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ORIGINAL ARTICLE

Molecular Detection, Genotyping and Associated Risk Factors of Hepatitis B Virus in Basrah Province, Southern of Iraq

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ABSTRACT

Background: *Hepatitis B Virus (HBV) infection is one of the most serious public health issues feared worldwide because of its long-term consequences in infected individuals.* ***Objective:*** *detect and genotype HBV among patients attending the Digestive System Hospital in Iraq and to identify associated risk factors.* ***Methodology:*** *This study was performed from November 2023 till February 2024 at Digestive System Hospital & Public Health Department laboratory, Iraq. All participants (n = 100) who were diagnosed with HBsAg positivity using ELISA were enrolled in the study.* ***Results:*** *95 samples were confirmed positive for HBV DNA after extraction and purity analysis. X-gene and S-gene were amplified through the polymerase chain reaction (PCR) molecular detection; the bands were approximately 139 bp and 417 bp. Among them, the genotyping analysis showed a wide variety of genotypes, among which chronologically, genotype C was the most common, followed by D, B, C2, D3, F, and I. The epidemiological analysis showed that the proportion of HBV infection was in males (59%) than in females (41%), and was more common in the ages between 31–45 years old (36%) and those who had blood group O+ (52%). There were also notable associations between HBV infection and weight, family history, smoking, and educational level. But there was no correlation seen between HBV infection and chronic disease, or length of illness.* ***Conclusion:*** *This study highlights the need for focused public health measures and improved surveillance measures to control transmission of HBV in Iraq.*

INTRODUCTION

Liver diseases, caused by a variety of factors including viral agents, inherited susceptibilities, and toxic exposures, represent a major public health issue worldwide. Control of viral hepatitis is of utmost importance, with Hepatitis B Virus (HBV) and Hepatitis C Virus (HCV) being the most prominent participants, which cause significant liver disease and serve as precursors to cirrhosis and hepatocellular carcinoma (HCC)[1].

HBV is an enveloped DNA virus from the Hepadnaviridae family with routes of transmission including parenteral, sexual, and perinatal. The persistence of covalently closed circular DNA (cccDNA) in infected hepatocyte nuclei supports viral latency and reactivation and underlies the obstacles to eradication[2,3]. HBV and HCV account for about 1.4 million deaths each year worldwide and are a significant source of morbidity and mortality globally[4,5]. Although HBV has a potent vaccine, a large fraction of the population, especially those in low-resource regions, are not immunized[6,7].

HBV can be divided into ten genotypes (A-J) exhibiting differences in geographical distribution and clinical relevance. The most common genotypes found in Asia are B and C, which are associated with an increased risk of developing HCC[8]. The World Health Organization (WHO) has established a 2030 elimination target for viral hepatitis with a focus on the introduction of comprehensive screening, diagnostic, and treatment strategies[6].

In Iraq, HBV and HCV are important public health problems and significant contributors to the burden of chronic liver disease and HCC. Although the prevalence and risk factors of such infections have already been studied[9-13]. Limited information regarding dominant genotypes and associated epidemiological factors has been provided. The present study aimed to diagnose, identify the molecular types of HBV mutation, and demonstrate the risk factors associated with HBV infection among a selected Iraqi community.

METHODOLOGY

Study Design and Sampling

This cross-sectional study was performed from November 2023 till February 2024 at Digestive System Hospital & Public Health Department Laboratory, Iraq. All participants (n=100) who were diagnosed with

HBsAg positivity using ELISA were enrolled in the study. Epidemiological data such as age, sex, blood group, weight, family history, smoking habits, chronic disease, and duration of illness, medication, and educational attainment were obtained.

Serological Testing

HBsAg was detected with the ADVANCED/TP 21003 ELISA kit (China), according to the manufacturer's instructions.

Molecular Diagnosis of HBV

DNA Extraction and Purity Assessment

The FavorPrep Viral RNA/Nucleic Acid Mini Kit (Favorgen Biotech Corp., Taiwan) was used to extract viral DNA. The purity of DNA was evaluated at 260/280 nm with a NanoDrop spectrophotometer.

