

THE DISTRIBUTION OF HEPATITIS B VIRUS  
GENOTYPES AND SUBGENOTYPES IN ACUTE AND  
CHRONIC INFECTIONS. AND THE IDENTIFICATION OF  
AMINO ACIDS SUBSTITUTIONS IN ADDITION TO  
ALLELE FREQUENCY

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**Abstract:** Infection with the hepatitis B virus (HBV) is a worldwide health concern, Genotyping HBV is important because it can help to guide treatment decisions, and the risk of developing severe liver disease can be predicted with the use of HBV genotyping, like hepatocellular carcinoma and cirrhosis. Our study aimed to determine HBV genotype and Allele frequencies in acute, and chronic HBV patients living in Al-Anbar governorate/Iraq. samples were analyzed by nested PCR for hepatitis B virus DNA. Round 1 PCR products revealed that all samples of hepatitis B patients showed a 1003 bp band, which confirmed that the current sample was infected with the virus, Second-run PCR demonstrated a pattern of nested PCR products with a 600 bp band. The amplicons from nested PCR were sequenced by the Sanger sequencing method and each viral genotype was investigated and ascertained by alignment with the reference sequence (GenBank acc. NC 003977.2) and blasted using the NCBI blastin database. The results showed the only type D genotype was found in all 80 HBV patients, with seven nucleic acid variants observed in the investigated samples in different positions, namely 348 T>C, 495 C>T, 501 A>C, 520 C>A, 522 G>A, 530 T>C, and 535 A>C. Additionally, ten variants of amino acids were found in different positions to exhibit effects on the S protein within the size of the amplified loci

**Keywords:** HBV, nested PCR, Sanger sequencing, Genotypes, S gene



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## Introduction

Hepatitis B virus (HBV) is a dangerous and widely prevalent infectious liver disease that affects millions of individuals worldwide. It is one of the main global public health problems. Leading to hepatitis infections, both acute and chronic, it is regarded as a virus that poses a serious threat to human life everywhere, resulting in high levels of mortality [1].

WHO estimates that 257 million individuals had HBV infection, and that HBV complications were responsible for 887,000 deaths in 2015 [1].

HBV is an enveloped virus, and classified in the Hepadnaviridae family, in the Orthohepadnavirus genus [2], HBV is a 42 nm partly double-stranded DNA virus. It is made up of a 27 nm nucleocapsid core (HBc Ag) and an outer lipoprotein coat (also known as the envelope) that is about 4 nm thick and contains the surface antigen (HBs Ag) [3], [4].

HBV-DNA sequence molecular evolutionary analysis revealed a list of ten genotypes known as (A-I, and J) with more than 8% genetic variability [5]. Such genotypes display heterogeneous distributions of global frequencies. The most frequently encountered genotype among Iraqi HBV patients is D [6], [7], followed by genotype B [8].

Hepatitis B genotypes play an important role in clinical manifestations of infection and response to antiviral therapy. They have also been reported to be responsible for differences in the natural history of chronic infections [9]. Some genotypes have a high rate of escape mutations, the presence of these mutations in these genotypes is associated with the incidence of hepatocellular carcinoma compared to other genotypes, in addition to the effect of geographical distribution on the occurrence of these mutations [10]. Accordingly, there is a growing need for HBV genotype frequencies to be determined in populations of different geographic regions and ethnicities.

However, viral mutations and genotypes of hepatitis B virus are major determinants in the management and treatment of this disease [11]. The outcome of hepatitis B infection is determined by the specificity of the virus and the host's immune response. It is generally believed that persistence of infection and liver damage result from viral genome variants, viral load, and inhibition of viral components against the host immune system [12].

## Methods

### Subjects

A cross-sectional study was conducted during January-October 2023 in Al-Anbar governorate of Iraq, to detect HBV genotypes in HBV-infected Iraqi individuals (both acute and chronic), and controls.

