

High Prevalence of Occult Hepatitis B Virus Infection among Frequently Blood Transfused Children: A Single Egyptian Center Experience

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Abstract

Background

Occult hepatitis B virus (HBV) infection (OBI) is a form of the disease which does not present with Hepatitis B surface antigens (HBsAg) in the serum of patients with HBV DNA being detectable in the serum and hepatocytes. OBI is an important risk factor for acquiring post transfusion hepatitis. This could lead to reactivation of the HBV, liver cirrhosis, and hepatocellular carcinoma. We aimed to examine the prevalence of OBI in frequent blood and blood product transfused Egyptian pediatric patients.

Materials and Methods: This case-control study was done in pediatric department, Minia University. Forty-five patients randomly selected from the blood transfusion unit in the central Minia blood bank were enrolled. Their age ranged from 3-18 years. Another 12 known hepatitis B positive age- and sex-matched patients were enrolled as controls. Routine AST, ALT, blood urea, serum creatinine, and hemoglobin levels were done, HBsAg, anti-hepatitis B surface antibodies (anti-HBsAb), anti-hepatitis B core antibodies (anti-HBcAb), and HBV DNA (by nested PCR) were examined for both patients and controls.

Results: Although anti-HBs serum levels were $\geq 10\text{IU/L}$, HBV DNA was present in 27 (60%) of the 45 HBsAg negative patients. Thirteen (48%) of the OBI patients were anti-HBcAb positive. No significant differences were found between HbcAb positive and negative OBI groups regarding age, gender, frequency of blood transfusion, presence of hepatomegaly, presence of splenomegaly, serum ferritin, AST, ALT, blood urea, serum creatinine, and hemoglobin levels. On the other hand, Hepatitis C Virus Ab positivity was significantly higher among HbcAb positive patients ($p=0.02$).

Conclusion

Based on the results, the risk of acquiring OBI is high in patients receiving frequent blood transfusions despite obligatory HBV vaccination.

Key Words: Chronic blood transfusion; Egypt; HBsAg; HBV; Thalassemia; Viral hepatitis.

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1- INTRODUCTION

Occult hepatitis B infection (OBI) is defined as the presence of HBV DNA in the hepatocytes and sera of individuals testing negative for hepatitis B virus (HBV) surface antigen (HBsAg). The molecular basis of OBI is closely related to the peculiar life cycle of the HBV, and in particular to the long lasting persistence of free viral genomes such as HBV covalently closed circular DNA (cccDNA) chromatinized episomes in the nucleus of the infected cells (1). The stability and long-term persistence of cccDNA molecules together with the long half-life of hepatocytes imply that HBV infection, once it has occurred, may possibly continue for life even in conditions of strong inhibition of viral functions (2). Many studies indicate that OBI is an important risk factor for hepatocellular carcinoma development (3-5). In fact, OBI may maintain most of the pro-oncogenic properties of the overt HBV infection, including the capacity to integrate into the host's genome, to produce proteins with transforming properties (even if at low levels), and a mild but persisting necro-inflammation. Although several OBI studies were conducted on high risk Egyptians children (6-10), data examining the prevalence of OBI among frequently transfused Egyptian children are scarce. In this regard, only one study at Cairo University (8) showed that OBI is present in 32.4% of 80 beta thalassemic children aged 2-12 years. This study aimed to detect OBI in frequently blood and blood product transfused pediatric patients at Minia Governorate, Upper Egypt. We aimed in this study to detect the frequency of OBI among frequently blood transfused Egyptian children.

2- MATERIALS AND METHODS

2-1. Study design and population

This case control study was carried out in the period from May 2013 to March

2014 and included 45 cases who were randomly selected from Minia Governorate regional blood bank. The age of the subjects ranged from 3 to 18 years. Another 12 positive age- and sex-matched control cases (known HBV positive patients) were enrolled from Minia fever hospital dialysis unit. Twenty-four out of the 45 cases (53.4%) were males while 21 (46.6%) were females. In the 12 control cases 7 (58.3%) were males and 5 (41.7%) were females.

