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Prevalence of Occult Hepatitis B Virus Infection in Hemodialysis Patients Using Nested PCR

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Abstract

Background: Occult hepatitis B infection (OBI) is defined as the lack of detectable HBsAg in serum, despite the presence of intrahepatic viral DNA, and low levels of covalently closed circular DNA (cccDNA). Since the hemodialysis patients are at a greater disadvantage if they are a carrier of Hep B, as it can lead to OBI this study was designed to determine the prevalence of OBI in hemodialysis patients residing in Zanjan, Iran.

Methods: We conducted an anti-HBc test (ELISA) on 166 HBsAg negative hemodialysis patient samples. OBI was evaluated using seropositive (anti-HBc and/or anti-HBs) and seronegative (anti-HBc and anti-HBs) using nested PCR.

Results: Out of the total hemodialysis patients sampled, the study consisted of 58.4% male and 41.6% female participants. The age of the study group ranged from 58.89±15.49, and had received approximately 28.27±27.43 years of dialysis. Additionally, 5.4% of patients had a history of blood transfusions, while 58.4% were vaccinated against the hepatitis B virus (HBV). Moreover, 23.5% patients were anti-HBc positive, while 76.5% patients tested negative. Lastly, 66.3% of the patients were positive for anti-HBs, whereas 33.7% were negative for anti-HBs. Overall, the study revealed that the prevalence of OBI was 6%, and HBV DNA was detected in 2.1% of individuals who were vaccinated against hepatitis B ($p < 0.01$).

Conclusions: Though no significant difference between the prevalence of OBI to the patients' age, sex, duration of dialysis, or history of blood transfusion was identified, however, a strong correlation between the prevalence of OBI to HBV vaccination was found.

Keywords: ELISA, Hemodialysis, Nested PCR, Occult hepatitis B infection.

Introduction

Chronic hepatitis B infection is a major health concern that affects 400 million people worldwide, especially developing nations (2). Unfortunately, there is a higher risk of developing cirrhosis, hepatic insufficiency, and hepatocellular carcinoma (HCC) in hepatitis B carriers. Each year, it is estimated that 200,000 deaths result from cirrhosis alone, and 300,000 deaths are related to HCC worldwide (2). However, most hepatitis B virus carriers may not experience any of the above liver complications, in fact, majority of those individuals (15 to 40%) may be susceptible to liver disease (3). In Iran, specifically, the prevalence of hepatitis B is 2.14% (2.55% in men and 2.03% in women), and the rate of infection is estimated to be 1.5 to 2.5 million people (4). Furthermore, numerous studies demonstrate that the rate prevalence of hepatitis B infection can differ amongst the provinces of Iran (4, 5).

Though the viral load of the hepatitis B surface antigen (HBsAg) in dialysis patients remains low and stable over time, the prevalence of a hepatitis B virus (HBV) infection in dialysis centers in developing countries is between 2 and 20% (6). Interestingly, one of the leading causes of HBV in adult hemodialysis patients dates back to occult HBV infection (OBI) (7). OBI is described as the lack of HBsAg in serum, despite the presence of HBV DNA in the liver and peripheral blood

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mononuclear cells (PBMCs), or additional HBV antibodies and antigens (8). Though, anti-HBc and anti-HBs are not detectable during an OBI seronegative HBV infection, an OBI seropositive HBV infection may be scrutable by the presence of anti-HBc and anti-HBs (1). The underlying cause of OBI may be due to low viral replication, and the strength of the patient's immune response, other concomitant infections, or a mutation that affects the expression of viral antigens (9).

OBI is among the most common transmissible diseases, through transplantation and transfusion, that can cause cirrhosis and liver cancer (8). Specifically, hemodialysis patients are at a higher risk of OBI, not only from the large amount of blood received, injections and invasive procedures, but due to the suppression of the immune system as well (10). Most importantly, many of these patients will become exposed to HBV during their routine hospital visits. Various reasons could account for their increased risk, including the placement of hepatitis patients in hemodialysis units together with hemodialysis patients either short-term or long-term, exposure to contaminated blood products, sharing of hemodialysis equipment, skin damage, prior immunodeficiencies and vaccination status, which is considered to be less than 50% amongst hemodialysis patients in any single ward (11, 12). Hemodialysis patients require ongoing dialysis due to impaired or non-functional kidneys (13), putting them at an increased likelihood of contracting opportunistic HBV infections (12). According to the literature, the prevalence of OBI in dialysis patients varies between 0 and 36% (10). In a study conducted by Kivani et al., the prevalence of OBI in hemodialysis patients was reported to be 4.49% (14). The present investigation was conducted to estimate the prevalence of OBI and anti-HBc in hemodialysis patients, in hopes of improving the quality of life of these patients in hospitals of Zanjan, Iran.

