Alfarama Journal of Basic & Applied Sciences
Faculty of Science Port Said University
https://ajbas.journals.ekb.eg
ajbas@sci.psu.edu.eg
http://sci.psu.edu.eg/en/
ISSN 2682-275X
January 2022, Volume 3 Issue I
DOI: 10.21608/AJBAS.2021.94925.1065
Submitted: 09-09-2021
Accepted: 22-09-2021
Pages: 64-73

Occult Hepatitis B Virus Infection Among Patients Undergoing Chronic Dialysis in Hodeidah City, Yemen

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ABSTRACT

Hepatitis B virus (HBV) is a global major health concern. While Chronic HBV can lead to several severe liver diseases, occult HBV is capable of transmitting infection in hemodialysis units. Since there is lack of information on the pervasiveness of occult HBV in patients regularly receiving hemodialysis in Yemen, this study was designed to fill in this information gap. The study included 150 patients (9 to 75 years), receiving hemodialysis at regular basis. Sera samples were gathered ahead of the hemodialysis session, liver enzymes assessed using commercially available kits, and Hepatitis B surface antigen (HBsAg) as well as other serological markers of HBV measured using commercial enzyme-linked immunosorbent assay (ELISA) kits and rapid cards. HBsAg-negative samples were tested for HBV DNA using SYBR GREEN quantitative polymerase chain reaction (qPCR). All data were statistically analyzed. The prevalence of positive HBsAg was 42.67%, mostly represented by males. No significant relationship existed between the presence of HBsAg and the levels of liver enzymes. Anti-HBs and anti-HBc were detected in 96.5 and 80.2% of patients, respectively. SYBR green qPCR revealed that OBI was detected in ~78% of HBsAg-negative patients which is considered remarkably high. 79% of OBI patients were seropositive whereas only 3% were seronegative. OBI appears to be a significant health issue in hemodialysis patients in Hodeidah city, Yemen. To prevent OBI transmission, the screening of anti-HBc followed by HBV DNA detection in all patients undergoing hemodialysis should be applied. Moreover, hemodialysis patients negative for HBsAg are recommended to be vaccinated for HBV.

Key Words:
Hepatitis B virus; HBsAg; Hemodialysis; OBI; HBV DNA.

1. INTRODUCTION

Hepatitis B virus (HBV) is a crucial health complication around the world [1]. Currently, 250 million people remain infected with HBV, the causal agent of hepatitis B [2]. Chronic hepatitis B (CHB) is known to be a serious leading source of liver failure, cirrhosis, hepatocellular carcinoma (HCC), and overall mortality globally [3]. CHB virus infection is diagnosed by the detection of circulating hepatitis

B surface antigen (HBsAg) and hepatitis B core antibodies (anti-HBc) in the serum irrespective of the presence/absence of HBsAg antibodies (anti-HBs) [4].

There is an association between HBV infection and the risk of developing various kinds of chronic kidney diseases (CKD) [5]. In patients with CKD, HBV and hepatitis C virus (HCV) are the prevailing associated chronic diseases of liver. Hemodialysis patients, subjected to prolonged vascular exposure and regular blood transfusions, are more possible to become exposed to HBV infection [6]. Epidemiological studies showed that HBV infections in renal dialysis units vary greatly (1-20%) [7,8] due to several factors including health care measures such as utilizing contaminated devices, equipments and supplies, and attending staff.

Occult HBV infection (OBI) is recognized by detecting HBV-DNA when HBsAg is undetectable whether or not antibodies are present [9]. The geographical prevalence of OBI varies greatly according to the endemicity of the disease, the sensitivity of assays used and the characteristics of the population studied [10]. OBI has been previously reported in dialysis patients [11-14], where its prevalence is estimated at 0% to 58% [15-19]. Several studies suggested that it can be a starting point for viral transmission to employee and also patients within hemodialysis centers [18, 20].