### Samples Collection

Regarding blood samples, five milliliters of venous blood were obtained from the patient and control groups. The collected blood samples were transferred to a plain tube and kept at room temperature for 20 minutes, then centrifuged at 4000g for 5 minutes, the separated serum was

collected in Eppendorf tubes, divided into portions, and stored at (-20 °C) to be sampled recently to detection HBV genotypes by sequencing.

### Detection of HBV Serological Markers

#### ELISA screening test for HBsAg

All serum samples were examined to detect HBsAg. Determination was performed by using HBs Ag ELISA KIT. Monoclonal antibodies that are specific for HBs Ag used in the solid-phase simultaneous sandwich immunoassay are coated on microtiter wells. Enzyme-conjugated polyclonal antibodies and a serum specimen are introduced to the Microtiter wells coated with antibodies.

#### Testing for HBV Serological Profiles

The HBV serological markers of the HBV-infected patients were determined using the Diagnostic Kit for HBV Infection (Colloidal Gold) test kits. It uses the dual-antibody sandwich method to measure HBsAg and HBeAg in whole blood or serum, and the dual-antigen sandwich method to measure HBsAb. Moreover, it uses the neutralization competitive inhibition approach to test HBcAb and HbeAb.

#### Identification of HBV Genotypes

Serum patients were extracted for total HBV viral DNA using the spin column method through a viral nucleic acid extraction kit (Genaid Viral Nucleic Acid Extraction Kit II, Korea). Nested PCR was employed to amplify our sequencing product to PCR runs or conducted the first amplify the outer region of the S gene and a different primer we used in a separate reaction to amplify the specific region for sequencing purposes.

**Table 1** The primer sequences of HBV For Nested PCR

	Nested PCR		Sequences	PCR product size (bp)
Hepatitis S region	Inner	Forward Primers	5'GGATTCCTAGGACCCCTGCT3'	600 bp
		Reverse Primers	5'ACCCAAAGACAAAAGAAAATTGGT3'	
	Outer	Forward Primers	5'ACCTTCCACCAAACCTCTGCA3'	1003 bp
		Reverse Primers	5'TGTTCTTGTGGCAAGGACCC3'	

The outer PCR reaction was conducted using 2x go Taq Green master mix Promega\ USA. 12.5 microns of go Taq master mix was added to a 0.5 ml clear PCR tube followed by 1 micron of (forward and reverse) outer primers. 2 microns of viral DNA template was added and the final volume was completed to 25 microns by adding nuclease-free water, and then run 1<sup>st</sup> PCR protocol by thermal cycler. The product of the first reaction (1003bp) was examined in 2 % agarose gel electrophoresis and stained with ethidium bromide to examine the band to ensure the product size and quality. This product was used as a template and then transferred to a second PCR reaction using the inner primer and the same other components for the PCR reaction.

Finally, The amplicons (600bp) from nested PCR were sequenced by the Sanger sequencing method using an ABL3730XL automated DNA sequencer each viral genotype was investigated and ascertained by alignment with reference sequence and blasted using the NCBI blastin database.

#### Statistical analysis

The Statistical Analysis System- [13] software was utilized to determine how different Groups affected the study's parameters. To compare means significantly, the Least Significant Difference (LSD) test (Analysis of Variation, or ANOVA) was employed. In this study, the chi-square test was utilized to compare percentages (0.05 and 0.01 the probability) statistically significant.

## Result and Discussion

This study dealt with 80 patients with HBV infection and 40 healthy people as a control in Al-Anbar province/Iraq. Their mean age  $\pm$  SD (standard deviation) of hepatitis B virus (HBV) infection in Acute patients was  $(39.25 \pm 2.17)$  years, and in Chronic patients was  $(36.35 \pm 2.56)$  years, while control had a lower age mean  $(26.97 \pm 1.49)$  years. males also females made up 50% of the people who had Acute HBV, While males made up 67.5% of the people who had Chronic HBV, and females made up 32.5%. In controls, proportions were reported (45 and 55%), respectively (Table 2).

