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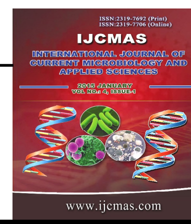

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Original Research Article

Occult Hepatitis B virus infection in patients with blood diseases

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Keywords

Occult hepatitis B virus infection, Blood Diseases, Blood Transfusion, Transmission, Total anti-HBc, Hepatitis B surface antigen, Hepatitis B virus-DNA.

A B S T R A C T

This study aimed to determine the frequency of occult HBV infection among patients with haematological disorders who received frequent blood transfusion. It included 60 HBsAg-negative patients with haematological disorders at the hematological department of Medical Research Institute Hospital who received frequent blood transfusion. Sera collected from all patients were tested for HBsAg, anti-HBc, anti-HCV by enzyme-linked immunosorbant assay and detection of HBV-DNA by PCR. The anti-HBc was detected in 43.3% of the patients; HBV-DNA was detected in 53.3% by SYBR green real time PCR in two different region of the genome with a high detection in the S region alone (28.3%) compared to the core region (13.3%). Conventional nested PCR amplifying the *pol* gene was positive in only 6 (10%) of the patients. With the use of HBsAg as the sole detection marker for HBV, there is a danger of HBV transmission through blood transfusion. Hence, more sensitive screening tests should be added to the routine blood donor screening test to eliminate most of the risk of unsafe blood donation.

Introduction

Hepatitis B virus (HBV) remains a major public health problem worldwide that accounts for significant morbidity and mortality. About one third of the world's population have serological evidence of past or present hepatitis B virus infection, and more than 350 million people may be affected by chronic HBV infection (World Health Organization, 2012).

The clinical evolution of HBV is variable, ranging from mild liver disease to fulminant hepatitis, cirrhosis, or hepatocellular carcinoma (Kennedy and Alexopoulos, 2010).

Implementation of hepatitis B surface antigen (HBsAg) in routine screening of blood donors in the early 1970s has greatly enhanced transfusion safety. The incidence

of transfusion-transmitted hepatitis B has been steadily reduced over the last four decades (Candotti and Allain, 2009). However, it was demonstrated that HBV transmission by blood components negative for HBsAg can still occur and HBV transmission remains the most frequent transfusion-transmitted viral infection; thus the study of blood products only by means of detecting HBsAg is not enough to recognize sample HBV infectivity since HBV infection may exist without detectable HBsAg (El-Sherif *et al.,* 2009; Niederhauser *et al.,* 2008).

Occult hepatitis B virus (HBV) infection is defined OBI is defined by detection of HBV DNA in serum and/or liver of patients and the absence of HBsAg, with presence or absence of anti-HBc or anti-HBs (Raimondo *et al.,* 2007; Said, 2011).

The prevalence of occult HBV is unclear and depends in part on the sensitivity of the HBsAg and DNA assays used as well as the prevalence of HBV infection in the study population (Raimondo *et al.,* 2013). OHB varies significantly between different geographical regions. Studies have shown that the prevalence of occult HBV infection is closely related to the endemicity of HBV infection. Patients from countries highly endemic for HBV are more likely to develop occult HBV infections (Schmeltzer and Sherman, 2010).

High incidence of transfusion associated hepatitis B virus was reported in patients receiving frequent blood transfusion like thalassemia and hemato- oncology patients (Arababadi *et al.,* 2012; Shaker *et al.,* 2012). This is mainly because of the blood from the donors with ‘occult’ HBV infection (OBI). Hence, more sensitive screening tests, such as PCR, should be employed to decrease the risk of post transfusion hepatitis.

Reports on the prevalence of OBI in the Egyptian population are limited. Therefore, this study aimed to determine the frequency of OBI among patients with hematological disorders receiving frequent blood transfusion and investigate its relationship with HCV infection in these patients. Also, to further highlight the need for donor screening strategy with supplementary molecular diagnostic tools for high risk population.

Materials and methods

This study was carried out during the period between March 2013 and November 2013. It included 60 HBsAg –negative patients with haematological disorders including; thalassemia, aplastic anemia, multiple myeloma, non-Hodgkin lymphoma and leukemia, who received frequent blood transfusion at the hematological department of Medical Research Institute Hospital, Alexandria University.

All relevant informations were collected from each patient (after obtaining full consent) including personal data as (age, sex, etc) as well as health data (history of blood transfusion, history of jaundice, previous surgical interference and dentistry). The study was carried out after receiving the approval of the Ethics Committee in the Medical Research Institute.