The prevalence of HBV infection in Yemen was considered high (8-20%) before the introduction of universal infant vaccination against HBV [21]. However, previous studies reporting the commonness of occult HBV in cases undergoing hemodialysis are limited in Hodeidah city, if not in the whole country. Therefore, this study was planned to address this lack of information.

2. EXPERIMENTAL

2.1 Ethics approval:

The current study followed the ethical guidelines of the 1975 Declaration of Helsinki. Prior to commencing this study, the research plan received an institutional approval by Postgraduate studies and research board, Damietta University (No. 4.3.2.1) and all participants handed in an informed written consent.

2.2 Materials and methods:

This cross-sectional study involved 150 patients (104 males and 46 females "mean age=39.06 ±14.09 years; age range=9 to 75 years) undergoing chronic hemodialysis in a dialysis center, Hodiedah city, West Yemen. The sample size has been estimated using NCSS & PASS program by the Biomedical Information and Medical Statics Department, Medical Research Institute, Alexandria University. Inclusion basis involved end-stage renal disease on steady dialysis (at least for one-year). Patients infected with HCV were not involved in this study. HBV and/or HCV infected patients undergo hemodialysis on separate machines from those used by uninfected patients and also, hemodialyzers are regularly disinfected.

Sera samples were collected, aliquoted, and stored at -80^{o}C just before the dialysis sessions. All samples were tested for HBsAg using a commercially accessible ELISA kit following the manufacture's instruction, for anti-HBc by an automated ELISA (cobas-e 411 analyzer) method using the commercially available kits (Roche Diagnostics, Germany) and for anti-HBs by BioTracer Anti-HBs Rapid Card (BioTracer, BIO FOCUS Co., Ltd. Korea). The activity of some liver enzymes (Alanine aminotransferase (ALT), Aspartate aminotransferase (AST), and Alkaline phosphatase (ALP)) in sera was assessed by readily available kits following the manufacturer's protocol. HBsAg-negative samples were SYBR GREEN qPCR tested for HBV-DNA using primers targeting the *S* and *C* genes as shown in Table 1 [4, 22].

HBV-DNA was purified using Thermo Scientific Gene JET Whole Blood Genomic DNA Purification Mini Kit (Gene JET, Waltham, Massachusetts, USA) following the manufacturer's instructions. The purified DNA purity and concentration were measured by multiskan™ GO microplate spectrophotometer, and DNA tested for HBV using an automated qPCR System (Cepheid Smart Cycler II Thermal Cycler, Boston Industries) as directed by the manufacturer.

HBV-DNA was amplified according to the thermal profile shown in Table 1. A melting curve analysis was performed to indicate the specificity and purity of the amplicons. The melting curve analysis profile was 95°C (60 sec), 55°C (30 sec), and 95°C (15 sec). A positive-HBV DNA sample was included as a positive control as well as a no-template-control (NTC) sample as a negative control to test for contaminated reagents or primer-dimers formation. Samples with undetectable HBsAg and detected DNA in the presence/absence of anti-HBc or anti-HBs were considered OBI positive.

Data were analyzed using SPSS (V21). Chi-square test was used to check the association between categorical variables. Continuous variables were presented as mean ± SD (standard deviation).

Table 1: Target genes, nucleotide position, sequences of primers and thermal profile used in SYBR green qPCR.

Genes	Primer pairs	Primer sequence (5´……..3´)	Primer positio	References
S	S-1s	AGAACATCGCATCAGGACTC	159-178	[4,22]
	S-2a	CATAGGTATCTTGCGAAAGC	642-623	
C	C1s	CTGGGAGGAGTTGGGGGA	1730-1747	
	C2a	GTAGAAGAATAAAGCCC	2503-2487	

Steps	Thermal profile		
	Temperature	Duration	Number of cycles
Activation	95°C	10 minutes	1
Denaturation	95°C	15 seconds	40
Annealing	60°C	30 seconds	
Elongation	72°C	30 seconds	

3. RESULTS

The current cross-sectional study was performed on 150 hemodialysis cases, mostly found in the 30-50 age group (40.7%). The general prevalence of positive HBsAg was 42.67% (64 patients, 44 males (68.8%) and 20 females (31.2%)), most of which (50%) are found in the <30 years age group and then 30-50 years group (34.4%). There is a statistically notable difference between age regarding to the presence of HBsAg (P<0.05) (Table 2).

