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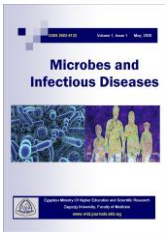

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Original article

Mutation frequency and drug resistance profiles in Hepatitis B Virus (HBV) genotypes in chronic carriers

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ABSTRACT

Background: Hepatitis B virus (HBV) continues to pose a significant threat to the global health sector. With D and E genotypes predominating in Iraq, antiviral resistance mutations in these genotypes have received a lot of interest. **Aims:** This study aimed to evaluate the mutation profile in polymerase gene of HBV among chronic carriers and its association with antiviral drug resistance, particularly lamivudine and tenofovir. **Methods:** A cross-sectional study was conducted during the period between June and November, 2024, in Hepatology and Gastroenterology Teaching Hospital in the Medical City-Baghdad- Iraq. One hundred serum samples were collected from chronic HBV carriers. HBsAg was detected using enzyme-linked immunosorbent assay (ELISA) kits and the polymerase gene was analyzed using nested PCR and Sanger sequencing. Mutation frequencies were determined, and their clinical significance was explored using database comparisons and statistical correlation with patient demographics and treatment history. **Results:** Lamivudine resistance mutations, such as rtL180M: 23% and rtM204V/I: 21/20%, were found to be quite prevalent compared to the less common tenofovir resistance mutation, such as rtA181T and rtA194T. Notably, novel mutations like as rtV173G, rtP217T and rtS219A, were identified underlining unique fingerprints of HBV in this region. **Conclusion:** The study reveals a high prevalence of lamivudine resistance mutations among chronic carriers in Iraq, while tenofovir resistance is lower. Novel mutations highlight unique regional evolutionary patterns, with older age, elevated viral load, and prior antiviral treatment being significant predictors of mutation emergence.

Introduction

Hepatitis B virus (HBV) is a significant global public health issue, affecting millions and resulting in chronic infections that can lead to liver cirrhosis and hepatic cell carcinoma. According to WHO Iraq was considered to be an endemic region of intermediate endemicity with a normal population seroprevalence of 3% for HBsAg [1]. Although the precise percentage of HBV exposure in Iraq is unknown, the available evidence points to a prevalence of 35–38 percent, with 32 percent having anti-Hepatitis B virus surface antibodies from natural conversion and 4 percent being carriers [2]. The virus exhibits considerable genetic diversity, with genotypes D and E predominating in Iraq and neighboring regions, each having unique clinical and therapeutic consequences [3]. These genotypes exhibit distinct molecular traits that affect the course of the disease and the treatment efficacy [4]. Because chronic HBV infection often leads to severe liver diseases, it has become a serious clinical issue that poses major challenges for the public health system throughout the world [5]. At the molecular level, the genome of HBV consists of

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overlapping reading frames. With a unique life cycle involving an error-prone polymerase, as demonstrated by its high mutation rates and its capacity to adapt to therapeutic pressure, the polymerase genes have contributed to resistance against antiviral therapy [6]. Many previous studies conducted in Iraq corroborate the predominance of genotype D among chronic HBV carriers, with mutation patterns in the polymerase genes linked to both therapeutic challenges and disease progression [7, 8]. Similar data obtained from India confirm the high prevalence of genotypes D and A, which further supports their dominant role in the regional epidemiology of HBV [9]. Mutations in the HBV polymerase gene significantly contribute to antiviral resistance, which impacts treatment efficacy. For instance, the rtM204V/I, which often co-occur with rtL180M, reduces drug binding efficacy, leading to lamivudine resistance. Similarly, tenofovir resistance, although rare, is linked to rtA194T and rtN236T mutations, posing a challenge to its high genetic barrier [10]. According to previous studies, individuals receiving long-term therapy are more likely to have lamivudine and entecavir resistance mutations, worldwide, however, with regional variations in mutation frequencies [11, 12]. Genotype-specific resistance profiles have been emphasized by regional analysis. For example, in China, high rates of lamivudine resistance mutations were observed in genotype B and C patients, underscoring the necessity of customized treatment plans[13]. In South Africa, tenofovir resistance was rare, although emerging rtA194T mutations were linked with prolonged therapy, reflecting selective pressures in different treatment contexts [14].

