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Serological and Molecular Detection of Hepatitis B virus among patients referred to Kurdistan Center for Hepatology and Gastroenterology in Sulaimani City/Kurdistan Region of Iraq

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ABSTRACT

Hepatitis B virus infection is caused by the hepatitis B virus, a major global health problem. This infection can lead to chronic conditions, followed by cirrhosis and hepatocellular carcinoma (HCC). The current study was aimed to detect HBV using serological and molecular techniques. During 2019, 300 blood samples were collected from Kurdistan Center for Hepatology and Gastroenterology in Sulaimani city. Enzyme-linked immunosorbent assay (ELISA) and real-time polymerase chain reaction (RT-PCR) techniques were used for the detection of HBsAg and HBV DNA, respectively. Obtained results were revealed that 92 out of 300 tested patients (30.66%) seropositive for HBsAg. Among 92 seropositive patients, 53 were shown positive results for HBV DNA by RT-PCR. Dental clinic visiting and dialysis were among the important risk factors for HBV transmission. The vast majority of positive results were among males. Smokers showed relatively high rates of positive results. One-third of the referred patients who had liver complaints were positive for HBsAg. More than half of the seropositive patients showed RT-PCR positive results. It was concluded that the molecular method (RT-PCR) is more sensitive and gives a more accurate result than serology (ELISA). Therefore, it can be used as a diagnostic tool for HBV detection.

Index Terms: Hepatitis B Virus, Hepatitis B Surface Antigen, Enzyme-linked Immunosorbent Assay, Gold In-tube, Real-time Polymerase Chain Reaction

1. INTRODUCTION

Hepatitis B virus (HBV) is a small DNA virus with a spherical structure and lipid coat membrane, a *Hepadnaviridae* family member. Cause acute liver inflammation in acute and chronic forms is followed by liver failure, cirrhosis, and hepatocellular carcinoma (HCC). More than 90% of patients with acute HBV infection recover although they have severe symptoms, whereas in chronic forms (can be asymptomatic); patients are unable to clear the virus [1]. Several transmission routes are now known, including tattooing, piercing, exposure to the infected blood and body fluids, saliva, vaginal discharge, and semen, perinatal transmission. However, it was noticed that HBV infection in infancy and early childhood could lead to chronic hepatitis in about 95% of cases [2].

It was reported that the sexually transmitted HBV infection also could lead to chronic hepatitis in 5% of unvaccinated men and women. Moreover, studies revealed that HBV could

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survive outside the body for at least seven days [2], [3]. It was confirmed that HBV infection is a global health problem, and more than 400 million people worldwide suffering from chronic HBV infections [4]. Hepatitis B surface antigen (HBsAg) is one of the essential antigens of the virus, located on the lipid membrane of the virus [1], [4]. It was reported that Hepatitis B e antigen (HBeAg) negative and anti-HBe positive results in the laboratory could be considered as evidence of chronic HBV infection [4]. Other investigators concluded that HBsAg and Anti-HBsAg antibodies are more likely indicators of detecting HBV infection [5]. The absence of HBsAg and detection of anti-HBsAg among HBV patients can be considered a sign of recovery from the disease, whereas if HBV DNA is still detected during this stage, this could be an indicator for chronic infection [4]. The vast majority of chronic cases may lead to HCC [6].

It was reported that the seropositive patients for HBsAg, HBeAg, and HBV DNA were more likely suspected with the chronic infection of HBV. Several mechanisms were known to lead to the conversion of chronic HBV infection to HCC [6]. Researches showed that if the immune system fails to clear HBV, the cycles of necrosis, inflammation, and reconstruction will repeat, causing hepatocytes may suffer from potentially epigenetic changes and carcinogenic mutations [7]. As a subsequence, the HBV genome may find in almost liver tumor cells, which may alter liver cell function. This can lead to changes in carcinogen-related genes, including cyclin A, telomerase reverse transcriptase, platelet-derived growth factor beta-receptor, and mitogen-activated protein kinase 1 [6], [7]. It was good understood that smoking and alcohol are risk factors for HBV infection [8]. HBV replication is a relatively complex mechanism. The HBV genome is converted to a relaxed circular DNA or a double-stranded linear DNA during replication. Each of them can be converted to covalently closed circular DNA (cccDNA) [9]. RNA pre-genome and HBV mRNAs are produced by cccDNA. The RNA pre-genome acts as the template for the synthesis of the negative-sense DNA strand and the positive-sense DNA strand is finally made based on the DNA negative-strand [9], [10]. As laboratory markers, the HBsAg, anti-HBs, HBeAg, anti-HBe, Hepatitis B core antigen HBcAg, and anti-HBc IgM/IgG are the most critical serological markers for the diagnosis of HBV [11]. HBsAg is the most significant marker for detecting HBV by ELISA [5], [6], [9], [10], [11]. Molecular methods for identifying HBV DNA according to the WHO standards include the quantitative polymerase chain reaction (qPCR) and the real-time polymerase chain reaction (RT-PCR) [10], [11]. The current study aims to determine the percentage rates of HBsAg seropositivity and HBV nucleic acid detection by RT-PCR among patients (who are enormously suffered from liver complaints) referred to Gastroenterology Center in Sulaimani City.

