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Occult Hepatitis B Virus Infection: A Killer Hidden in Transfused Blood, Sudan

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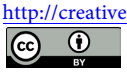

Abstract

Introduction: Transmission of hepatitis B virus (HBV) through the blood is a significant risk, especially in poor countries with high endemicity. Occult transmission of HBV (OBI) is an important acquisition scenario. **Objective:** A cross-sectional laboratory-based study followed to detect OBI in blood of accepted donors admitted to Mad Medani Blood Bank, Sudan. **Methods:** During the study, 200 accepted blood units were examined for HBsAg by ELISA technique and negative samples were tested for total anti-HBc antibodies using electroluminescence immunoassay (ECLIAS). HBV DNA amplification was performed for units that showed total anti-Hbc positivity. **Results:** Of the 200 blood units, 3 appeared positive by ELISA. Total anti-HBc antibodies were present in 34% (67/197) of blood units. HBV DNA was successfully amplified in 52.2% (35/67) of total anti-HBc positive samples. A significant association was observed between reactive total anti-HBc and age group ($p < 0.001$), marital status ($p < 0.001$) and HBV vaccination (p-value 0.012) of blood donors. **Conclusion:** OBI was recorded at a high rate in the blood of donors, which necessitates the implementation of detection methods to protect the recipients.

Keywords

OBI, Blood Donors, Nested-PCR, Sudan

1. Introduction

Hepatitis B virus (HBV) remains one of the major blood-borne pathogen contribute to liver health world-wide. Several methods of transmission of HBV to

humans have been indicated, including through blood transfusion [1]. However, the acquisition of hepatitis B virus can be prevented by donating blood, and therefore the safety of transfused blood has improved since the early 1970s, after the introduction of serological detection to screen the virus in the blood, e.g. hepatitis B surface antigens (HBsAg) [2]. It is important to know the results of other serological tests to know the immune conditions in which HBsAg test is negative; such as natural infection and vaccination, these markers include hepatitis B surface antibody (anti-HBs) and total hepatitis B core antibody (anti-HBc) [3]. A serious pathological condition related to hepatitis B virus infection is the phenomenon of hepatitis B virus inside the body in its mysterious infectious form known as OBI [4], and the possible cause is due to several factors related to the virus and the susceptible host [5]. Furthermore, OBI represents a clinical category of hepatitis B virus infection associated with the presence (seropositive) or absence (seronegative) of hepatitis B surface antibodies which are the major infection-associated antibodies [6].

In practice, HBV-DNA has been detected in small quantities in the liver and serum of individuals, although HbsAg is not present with the available techniques [7]. Developing countries and Africa are more susceptible to infection with OBI, with high rates of seroprevalence of HBV [8], and it should be noted that factors that enhance the spread of the virus in countries such as those in sub-Saharan Africa are the endemicity and the performance of serological screening methods [9].

Although blood transfused saves people's lives, the risks of transmitting diseases through it require further study to improve protective measures, in this context, hepatitis B virus stability has led to environmental transmission and integrity of DNA in blood, serum, and surgical devices [10] [11]. Some studies have indicated the prevalence rates of OBI in donor's blood and have recommended laboratory testing of viral DNA as part of routine work [12] [13], on this occasion, this recommendation is difficult to achieve in poor countries due to the lack of logistical capabilities. Therefore, transmission of the virus in developing countries is a real risk [13] [14] [15].

Little is known about the status of occult hepatitis B infection in Sudan, however, a recent study in Gezira State highlighted to concern about the frequency of OBI among patients with chronic kidney diseases [16]. We can assume that a considerable proportion of blood donors are OBI carriers, because the screening tests used are only directed against HbsAg. The goal of this study was to detect the rate of OBI among blood donors attending Wad Medani Blood Bank in Gezira State, using serological and molecular methods.

2. Methods

2.1. Ethics

This study was approved by two bodies; Ministry of Health, Gezira State and Faculty of Medical Laboratory Sciences, University of Gezira. Participants were informed by research objectives.

2.2. Study Design and Settings

Cross-sectional laboratory based study was followed in the central blood bank of Gezira State in Sudan during 2017 to 2019. According to the required characteristics purposive sampling was followed, two hundred blood volunteers, met the criteria for blood transfusion, attending the blood bank with negative results for rabid screening test for HBsAg, HCV and HIV during study peroide were participated. Risk factors for HBV infection were recorded for each participant; marital status, history of blood transfusion, alcohol intake, vaccination, history of surgical operation and history of jaundice.

2.3. Sampling

Five ml of venous blood were obtained from each volunteer after alcoholic sterilization of skin, samples were transferred in containers with EDTA anticoagulant separated plasma specimens were kept at −20˚C until used.