In Iraq, the unique resistance patterns driven by genotype D and E prevalence have yet to be completely explored. This supports the necessity for regional studies to tackle distinct treatment challenges [15]. Novel approaches, such as deep sequencing, have revealed rare mutations like rtT301A that may alter drug efficacy, emphasizing the intricacy of resistance mechanisms [16]. Thus, this study aimed to assess the mutation frequency of the HBV in chronic carriers in Iraq, with an emphasis on genotypes D and E. In addition to examining the correlation between the detected mutations and antiviral resistance, generating robust evidence to guide antiviral treatment strategies, ultimately contributing to improved outcomes for chronic HBV carriers in Iraq and other endemic regions.

Patients and Methods

Patients

This investigation was performed on 100 chronic HBV adults (aged between 18-76 years old, 52 were males *vs* 48 females) who visited the Hepatology and Gastroenterology Teaching Hospital in the Medical City -Baghdad-Iraq, during the period extended between 1st June and 31st November 2024. This study was approved by the Scientific and Ethical Committee of the University of Diyala/ College of Medicine with ethical code 2025SRH903. Only those patients who were over 18 years old and tested positive for HBsAg at least six months before the study were included. The detection of HBsAg is done using enzyme-linked immunosorbent assay (ELISA) kits (Sure Bio-Tech, Hong Kong). Both patients on antiviral therapy and treatment-naïve are included to have the representative clinical spectrum of patients. Informed consent was obtained from participants before enrollment and the study was approved by a relevant institutional review board.

Sample Collection

Approximately 5 ml of whole blood was drawn by venipuncture from each participant and left for serum separation. Sera were separated by centrifugation for 10 minutes at 3000 rounds per minute (rpm). The serum was immediately divided into 0.5 ml aliquots and stored at −20 °C. To maintain participant confidentiality and to track each sample throughout the study, each sample was labeled with a unique identifier. Aside from sample collection, demographic and clinical data were recorded as age, sex, geographic origin, treatment history, and viral load levels, for each participant. We then linked these data to corresponding serum samples for correlation analyses.

HBV DNA extraction and amplification

HBV DNA was extracted according to the manufacturer's instructions of the QIAamp Viral DNA Mini Kit (QIAGEN, Germany). The eluted purified viral DNA was mixed in 50 µL of nuclease-free water and stored at -20°C for further analysis. The Hepatitis B virus polymerase gene was amplified by PCR using a two-step nested PCR approach to enhance the sensitivity and specificity of amplification. Amplification was done using two sets of primers, Table (1). The primers used in the study were designed based on the conserved and mainly targeted nucleotide sequences in the RT domain avoiding the break point in in genotype D

(GenBank: GQ205441.1 available at: http://ncbi.nlm.nih.gov/nuccore/GQ205441.1). The first round of PCR was performed with outer primers. An extracted DNA of 2 µL, each at 0.2 µM of each outer primer, 200 µM of each four dNTP, 1× PCR buffer, 2.5 mM $MgCl_2$, and 1.25 U of Taq DNA polymerase (Thermo Fisher Scientific, USA) were mixed to obtain a total reaction volume of 25 µL. Thermal cycling conditions were used for the reaction mixture including initial denaturation at 95°C for 3 minutes, 35 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, extension at 72°C for 1 minute, and final extension at 72°C for 7 minutes. Inner primers designed to amplify a 1,000 bp segment of the polymerase gene were used in the second round PCR of 2 µL of the first round PCR product itself as a template. Reaction conditions and thermal cycling parameters were maintained as per the first round protocol, with the exception of the annealing temperature, which was modified based on primer-specific requirements. Gel electrophoresis was used to confirm successful amplification and to measure product size using a DNA ladder. The DNA fragments were visualized using UV illumination, using a 1.5% agarose gel stained with ethidium bromide.