2. MATERIALS AND METHODS

2.1. Study Population

The study's population included people visiting the Kurdistan Center for Hepatology and Gastroenterology in Sulaimani city from June to November 2019. All patients had liver problems, and they had experienced specific symptoms included fever, chills, abdominal pain, nausea, diarrhea, dark urine, loss of appetite, jaundice, and fatigue. The sample size of the current study was 300 patients included 160 males and 140 females.

2.2. Sample Collection

Fresh venous blood samples were collected and transferred to the laboratory using cool boxes for the desired Lab. investigation. Serum samples were kept until HBsAg and HBV DNA detection by ELISA RT-PCR.

2.3. HBsAg Detection by ELISA

Sandwich-ELISA method was depended to detect HBsAg using a special ELISA kit (Elab-Science, China) with a pre-coated microtiter plate well with recombinant HBsAb. All preserved sera samples were transferred to room temperature for about 30 min. 100 µL of each sample, standard, and blank were added to the desired wells. The plate was covered with sealer and incubated for 90 min at 37°C. The wells were aspirated and washed by an ELISA washer as directed by the supplied company. 100 µL of Biotinylated Detection Ab Working Solution were added to each well, and the plate was covered and incubated for 60 min at 37°C. The plate was washed. To each well, 100 µL of HRP Conjugate Working Solution was added except for the blank control and incubated q for 30 min at 37°C. The plate was washed after that, 90 µL of Substrate Solution was added to each well and mixed, then incubated at 37 °C for 15 min. 50 µL of stop solution were added for the wells and mixed thoroughly. The optical density was measured at 450 nm using ELISA Microplate Reader.

2.4. Viral DNA Extraction and Amplification

High molecular weight genomic viral DNA was extracted by a fully automated magnetic beat nucleic acid extraction system (Zinexts, Taiwan). The viral nucleic acid extraction kit (Zinexts) was used to separate viral genomic viral DNA using 400 µl of serum and 100 µl Elute volume. The

extracted viral DNA was stored at -80°C until the day of examination by RT-PCR using an RT-PCR machine (Line Gene 9600 PLUS -Bioer, China-). A special Kit (Fluorion HBV QNP 2-0 -Inotek, Turkiye) was used for the detection of HBV DNA.

2.5. PCR Reaction

A total volume of PCR Mix, internal control, aH2O, and Extracted viral DNA (25 µl) was prepared as directed by the supplied company as summarized below:

Items	Volume
PCR Mix	12.5 µl
Detection Mix 1	1.4 µl
Detection Mix 2	1.1 µl
Internal Control	1 µl
dH2O	4.0 µl
Extracted viral DNA/Standard/ Negative/Positive Control	5 µl
Total Volume	25.0 µl

The process of PCR programming for detecting HBV nucleic acid was performed starting with the denaturation step, renaturation, annealing, elongation, and data collection as summarized in the below table.

Step	Temperature (°C)	Duration	Cycle
Initial denaturation	95	15 min	1
Denaturation	95	30 s	50
Annealing, elongation, and data collection	54	1:30 min	
Infinite hold	22	∞	

2.6. Statistical Analysis

The results were analyzed using Chi-square and Mann–Whitney U-tests through SPSS V. 25 software (SPSS Inc., Chicago, IL, United States). Statistically, the *P*-value was considered to be <0.05 ($P < 0.05$) significant.

3. RESULTS

The patients who participated in this study who suffered from health complaints were distributed on males (160) and females (140). The mean age of them was 38.16 ± 15.24 years. All patients who were referred to the GIT center were clinically suspected of having hepatitis and liver complaints. Several symptoms were depended and recorded among the tested patients who were submitted for serologic and molecular detection of the Hepatitis B virus. Pre-diagnosed liver caser patients, blood transfusion also was included in addition to their residency (Table 1).