Five milliliters of blood was withdrawn from each patient, and the sera were separated and aliquoted, then stored at -80°C until use.

Determination of liver function tests including: aspartate aminotransferase (AST), alanine aminotransferase (ALT), and total serum bilirubin were measured by automated spectrophotometric apparatus

Serological Testing

All samples were tested for HBsAg, anti-HBc, anti-HCV by enzyme-linked immunosorbant assay (Abott Murex Diagnostic Division, Kent, UK) according to the manufacturer's instructions.

DNA extraction

DNA was extracted from 200 µl of serum using the QIAamp viral DNA mini kit (Qiagen Inc., Valencia, California, USA) following the manufacturer’s instructions. Briefly, samples were incubated with protease and lysis buffer. After incubation, there were two washing steps, and the nucleic acids were eluted in a volume of 50 µL of elution buffer. The eluted DNA was stored at −20°C until tested.

Amplification of S and C genes by SYBR green Real Time PCR:

Detection of serum HBV DNA was done by SYBR Green real time PCR amplification of the surface antigen gene using specific forward (5'AGAACATCGCATCAGGAC TC-3'; nt: 159–178) and reverse (5'CATAGGTATCTTGCGAAAGC-3'; 642–623) primers and amplification of the core gene using specific forward (5' CTGGGAGGAGTTGGGGGA -3'; nt: 1730 –1747) and reverse (5' GTAGAAGAATAA AGCCC -3'; 2503–2487) primers. The amplified products were detected with SYBR Green dye (Applied Biosystems Inc., Foster city, California, USA) (Kao *et al.,* 2002).

The amplification reaction consisted of 12.5 µl SYBR green universal PCR master mix two-fold (Applied Biosystems Inc., Foster city, California, USA), 0.3 µl (30 pmol) of S or C gene sense primers, 0.3 µl (30 pmol) of S or C gene antisense primers, 10 µl of Qiagen extracted DNA, and nuclease-free water was added to bring the reaction to a final volume of 25 µl.

Thermal profile: AmpliTaq activation for 95°C for 10 min, followed by 40 cycles of PCR amplification, including denaturation at 95°C for 15 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min, followed by melting curve analysis to determine the purity and specificity of the amplification product. The melting curve analysis profile was 95°C for 1 min, 50°C for 30 s, and 95°C for 15 s.

Hepatitis B Virus Specific Nested PCR for pol gene:

The presence of HBV DNA was examined in all samples by nested PCR using primer pairs designed from the highly conserved overlapping regions of the S and P genes of the HBV genome: outer primer pairs were HBPr134 (sense) 5′-TGCTGCTATGCC TCATCTTC-3′ and HBPr135 (antisense) 5′-CAGAGACAAAAGAAAATTGG-3′ and the inner primer pairs were HBPr75 (sense) 5′-CAAGGTTATGTTGCCCGTTTGTCC-3′ and HBPr94 (antisense) 5′-GGTATAAAGGGACTCACGATG-3′. PCR amplifications for the first run were carried out in 25 µL reaction consisted of 12.5 µl universal PCR master mix 2-fold (Thermo Scientific, Fermentas, Vilnius, Lithuania), 0.3 µl (30 pmol) of each of the sense primers and antisense primers, 10 µl of Qiagen extracted DNA, and nuclease-free water to bring the reaction to a final volume of 25 µl.

Thermal cycling parameters were initial denaturation at 95°C for 4 min, followed by 40 cycles of 30 sec at 95°C denaturation, 30 sec at 50°C annealing temperature, and 30 sec at 72°C extension, followed by a final extension of 5 min at 72°C. Three microliters of the first-round products was re-amplified using inner sense

and antisense primers with the same amplification protocol and thermal cycling parameters except for the number of cycles that was 35 cycles of amplification to yield a final amplicon of 341 bp. The PCR products of the second round of PCR were analysed by electrophoresis in 2% agarose gels stained with ethidium bromide, and were observed under ultraviolet light to yield a 340 bp fragment.

Statistical analysis

Data were analyzed using the Statistical Package for Social Sciences (SPSS ver.20Chicago, IL, USA). Categorical data were expressed as frequencies, whereas continuous data were presented as means±SD. We used Pearson Chi-square test to test the association between 2 qualitative variables , and Fishers Exact test was used if (>20% of cells have expected count <5). We used a significance level of 0.05,below which the results considered to be statistically significant.