HBV PCR Detection and Genotyping

Conventional-PCR was performed to estimate the expression of (x-gene) and (s-gene) using the primers listed in table 1, The PCR master mix was prepared according to the instructions of company (Cat. M7822, Promega, USA). The program was used to amplify (X-gene and S-gene) as table 2.

Table 1: Sequence of primer and their length

No.	Primers	Primer Sequence	Length (bp)
1	F-X gene	GTCCCCTTCTTCATCTGCCGT	21
	R-X gene	GTTCACGGTGGTCTCCATG	19
2	F-S gene	CGTGGTGGACTTCTCTCAATTTTC	24
	R-S gene	GCCARGAGAAACGGRCTGAGGCCC	24

Table 2: Steps program of PCR (S gene) and (X gene)

No	Step	S gene			X gene		
		Temperature	Time	Cycle	Temperature	Time	Cycle
1	Initial denaturation	95 °C	5 min	1	95°C	5 min	
2	Denaturation	94 °C	30 sec	35	94°C	30 sec	35
3	Annealing	61 °C	30 sec		58.2°C	30 sec	
4	Extension	72 °C	30 sec		72°C	30 sec	
5	Final extension	72 °C	5 min	1	72°C	5 min	

DNA Sequencing and Genotyping

HBV genotypes were analyzed by Nucleotide BLAST in the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) database. Hepatitis virus was identified by Basic Local Alignment Search Tool (BLAST) followed by national center for Biotechnology Information (NCBI), since the sequence of nucleotide was copied and pasted in the "BLAST" after proofreading, then the program was identified the hepatitis virus with others by comparing sequences together.

Statistical Analysis

Analysis was performed using the SPSS software. Association between HBV infection and epidemiological variables: $\chi 2$ tests. Statistical significance was accepted at a $p<0.05$.

RESULTS

Serological and Molecular Detection

One hundred samples that were positive for HBsAg by ELISA were confirmed positive for HBV DNA by PCR. 95 samples were confirmed positive by PCR. The x-gene (139 bp) (Figure 1) and s-gene (417 bp) (Figure 2) were detected successfully by PCR amplification in 95 samples.

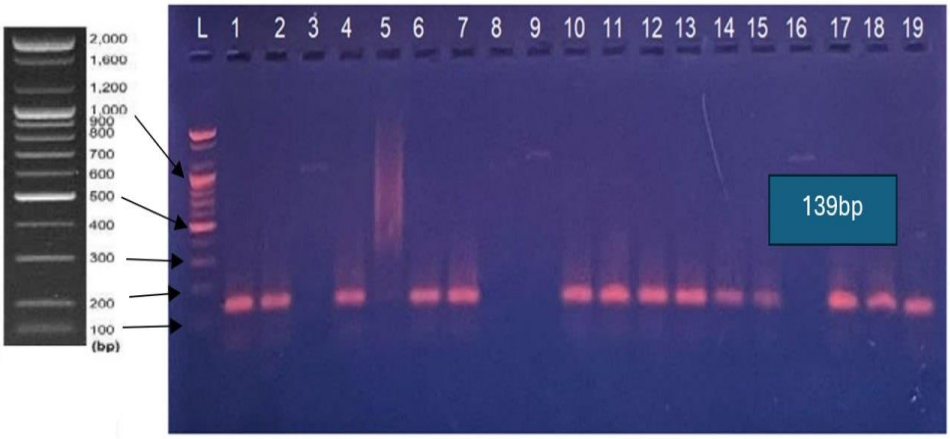

Fig. 1: Agarose gel electrophoresis (1.5%) showed a model of amplified X-gene (139 bp). Lane L: 100 bp Marker, Lane 1-19: x-gene bands for HBV. In above Figure, all wells positive except well 3, 8,9,16, which was negative

