants.

In **conclusion**, genomic analysis of HBV *Pol* gene isolated from CHB could reveal abundant information including mainly the presence of HBV drug resistant mutants 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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