2.4. Serological and Molecular Tests

Hepatitis B virus was screened for HBsAg and total anti-HBc. HBsAg which indicates active cases was examined by sandwich enzyme linked immune sorbent assay (ELISA), a micro-plate reader (Awareness Technology, Model: 303 PLUS, USA) used for absorbance measurement at primary wavelength 450 nm, and reference filter at 630 nm. The results were calculated and evaluated using cut-off value.

Total hepatitis B core antibodies which indicates acute and recovered cases were quantitatively tested using electrochemiluminescence immunoassay (ECLIAS) technique, the machine used was fully automated cobase 411 (Hitachi High-Technology Corporation, Japan).

OBI was identified by DNA testing; extraction was accomplished by Phenol chloroform isoamyl alcohol (25:24:1). Steps were: 250 µL of plasma sample was mixed with 100 µL of 5% sodium dodecyl sulphate (SDS) and 20 µL of proteinase K. The mixture was incubated at two different temperatures first at 56˚C for 1 hour followed by 10 minutes at 95˚C. After incubation phenol chloroform isoamyl alcohol (25:24:1) were added as 500 µL. The mixture was centrifuged, and the upper layer of was put into a new eppendorf tube. 9% NaCl and cold absolute ethanol were added as 20 µL and 1000 µL respectively. The mixture was incubated for 24 hours at −20˚C. Next, the mixture was centrifuged and supernatants were discharged to yield DNA pellet. Tubes were left at room temperature to dry and 200 µL of 70% ethanol was added. The tubes were vigorously shaken until the pellet disappeared. The tubes were centrifuged and the supernatants were discharged. Lastly tubes were facedown open for 2 hours after that re-suspend in 50 µL of sterile distilled water. The extracted DNA was preserved at −20˚C.

Nested PCR done to detect hepatitis B virus DNA using primer pairs (**Table 1**) (Macrogen, Seoul Korea). Commercial PCR master mix was used (iNTRON

Table 1. Sequences of used primers for HBV DNA.

Forward/reverse	5' - 3'
Nested forward	GTTGCCCGTTTGTCCTCTAA
Nested reverse	AAGCCCTACGAACCACTGAA

biotechnology Soul, Korea) contained 5 µL of 10× PCR buffer, Taq DNA polymerase, MgCL and 10 mMd NTPs, 2 µl of DNA was added, followed by 1 µL of each 10 P mol/ml forward and reverse primer, deionized sterile water was added to complete the volume to 20 µL.

PCR machine (9700 thermocycler, Singapore) used to amplify DNA, the PCR program started with a first denaturation step 95˚C for 5 minutes; followed by 30 cycles at 95˚C for 1 minutes, 56˚C for 1 minute, 72˚C for 2 minutes, and a final extension at 72˚C for 5 minutes. Yielded amplicons were visualized in 1.5% agarose using Cleaver Scientific Ltd. gel documentation system (Model: OMNIDOC). Length of target amplified region showed 250 bp.

2.5. Data Collection and Analysis

Primary data obtained were the results of laboratory investigations. Secondary data of participants; socio-clinical information and risk factors were collected from blood bank records using a questionnaire. Statistical analysis was performed by SPSS version 20 computer program, and presented as a tabular and graph. The significance of the association between variables was measured by Chi-square test, and a p value of less than 0.05 was considered significant.

3. Results

The registered blood donors were males between 17 and 41 years of age with a mean of 26 years. For mandatory screening against HIV, HBsAg antigen, HCV, and VDRL, all participants showed negative rapid immunochromatography tests. The confirmatory ELISA method for HBsAg showed that 15% (3/200) was positive. Total anti-HBc antibodies were examined for 197 samples that gave negative ELISA, 34% (67/197) were reactive (Table 2), the 67 blood samples were subjected to HBV DNA extraction, successful amplification was achieved in 52.2% (35/67), and the overall percentage was 16.2% (32/197) (Figure 1) (Table 3). Number of samples positive for both Total anti-HBc antibodies and HBV DNA was 47.8% (32/67). Significant association was observed between reactive total anti-HBc antibodies and age group ($p < 0.001$), marital status ($p < 0.001$) and HBV vaccination (p-value 0.012) of blood donors (Table 2). No association was observed between socio-demographics of study subject and OBI (Table 3).

4. Discussion

Although blood is a vehicle for the transmission of many viral, bacterial and parasitic diseases, mandatory screening tests for blood transfusion in Sudan are

Table 2. Socio-demographics association and distribution of HBV anti-HBc antibodies in blood donors. No. 197.