**Table 2** Distribution of sample study according to Gender, and Age in different groups

Variables	Acute N (%)	Chronic N (%)	Control N (%)	P-value
<b>Gender</b>				--
Male	20 (50.00%)	27(67.50%)	18(45.00%)	0.0392 *
Female	20 (50.00%)	13(32.50%)	22(55.00%)	
<b>Age (mean <math>\pm</math>SE)</b>	39.25 $\pm$ 2.17 a	36.35 $\pm$ 2.56 a	26.97 $\pm$ 1.49 b	0.0002 **
Means having different letters in the same column differed significantly. * (P $\leq$ 0.05), ** (P $\leq$ 0.01).				

To detect the genotype results revealed, the DNA from the nested PCR was sequenced directly, After running NCBI blastin on these PCR amplicons, The NCBI blastin engine revealed 98% to 99% sequence similarities between the sequenced samples and the targeted reference target sequences with regard to the 600 bp amplicons. Accurate locations and other features of the obtained PCR fragments were discovered by comparing the observed nucleic acid sequences of these studied samples with the reference nucleic acid sequences (GenBank acc. NC 003977.2).

Depending on the virus sequence homogeneity, The results in this study of the HBV genotype in Al-Anbar patients were determined by using the Sanger sequencing assay, The only type D genotype found in all 80 HBV patients whose DNA was examined, 47 (58.75%) males and 33 (41.25%) females carrying it, and the subgenotype was D1, D3 (92.5%, 7.5%) respectively.

Our results agreed with Shafiq (2012) showed that all acute and chronic hepatitis infections in the Nineveh governorate were genotype D. In Basra, research was conducted. It is estimated that 100% of individuals enrolled have genotype D. [14]. Additionally, in Samara, [15] found that the D genotype was the predominant genotype in more than 93% of samples.

Moreover, the determination of HBV genotypes in Iraq's neighboring countries raises a study in Syria [16], which found that all patients had genotype D, which is associated with a low response to interferon-based therapy, also another study revealed that 97% of patients have D genotype [17]. In Jordan, the dominant genotype was D, which was found in all HBV-infected Jordanians [18]. Also, Another study conducted in Turkey discovered that all patients studied had genotype D, Of the 63 patients investigated by [19], There were also a lot of studies done in Iran that showed that genotype D was found in all of the patients [20]–[23].

According to all of the studies mentioned above, genotype D of the Hepatitis B virus is the most common in the Middle East region. Our findings that genotype D is the only genotype are consistent with other studies in Iraq, particularly in the west of Iraq, such as Nineveh province and also the Duhok Kurdistan region in the north-west of Iraq, as well as studies in Syria and Jordan adjacent to our study region.

The high prevalence of HBV in this study indicated the high endemicity of genotype D in our community. In Iraq, drug abuse increased with the invasion of Iraq in 2003, and is still a growing

problem. Other causes may be a low level of education about the way of disease transmission, lack of interest in sterilization in dental clinics, and negligence in using disposable materials, makeup, and hairdresser salons may be another factor for coinfection with different HB virus genotypes [24]–[26]. Additionally, HBV replicates unequally via reverse transcription of an RNA intermediate [27]. Furthermore, because the viral polymerase is not involved in editing, mutations are frequent and contribute to the genetic variability of HBV. The Hepadnavirus genome is expected to have a mutation rate of  $2 \times 10^{-4}$  base substitutions/site/year, which is approximately 100 times greater than other DNA viruses but 100–1000 times lower than comparable RNA viruses [28].

### HBV Nucleic Acid Variation and Allele Frequency

When comparing the alignment findings of the 600 bp for 80 samples to the HBV reference nucleic acid sequences (GenBank acc. NC 003977.2), seven nucleic acid variants were found in all the sequences represented by seven positions.

Our results as shown in (Table 3) indicated the presence of seven nucleic acid variants observed in the investigated samples, namely 348 T>C, 495 C>T, 501 A>C, 520 C>A, 522 G>A, 530 T>C, and 535 A>C. The observed variation was found in all samples with the same position. Whereas other variations were detected in different investigated samples in different positions but not in all samples.

**Table 3** The pattern of the mutations in the sequences in comparison with the NCBI referring sequences (GenBank acc. KF170747.1).