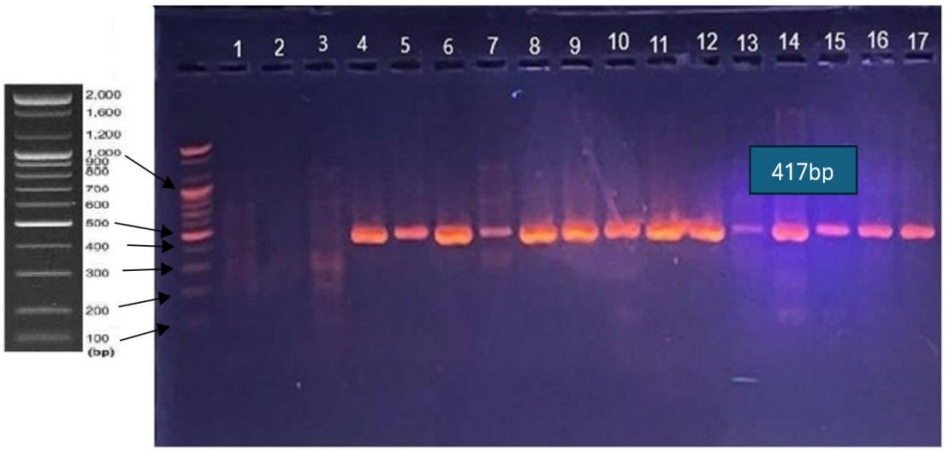

Fig. 2: Agarose gel electrophoresis (1.5%) showed a model of amplified S-gene (417 bp). Lane L: 100 bp Marker, Lane 1-19: S-gene bands for HBV. In above Figure, all wells positive except well 1, 2, 3, which was negative.

Sequencing of the HBV X-gene

The DNA sequencing results for the HBV X-gene, obtained from 28 out of 30 samples sent to Macrogen Company in Korea, demonstrated high similarity rates with the isolates in the GenBank database.

Distribution of HBV Infection According to Tested Risk Factors

The HBV infection rate was significantly higher in males (59%) than in females (41%) ($p< 0.05$). Whereas the apex of infection rates was discerned within the demographic cohort aged 31-45 years (36%) ($p< 0.01$) and among individuals possessing blood group O+ (52%) ($p< 0.01$). Examination of weight categories revealed that the highest infection prevalence was situated within the 81-90 kg range (30%) ($p< 0.01$). Most of the infected people had no family history for HBV (78%) ($p< 0.01$). Infection rate was higher in non-smokers patients (males and females) 61% ($p< 0.05$). While the results revealed that the smoker male have more incidences (39) when compared with nonsmoker males (20) with significant differences between them ($p< 0.05$). However, no statistically significant correlation emerged between HBV infection and the presence of chronic diseases, treatment or the duration of illness ($p> 0.05$) (Table 3).

Table 3: Distribution of HBV infection Based on Tested Risk Factors

Risk factors		HBV infections	*p*-Value
Sex			
Male		59	*p*< 0.05
Female		41	
Age group			
16-30		13	*p*< 0.01
31-45		36	
46-60		30	
>60		21	
Blood group			
O+		52	*p*< 0.01
B+		24	
A+		15	
AB+		4	
B-		4	
A-		1	
Weight group			
50-60		6	*p*< 0.01
61-70		20	
71-80		21	
81-90		30	
>90		23	
Chronic illness			
Yes		46	*p*> 0.05
No		54	
Family history			
Yes		22	*p*< 0.01
No		78	
Period of illness			
Old		56	*p*> 0.05
New		44	
Treatment			
Yes		55	*p*> 0.05
No		45	
Educational level			
Uneducated		70	*p*< 0.05
Educated		30	
Smoking			
Yes	Male	39	*p*< 0.05
	Female	0	
No	Male	20	
	Female	41	

HBV Genotyping

The most common genotype was genotype C (n=16), followed by genotype D (n=5), genotype B and C2 (n=2 each), genotype D3, F, and I (n=1 each). Distribution of the genotype differed markedly with respect to sex, age, blood group, and weight (Table 4).

