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ORIGINAL ARTICLE

Detection of Hepatitis B Virus (HBV) Genotypes by Sequence in Diyala Province

MARWA HADI MUSTAFA[1], ZAHRAA JAAFAR JAMEEL[2]
[1]*University of Diyala, College of Science, Department of Biology*
[2]*University of Al-karkh, College of Energy and Environmental Science, Department of Environment*
Correspondence to: Marwa Hadi Mustafa, Email: scibioms23@uodiyala.edu.iq, Cell: 009647717028029

ABSTRACT

Background: To put it simply, hepatitis is liver inflammation. Hepatitis B virus (HBV) infection is a public health problem because it can lead to serious liver conditions like hepatocellular carcinoma and cirrhosis. Ten HBV genotypes (A-J) were distinguished based on viral sequence similarity.

Objective: detection of HBV utilizing polymerase chain reaction (PCR) technique, followed by viral nucleotide sequence and phylogenetic analysis.

Methods: A cross-sectional study was conducted in170 reviewers (103 males and 67 females) who attended the Baquba Teaching Hospital, Teaching Laboratories, the Main Blood Bank, and Baladrooz General Hospital. Age ranging 13-75 years. The samples were collected from Diyala Province of Iraq; person data was collected by using questioner, including their name, age, gender, address, academic achievement, source of infection, take vaccine of HBV, and corona infection. Enzyme-linked immune sorbent assay test (ELISA) was used to detect hepatitis B surface antigen (HBsAg) in serum. Amplification Polymerase Chain Reaction (PCR) of HBV p gene of serum samples was performed to detect HBV from serum samples. The sequence of the PCR products of the polymerase gene was sent for Sanger sequencing using ABI3730XL, an automated DNA sequencer, by Macrogen Corporation – Korea for the study of mutations and genotype identification. The results were received by email and then analyzed using geneious software.

Results: The positive samples of HBV by ELISA were 70 (41.20%), and 100 (58.80%) samples were negative. While positive samples for HBV-DNA PCR were 14 (8.20%). When compared the sequence of local isolates with NCBI-Blast Hepatitis B virus showed that all isolates were D genotype. Variation result showed that the highest type of mutation was Transition when compared with the NCBI referring sequences.

Statistically: The present study revealed there was a significantly different ($p<0.05$) in the positivity of HBV detected by ELISA and PCR
between study groups. Additionally, there was a significantly different ($p<0.05$) between the positivity of HBV detected by ELISA and PCR
and age groups, gender, Social status, address, education , jaundice infection of patients, the HB vaccination, and infection source of
patients.

Keywords: HBV, ELISA, PCR, Sequence, Variation, Phylogenetic Tree, Diyala, Iraq.

INTRODUCTION

Hepatitis is an inflammation of the liver. Hepatitis is a worldwide health issue that causes liver damage (Al-Absi et al., 2018). Various viruses, including hepatitis A, B, C, D, and E, cause human viral illnesses. Although viral infections, including HBV, are the most common cause of hepatitis, other non-viral factors such as toxins, medications, autoimmune illnesses, bacterial infections, and parasites can also cause hepatitis (Sayre and Thompson, 2021). Infection with Hepatitis B is one of the most significant public health concerns, particularly in poor countries (Lim et al., 2020). The infectious form, known as the Dane particle, has a diameter of 42nm and consists of a nucleocapsid, a large (L) envelope protein, a middle (M) envelope protein, and a small (S) envelope protein. The DNA genome is circular and partly double-stranded, and it is linked to a polymerase (Lamontagne et al., 2016). The HBV genome comprises about 3,200 nucleotides (Matsuura et al., 2009).

HBV genotypes are differentiated by an 8% variation in the nucleotide sequence, whereas subtypes within each genotype have a 4% divergence (Sunbul, 2014). Molecular evolutionary analysis of HBV-DNA sequences showed ten genotypes labelled A–J, with more than 8% genetic diversity (Choga et al., 2019). Iraq is considered a low-endemic country for HBV and HCV compared to its neighbors. The rising frequency of all varieties of hepatitis in Iraq could be related to the security situation and the overcrowding of refugees and migrants, as well as a lack of vaccine availability (Merzah et al., 2019).