Socio-demographic		Anti-HBc positive	Anti-HBc negative	Total	P-value
Age group	17 - 25 yrs	17	95	112	<0.001
	26 - 35 yrs	45	30	75	
	36 - 41 yrs	5	5	10	
	Total	67	130	197	
Marital status	Yes	39	111	150	<0.001
	No	28	19	47	
	Total	67	130	197	
History of blood transfusion	Yes	2	1	3	0.229
	No	65	129	194	
	Total	67	130	197	
Alcohol intake	Yes	5	7	12	0.563
	No	62	123	185	
	Total	67	130	197	
Vaccination	Yes	5	28	33	0.012
	No	62	102	164	
	Total	67	130	197	
History of surgical operation	Yes	3	3	6	0.401
	No	64	127	191	
	Total	67	130	197	
History of jaundice	Yes	2	1	3	0.229
	No	65	129	194	
	Total	67	130	197	

Table 3. Socio-demographics association and distribution of HBV DNA in blood donors. No. 67.

Risk factor	Yes/No	HBV-DND Positive	HBV-DND Negative	Total	P-value
Marital status	Yes	16	23	39	0.193
	No	16	12	28	
	Total	32	33	67	
History of blood transfusion	Yes	0	2	2	0.170
	No	32	33	65	
	Total	32	35	67	

Continued

Alcohol intake	Yes	2	3	5	
	No	30	32	62	0.718
	Total	32	35	67	
Vaccination	Yes	1	31	32	
	No	4	31	35	0.169
	Total	5	32	67	
History of surgical operation	Yes	0	3	3	
	No	32	32	64	0.090
	Total	32	35	67	
History of jaundice	Yes	0	2	2	
	No	32	33	65	0.170
	Total	32	35	67	

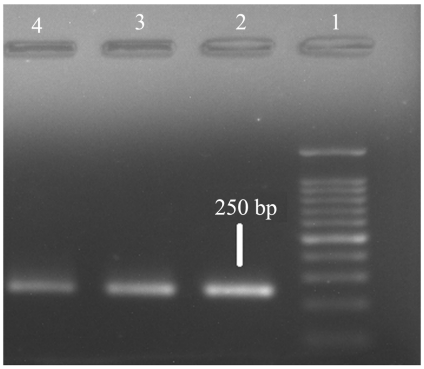

Figure 1. Nested-PCR amplification of HBV DNA on 1.5% agarose gel electrophoresis. Lane 1, DNA ladder. Lane 2, 3 and 4 showing typical band size of (250 bp) corresponding to the molecular size of amplified region.

limited to HBV, HCV, VDRL and HIV. And the reliability of the tests used in the detection of the above-mentioned diseases is not sufficient and does not meet the required measures for blood safety. One of the measures that has increased the safety of transfused blood in developed world, such as many European countries and the United States of America, is the comprehensive screening of HBV, including implementation of DNA amplification approaches [17], and not limited to the detection of HBsAg, as is followed in developing countries [18].

The spread of HBV infection in Sudan has been indicated by many references based on the sero-positivity [19] [20] which could be an indicator for the hypothesis of the existence of other forms of the disease, such as endemicity and OBI. The OBI rate among blood donors in this study by nested PCR represents the first documentation in the study area, which is lower than the rate recorded in 2013 in Khartoum State, Sudan for blood donors which amounted to 38% using

a more sensitive technique; R.T PCR [21]. But, more than the frequency revealed in the Sudanese border area of Sudan in Southern Darfur State during 2017 which reached 6% by the nested PCR method [22]. The subsequent increase is attributed to the prevalence of hepatitis B virus infection in Gezira State [23], as well as the disparity in population.

From this study, donors who showed total core antibodies positive to hepatitis B virus and, negative for HBV DNA and HBsAg did not present a transmission risk, and this was confirmed by similar studies [3] [24]. The significant association documented from the current study between total anti-HBc antibodies and HBV vaccination is a good predictor for HBV exposure, therefore, 92.5% (62/67) of donors with reactive total anti-HBc were not vaccinated. This finding is consistent with studies that recommended the inclusion of total anti-HBc test to examine [25] of the immune response after vaccine administration [26].

The prevalence of total anti-HBc is known to increase with age, and this has been observed in both endemic and blood donor populations [25] [27], in line, the current study found that the younger age group—which is the most frequent—showed a lower positivity rate with percentage of 15.2% (17/112) than the older age groups. From this result we can clarify that OBI screening in young individuals does not reflect the recent state of the disease in the community. In this study the high prevalence of OBI in the targeted population, which was 16% (32/197), must be taken into account from several aspects. Firstly, it is an indicator of the occurrence of OBI in the various components of the Sudanese community. Secondly, it presents a masked killer in the transfused blood. Thirdly, its detection requires the implementation of appropriate laboratory investigation methods.

5. Conclusion

Blood transfusion in the study area constituted a method of OBI transmission due to the lack of advanced technology for OBI detection, and thus, transfusion safety measures against important blood-borne pathogen; HBV are insufficient.

6. Study Limitation

The study measured total anti-Hbc IgM and IgG, and thus did not differentiate between recent IgM-positive HBV infections and previous cases. The study also did not use an additional highly sensitive technique for examination of OBI such as RT. PCR.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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