Sanger Sequencing and sequence alignment

The therapeutically relevant mutations in the HBV polymerase gene were identified by using Sanger sequencing of the amplified gene segments. PCR products were sent for sequencing using BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Analysis of isolates was performed using purified nested PCR products as the templates for sequencing reactions after screening their quality by using the parameters established in the Sequence Scanner Software v2.0 (Applied Biosystems, USA). The pure sequences were further aligned and assembled to yield a consensus sequence of each sample using Molecular Evolutionary Genetics Analysis (MEGA) software X, loaded in FASTA file format. Multiple sequence alignment using the ClustalW algorithm of MEGA software was performed. The HBV polymerase gene sequences were aligned to the wild-type HBV polymerase gene sequence as the reference sequence from (GenBank accession NC_003977.2 which is available at the NCBI information site on the following website http://blast.ncbi.nlm.nih.gov/Blast.cgi). Sequences were then used to cross-reference with known drug resistance mutations known from established Resistance databases, HepSeq and the Stanford HBV Drug Resistance Database. A detailed analysis was conducted to predict the impact of the novel mutations on protein function themselves using bioinformatic tools.

Statistical Analysis

All analyses were performed using SPSS, version 25.0 (IBM Inc., Armonk, NY) as well as R programming language to bring about robust, reproducible results. Descriptive data were presented in measures of frequency, mean, and median, together with standard deviations. All tests were performed with a significance threshold of $p < 0.05$. A logistic regression analysis was performed to explore the relationship between predictors and mutation prevalence levels. The predictors included Viral load, age, sex, and treatment history. The association between predictors and mutation prevalence is presented as odds ratios (ORs) and 95% CIs. The significance of these relationships was assessed using their beta coefficients, standard errors, and P-values. Phenotypic odds ratio estimated in accordance with Altman, (1991) by the aid of medcalc.net.

Results

Sociodemographic characteristics of patients and viral load

A total of 100 chronic HBV patients were enrolled in this study with mean±SD age (46.5+ 5.35) and mean±SD viral load (4,489,276.5 + 448,917.5). Of them, 48% were females and 52% were males. Treatment history indicated that 38% of them are naïve, 30% under Lamivudine, 14% under Tenofovir, and 18% were under both medicines, Table 2.

Mutation Frequency and Amino Acid Substitution in Hepatitis B Virus Polymerase gene

The *rt* region in100 samples was amplified with amplicon size 1220 bp as shown in Figure 1. A detailed overview of mutations found in the HBV polymerase gene detected in study participants is presented in the Table 3. The table shows the sequence variation and graphical representation of nucleotides and amino acids changes between wild-type and mutants. Frequencies of resistance mutation patterns at the sites of the polymerase rt domains were estimated and the amino acid substitution with which it binds are listed. Results show that mutations closely associated with

lamivudine resistance (rtM204V, rtM204I, rtL180M) were among most common, with frequencies of 20%, 21%, and 23%, respectively. These mutations are common lamivudine resistance markers. On the other hand, these mutations associated with tenofovir resistance, rtA181T, rtL180P and rtA194T, were less common, with frequencies of 12, 8, and 6%, respectively, which implies a limited increase of resistance to this antiviral in the study cohort.

The sequence variation and graphical representation of nucleotides and amino acids changes between wild-type and mutants were presented in Figure 2.

As shown in Table 4, new mutations in the HBV polymerase gene found within our study population. However, these mutations were not listed in existing resistance databases nor in the previously published known resistance profiles for these virus strains, indicating that these mutations may represent new genetic variations of these circulating virus strains in the study area region. These mutation, amino acid substitution, predicted impact and prevalence were listed. The structural and functional effects of each mutation were evaluated using bioinformatics tools after considering their location within the polymerase protein. A predicted polymerase stability affecting mutation rtV173G (V to G substitution) was rtV173G. The prevalence of this mutation was small, composed in 2.5% of cohort suggesting that it may be an infrequent but potentially important change. Thus, the rtP217T mutation, substitution from proline to threonine, hypothesized to cause structural changes to the polymerase enzyme, but with an unknown effect on drug resistance. The mutation was rare, and was found in 1.8 percent of the study participants. We also predicted the rtS219A, wherein serine is changed to alanine, to change the structure of the polymerase, possibly also impacting viral function or resistance mechanisms. The prevalence of this mutation in the study population was 2.0%.