Result and Discussion

This study included 60 HBsAg –negative patients (confirmed by ELISA for HBsAg) with haematological disorders who received blood transfusion at the haematology department of Medical Research Institute Hospital, Alexandria University. 15 (25%) of the patients had thalassemia, 14 (23.33%) were diagnosed as acute leukaemia, 15 (25%) were diagnosed as chronic leukemia, 5 patients (8.33%) had non-Hodgkin lymphoma, 3 patients (5%) had multiple myeloma and 4 patients (6.66%) had aplastic anaemia.

Among our patients the mean age ranged from 33.5 ± 11.82 in aplastic anemia to 53.3 ± 21.5 among multiple myeloma. 35 (58.3%) were females and 25 (41.7%) were males.

Among our patients 10 (16.7%) were anti-HCV positive and 26 (43.4%) were anti-HBc positive, Anti-HBcore positivity was higher in thalassemia and aplastic anaemia patients (60%), while the 3 multiple myeloma patients were anti-HBcore negative followed by non-Hodgkin lymphoma where anti-HBcore was detected in only 20%.

As presented in Figure 1; Anti-HBcore positivity was significantly lower (26.1%) when the frequency of blood transfusion was lower than 10, compared to 50 – 57.9% with higher frequency of blood transfusion. HBV-DNA was detected in only 3 (11.5%) out of the 26 Anti-HBc positive hematological patients, compared to 3 (8.8%) out of the 34 Anti-HBc negative patients.

Thirty two (53.3%) out of the 60 hematological patients were HBV-DNA positive by SYBR green real Time PCR Figure 2. Of them; 17 (28.3%) were positive for s gene alone, 8 (13.3%) for c gene alone, and 7 (11.7%) were positive for both genes. As listed in Table 1; the presence of HBV-DNA was not significantly related to the frequency of blood transfusion among hematological patients.

Conventional PCR with amplification of pol gene was positive in only 6 patients (10%) compared to 32 patients (53.3%) positive by real time PCR for S and/or C gene as presented in Table 2.

Only 9 (15%) patients had abnormal ALT ranging from 50% in aplastic anemic patients to 7.1% out the acute myeloid leukemia patients. No abnormal ALT was found among the 3 multiple myeloma patients. 30 (50%) of the patients had elevated bilirubin level, the highest percentage 13 (86.7%) was found among the 15 thalassemic patients.

Hepatitis B virus (HBV) is a serious risk as a disease that can be spread through blood transfusion. Occult hepatitis B infection (OBI) is defined in a patient with the presence of HBV-DNA but a lack of HBsAg in the serum and hepatocytes. OBI can be considered as a high potential risk factor for inducing post transfusion hepatitis (PTH), hepatocellular carcinoma (HCC), cirrhosis, and reactivation of the HBV. Recently, several investigations from various regions of the world have reported PTH as well as HCC and cirrhosis among blood recipients with diseases such as thalassemia and other disorders requiring regular hemodialysis. This form of hepatitis also causes complications for individuals that are co-infected with other viruses such as HCV and HIV. Because of its extreme disease potential, OBI can be considered a high risk for PTH, HCC, and cirrhosis. Therefore, an understanding of the prevalence of OBI among blood donors is a critical strategy in most transfusion services (Sabat *et al.,* 2014).

The presence of HBV- DNA in HBsAg negative blood units has been reported from many parts of the world. Patients from countries highly endemic for HBV are more likely to develop occult HBV infections (Said, 2011). OBI may follow recovery from infection, displaying antibody to hepatitis B surface antigen (anti-HBs) and persistent low-level viraemia, escape mutants undetected by the HBsAg assays, or healthy carriage with antibodies to HBe antigen (anti-HBe) and to HBV core antigen (anti-HBc). OBI seems to be the most likely mechanism for post transfusion hepatitis (PTH) in permanent blood recipients including, thalassemia and blood diseases (Shaker *et al.,* 2012, Said *et al.,* 2009).

In the present study 26 (43.3%) of the 60 patients were anti-HBc positive. Anti-HBc positivity was higher among the thalassemic patients as 9/15 (60%) of them were anti-HBc positive. Thalassemic patients are "at risk" because they receive high volumes of transfused blood and its components. Although, there are several publications regarding HBV infection prevalence in thalassemic patients (Mirmomen *et al.,* 2006; Khakhkhar and Joshi, 2006), there are only few publications regarding the prevalence of the OBI form of HBV infection among these patients.