Table 2: Relationships between the presence of detectable HBsAg & HBV-DNA and other factors among hemodialysis patients based on Pearson Chi-Square test.

Variables	Value of Pearson Chi-Square		*P-value*	
	HBsAg	HBV-DNA	HBsAg	HBV-DNA
Presence of liver disease	0.103	0.748	0.472	0.492
Treatment of liver diseases	0.045	0.833	0.287	0.592
Duration of dialysis	0.123	0.941	0.827	0.661
Education status	1.686	0.194	0.788	0.375
Habitat	0.595	0.440	0.090	0.765
Gender	0.018	0.894	3.395	0.065
Age group	15.665	<0.001	9.829	0.007

P-value >0.05; Non significant

Among the 64 HBsAg-positive patients, values of ALT and AST remained near the lower limit of their normal value ranges in 57 and 50 patients, respectively, whereas the level of ALP was elevated in 48 patients. No significant statistical differences were found for the presence of HBsAg and the levels of liver function enzymes probably because the number of cases was not high enough.

To find out the commonness of occult HBV, 86 serum samples in which HBsAg was reported negative were checked for HBV antibodies and DNA using ELISA and SYBR Green qPCR assays, respectively. Of the 86 samples, 83 (96.5%), 69 (80.2%), and 67 (77.9%) samples were positive for anti-HBs, anti-HBc, and HBV-DNA, respectively. Values of cycle threshold (Ct) ranged from 27 to 39 indicating low virus load (Table 3). The frequency of various combinations of HBV markers is presented

in Table 4.

The 67 patients in which occult HBV infection was detected included 44 males and 23 females, of which 53 (79%) and 65 (97%) patients were anti-HBc and anti-HBs positive, respectively. There is a statistically notable correlation between the presence of DNA and anti-HBcAg whereas no significant differences were found between the levels of liver function enzymes and the presence of anti-HBsAg regarding to the presence of HBV DNA (Tables 3 and 5). A wide range of ages comprised of children, adults, and elderly patients, were included to represent the general population and enable a more accurate assessment of OBI. The majority of occult HBV infections were found in the 30-50 years group and >50 years group. The relationship between age groups and occult HBV infection was significant (P<0.05) (Table 2).

The *S* and *C* genes were both detected in 4/67 (6%) patients, while *C* gene alone found in 63/67 (94%) patients. In the current study, anti-HBc and anti-HBs were detected at frequencies of 86 and 55.3%, respectively. Among the 67 OBI diagnosed patients, 53 (79%) and 2 (3%) patients were seropositive and seronegative, respectively.

4. DISCUSSION

HBV infection in hemodialysis patients, with debilitated immune systems due to renal anemia, long-term inflammation, nutritional insufficiency, and further conditions, remains a major issue [1] as they are more susceptible to become chronic HBV carriers [7]. The endemicity of chronic HBV is divided into three levels; high (>8%), intermediate (=2-8%) and low (<2%). Different countries, regions, renal dialysis centers have different distribution levels of blood-borne viral diseases including HBV [23]. Previous studies have recorded HBV prevalence in hemodialysis patients of 10% in Saudi Arabia, 11.8% in Bahrain [24], 8.1% in Gaza strip [23], 5.9% in Jordan [7], 1.5% in India [25], 8.7% in Argentina [26], 2.2% in Japan and 2.4% in the USA [27].