Effects of the Predictor Variables on the mutation prevalence

Logistic regression analysis was performed to determine the predictor variables associated with some mutations in the HBV polymerase gene. The results in Table 5 showed that mutation prevalence is positively associated with age (OR= 1.05, 95% CI=1.01-1.09, P= 0.012) meaning for every additional year of age the odds of having a mutation increases by about 5%. This finding also held true for viral load, which was strongly positively associated with mutation prevalence, with slightly elevation in viral loads can increase the likelihood of mutation presence (OR = 1.30288343E-8, 95% CI = 4.34294477E-9 - 2.17147235E-8, P = 0.003). Treatment history, however, is also highly significant (OR=1.50, 95% CI = 1.10-2.04, P = 0.012), since patients who were treated for their infection prior to being enrolled in the study are 50% more likely than treatment naïve individuals to have mutations. Whereas, sex does not have statistically significant association with mutation prevalence in this analysis (OR=1.20, 95% CI = 0.92-1.56, P = 0.183). These data show that age, viral load and treatment history are potent predictors of mutation prevalence in HBV polymerase genes but that there is lesser impact of sex in this setting.

Cross-referenced comparison between detected mutations and resistance databases

Table 6 provides a detailed comparison of the identified mutations in the study and their associated resistance annotations with established resistance databases to evaluate their presence in the global context and their impact on antiviral drug resistance. The results show that mutations (rtM204V, rtM204I**,** rtL180M, rtA181T and rtA194T) identified in the study are registered in the resistance database with their impact on drug resistance. However, the rtL180P mutation, a rare mutation identified in the study, was not found in the existing resistance databases. The clinical significance of this mutation remains unclear, and further studies are needed to determine its potential impact on antiviral efficacy and its role in HBV resistance.

Table 1. Primer sequences used to amplify conserved HBV polymerase region, genotypes D and E.

Round	Primer
1st round (outer)	**P1 (F): 5'-GAG GGA TTA TTA GAG GAC AG-3'**
	P2 (R): 5'-ACC CAG GAA GGG ATG AAG-3'
2nd round (nested)	**P3 (F):** 5'-GAG ATG GAG TGG GGG TGG-3'
	P4 (R):5'-CGA GTT GGT GGC AGT GAA-3'

Table 2. Patient demographics and viral load data.

Variable	Mean ± SD	Median	Frequency	Percentage
Age (years)	46.5 ± 5.35	45.0	-	-
Sex				
Female	-	-	48	48%
Male	-	-	52	52%
Treatment history				
Naïve	-	-	38	38%
Lamivudine	-	-	30	30%
Tenofovir	-	-	14	14%
Both	-	-	18	18%
Viral Load (IU/mL)	4489276.5 ± 448917.5	444524.0	-	-

Table 3. Gene Mutations, Sequence Variations Mutation Frequency and Amino Acid Substitution in Hepatitis B Virus Polymerase.

Mutation Position	Wild-type Sequence	Mutant Sequence	Amino Acid Substitution	Drug Resistance Association	Frequency (%)
rtL180M	CTG	ATG	Leucine → Methionine	Lamivudine	23
rtM204I	ATG	ATA	Methionine → Isoleucine	Lamivudine	21
rtM204V	ATG	GTG	Methionine → Valine	Lamivudine	20
rtA181T	GCC	ACC	Alanine → Threonine	Tenofovir	12
rtL180P	CTG	CCC	Leucine → Proline	Tenofovir	8
rtA194T	GCC	ACC	Alanine → Threonine	Tenofovir	6

Table 4. Identification of Novel HBV Polymerase Gene Mutations and Their Predicted Impacts.

Mutation Position	Amino Acid Substitution	Predicted Impact	Prevalence (%)
rtV173G	Valine to Glycine	Possible effect on polymerase stability	2.5
rtP217T	Proline to Threonine	Structural alteration, resistance impact unknown	1.8
rtS219A	Serine to Alanine	Potential structural alteration, resistance unknown	2

Table 5. Logistic Regression Analysis of Demographic Variables and Mutation Prevalence.