2-2. Inclusion and exclusion criteria

Inclusion criteria included: subjects negative for HBsAg and frequently blood and/or blood products transfused children (Thalassemia, chronic hemolytic anemia, etc.). All patients and controls were subjected to a full medical history taking regarding different variables e.g. age, gender, residence, frequency of blood transfusion in the last year, presence of any liver disease, positive family history of HBV infection, positive family history of repeated blood transfusion, and vaccination history for HBV including the obligatory vaccination and any booster doses.

2-3. Methods

All subjects were examined clinically and by ultrasonography stressing on signs of liver affection such as jaundice, hepatomegaly and/or splenomegaly.

2-4. Measuring tools

All cases and controls were tested for HBV DNA, HBsAg, anti-HBcAb, anti-HCV-Ab by ELISA, serum ferritin by Minividas system (Biomerieux, France), serum ALT, serum AST by Automated Clinical Chemistry Auto-analyzer System (Dimension ES, Dupont Medical products, Finland), blood urea, serum creatinine by automated spectrophotometric apparatus and CBC by electronic counter (SysmexKx – 21N) at Minia Medical Insurance Hospital.

2-4-1. Blood sample collection and storage:

Three to five ml of venous blood were collected in sterile tubes, and centrifuged at 3000 rpm for 10 min. The blood samples were collected at least 15 days after the last blood transfusion. The sera were collected in two screw capped sterile cryovials (Greiner Bio-one GmbH, Frickenhausen, Germany). The serum aliquots were stored at -20 °C until PCR analysis and other testing. The test has a lower limit of detection of 1ng/ml with a sensitivity of 98.8% and specificity of 99.8%. The assay was performed according to manufacturer's instructions.

2-4-2. Detection of HBV core antibody:

Commercially available rapid test for HBcAb (one step Hepatitis B core antibody Test Device) that can be used with serum or plasma (ABON Biopharm (Hangzhou) Co., Ltd., China) was used to detect HBcAb. The assay was performed according to the manufacturer's instructions.

2-4-3. Quantitation of HBsAb:

All the study subjects were examined by quantitative HBsAb ELISA to determine the level of HBsAb in their sera according to the manufacturer's instruction (Diasourin, Italy).

2-4-4. HBV DNA Extraction and

Detection: All samples were tested for HBV DNA detection using nested-PCR assay. For each patient who was negative for HBsAg, plasma was explored for the presence of HBV DNA by DNA extraction and PCR amplification. Viral DNA was extracted from 150µl of plasma using Viral Gene-spin TM Viral DNA/RNA extraction kit according to manufacturer's instructions (iNtRon, Korea). For the amplification of HBV DNA, a nested PCR method was employed using a thermal cycler (Progene, Technie; Cambridge LTD, UK). Two pairs of oligonucleotide primers within the highly conserved S/pol region of HBV genome were used for detection of the S region as previously

described (11). The sequences of the primers used in the nested PCR were as follows: Outer -F: 5'-GGT TAT CGC TGG ATG TGT CTG C-3' (22): 365- 386. Outer R: 5'-CCA CAA TAC GTT GAC AGA CTT TCC-3' (24): 980-1003. Inner F: 5'-CTC TTC ATC CTG CTG CTA TGC CTC-3' (24): 404-427. Inner R: 5'-TGG TAA CAG CGC TAA AAA GGG ACT C-3' (25): 781-805. The amplified products were visualized on an agarose gel stained with ethidium bromide. We used HBV DNA positive controls from HBV DNA positive patients and negative controls containing no template and/or water at each PCR testing. The cut off of HBV DNA detection was 5 IU/ml. The cases that showed positive results were retested for confirmation of HBV DNA positivity.

2-5. Ethical consideration

The study was conducted according to the declaration of Helsinki and the study protocol was approved by Minia Faculty of Medicine Review Board (ID-code: 34/513). The study was explained to all participants using the consent form.