Table 4: Distribution of HBV infection according to genotypes

Genotypes	Infected	Percentage
C	16 *	57.3%
D	5	17.8%
B	2	7.2%
C2	2	7.2%
D3	1	3.5%
F	1	3.5%
I	1	3.5%
Total	28	100%
p-value	0.000	

Distribution of HBV Genotypes Based on Tested Risk Factors

According to the sex, HBV genotype C was the most common among both males and females, followed by genotype D. Genotypes B, C2, D3, F, and I were not recorded among females. The current study also found that the highest percentage of genotypes was in the (31-45) years' age group, recording 10 genotypes. This was followed by the (46-60) years and >60 years' age groups, each recording 7 genotypes. The (16-30) years' age group recorded 4 HBV genotypes.

The study revealed that patients with blood group O+ had the highest percentage of genotypes, recording 18 genotypes (8 of them were genotype C). This was followed by blood group A-, which recorded 6 genotypes. Blood groups B+ and B- recorded the lowest percentage of genotypes. The distribution of HBV genotypes according to weight showed that the highest rate was in the (71-80) kg weight group, which recorded 9 genotypes (genotype C was 5). This was followed by the (81-90) kg weight group, recording 7 genotypes. The (60-70) kg and >90 kg weight groups each recorded 6 genotypes. Regarding the correlation between genotypes and presence of chronic illness, the study showed that HBV infections in patients without chronic diseases recorded 15 genotypes, while infections with chronic diseases patients recorded 13 genotypes. (Table 5).

Table 5: Distribution of HBV Genotypes According to Tested Risk Factors

Risk factors	Genotype							Total
	B	C	C2	D	D3	F	I	
Sex								
Male	2	12	2	2	1	1	1	21
Female	0	4	0	3	0	0	0	7
Age group								
16-30	0	3	0	1	0	0	0	4
31-45	0	6	1	2	0	0	1	10
46-60	1	4	1	0	1	0	0	7
>60	1	3	0	2	0	1	0	7
Blood group								
O+	2	8	1	5	1	1	0	18
A-	0	5	1	0	0	0	0	6
B+	0	2	0	0	0	0	1	3
B-	0	1	0	0	0	0	0	1
Weight group								
60-70	1	4	0	1	0	0	0	6
71-80	0	5	1	2	0	0	1	9
81-90	1	4	0	2	0	0	0	7
>90	0	3	1	0	1	1	0	6
Chronic illness								
Yes	2	8	1	2	1	1	0	15
No	0	8	1	3	0	0	1	13

DISCUSSION

All of the patients gave their informed consent. Using both serological (ELISA) and molecular (PCR) approaches, we were able to characterize HBV infection status, identifying strong concordance but important differences between the two techniques.

Out of the 100 samples screened with ELISA for HBsAg detection, 95 came out positive by PCR as well. Which is consistent with study, in India, which documented 24 cases for HBV surface antigen (HBsAg) by ELISA and only 12 cases for HBV DNA[14].This difference, although small, is important in clinical and epidemiological settings. This could lead to a lower detection rate of PCR positive samples compared to ELISA positive samples due to a number of factors[15].

ELISA is sensitive but can also yield false positive results due to cross-reactivity with other antigens[16]. Second, only because HBsAg is present does not indicate active viral replication, which is detected by PCR. Resolved HBV infection or inactive carrier state: these can lead to (very) low but still detectable HBsAg; in such cases, no viral DNA is detectable.

The observation of low HBV-DNA levels in some high-level HBsAg carriers is particularly interesting to note. This implies that levels of HBsAg and HBV DNA may not always be correlated, possibly as a result of an immune-mediated inhibition of viral replication. This phenomenon highlights the importance of utilizing both serological and molecular assays to effectively diagnose and monitor HBV infection[17]. Additionally, reactivity studies show that the combined use of ELISA and PCR improves diagnostic accuracy and decreases false-positive results, in addition to providing insight into the monitoring of individuals with active viral replication.