In agreement with our findings Shaker et al (Shaker *et al.,* 2012) from Egypt reported that OBI had a high prevalence (32.4%) among thalassemic patients. Similar results were reported by Singh et al (Singh *et al.,* 2003) who found that HBV-DNA was present in 23(32.8%) out of 70 thalassemic children with only one positive for HBsAg.

The results of the present study are consistent with the study of Sabat *et al.* (2014) who detected HBV DNA in 50 % of subjects having anti HBc as the only marker (Occult HBV infection).They concluded that 50 %of occult HBV infection is a major concern suggesting inclusion of viral DNA amplification test along with antigen/antibody detection On the other hand Arababadi *et al.* (2008) showed that there were no OBI cases among 60 Iranian thalassemic patients. The probability of these patients contacting HBV infection depends on the number of transfusions they receive and the prevalence of HBV infection in blood donors (Hollinger and Sood, 2012)

Figure.1 Distribution of anti-HBcore according to the frequency of blood transfusion and hematological diseases

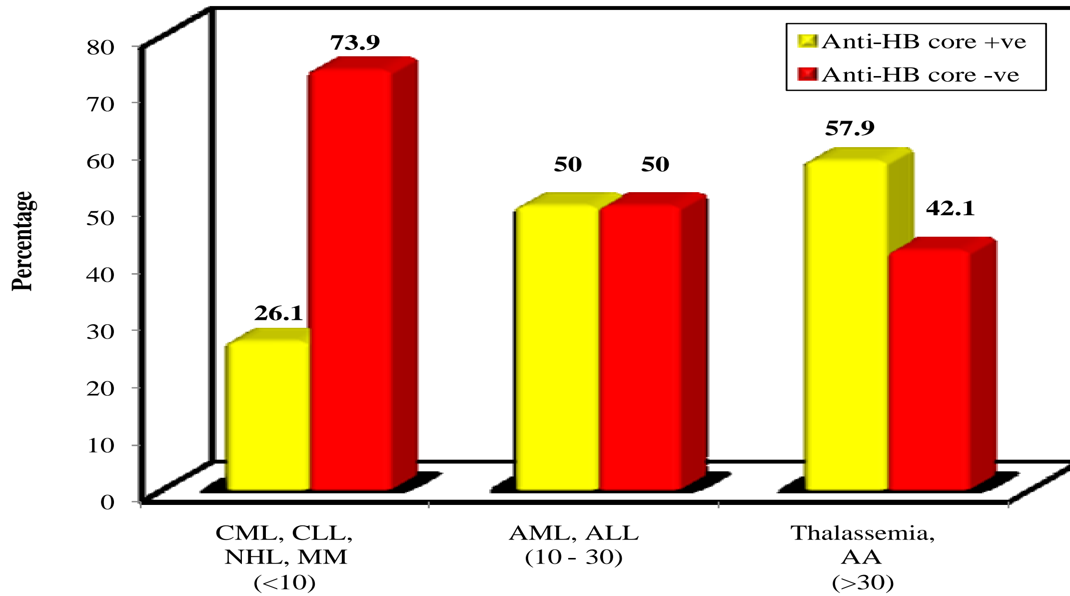

Figure.2 Distribution of s and c genes by SYBR green real time PCR among the 60 patients with hematological diseases