2-6. Data Analysis

Statistical analysis was conducted with the Statistical Package for the Social Sciences (SPSS) software version 16.0 (IBM SPSS, NY, USA). The results were expressed as percentages, means \pm standard deviation (SD) or median (max-min). Results were compared using the chi-square test for categorical variables and student's *t*-test, analysis of variance (ANOVA), and multivariate analysis for quantitative variables. All comparisons were two-tailed, and *p*-value less than 0.05 was considered statistically significant. Mann-Whitney U test was used for comparing the continuous variables that did not have normal distribution.

3- RESULTS

3-1. Characterization of the study subjects

The baseline and clinical characteristics of the 45 patients and 12 control subjects are shown in **Table.1**. As shown, history of blood transfusion was found in patients with age range from 3 to 17 years (mean =10±4 years), while the age of the controls ranged from 4 to 18 years (mean =11±3.6 years). Family history of blood transfusion ($p=0.006$), and HBV infection ($p=0.46$) were found in 19 (42%), and 2 (4%) of the 45 patients, respectively. These parameters were not found in the controls. All patients and controls (100%) were vaccinated against HBV. No significant differences were found between patient and controls regarding history of routine Hepatitis-B vaccination ($p=1$). Hepatomegaly was found in 15 (33%) patients and in 9 (75%) controls ($p=0.01$) while splenomegaly was found in 19 (42%) of the 45 patients and in 4 (33%) of the 12 controls ($p=0.56$; **Table. 1**). The laboratory characteristics of the

studied pediatric patients and controls are shown in **Table.2**. As shown, significant differences were found between cases and controls regarding HBV DNA test ($p=0.001$), HBsAg ($p=0.001$), HBcAb ($p=0.001$), serum ferritin ($p=0.001$), AST ($p=0.005$), and ALT ($p=0.001$) levels. For the studied patients, AST level of cases ranged from 14 to 218 IU/L with a mean of 59 ± 47.2 IU/L and ALT level among cases ranged from 8 to 245 IU/L with a mean of 63 ± 56.4 IU/L. The hemoglobin (HB) level was 6-10 g/dl with a mean of 8 ± 1.1 g/dl while among controls, it ranged from 10-13 g/dl with a mean of 12 ± 0.9 g/dl. Serum ferritin levels ranged from 6 to 7485 ng/ml with a mean of 2170 ± 1547.8 ng/ml in the studied patients and 14-42 ng/ml with a mean of 28 ± 9.9 among controls. Hepatitis C virus (HCV) antibodies (HCV-Ab) were found positive in 13 (29%) of patients and 6 (50%) of the controls with no significant differences between the two groups ($p=0.17$; **Table.2**).

Table-1: Demographic and clinical characteristics of the studied patients and controls.

Parameter		Cases n=45	Controls n=12	P-value
Age, year	Range	3-17	4-18	0.44
	Mean ± SD	10±4	11±3.6	
Gender	Male	24 (53%)	7 (58%)	0.76
	Female	21 (47%)	5 (42%)	
Positive family history of blood transfusion		19 (42%)	0 (0%)	0.006
Positive family history of HBV infection		2 (4.4%)	0 (0%)	0.46
Positive routine HBV vaccine		45 (100%)	12 (100%)	1
Presence of hepatomegaly	Positive	15 (33%)	9 (75%)	0.01
	Negative	30 (67%)	3 (25%)	
Presence of splenomegaly	Positive	19 (42%)	4 (33%)	0.56
	Negative	26 (58%)	8 (67%)	

SD: Standard deviation.

Table-2: Laboratory data of studied pediatric patients and controls.