Materials and methods

The study was conducted at the Vali-e-Asr Hospital in Zanjan, Iran on 166 HBsAg negative hemodialysis patients, between March of 2015 to September of 2016. This study was approved by the Ethical Committee of Zanjan University of Medical Sciences (No. ZUMS.REC.1395.83). We collected data, from patient records, including the patients' age, sex, duration of dialysis, history of vaccination, transfusion, and liver enzyme test results (ALT and AST). Then, we collected 10 ml of blood and transferred the vials to the technical department for centrifugation and serum separation. Anti-HBc assays were performed on samples that were HBsAg negative using an ELISA (Dia.pro, Italy). OBI was evaluated using seropositive (anti-HBc and anti-HBs) and seronegative (anti-HBc and anti-HBs) using Nested PCR. The current study also was approved by the Ethical Committee of Zanjan University of Medical Sciences NO. ZUMS.REC.1395.83.

DNA extraction

Patient DNA was extracted from 200 ul serum samples according to the manufacturer's manual (South Korea, Exgene Cell SV- mini), and stored at -20 °C for nested PCR.

Nested PCR

We used nested PCR to amplify the viral genome. Two pairs of internal and external primers were used to target the S-region of the HBV genome. The specific primer sequences used can be accessed in Table 1. The first reaction consisted of the following mixture: Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Denmark), 0.2 ul from external primers (Table 1), sterilized water and 2 ul from the extracted DNA sample. The final volume was 20 ul. The second reaction consisted of the following mixture: Taq DNA Polymerase 2x Master Mix RED (Ampliqon, Denmark), 0.2 ul from internal Primers (Table 1), sterilized water and 0.5 ul from the first reaction product. Both the first and second reactions were performed under the following conditions: initial denaturation at 94 °C for 5 minutes, 40 cycles at 94 °C for 30 seconds, 60 °C for 30 seconds, 72 °C for 30 seconds and the final incubation was performed at 72 °C for 5 minutes. For all steps, distilled water was used as a negative control, and patient DNA with chronic HBV was used as the positive control. The product from the second reaction was electrophoresed on 2% agarose gel and the results were analyzed using Gel Doc.

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Table 1. Primers used in Nested PCR.

	Primers	Sequence (5' → 3')	PCR product size (Pair)	Reference
External primers	S1-F	CATCAGGATTCCTAGGACCCCT	311	1
	S3-R	AGGACAAACGGGCAACATAC		
Internal primers	S2-F	CTTGTTGACAAGAATCCTCACA	227	1
	S4-R	CCAACAAGAAGATGAGGCATA		

Statistical analysis

Data obtained from the ELISA and nested PCR were analyzed using SPSS 16 software. Chi-square test was applied to determine the association between nominal data. We utilized the Fisher exact test, when the frequency of a cell in contingency (cross-tab) tables was zero. To compare the continuous data between two groups we applied the Student t-test if the related data had a normal distribution, otherwise we utilized Mann-Whitney test. Furthermore, ANOVA or Kruskal-Wallis tests were performed to compare the data among more than two groups, when the associated data had a normal or non-normal distribution, respectively. The significant level for all analyses was considered less than 0.05.

Results

After extracting critical data from the hospital's patient records, our analysis showed that 58.4% (97) of patients were male, while 41.6% (69) were female. We found that the mean age of our cohort, in years, was 58.89±15.49, and that prior to this study, this group had already undergone 28.27±27.43 years of dialysis treatment. In addition, 5.4% (9) of patients had blood infusion, and 58.4% (97) had been vaccinated against the hepatitis B virus (HBV). 39 (23.5%) patients were anti-HBc positive and 127 (76.5%) patients were anti-HBc negative. Moreover, 110 (66.3%) patients tested positive for anti-HBs and 56 (33.7%) patients were anti-HBs negative. According to the serological patterns against HBV, understudy patients were categorized into four subgroups including HBs Ab-/HBc Ab-, HBs Ab-/HBc Ab+, HBs Ab+/HBc Ab-, HBs Ab+/HBc Ab+. We, then, looked at the prevalence of these serologic markers and found that there was no significant correlation to the patients' age, sex, duration of dialysis, vaccination against HBV, and history of transfusion (Table 2). Interestingly, ten patients had OBI (Fig. 1, Table 3). We found that the prevalence of OBI in seronegative-OBI individuals were higher compared to seropositive-OBI individuals, and HBV DNA was not detected in HBc Ab+ individuals. The total number of anti-HBc+ and anti-HBs+ patients was 31, 29 of which, were HBV DNA negative, which relates back to their previous history of HBV infection. In our cohort study we observed 94.9% immunized patients against HBV (positive for anti-HBs and negative for HBV DNA) (Table 4). Although, there was no correlation between the prevalence of OBI to the patients' age, sex, duration of dialysis, or history of transfusion, there was a significant correlation between the prevalence of OBI and vaccination status of patients ($p< 0.011$). Overall, we found that the prevalence of OBI in patients who received the HBV vaccine was lower than those who did not receive the vaccine (Table 4).