TABLE 1: Distribution of the tested patients according to the different variables

Variable		Number (%)
Gender	Males	160 (53.33)
	Females	140 (46.67)
Smoking	Smokers	180 (60.00)
	Non-smokers	120 (40.00)
Age mean	Mean±SD	38.16±15.24
Clinical Symptoms	Fever	260 (86.67)
	Chills	249 (83.00)
	Abdominal pain	171 (57.00)
	Nausea	133 (44.33)
	Diarrhea	120 (10.00)
	Loss of appetite	110 (36.67)
	Jaundice	73 (24.33)
	Dark urine	35 (11.67)
	Fatigue	30 (10.00)
Liver cancer (HCC)	Yes	6 (2.00)
	No	294 (98.00)
Residency	Urban	130 (43.33)
	Rural	170 (56.67)
Blood transfusion	Yes	13 (4.33)
	No	287 (96.67)

Serologic tests by ELISA revealed that out of 300 tested patients, 92 (30.66%) were seropositive for HBsAg. The vast majority of positive results were among males (50 patients - 54.34%), whereas the percentage rates of seropositive cases were lower among females (42 patients - 45.66%). When the results were analyzed statistically, it was noticed that gender has a valuable effect on the seropositive results. The majority of the seropositive cases were smokers (67.4%). There was a significant difference between smokers and non-smokers ($P < 0.05$), whereas it has appeared that the marital status has no significant effects on the HBsAg seropositive results ($P > 0.05$).

Studying several risk factors for HBV transmission indicated that visiting dental clinics and dental surgery (927.17%), followed by dialysis (21.73%), were among the highest risk factors. Statistical analysis showed that dental visiting and dialysis significantly affect the seropositive results ($P < 0.05$). Although relatively a large number of the seropositive patients were within uncertain transmission routes, 39 patients out of 92 (42.4%). Finally, it has appeared that seropositive results were higher (58.7%) outside Sulaimani city center (rural) while the percentage was lower (41.3%) in the city center (urban). It was concluded that the patient's residency has valuable effects on the obtained results ($P < 0.05$) (Table 2).

Depending on HBV DNA detection by RT-PCR, it was noticed that out of the 92 seropositive patients, 53 (57.6%) were positive for HBV DNA (Table 3). Positive results

were higher among males (62.26%) when compared with females (37.74%). Similarly, DNA detection among smokers was relatively higher (64.15%) comparing to non-smokers (35.85%). The positive results among married patients were elevated (56.6%) if compared with single patients (43.4%). Half of the patients with liver cancer were seropositive for HBsAg, whereas two-third of them was HBV DNA positive. Moreover, patient's residency showed significant effects on the positive results where the percentage rates of positive results were higher among patients outside Sulaimani city center (Rural) (58.5%) comparing to those from the city center (41.5%) (Urban). Statistical analysis indicated that gender, smoking, marital status, and liver cancer have significant effects on HBV DNA detection results ($P < 0.05$) (Table 3). Studying the modes of transmission indicated that dental clinics and dental visiting have a significant effect on the positive results of RT-PCR ($P < 0.05$) and can be considered as an important risk factor for HBV transmission, followed by dialysis, which is also can be considered as a risk factor after dental clinic visiting (Table 3).

TABLE 2: Represents different risk factors for HBsAg seropositivity by ELISA

Variable		Number (%)	*P* value
Gender	Males	50 (54.34)	<0.05
	Females	42 (45.66)	
Smoking	Smoker	62 (67.4)	<0.05
	Non-smoker	30 (32.6)	
Marital status	Married	48 (52.17)	>0.05
	Single	44 (47.83)	
Liver Cancer	Yes	3 (50%)	>0.05
	No	3 (50%)	
Mode of Transmission	Dental visiting	25 (27.17)	<0.05
	Surgery	1 (1.88)	
	Dialysis	20 (21.73)	
	Blood transfusion	2 (2.16)	
	Sexual route	2 (2.16))	
	Animals modes	1 (1.08)	
	Barbershop	1 (1.08)	
	Familial history	1 (1.08)	
	Uncertain	39 (42.4)	
Residency	Urban	38 (41.3)	<0.05
	Rural	54 (58.7)	

TABLE 3: Studying risk factor for HBV DNA detection by RT-PCR

Variables	Explanation	Number (%)	*P* value
Gender	Male	33 (62.26)	<0.05
	Female	20 (37.74)	
Smoking	Smokers	34 (64.15))	<0.05
	Non-smokers	19 (35.85))	
Marital state	Married	30 (56.6)	<0.05
	Single	23 (43.4)	
Liver cancer	No	1 (33.33)	<0.05
	Yes	2 (66.67)	
Modes of transmission	Dental visiting	16 (30.18)	<0.05
	Dialysis	7 (13.2)	
	Surgery	2 (3.77)	
	Blood transfusion	2 (3.77)	
	Sexual	1 (1.88)	
	Animals	1 (1.88)	
	Barbershop	1 (1.88)	
	Familial	1 (1.88)	
	Unknown	22 (41.5)	
Residency	In City center	22 (41.5)	<0.05
	Outside the city center	31 (58.5)	

The positive results were relatively higher among males for HBsAg and DNA detection (54% and 62.26%), respectively, when compared to female patients who showed lower positive results (46% and 37.74%), respectively. It was noticed that relatively a large number of patients were seropositive for HBsAg while they were negative for HBV DNA detection by RT-PCR (Fig. 1).