MATERIALS AND METHODS

Specimen collection: One hundred and seventy reviewers' veins were punctured with a disposable syringe to collect five millimeter of venous blood under a septic technique. Serum was extracted from each blood sample by centrifuging it at 4000 rpm for 4 minutes after being collected in a sterile tube without anticoagulant. The serum was separated and stored in multiple marked clean tubes, then stored at -20ºc to be used for ELISA, DNA extraction, and PCR technique.

Hepatitis B virus screening test by ELISA: One hundred and seventy samples were tested for the detection of Hepatitis B surface antigen (HBs Ag) by using an ELISA kit (fortress - U.K). This kit employs monoclonal antibodies (IgG antibody to HBsAg) in the sandwich type method. All steps were taken in accordance with the manual supplied by the manufacturer. When we ran the Kit, we also included a blank, three negative controls, and two positive controls. Each well's optical density (O.D.) was determined at a wavelength of 450 nm. The cut-off is set at the median absorbance of three negative controls ×2.1.

Comparisons were made between the samples' calculated absorptions and the threshold value. Samples with an absorbance higher than or equal the cut-off value indicate positive results. Negative results samples giving an absorbance less than the Cut-off value.

Hepatitis B virus (HBV) genotypes

DNA extraction: Serum samples were processed using extraction kits (Maxwell® 16 Viral Total Nucleic Acid Purification Kit, CAT.# AS1150, Promega, USA) that contained enough reagents for 48 purifications of viral genomic DNA.

PCR: All DNA that has been extracted from HBV samples in the current study went through PCR procedure in order to target p gene that is under study. Primers used to amplify the p gene were listed in table format and used to prepare the reaction mix per the manufacturer's instructions (Promega, USA), as shown in table (1). PCR thermocycler condition included an initial denaturation step for 5 minutes at 95°C, then a cycling step including 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72º C for 30

seconds, followed by a final extension for 7 minutes at 72º C, and hold for 10 minutes at 10º C.

Table-1: The PCR primers with their sequence and amplicon size

Primer	Sequence (5'-3')		Amplicon Size
First PCR –HBV-universal sense Primer	P1	TCA CCA TAT TCT TGG GAA CAA GA	1063bp
First PCR –HBV-universal antisense Primer	S1-2	CGA ACC ACT GAA CAA ATG GC	

HBV genotyping by sequence: Macrogen Corporation, Korea's ABI3730XL automated DNA sequencer was used to sequence the deoxyribonucleic acid (DNA) of hepatitis B virus clones 7, 26, 11, 37, 40–46, 54–58, and 65 using the Sanger sequence. The data was sent to us via email and analyzed by geneious software. After performing an NCBI blastn for these PCR amplicons, the sequencing reactions pinpointed their precise locations in relation to the 12 of 14 HBV samples under study.

Statistical analysis: In order to analyze the data from the current study, we used SPSS (version 25.0) and Graph Pad Prism (version 6), two statistical packages designed specifically for the social sciences. Categorical variables were compared by Pearson-Chi-square test or two-tailed Fisher exact probability (p). A level of P values ≤0.05 was considered statistically significant.

RESULTS

Relation of positivity of ELISA and PCR with Age groups: The present study included 103(60.60%) male and 67(39.40%) female reviewers. Age ranging 13-75 years. The present study revealed there was a significant different (p<0.05) between the positivity of HBV detected by ELISA and PCR and age groups of patients, where it was found that the positivity of HBV detected by ELISA was the highest percentage (30.0%) at age group 21-30 year and least percentage (7.1%) age group ≤20 years, while the positivity of HBV detected by PCR was the highest percentage (28.60%) at age group >60 years and least percentage (7.1%) age group 51-60 year. Additionally, we found there was a significantly different (p<0.05) between the positivity of HBV detected by ELISA and PCR and all age groups except ≤20 years age group, as shown in table (2).