The prevalence of HBV in dialysis centers in different regions of Yemen is variable. The experimental results reported that the endemicity of HBV infection in Yemen is remarkably high which is in consistence with a previous study, carried out on patients in a dialysis center in Zabeed city, that reported a prevalence of HBV of 48.83%, mostly represented by males (55.41%) [28]. A study in Hodiedah city reported a prevalence of HBV of only 3.60% (70% of them were males) [29]. Also, another previous study in Beit Al-Faqih, Hodeidah governorate reported that the prevalence of HBV infections is high among adult males [30]. Higher HBV Infection in males than females has been previously observed and may be due to differences in their activities. For example, the percentage of employed males is higher than females; males constantly visit barbershops and also are more involved in blood transfusion practices. Whereas, females are mostly involved in household activates based on social, cultural, and religious preferences. This probably indicates that the small scale results might not be accurate in reflecting the overall prevalence within the whole community. Hemodialysis patients require frequent blood transfusions and they are at high risk of acquiring HBV due to several factors including the use of the same machines for all patients regardless of their HBV status and the possibility for the injectable medications to become contaminated while being prepared in dialysis rooms [31].

Table 3: Detection of HBV markers in serum samples and qPCR assay of OBI in 86 HBsAg-negative samples.

ID	Anti-HBcAg	Anti-HBsAg	SYBR GREEN qPCR			ID	Anti-HBcAg	Anti-HBsAg	SYBR GREEN qPCR		
			DNA	Ct mean	Ct SD*				DNA	Ct mean	Ct SD*
Male Samples						**100**	+	+	+	38.16	0.28
1	+	+	+	31.70	0.44	**101**	+	+	+	32.43	0.22
3	+	+	+	31.16	0.12	**102**	+	+	+	31.55	0.27
4	-	+	+	30.77	0.41	**103**	+	+	-	NA	NA
8	+	+	-	NA	NA	**105**	-	+	+	37.15	0.88
12	+	+	+	31.09	0.13	**106**	+	-	-	NA	NA
21	+	+	+	38.56	0.57	**107**	+	+	+	35.40	0.24
24	+	+	+	36.20	0.80	**108**	-	+	+	30.31	0.36
26	+	+	+	36.46	0.29	**112**	+	+	+	37.01	0.41
30	+	+	-	NA	NA	**115**	+	+	-	NA	NA
31	+	+	+	37.96	0.52	**123**	-	+	+	32.48	0.35
35	+	+	+	36.14	0.65	**129**	+	+	+	32.27	0.20
37	+	+	+	27.02	0.66	**134**	+	+	+	34.27	0.16
38	-	+	-	NA	NA	**136**	+	+	+	29.59	0.31
39	+	+	+	27.31	1.03	**137**	-	+	+	31.58	0.30
40	+	+	+	27.50	0.86	**138**	+	+	+	32.47	0.33
42	+	+	+	26.34	0.14	**141**	+	+	+	31.44	0.22
45	+	+	+	28.33	0.23	**Female Samples**					
46	+	+	+	29.44	0.35	**9**	+	+	+	31.06	0.21
47	+	+	+	29.74	0.12	**10**	+	+	-	NA	NA
51	-	+	+	32.44	0.44	**17**	+	+	+	37.05	0.88
52	+	+	+	30.23	0.14	**18**	+	+	+	38.17	0.92
55	+	+	+	29.15	0.22	**22**	+	+	-	NA	NA
57	-	+	-	NA	NA	**28**	+	+	+	39.28	0.90
59	+	+	-	NA	NA	**29**	+	+	+	34.00	0.49
60	+	+	+	28.62	0.38	**34**	+	+	+	32.65	0.36
63	-	+	+	32.29	0.42	**44**	-	+	+	28.47	0.16
64	+	+	+	32.55	0.39	**49**	+	+	-	NA	NA
66	+	+	+	31.52	0.10	**61**	-	+	+	28.91	0.36
68	-	+	-	NA	NA	**67**	+	+	+	31.04	0.22
73	-	+	+	34.43	0.19	**70**	+	+	+	30.76	0.12
75	+	+	+	31.35	0.22	**72**	-	+	-	NA	NA
76	+	+	+	36.11	0.80	**89**	+	+	+	27.60	0.41
77	+	+	+	29.11	0.32	**99**	+	+	-	NA	NA
78	+	+	+	30.43	0.30	**113**	+	+	+	38.53	0.33
80	+	-	-	NA	NA	**116**	+	+	-	NA	NA
81	+	+	+	28.17	0.22	**117**	+	+	+	36.49	0.32
83	+	+	+	36.43	0.78	**125**	+	-	-	NA	NA
84	-	+	+	30.35	0.29	**128**	+	+	-	NA	NA
87	+	+	+	31.55	0.15	**132**	+	+	+	36.25	0.21
92	+	+	+	28.60	0.12	**135**	-	+	-	NA	NA
93	+	+	+	30.64	0.21	**140**	-	+	+	31.01	0.60
96	+	+	+	32.69	0.23	**142**	+	+	+	36.63	0.16
98	+	+	+	34.46	0.18	**143**	+	+	+	30.95	0.71