Table 2. The descriptive results of HBV serologic patterns with duration of dialysis, history of blood transfusion and vaccination.

Variables		HBsAb-/ HBcAb-	HBsAb-/ HBcAb+	HBsAb+/ HBcAb-	HBsAb+/ HBcAb+	p< 0.05
Frequency of HBV serological patterns: n (%)		48 (28.9)	8 (4.8)	79 (47.6)	31 (18.7)	
Average age of patients (Means ± SD€)		61.29 ± 15.0	61.62 ± 6.88	57.93 ± 16.32	59.93 ± 15.67	* 0.883
Gender: n (%)	Male	29 (29.9%)	3 (3.1%)	49 (50.5%)	16 (16.5%)	£ 0.469
	Female	19 (27.5%)	5 (7.2%)	30 (43.5%)	15 (21.7%)	
Duration of dialysis (Means ± SD)		19.72 ± 16.67	44.50 ± 30.76	31.50 ± 29.21	29.09 ± 32.38	* 0.210
History of blood receiving: n (%)	Yes	2 (22.2%)	–	5 (55.6%)	2 (22.2%)	** 0.886
	No	46 (29.3%)	8 (5.1%)	74 (47.1%)	29 (18.5%)	
Vaccination: n (%)	Yes	29 (29.9%)	3 (3.1%)	50 (51.5%)	15 (15.5%)	£ o.850
	No	19 (27.5%)	5 (7.2%)	29 (42%)	16 (23.2%)	

* Kruskal-wallis test was used. £ Chi-square test was used.

** Fischer exact test was used.€ Standard deviation test was used.

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Table 3. Demographic information of negative and positive HBV DNA patients.

Variables		HBV DNA+	HBV DNA-	p< 0.05
Average age of patients (Means ± SD)		60.70±14.00	58.78±15.61	0.946
Gender: n (%)	Male	4 (4.1%)	93 (95.9%)	0.222
	Female	6 (8.7%)	63 (% 91/3)	
Duration of dialysis (Means ± SD)		30.80±22.26	28.11±27.78	0.387
History of blood receiving: n (%)	Yes	-	9 (100%)	0.435
	No	10 (6.4%)	147 (93.6%)	
HBV vaccination: n (%)	Yes	2 (2.1%)	95 (97.5%)	0.011
	No	8 (11.6%)	61 (88.4%)	

Table 4. Identification of HBV DNA among Negative HBsAg Hemodialysis Patients with diverse Serological Patterns of HBV.

Diverse Serological Patterns of HBV	Patients	Occult HBV DNA+	Occult HBV DNA-	p< 0.05
	n (%)	N (%)	n (%)	
HBs Ab-/HBc Ab-	48 (28.9)	4 (8.3)	44 (91.7)	0.857
HBs Ab-/HBc Ab+	8 (4.8)		8 (4.8)	
HBs Ab+/HBc Ab-	79 (47.6)	4 (5.1)	75 (94.9)	
HBs Ab+/HBc Ab+	31 (18.7)	2 (6.5)	29 (93.5)	
Total	166 (100)	10 (6)	156 (94)	