Table 2: Relation of positivity of ELISA and PCR with Age groups of the study population

Positivity of ELISA and PCR			ELISA	PCR	P value
Age groups	≤20	N	5	2	p>0.05
		%	7.10%	14.30%	
	21-30	N	21	2	P<0.001*
		%	30.00%	14.30%	
	31-40	N	14	3	P<0.001*
		%	17.10%	21.40%	
	41-50	N	12	2	P<0.001*
		%	20.00%	14.30%	
	51-60	N	7	1	P<0.001*
		%	10.00%	7.10%	
	>60	N	11	4	P<0.01*
		%	15.70%	28.60%	
P value			p<0.05*	p<0.05*	

"Significant difference between proportions using Chi-square test at 0.05 level".

Relation of positivity of ELISA and PCR with vaccine: The present study revealed there was a significantly different (p<0.05) between the positivity of HBV detected by (ELISA, PCR) and the HB vaccination, we found the positivity of HBV detected by (ELISA and PCR) were the highest percentage (90.00% and 100.00%) in patients without HB vaccination than patients with the HB vaccination, as shown in table (3).

Relation of positivity of ELISA and PCR with Infection Source: The present study revealed there was significant different (p<0.05) between positivity of HBV detected by ELISA and PCR and infection source of patients. We found the positivity of HBV detected by ELISA and PCR were highest percentage (48.6% and 50.0%) in teeth patients than others sources, as shown in table (4).

Table 3: Relation of positivity of ELISA and PCR with vaccine

Positivity of ELISA and PCR (n=70)			ELISA	PCR	P value
HB vaccination	Yes	N	7	0	1
		%	10.00%	0.00%	
	No	N	63	14	P<0.001*
		%	90.00%	100.00%	
P value				P<0.001*	1

*"Significant difference between proportions using Chi-square test at 0.05 level".

Table 4: Relation of positivity of ELISA and PCR with Infection Source

Positivity of ELISA and PCR(n=70)			ELISA	PCR	P value
Infection source	Unknown	N	7	1	P<0.001*
		%	10.00%	7.10%	
	Teeth	N	34	7	P<0.001*
		%	48.60%	50.00%	
	Tattoo	N	4	0	1
		%	5.70%	0.00%	
	General surgery	N	20	4	P<0.001*
		%	28.60%	28.60%	
	Blood transfusion	N	5	2	p>0.05
		%	7.10%	14.30%	
P value			P<0.001	P<0.001*	

*"Significant difference between proportions using Chi-square test at 0.05 level".

Genotyping By Sequence: The current results showed that (7,54) (11,40,43) (26,65) isolates had the same sequence while the remaining 37,44,45,46,56,57,58 isolates each one had its own sequence. When compared the sequence of local isolates with NCBI-Blast Hepatitis B virus showed that all isolates were D genotype. In order to locate similar and dissimilar positions within these sequences, which will help in identifying HBV genotypes and patterns of observed variations.

Variation and Analysis of Phylogenetic Tree: The exact locations of the observed mutations in the sequenced 1063bp fragments were summarized in table (5).

Table 5: The pattern of the observed mutation in the 1063 bp of polymerase (P) amplicons in comparison with the NCBI referring sequences

Sample No.	Mutations No.				GenBank acc. no.
	Transition	Transversion	Deletion	Insertion	
7	2	2	1	4	KF170747.1
11	3	8	1	2	MW601313.1
26	6	1		4	MN702719.1
37	16	11		3	MZ097786.1
40	1	1	1	2	MW601313.1
43	1	1			MW601313.1
44	5	4		5	MZ097849.1
46	7	3		5	MZ097843.1
54	3	3	1	3	KF170747.1
56	3	3		3	MZ097840.1
58	1	1		1	MZ097756.1
65	7	5		3	MN702719.1

In this study, nucleic acid variations in the amplified 1063bp of the HBV samples were used to construct a robust phylogenetic tree. This phylogenetic tree included nucleic acid sequences from the HBV virus and a total of twelve isolates. Only HBV, an organism not found in any other part of the tree, was incorporated into this one.