Parameter		Cases n=45	Controls n=12	P-value
HBV DNA by PCR	Positive	27 (60%)	12 (100%)	0.001
	Negative	18 (40%)	0 (0%)	
HBsAg	Positive	0 (0%)	12 (100%)	0.001
	Negative	45 (100%)	0 (0%)	
HBcAb	Positive	13 (29%)	12 (100%)	0.001
	Negative	14 (31%)	0 (0%)	
	Not done	18 (40%)	0 (0%)	
HCV-Ab	Positive	13 (29%)	6 (50%)	0.17
	Negative	32 (71%)	6 (50%)	
Serum ferritin (ng/ml)	Range	6:7485	14:42	0.001
	Mean ± SD	2170±1547.8	28±9.9	
AST (IU/L)	Range	14:218	15:33	0.005
	Mean ± SD	59±47.2	22±5.7	
ALT (IU/L)	Range	8:245	12:39	0.001
	Mean ± SD	63±56.4	23±7.8	
HB level (g/dl)	Range	6:10	10:13	0.09
	Mean ± SD	8±1.1	12±0.9	

SD: Standard deviation, HBV DNA by PCR: hepatitis B virus DNA by polymerase chain reaction, HBsAg: hepatitis B surface antigen, HBcAb: hepatitis B core antibodies, HCV-Ab: hepatitis C virus antibodies, AST: aspartate aminotransferase, ALT: alanin aminotransferase, HB: hemoglobin.

3-2. High OBI among frequently transfused Egyptian children

PCR testing of HBsAg negative frequently transfused cases for HBV DNA showed that 27 (60%) subjects were positive. Among the 27 HBV DNA positive cases, 6 (22.2%) were very weak positive, 9 (33.3%) were weak positive and 12 (44.4%) were strong positive. On the other hand, all the 12 controls (100%) were strong positive for HBV DNA by PCR. A representative example of PCR amplification of HBV DNA is shown in **Figure.1**. All 45 cases (100%) were negative for HBsAg while all 12 control

cases (100%) were positive. HBcAb was detected in 13 patients out of the 27 (48%) OBI patients and were considered HBcAb seropositive OBI subjects and 14 patients (52%) were negative and were considered HBcAb seronegative OBI subjects. The 18 subjects who tested negative for HBV DNA (40%) were not tested for HBcAb. On the other hand, all controls (100%) were positive for HBcAb. Anti-HBs was measured by quantitative ELISA in the patients and control groups. Although anti-HBs serum levels were ≥ 10 IU/L, HBV DNA was present in 27(60%) of the 45 HBsAg negative patients.

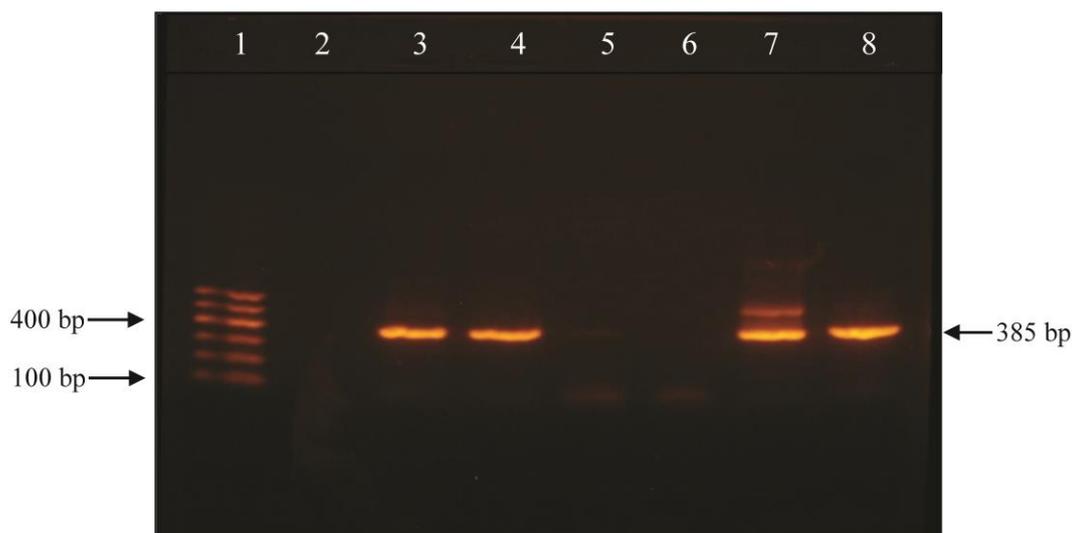


Fig.1: Detection of HBV DNA in frequently blood transfused Egyptian children. Viral DNA was extracted from the patients' sera and amplified by nested PCR as described in the Patients and Methods section. Lane 1: DNA ladder (100 bp ladder), Lane 2: negative control, Lane 3: positive control, Lanes 4, 7, and 8 are positive samples, Lane 5: a very weak occult HBV sample, and Lane 6: a negative sample.