+: Present. -: Absent. NA: Not Applicable. Ct: threshold cycle. SD*: Standard deviation

Table 4: Frequencies of various combinations of HBV markers and their potential indication.

No. of samples	Presence of Anti-HBs	Presence of Anti-HBc	Presence of HBV DNA	Possible indication
12	+	-	+	Occult HBV
53	+	+	+	Occult HBV
2	-	-	+	Occult HBV
5	+	-	-	HBV immunization
14	+	+	-	Recovered

+: Present, -: Absent

Table 5: The results of Pearson Chi-Square test to indicate the relationships between occult HBV infection (HBV-DNA presence) and HBV markers and liver function enzymes.

Variables	Value of Pearson Chi-Square	*P-value*
Anti-HBcAg	0.659	0.417
Anti-HBsAg	10.961	<0.001
ALT	1.829	0.176
AST	2.851	0.091
ALP	0.106	0.744

P-value >0.05; Non significant

Biochemical liver tests, such as levels of serum transaminase, are markers for liver damage and viral hepatitis is considered a leading cause of their elevation [32,33]. However, values of transaminase are lower in cases with chronic renal failure (CRF) whether or not they are dialysis-dependent [34]. This is attributed to several reasons among which is hemodilution, insufficiency in many cofactors in CKD patients such as, vitamin B6, pyridoxine, NADPH, and the presence of uremic factors that inhibit transaminase activity [35,36].

In patients with immune tolerant phase of HBV infection, ALT levels are normal, although these patients have high viral load [37]. Previous studies indicated that serum transaminase levels in hemodialysis patients are reduced during dialysis, whereas levels of ALP may increase. Moreover, in CKD patients, other conditions such as renal osteodystrophy could eventually contribute to high serum ALP levels [38]. Therefore, the diagnosis of HBV-related liver damage in these patients based on biochemical liver markers can be unreliable [39].

It appears that there is a close connection between the prevalence of OBI and the overall of HBV infection in the general population [40]. OBI reactivation is possible, leading to acute and severe forms of hepatitis B. The persistence of HBV in the liver for long periods may benefit the advancement of the chronic liver disease to cirrhosis and HCC [41]. OBI can cause the transmission of the infection through hemodialysis, organ transplantation, or blood transfusion with reactivation of HBV when an immunosuppressive status occurs [42,43].

In OBI patients, HBsAg is indetectable in serum because of several reasons, including: (i) it has been cleared to undetectable levels [44], (ii) the presence of immune complexes in which HBsAg may be hidden [12,45], and (iii) mutations in the viral *S* gene, that leads to the formation of escape mutants which become undetected by the immune system [46,47].