Predictor Variable	Odds Ratio (OR)	Standard Error (SE)	z-value	p-value	95% (CI)
Age	1.05	0.02	2.50	0.012	[1.01-1.09]
Viral Load	1.30288343E-8	- 8	3.00	0.003	[4.34294477E-9 - 2.17147235E-8]
Sex (Male vs Female)	1.20	0.15	1.33	0.183	[0.92-1.56]
Treatment History	1.50	0.20	2.50	0.012	[1.10-2.04]
Footnote: A two-tailed test was used, with statistical significance set at a p-value < 0.05.					

Table 6. Comparison Table of Mutations, Database Presence, and Resistance Annotations.

Mutation	Database Presence	Resistance Annotation
rtM204V	Present	Associated with resistance to lamivudine and reduced susceptibility to entecavir.
rtM204I	Present	Confers resistance to lamivudine; often observed in conjunction with rtL180M.
rtL180M	Present	Enhances replication fitness of rtM204V/I mutations; contributes to lamivudine resistance.
rtA181T	Present	Linked to resistance against adefovir and partial resistance to tenofovir.
rtA194T	Present	Associated with reduced susceptibility to tenofovir; clinical significance under study.
rtL180P	Absent	Rare mutation; potential impact on antiviral therapy efficacy remains unclear.

Figure 1. The PCR product of Pol gene of HBV electrophoresed on 2% agarose gel and stained with Ethidium Bromide M= 100bp DNA marker.

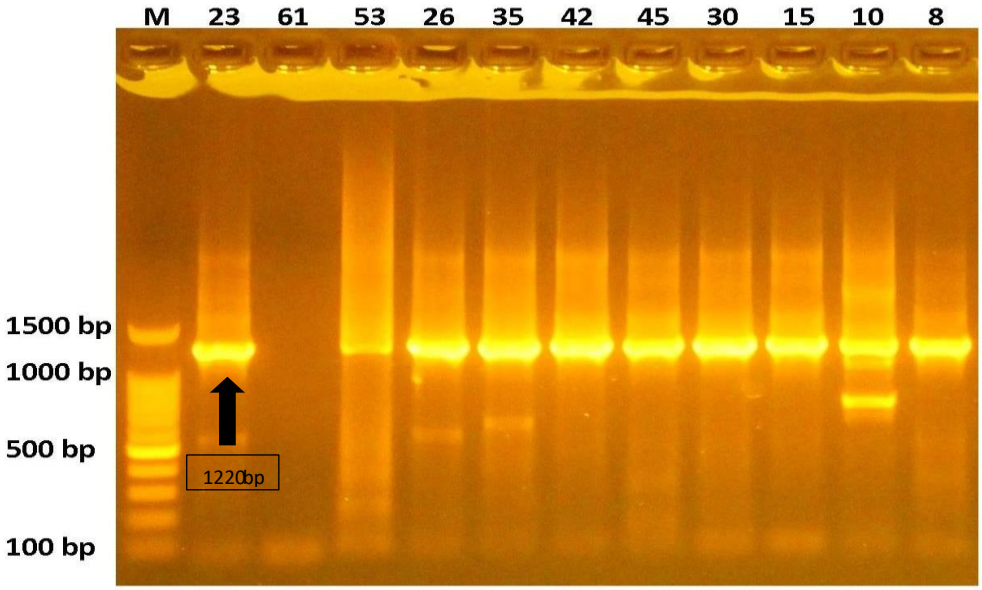


Figure 2. Graphical Representation of Nucleotide and Amino Acid Changes.