3-3. Comparison between HBcAb positive and negative OBI cases

Table 3 shows that there were no significant differences between HBcAb positive and HBcAb negative OBI groups regarding HBV DNA testing results, serum ferritin, AST, ALT, and hemoglobin levels ($p=0.68$). Also, age ($p=0.31$), gender ($p=0.84$), blood urea, serum creatinine

levels, the frequency of blood transfusion ($p=0.13$), presence of splenomegaly ($p=0.82$), and presence of hepatomegaly ($p=0.88$) were not significantly different between these two groups (not shown). By contrast, a significant difference ($p=0.02$) was found between HBcAb positive and negative OBI children regarding HCV-Ab (**Table.3**).

Table-3: Comparison between HBcAb positive and negative occult HBV cases regarding Laboratory data.

Parameters		HBcAb positive n=(13)	HBcAb negative n=(14)	P-value
HBV DNA by PCR	Negative	0 (0%)	14 (100%)	0.82
	Positive	13 (100%)	0 (0%)	
HCV-Ab	Positive	6 (46%)	1 (7%)	0.02*
	Negative	7 (54%)	13 (93%)	
Serum ferritin (ng/ml)		2605 ± 1923.3	1720 ± 1029.7	0.15
AST (IU/L)		60 ± 38.1	68 ± 66.2	0.7
ALT (IU/L)		77 ± 61.2	72 ± 67.6	0.86
HB level (gm/dl)		8 ± 1.3	8 ± 1.1	0.68

*Significant ($p < 0.05$), HBV DNA by PCR: hepatitis B virus DNA by polymerase chain reaction, HCV-Ab: hepatitis C virus antibodies, AST: aspartate aminotransferase, ALT: alanine aminotransferase, HB: hemoglobin, SD: Standard deviation.

4- DISCUSSION

The aim of study is to detect the frequency of OBI among frequently blood transfused Egyptian children. In this study, OBI (positive HBV DNA test) was detected in 60% of our 45 studied although hepatitis B surface antigen (HBsAg) - negative frequently transfused pediatric patients. In this study, OBI (positive HBV DNA test) was detected in 60% of our 45 studied HBsAg negative frequently transfused pediatric patients. In this regard, a recent report from Iran (12) showed OBI in 36% of HBsAg negative patients. Also, OBI was reported in 32.8% of thalassemic children in India with only one HBsAg positive patient (13). Another report showed that HBV DNA was detected in 32.4% of thalassemic Egyptian children (8). Both OBI and HCV were reported in 38% of the studied multi-transfused immunocompromised Egyptian children with hematological disorders (10).

This rate of co-infection was higher than our study where only 7 (15.6%) of studied children had both OBI and HCV antibodies (Table 3). Also, OBI was reported in 22.5% of HCV infected Egyptian children (14). This rate is similar to our findings where 7 (26%) of the OBI patients were HCV-Ab positive, of whom 6 were HBcAb positive. From these findings, we may conclude that HCV-infected children should be investigated for OBI and vice versa aiming at early detection and early therapeutic interventions. OBI prevalence rates among HBV-vaccinated children differs in diverse risk groups based on the local HBV incidence irrespective of anti-HBs sero-status (7). In agreement with the findings of the present study, it was previously concluded that the estimated risk of acquiring hepatitis infection in children receiving multiple blood transfusions is surprisingly higher than generally accepted (15, 16), especially in immunocompromised children (17).