In the constructed genius prime software program, the external branch includes Hepatitis B virus isolate 11-p1.ab1 is identical to 40-p1.ab1 and the other cluster includes 26-p1.ab1 is identical to 65-p1.ab1, as shown in figure (1).

Hepatitis B virus (HBV) genotypes

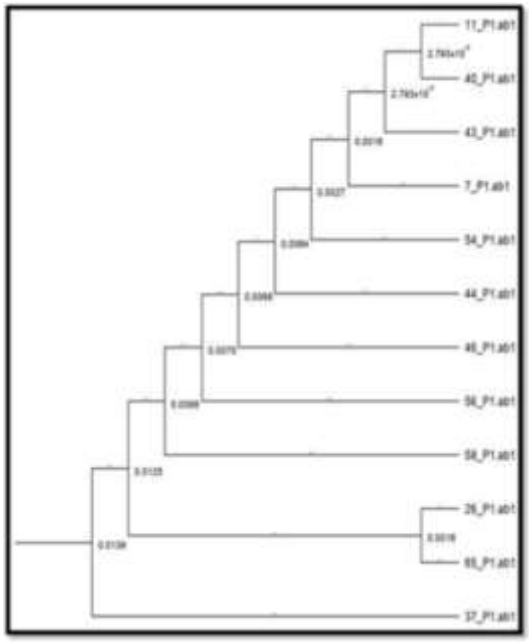

Figure 1: Geneious Phylogenetic Tree showing the 12 hepatitis B virus P gene sequences

DISCUSSION

The results of present study revealed there was significant different among age groups and HBs Ag positivity, table (1), where the 21-30 and 41-50 scored highest HBs positivity and These results were in agreement with those reported by other Iraqi studies (Masoumeh et al., 2019; Salman et al., 2021) in Al-Diwaniya and Diyala respectively. Additionally, worldwide studies reported results consistent with present results (Mohammad et al., 2019; Majed et al., 2020) in Iran and Jordan respectively. The present study showed there was significant different between positivity of HBV detection by PCR and age groups, where the age group >60 years scored highest percentage than others age groups. The differences among positivity of HBV detection by PCR and age groups due to chronic diseases, and immune efficiency that decreases with age progression. Probably the most common source of HBV infection among all age groups from the high frequency of nosocomial infection in hospital, contamination of blood and blood products, as well as a lack of health knowledge, are factors that may lead to HBV transmission among these ages (Ghanim, 2019). The present study showed the infection rate of HBV in patients with HB vaccination was low compared to patients without HB vaccination and that refer to efficacy of vaccine against HBV, table (2). Wijaya et al., (2021) showed the HB vaccination stimulates enhanced Natural Killer cells (NKs) degranulation on re-exposure to the vaccine antigen hepatitis B surface antigen (HBsAg), regardless of time since vaccination. A previous study found that having a family member who had hepatitis B increased the risk of contracting the disease among those who had not been vaccinated, but this association was not seen among those who had received the vaccine. The fact that vaccination has been shown to reduce the risk of hepatitis B infection in the general population may explain this finding. Future research should focus on examining this connection further (Moghadami et al., 2020). Multiple ways are now being studied to improve the immunogenicity of the HBV vaccine, including vaccine adjuvants, recombinant vaccines, and immune enhancement via dendritic cell upregulation (Saco et al., 2018).