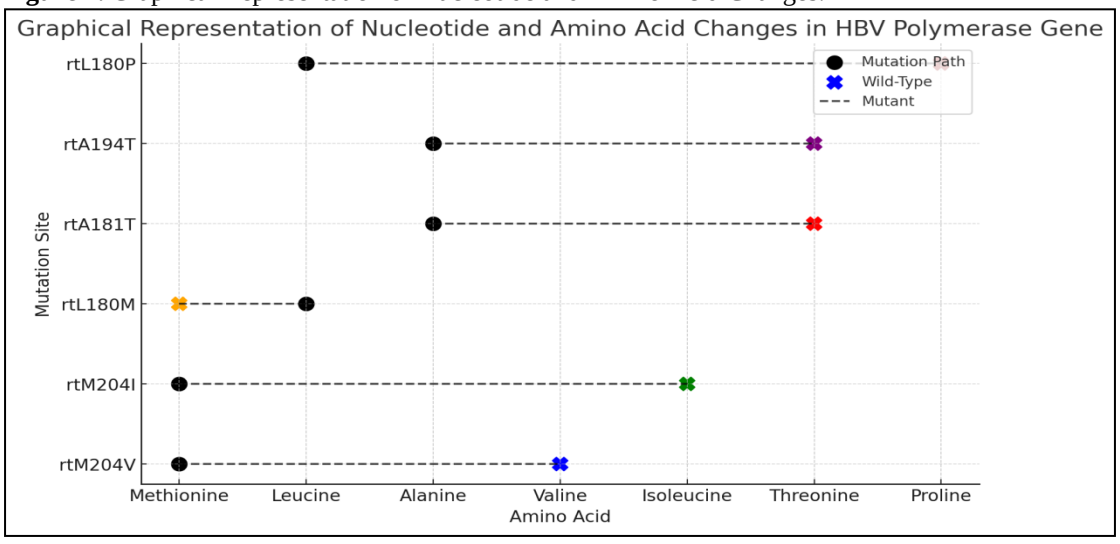
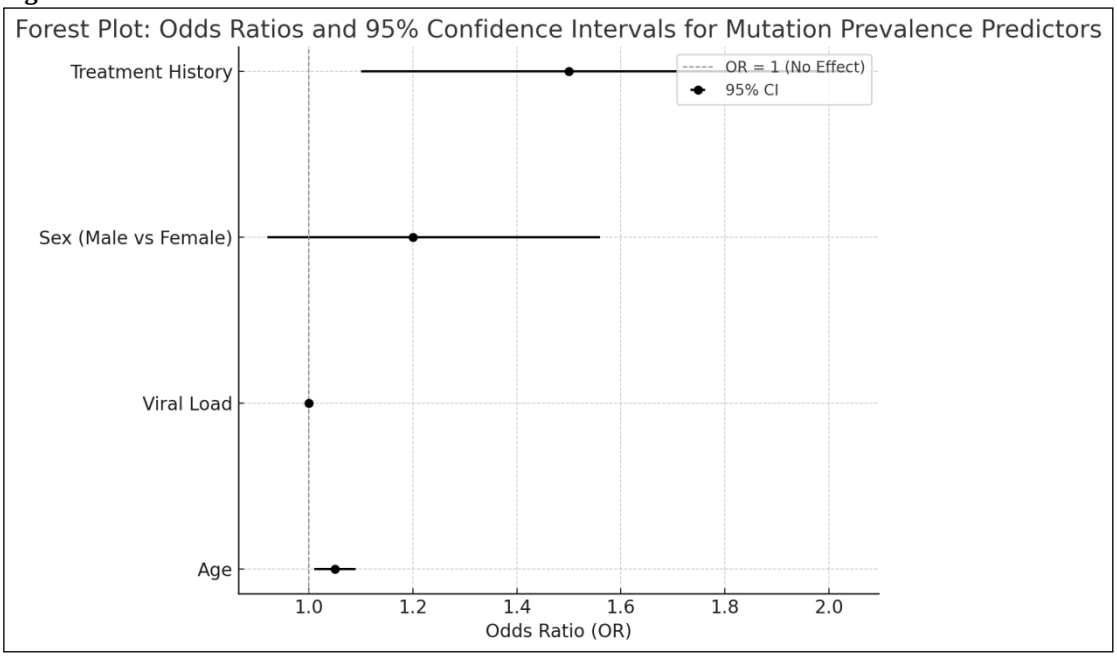


Figure 3. Forest Plot: Odds Ratios and 95% Confidence Intervals for Predictors of Mutation Prevalence.