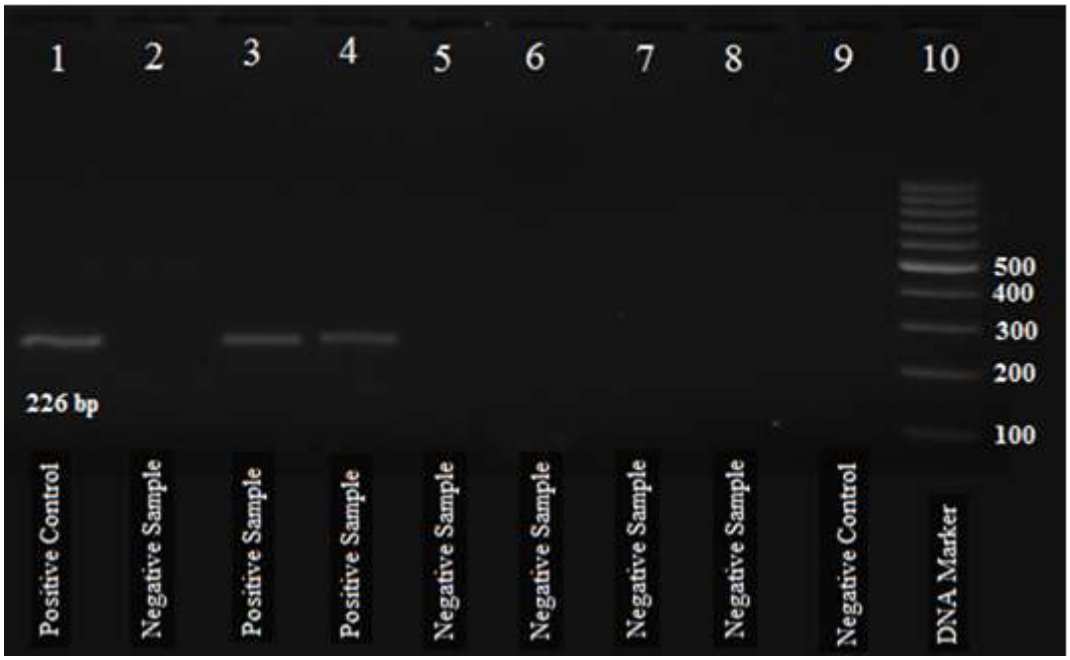

Fig. 1. Electrophoresis of Nested PCR product on agarose gel. Lane 1: positive control, lanes 2 to 8 samples, lane 9 is negative control, and lane 10 is DNA ladder. Samples 3 and 4 are positives show a 226-bp amplicon.

Discussion

OBI is characterized by the number of HBV DNA copies in the serum, in the absence of HBsAg, and regardless of the presence of other serological HBV markers, including anti-HBs and anti-HBc. Moreover, OBI patients are categorized as either seropositive-OBI or seronegative-OBI (15). The present study investigated seronegative and seropositivity of HBV markers in hemodialysis patients. Based on previous studies, 20% of individuals with OBI were seronegative and 80% were seropositive (15). In our study, only 20% of OBI cases were seropositive. Additionally, 6% of hemodialysis patients had OBI. Similar to our findings, Esmat et al. demonstrated that the prevalence of OBI in negative and positive HCV RNA cases were 6% and 8%, respectively (16). A North American study led by Minuk et al. reported the prevalence of OBI as 3.8% in seropositive cases (17). Similar to our observations, the prevalence of OBI was

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higher in seronegative patients. The difference in results may be related to the fact that North America is classified as a hypoendemic region, in regards to the prevalence of HBV (18). In contrast, Hashemi et al. reported the prevalence of OBI as 11% (19). This difference could be linked to the duration of dialysis, which according to their observations, was 47.46 ± 27.78, compared the duration of hemodialysis in our study, which was 28.27 ± 27.43. Further, the difference in prevalence (15) in their study could be related to the prevalence of HBsAg, which was reported to be between 7 and 16% (19). Similar to our study, the prevalence of OBI in seronegative individuals was higher than in seropositive individuals. Rastegarvand et al. found that 2.3% of hemodialysis patients had OBI (20). In another study led by Neisi et al, the prevalence of OBI was determined to be 4% (21). Ramazani et al. reported the prevalence of isolated anti-HBc as 2% and OBI as 1% and further identified OBI to be prevalent in isolated anti-HBc+ individuals (22). To account for this difference, in comparison to our study, we speculated that the type of population could play a profound role. Jardim et al. investigated the prevalence of OBI in HBsAg negative hemodialysis patients and the prevalence of anti-HBc+, anti-HBs+, and anti-HBc+ serologic markers, which was reported to be 0%, similar to our findings (23). Furthermore, Tabrizi et al. reported the prevalence of OBI as 0% among hemodialysis patients with a history of receiving the HBV vaccine, however, in our study, the prevalence of OBI in hemodialysis patients receiving the HBV vaccine was only 1.2% (24). The reason for this difference could be related to the efficacy of vaccines, the frequency and timing of vaccination. Since dialysis patients are typically older and the efficacy of vaccines and dialysis declines with age, the number of OBI cases were expected (25, 26). The results could also be influenced by the sensitivity and specificity of the methods used (22). In another investigation led by Fabrizi et al, the prevalence rate of OBI in positive anti-HBc samples was reported to be 0%, similar to the results of our study (27). Conversely, Zaki MS et al. reported the prevalence of OBI to be 18.8% in hemodialysis patients, which we found to be the greatest prevalence rate of HBV DNA in patients negative for serologic HBV markers (28). In this particular study, they also found a significant relationship between OBI and duration of dialysis, which, consequently, would increase the probability of OBI. Lastly, Fontenele et al. reported the prevalence rate of OBI to be 2.3% in hemodialysis patients, all of which, received four doses of the HBV vaccine (29). Furthermore, there was a significant correlation between the outcome of their study with our present study.

We found that the prevalence of OBI was 6% in patients undergoing hemodialysis. There was no significant relationship between the prevalence of OBI to the patients' age, sex, duration of dialysis, and history of blood transition. However, there was a significant relationship between vaccination and the prevalence of OBI. Regarding the prevalence of OBI in the dialysis center, we recommend that OBI be screened together with HBsAg hepatitis B in hemodialysis patients.

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The authors declare that they have no conflict of interest.

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