Must be available to inform people about the infectious environment of HBV and how it is transmitted. Because HBV is a vaccine-preventable disease, it is critical to motivate people, particularly high-risk populations, to be vaccinated (Rana et al., 2019). Present study showed that teeth and surgery scored highest percentage of HBV infection compared to others sources, table (3). In Saudi Arabia, Al Humayed et al., (2016) in their extensive research, the authors came to the conclusion that dental treatment raises the chance of contracting both HBV and HCV, although that risk might be effectively minimized by taking usual precautions. In Ethiopia, Asemahagn, (2020) demonstrate a significant prevalence of HBV positive among surgeons, and these findings corroborated our findings. In Pakistan, Ullah et al., (2020) showed that there is a relatively high prevalence of HBV infection in hospitalised patients; this may be because of insufficient screening before surgery. Dental workers are more likely to suffer needlestick wounds that expose them to potentially infectious patients' blood and bodily fluids. Unvaccinated people have a high risk of contracting HBV by HBV-contaminated needles, ranging from 12 to 60 percent (Nagao et al., 2021). The lack of trained personnel, as well as the use of non-sterilized surgical instruments and blood products, could be to blame. The present study showed there is significant different between positivity of HBV detection by PCR and infection sources.

Genotyping By Sequence: Genotype and subgenotype determination of the hepatitis B virus is useful for developing vaccination schedules, designing antiretroviral treatment plans, and establishing baselines for disease diagnosis and prevention (Mohammad et al., 2019). HBV genotypes and mutations can affect viral pathogenesis by impacting host immune recognition, viral replication and virulence, cell adhesion and penetration, and hepatocarcinogenesis (Lin and Kao, 2020). HBV genotyping can be done using a variety of approaches. The gold standard for genotype determination is a full-length HBV genome sequencing and phylogenetic analysis (Garcia-Garcia et al., 2021).

Variation and Analysis of Phylogenetic Tree: Mutation refers to the variation in the nucleotide sequence of an organism's genome. HBV mutations are primarily caused by a loss in HBV reverse transcriptase proofreading activity and by immune system pressure from the host (Desmond et al., 2012). Additionally, because the HBV genome contains overlapping reading frames, changes brought on by antiviral selection pressure may have an impact on the neutralisation epitopes found in HBsAg (Pollicino et al., 2009). According to reports, HBV genomic mutations can have an impact on the effectiveness of treatments as well as viral replication and disease progression (Al-Qahtani et al., 2017). The extensive distribution of HBV mutations with clinical implications is a possible danger to the long-term viability of mass vaccination programmes and a significant issue for the design of diagnostic tests and current treatment approaches (Yan et al., 2017). These mutations may cause resistance to lamivudine and famciclovir. Lamivudine, a nucleoside analogue taken orally, prevents HBV replication (Marcellin et al., 2004). In the majority of patients, it can considerably reduce serum HBV DNA levels, normalize alanine aminotransferase (ALT) levels, and decrease liver necroinflammatory activity (Lai et al., 1998). Continuous lamivudine treatment may slow clinical progression in individuals with CHB and advanced fibrosis or cirrhosis by greatly lowering hepatic compensation and the risk of hepatocellular carcinoma (Wands, 2004). Drug-resistant HBV mutations are the most significant drawback of lamivudine treatment, which is also associated with an increase in ALT, DNA, and poor histology in some people. Liver disease episodes have also been linked to this tyrosine-methionine-aspartate-aspartate (YMDD) motif mutation in the C domain of the HBV DNA polymerase gene (Dienstag et al., 1999). The phylogenetic tree clearly demonstrated that the analyzed HBV isolates belonged to genotype D. This finding demonstrated the unique function of the resulting phylogenetic tree in the precise detection and genotyping of HBV samples.

CONCLUSIONS

The most frequent age affected for HBV patients was the twentieth and the fortieth decades. HBV infection in patients without HB vaccination more than patients with HB vaccination, and dental

patients more than others sources of infection. The HBV genotype D was the most frequent among the investigated sample of HBV patients. Variation result showed that the highest type of mutation was Transition, followed by Transvertion, Insertion, and then Deletion when compared with the NCBI referring sequences. These mutations may cause resistance to Drugs.

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