OBI was reported in 10.9% among HBV-vaccinated children in Taiwan (18). This is lower than the results of this study, where we reported OBI in 60% of the studied children despite their routine vaccination with HBV vaccine. In one study, this was attributed to suppressed antibody response to vaccination caused by decreased immunologic response in such patients (18). Also, vaccine failure may be due to maternal transmission, vaccine escape variants of the virus, and hypo- or non-responsiveness to the vaccine (19). However, after nationwide vaccination, the prevalence of HBV infection in children decreased from 10% to <1% in Taiwan (19). Moreover, the prevalence of OBI was 0.8% among young adults in China, all of whom received three doses of HBV vaccine (20). We speculate that the high prevalence rate of OBI (60%) in this study among frequently blood transfused Egyptian children, which is almost two-fold that of another report in Cairo (8), could be explained by the presence of viral escape mutants. This hypothesis is supported by the data showing that all the 27 OBI patients had ≥ 10 IU/ml of anti-HBs in their sera. In our study, there was no significant difference regarding the prevalence of OBI in male and female multi-transfused children.

However, a significantly higher prevalence of OBI among males was reported (21). OBI detected in our HBsAg negative children is most likely due to reception of blood from OBI infected donors. Blood donors with OBI may represent the main source of HBV infection among these children as they are not routinely screened for OBI. In this regard, it has been shown that among 3600 Brazilian donations, 799 were anti-HBc reactive (22.2%) (22), and that anti-HBc (IgM) reactivity was 0.03% among voluntary blood donors, 0.35% in replacement donors and seroprevalence was significantly higher among replacement donors than the voluntary

ones (23). In Egypt, prevalence of anti-HBc antibody in HBsAg negative blood donors was found to be 13.3%, of which 10% were HBV DNA positive (24). In another large Egyptian study among the accepted blood units for donation in blood banks, anti-HBc antibody was found in 78 out of 712 units (10.96%) (25). The clinical significance of isolated anti-HBcAb in serum (i.e. anti-HBc-positive status in HBsAg-negative patients) is still unclear. Some reports suggested that anti-HBcAb-positive status implies a potentially infectious state. Several studies of HBV transmission in recipients of blood (26), liver and kidney grafts from anti-HBc-positive donors have been reported (27, 28). In this study, a significant difference was found between cases and controls regarding serum ferritin levels ($p=0.001$). Increased gastrointestinal absorption of iron has been reported because of the associated chronic hemolysis, and it is also thought that repeated red cell transfusion consequent to chronic hemolysis and anemia cause's excessive iron and ferritin levels (29).

Also, in this study, significant differences were found between cases and controls regarding AST and ALT levels. It was reported that in the highly detectable HBV viral loads category, liver function tests overall were not good predictors of HBV infection (30). No relationship was found between increased biochemical liver tests and anti-HBV core-seropositive status (31). In the present study, only two patients of the 45 cases group had family history of HBV infection. This is in contrast to the data showing that OBI seems to be relatively frequent in immunized children born to HBsAg-positive mothers (32). HBV DNA was detected in 28% of Chinese children when the sera of 75 children born to HBsAg-positive mothers previously immunized by HBIG and prophylactic vaccine regimen were assayed for HBV DNA by real-time

PCR (33). It was shown that among 186 surveyed high-risk infants born to HBsAg-positive mothers, although all infants received hepatitis B-vaccination after birth, 9 (4.8%) infants tested positive for OBI with serum HBV DNA detected by-genome sequences with anti-HBs present in 6 of the 9 cases. Escape mutations and high maternal viral loads were associated with OBI among these children (33).

4-1. Limitations of the study

Unavailability to perform liver biopsy for the enrolled patient and limited number of patients agreed to share in the study due to cultural issues.

5- CONCLUSION

In conclusion, a high OBI prevalence rate (60%) was found among frequently blood transfused Egyptian children related to blood transfusion acquiring infection, frequent blood sampling and intravenous catheter intervention. Based on our findings, we recommend that frequently transfused children must be regularly screened for OBI by a sensitive PCR for HBV DNA. HBsAg negative blood donors should be screened to exclude HBV DNA positive ones. Prospective studies with a larger number of such patients are important to gain more insight into this important topic.

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7- CONFLICT OF INTEREST: None.

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