Successful amplification of both HBV x-gene and s-gene in samples only previously positive for HBV further confirms the sensitivity and specificity of the PCR assay used. The x-gene encoding HBx protein is essential for both viral replication and hepatocarcinogenesis[18]. The polymerase and surface proteins are further encoded by the plasmids of the core (x-gene) and the surface protein (s-gene), with the latter (HBsAg) being required for assembly/infectivity[19]. The evidence of these genes proves the existence of intact HBV DNA in the samples we studied.

In this study, genotype C was found to be the most common HBV genotype which corroborates other reports from Asia and the Middle East[20]. And differenced with other study, which did recorded genotype D is the highest infection among other types[21]. While in Baghdad, the B and F genotypes were the most common[22]. Genotypes B, D, C2, D3, F, and were also detected in both methods, indicative of the genetic diversity of HBV in the region. Genotype distribution may affect disease progression, response to treatment,

and risk of developing HCC. Genotypes B and C, for example, have been correlated with HCC risk. The variations in genotype distribution across demographic groups (sex, age, blood group, and weight) observed indicate the impact of varying transmission routes and risk factors[23].

More investigation is needed to outline that there is a potent relationship between HBV infection with gender (male), this agreed with study, in Dhi-Qar Province[24], The same finding was also reported in several Mediterranean countries[25], age (31-45) years, which agreed with study in Misan Province, it was shown most effect age group (25-50) years[26], but it differed with other studies in nighouber countries such as Saudi Arabia, incidence rates of HBV were substantially higher in individuals aged 45 years and older[27]. Blood group (O^+), this result agreed with study in Anbar Province[28], Saudi Arabia[29] and Jordan[30]. Our results indicated that blood group O^+ were at higher risk of HBV infection than non (O^-) blood group, weight (higher) which agreed with several studies[31], but it differed with study, which found inversely correlated between HBV infection and obesity[32], family history (absent), This result agreed with study showed no significant correlation was observed between the patient's family history and hepatitis B virus infection[33], smoking (non-smoker), and education levels (lower) as summarized or shown in our results.

The greater prevalence among males may represent differences in risk behaviors or occupational exposures. This highlights potential exposures during early adulthood, as the (31-45) age group shows the highest rates of infection. Further studies are needed to determine the mechanism of action for blood group O^+ association.

The observation that there were greater numbers of HBV in non-smokers may simply be the result of other factors which are seen more often in non-smokers or perhaps is a confounding variable. This association with decreased education levels highlights the need for education and awareness campaigns about public health.

Our current results should be confirmed in future studies in larger, population-based cohorts, and the long-term clinical relevance of HBV genotypes should be clarified. Moreover, exploring the impact of social determinants of health and particular risk behaviors could be important towards a better understanding of HBV transmission dynamics in Iraq.

CONCLUSION

There is an enormous burden of HBV infection in the studied Iraqi population as highlighted by the findings of this study. The increased prevalence of HBV in both males and the (31–45) age group highlights the importance of targeted interventions. Genotype C was predominant, consistent with previous reports from the region. The divergent results between ELISA and PCR call for both approaches for confirming HBV infection. These results highlight the need for the use of serological as well as molecular approaches in both the diagnosis and monitoring of HBV infection. Vaccination, screening, and education, targeted public health interventions can help to reduce the burden of HBV in Iraq. The observed associations between HBV genotypes and clinical outcomes underline the public health relevance of elucidating the long-term clinical relevance of HBV genotypes for the rational development of HBV control and elimination strategies.

Conflict of interest

Both authors declare that there is no conflict of interest in this study.

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Ethics statement

The Ethical Committee of the College of Science - University of Basra approved this study, based on the verbal consent of all patients participating in this study, under protocol number 27\2023 in date 10-10-2023.

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