Discussion

In the present study, lamivudine resistance mutations were the most significant resistance patterns in the HBV polymerase gene, and in particular, mutations rtM204V, rtM204I, and rtL180M emerged as the most widespread. The mutations are key because they are linked with high levels of resistance to lamivudine and some cross-resistance to other antivirals including entecavir. In contrast to tenofovir resistance mutations, rtA181T and rtA194T mutations were less prevalent, consistent with tenofovir's high genetic barrier to resistance. Identification of these patterns reflects the in-flight consequences of selective treatment pressure and the need for bespoke therapeutic strategies.

These findings align with global patterns when comparing them to recent studies. For example, a study of lamivudine resistance that found rt180M and rt204V common to lamivudine, entecavir, and telbivudine overdosing in two case studies in the country of Mexico reported the global impact of lamivudine resistance [10]. A study in Pakistan also demonstrated that lamivudine-resistant mutations, rtM204V and rtL180M, are predominant among treated patients [18]. In the same line, the Iraqi study showed the presence of rtM204V/I, rtL180M, and rtA194T, however, rtA181T was not detected [19]. This uniformity underscores the general selection of similar resistance profiles in the presence of lamivudine treatment pressures. However, the low tenofovir resistance mutation prevalence in the study is consistent with the findings from South Africa where tenofovir-resistant strains were only reported sporadically in this work and such continues to attest to the efficacy of tenofovir for HBV infections [14]. Suzuki *et al*, (2021) reinforce this with a case report that tenofovir is capable of controlling viral loads in patients with prior lamivudine and entecavir resistance [20] It is interesting that the discovery of new mutations, such as rtV173G found in the present study, contributes to the global data bank, similar to the finding of new mutations in HBV strain reported among HBV co-infected patients with HIV in Namibia [17]. The results suggest that local surveillance is essential for identifying new patterns of resistance with potential global importance. The variation in mutation frequencies among different studies could be from regional differences in treatment practice and HBV genotypic distribution. For example, the higher frequency of lamivudine resistance mutations in this study reflects extended and broad use of this drug in the study area (as is found in Colombia where high lamivudine resistance rates were associated with preferential monotherapy [12]. The findings of this present study are close to global resistance patterns where lamivudine resistance is the dominant challenge. The low prevalence of tenofovir resistance is concordant with its high genetic barrier and efficacy. The identification of novel mutations reinforces the critical need for localized studies to

monitor shifts in patterns. Collectively this represents evidence for the strategic use of antivirals, routine resistance testing, and utilization of next-generation sequencing to improve HBV management and impair the development of resistance globally. In the present study, we identified several novel mutations in the HBV polymerase gene, including previously unstudied mutations that include amino acid substitutions and deletions. These mutations are predicted to compromise both antiviral drug efficacy and viral replication, as well as immune escape mechanisms. The detection of such mutations, with their clinical implication, underscores the dynamic nature of HBV's genetic evolution and underscores the possibility that HBV can render ineffective existing treatment regimens that depend on nucleos(t)ide analogs. These findings are compared with recent studies in which novel mutations in the polymerase gene are consistently reported in different geographic regions. For example, in India, there are 46 unique patterns of mutation, of which, for example, adaptively selected substitutions such as rtL180M and rtM204 are known to confer resistance to lamivudine and entecavir. However, these mutations frequently were observed to be co-occurring with surface antigen escape variants, which further complicates both treatment and diagnosis [21]. Likewise, Vietnamese researchers also found mutations in the PreS/S gene linked to hepatocellular carcinoma progression, highlighting the clinical significance of HBV genetic variability [22]. According to a Nigerian study, genotype E mutations, including novel changes in the polymerase gene that confer immune escape and drug resistance, are common. It found that mutations such as G145R and D144A not only decreased vaccine efficacy but also impaired antigen detection [23].