Position	348	495	501	520	522	530	535
Wild type	T	C	A	C	G	T	A
Mutant	C	T	C	A	A	C	C
	T>C	C>T	A>C	C>A	G>A	T>C	A>C

To find out if these substitutions could lead to alterations in the corresponding positions in the S protein, more analysis was done on the discovered nucleic acid alterations. All nucleic acid sequences of our samples were translated to their corresponding amino acid sequences using software (Geneious Prime). Following that, Amino acid alignment was done of these amino acid sequences with the HBV reference amino acid sequences (GenBank acc. NC 003977.2), and ten variants were found in different positions to exhibit effects on the S protein within the size of the amplified loci.

These synonymous variants were detected in most of the samples in four positions, and exemplified in the entire S protein sequences, namely p82.Pro>Leu, p84.Gln>Pro, p91.Gly>Glu, and p33.Leu>Pro. Meanwhile, six variants were detected in various samples in the identified variants. These variants are: in sample number 38 in position 42 of the HBV reference amino acid sequence, the amino acid Alanine (Ala) substitutes for Valine (Val), and in sample number 11 there are 3 variations found p70.Lys>Thr, p99. Glu>Ala, and p100.Pro>Thr, while in position 71 the samples (8, 10, 14, 19, 29, 41, 45, 47, 48, and 70) have substitutes Val>Glu, finally in sample number 105 in position 83 the amino acid Glutamine (Gln) substitutes for Proline (Pro). Alignment charts of all samples were described in Appendix 3. However, it's possible that the invasive viruses generated these amino acid alterations as a response to medications that target their big S protein [29].

In the current study, a phylogenetic tree was created based on nucleic acid variations found in the amplified 600 bp of the S gene amplicons. This allowed for a phylogenetic understanding of the actual distances between our investigated samples and the identification of relatives based on mutations and genotypes. The phylogenetic tree contained the samples studied within the cladogram and was designed by the maximum likely hood tree. boot strap method/Jukes-cantor model/neighbor joining.

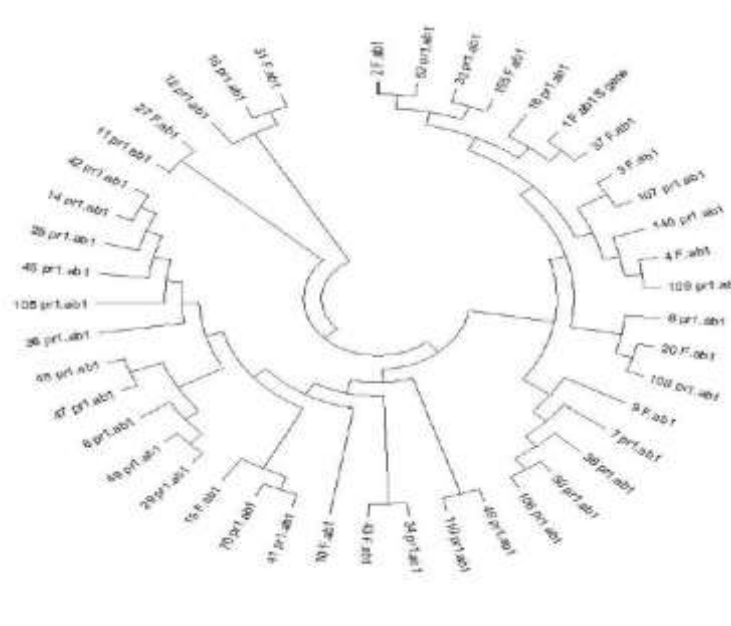


Figure 1: The cladogram phylogenetic tree of genetic variants of the S gene fragment of the Hepatitis B virus.

Due to its ability to explain the real neighbor-joining-based positioning in such reported differences, the current observation of this tree has corroborated sequencing reactions. Therefore, there was no discernible evolutionary impact on the current placement of the studied samples due to the recently detected nucleic acid changes, which were merely a slight tilt within the same viral genotype. The results of this study strongly implied that the samples might be known genetic variations of the genotype-D within the sequences of the hepatitis B virus.

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