4. DISCUSSION

Life-threatening infection by HBV stills a global cause of health concern. It was estimated worldwide that over 257 million people are under the risk of liver cirrhosis and HCC due to chronic hepatitis B virus (HBV) infection [12]. The high seropositivity rates of HBV infection among studied cases in the current study can be explained where all patients submitted to this research were with a chronic history of liver problems. All of them were previously referred by their specialist physicians to the gastroenterology center for checking and laboratory investigations. They were suspected of having hepatitis. Several studies and investigators reported a lower prevalence of HBV infections than our observation. In a previous study, it was reported that the prevalence of HBsAg seropositivity among a population of 345 tested cases was relatively lower [13]. In the current study, blood transfusion was considered as an important

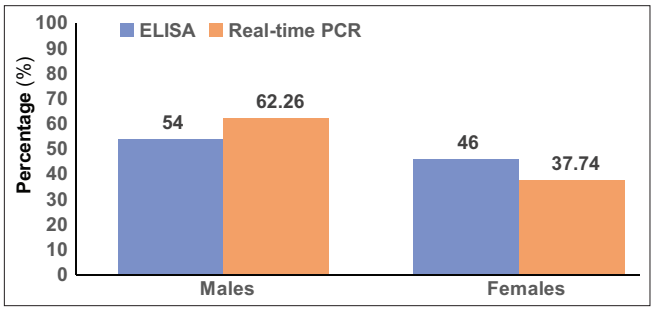

Fig. 1. Comparison between positive results of HBV using ELISA (seropositivity) and RT-PCR techniques.

risk factor for HBV transmission, which was also common among thalassemic patients. These observations were agreed with observation reported by others [14]. In another study done in Sulaimani, it was found that occupation, education level, history of jaundice, smoking, and alcohol drink had a significant effect on viral hepatitis infections, especially HCV infection [15]. Our observations were parallel with their conclusion considering some factors, including smoking. The results of the current study were different from the results reported by the Iranian research group who reported a relatively lower prevalence of HBV infection (7.4%) [16]. Moreover, our conclusions agreed with results reported in a study in Switzerland who reported relatively high HBV prevalence (32.4%) [17]. Ott *et al.* in 2012 found that the global HBeAg prevalence varied between 20 and 50% [18]. The current observations were in this range and agreed with their results. Similar to our results, a high prevalence of HBV infections (30.4%) were reported in Spain [19].

Studies done in Italy reported higher prevalence rates (52.7%) [20] than our observations. Moreover, studies done in Australia reported lower HBV prevalence in comparison to the current results, although it was relatively high compared to other related studies in other countries [21].

The high prevalence rates of HBV seropositivity among males in our study may be due to that they engaged in a range of parenteral (sharp objects sharing), especially in barbershops, which expose them to HBV infection. Our results were agreed with conclusions reported by other investigators in 2018 [9]. The risk of HBV infection among males was agreed with other epidemiological studies conducted in different areas among different groups and populations who observed that HBV infection is strongly associated with increased age among males [22], [23], [24], [25].

Results reported in the current study suggested that occupational transmission of HBV infections in dental settings, which sometimes found frequently, and high prevalence of HBV seropositivity among dental clinics and patients who referred to these clinics may be due to inadequate sterilization of the surgical tools used in dental clinics, and sharing some tools between the patients [26]. Similarly, inadequate sterilization and cleaning of materials and machines used in hemodialysis might be directly contributed to the prevalence of HBV infection among patients with hemodialysis.

The current study showed that not all HBsAg seropositive patients were positive for HBV DNA detection by RT-PCR, which was agreed with observations reported by a study done in Nairobi (Kenya) in 2017 by Mathai *et al.* [27]. They reported that the seropositive results of HBsAg and HBV DNA detection are totally different, and none of ELISA and RT-PCR cannot be alternative for each other [28]. Unlike conclusions of the current study, other researchers found that HBsAg detection and quantification by ELISA is more sensitive and gives more accurate results in detection of HBV infection, and they found that ELISA can be considered as an acceptable or adequate method in the diagnosis of HBV infection and HBsAg detection [29].

5. CONCLUSION

The percentage rates of HBsAg were relatively high (30.66%) among patients referred to the Gastroenterology center in Sulaimani who have suffered from liver complaints. More than half of the seropositive patients showed RT-PCR positive results for HBV nucleic acid detection. The gender, smoking, and marital status, and living places were significantly increased the incidence rate of HBV infection. Visiting dental clinics, dental surgery, and dialysis were among significant risk factors that facilitate the transmission of HBV infection.

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