As with Lehmann *et al* . (2023), unique insertions in the surface antigen gene were reported as inducing hyperglycosylation, a diagnostic escape. These findings highlight the ability of the HBV to evade host's immune responses and medical interventions [24]. Mutations in the reverse transcriptase region were characterized among both treated and untreated Chinese chronic HBV patients, with treated patients having a higher prevalence of complex mutations, which may indicate a role for antiviral selection pressure accelerating HBV genetic diversification [25]. In another study by Olusola *et al* . (2021), the authors identified genotype E-specific mutations associated with vaccine escape and occult infection, suggesting the clinical relevance of studying regional mutation patterns [3]. These comparisons show that novel mutations in the HBV polymerase and associated genes are reported worldwide, but the prevalence and particular types of mutations are influenced by the prevalence of regional treatment practices, genotypic distribution, and selective pressures. Consequently, the findings of the present study help to extend our knowledge regarding HBV evolution and its clinical implications. Such evidence makes clear that continuous surveillance, development of next generation therapeutics, and refinement of diagnostic tools are essential to effectively manage HBV.

The present study used logistic regression analysis to investigate the relationship between demographic variables and the prevalence of HBV polymerase gene mutation. The findings showed that older age, viral load, and history of antiviral therapy were all strongly associated with a higher chance of mutations. These results emphasize the main role of demographic and treatment factors on HBV mutation evolution and underline the need for targeted monitoring and intervention strategies in specific population subsets. This relationship between demographic factors and HBV mutation prevalence is corroborated in recent studies. In a Chinese hospital-based survey, the HBV genotype and old age were statistically significantly associated with the incidence of mutations [26]. A cohort study from China further provided additional evidence that baseline viral load was an independent predictor for mutation development as was prolonged entecavir treatment [27]. However, there were some differences, for example in South Africa, longer antiviral treatment and younger age were found to predict the persistence of HBV viremia and often associated with mutation in the HIV/HBV co-infected patients [28]. Much like in Thailand, research also found that male gender and younger patients with elevated HBV DNA levels were more likely to have A1762T/G1764A mutations that are related to active hepatitis and increased risk for disease progression [29]. Also, Liu *et al* . (2022) have investigated a link between demographic factors, age, and occupation, to increased risk of HBV-related liver disease mutations, and logistic regression confirmed these factors to be independent risk factors, adding to the demographic susceptibility [30]. Collectively, these studies

confirm the findings made in the present study, and highlight that demographic factors – including age, viral load, and treatment history – are important determinants of HBV polymerase gene mutations. These variations emphasize the need to tailor therapy and routine monitoring on a personalized level to target patient demographics at high risk for resistance and maximize the outcome.

Conclusion

This study provides critical insights into the molecular epidemiology of HBV genotypes D and E among chronic carriers in Iraq, indicating a significant incidence of lamivudine resistance mutations (rtM204V/I, rtL180M) and comparably low tenofovir resistance (rtA181T, rtA194T). Novel mutations (rtV173G, rtP217T, and rtS219A) have been found, highlighting distinct regional evolutionary patterns that may affect viral fitness and drug susceptibility. Importantly, the occurrence of mutations was significantly predicted by older age, higher viral load, and previous antiviral therapy. Taken together, these findings indicate that tailored treatment strategies, routine resistance testing, and next generation sequencing as a means of combating the problems of antiviral resistance would be justified. Research and surveillance, continued on these patients, will help to further refine therapeutic approaches and improve outcomes in HBV infected individuals and endemic regions.

Conflict of interest

The authors declare that there is no conflict of interest.

Financial disclosure

The authors declare that no funding was received. The authors depend on self-funding to complete this study.

Ethical approval

This study was approved by the Scientific and Ethical Committee of the College of Medicine/University of Diyala (approval number: 2025SRH894).It also follows the principles of the Declaration of Helsinki, Directive 2001/20/EC (Europe) and Regulation No 536/2014 [31].

Data availability

The authors confirm that the data are available from the corresponding author upon reasonable request.

Authors' contribution

Both authors contributed to the study design, data collection, and analysis. The first draft of the manuscript was written and revised by Al-Salihy. The second author, Motib, participated in a critical review. The final manuscript was read and approved by both Al-Salihy and Motib.

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