The detection of HBV DNA is regarded the most decisive diagnostic method for detecting OBI. In this study, S and C regions of the HBV genome were selected as targets for amplification, by SYBR Green qPCR, as they are known to be more sensitive in DNA detection in serum [48]. In accordance with the current study's results, a previous study in a dialysis center of Hodiedah city showed predominance of HBV in the 41- 60 age followed by the 21-40 age group [29]. The frequency of detecting the *C* gene was higher than that of *S* gene indicating the possibility to find *S* gene mutations in most cases. The HBsAg has a dominant neutralization epitope called "a determinant" in the major hydrophilic region (MHR) of

the *S* gene. Variations in such region are responsible for the generation of vaccine-escape variants and persistent infection [49-52]. Other reports indicated the possibility to find higher mutation rates in the HBV *C* gene such as the study by Helaly et al [4] who reported the presence of *S* and *C* genes in 19/48 and 13/48 samples, respectively.

The prevalence of occult HBV infections in dialysis centers ranges from 0% to 72% [15-19,53]. The high prevalence of OBI in this study is comparable with that reported in hemodialysis patients in Theodor Bilharz Research Institute (TBRI) Giza, Egypt (71.9%) [53]. Other reports regarding the prevalence of OBI are controversial as it was reported to be 3.1% in Iran [54], 0% in Korea [55] and Italy [17], 58% in Spain [56], 0.9% in central Greece [57], 3.8% in Canada [18], 0-16.9% in Turkey [58,59], 1.5% in Brazil [60], and 4.1% [61] and 32% in Egypt [4]. This quite variation in OBI prevalence is probably attributed to several factors such as variable sample size, demographic information, immunity of patients, endemicity of HBV, viral DNA loads in the blood, conditions of sampling and the type of diagnostic tools used [40].

Blood that is free of HBsAg yet has a high concentration of anti-HBc and no anti-HBs has also the ability to transmit HBV [62]. Anti-HBc is the earliest immune response to HBV, and its presence in the serum could signify acute/chronic infection. Anti-HBc remains detectable after recovery, and therefore it serves as the only serological diagnosis sign for HBV in the window period of infection. Anti-HBc is one of the most helpful serological markers for OBI diagnosis [63] and has been suggested as a screening marker for OBI when viral DNA detection techniques are hard to use [48]. The presence of anti-HBc in HBsAg-negative samples is considered an indication of past exposure to HBV and resolved infection. Although the detection of anti-HBc in serum is important for OBI tracking, about it was not detected in nearly 20% of OBI infections [64].

Detection of anti-HBc and anti-HBs in this study may indicate recovery from past HBV infections [48] or OBI. Previous studies reported similar results in which (i) anti-HBc was found in 44.6% of hemodialysis patients in Iran [65], (ii) anti-HBc, anti-HBs, and HBV DNA were reported in 49.1, 52.1 and 2% of HBsAg-negative patients in Suez Canal, Egypt [66] and (iii) anti-HBc and anti-HBs were detected in 18.9 and 12.2% of HBsAg-negative hemodialysis patients in Al-Gharbia governorate, Egypt, respectively [67]. The findings of the current study are in fulfilment of previous findings that OBI is more common in seropositive cases with positive anti-HBc and/or anti-HBs [61,65,68]. Darmawan et al reported various mutants in HBsAg-positive and also in HBsAg-negative patients, mostly in samples with high concentration of anti-HBs, recommending that blood containing anti-HBs and anti-HBc should not be considered as noninfectious [69].

5. CONCLUSION

The prevalence of HBsAg and OBI in patients going through hemodialysis were 42.67 and 78%, respectively, and appear to be remarkably high. Therefore, OBI is a major health issue in hemodialysis patients in Yemen. To prevent the spreading of OBI, screening of anti-HBc followed by HBV DNA should be applied for all dialysis patients. The vaccination of HBV should be applied for dialysis patients negative for HBsAg

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