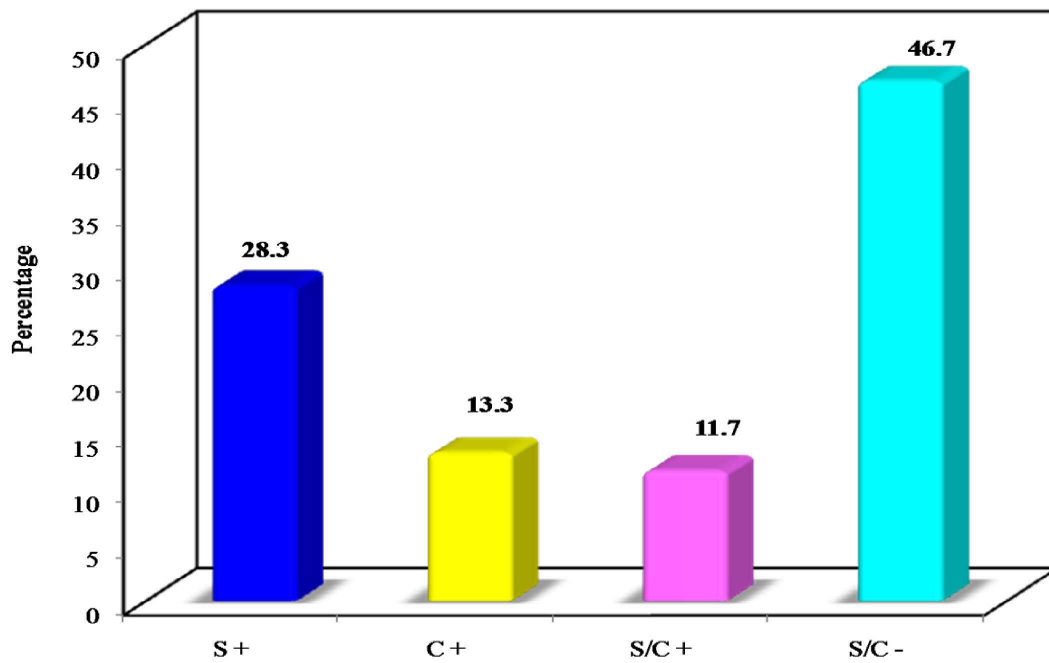

Table.1 Distribution of HBV-DNA according to the frequency of blood transfusion among hematological patients

Diseases	No of blood transfusion	HBV-DNA +ve	HBV-DNA –ve	χ^2	P
CML, CLL, NHL, MM	<10	14 (60.9%)	9 (39.1%)	0.268	0.604
AML, ALL	10 – 30	8 (44.4%)	10 (55.6%)	1.564	0.211
Thalassemia, AA,	>30	12 (63.2%)	7 (36.8%)	0.477	0.490

Table.2 Detection of HBV-DNA by conventional and real time PCR

	+ve Pol		**Total**	
	No	**%**	**No**	**%**
Gene				
S +	3	17.6	17	100.0
C +	1	12.5	8	100.0
S/C +	0	0.0	7	100.0
S/C -	2	7.1	28	100.0
Total	**6**	**10.0**	**60**	**100.0**

In the present study, the frequency of blood transfusion among thalassemic and aplastic anemia was higher than thirty (>30) this was significantly associated with a high anti-HBcore positivity (57.9%) compared to hematological patients with lower frequency of blood transfusion (<10) who showed anti-HB core positivity of only (26.1%).

These findings are supported by the work of Sabat *et al.* (2014) who observed that HBV seropositivity increased with increase in number of transfusions. They concluded that thalassemic subjects need detailed screening of transfusion products.

Other studies have indicated that 20% of the patients with occult HBV infection are negative for all HBV markers (Gutiérrez-García *et al.,* 2011). This was confirmed by Said *et al.,* (2009) who reported that only four (19%) out of 21 patients with occult HBV infection had anti-HBcore whereas 20 patients (95.2%) had a positive HBV-DNA sequence in the core region.

In the present study steps were taken to avoid false positive results using SYBR Green dye which can bind to any dsDNA. A hot start which requires incubation at 95°C for 10 min for to activate *Taq* polymerase was applied. The specificity of PCR was

verified by the performance of melting curve analysis for the PCR product, which depends on its GC contents, length, and sequence.

In the present study HBV-DNA was detected in two different regions of the genome with a high detection in the S region alone (28.3%) compared to the core region (13.3%). *S&C* positivity was detected together in (11.7%). HBV-DNA was detected in (53.3%) of the 60 hematological patients by SYBR green real time PCR.

Toyoda et al (2004) found that occult HBV infection appears to have no significant clinical impact when the infection is evaluated by the HBV detection for the *C* region. On the contrary, occult HBV may increase serum ALT levels, which indicates severe liver damage when HBV-DNA is positive by PCR for the *S* region. Further study will be required to clarify the difference in clinical significance of HBV occult infection between PCR positive for the *C* region and that positive for the *S* region.

OBI may be further classified into seropositive or seronegative categories with the seronegative subjects being negative for both anti-HBc and anti-HBs. The HBV DNA levels are lowest in these subjects (Hassan *et al.,* 2011; Ramia *et al.,* 2008).

In the present study, conventional nested PCR amplifying the *pol* gene was positive in only 6 (10%) of the hematological patients compared to 32 (53.3%) in real time PCR.

In the present study HBV-DNA was detected by conventional PCR in (11.5%) among anti-HBcore positive patients compared to (8.8%) in anti-HBcore negative patients, Higher rate of HBV-DNA detection (65.4%) among anti-HBcore positive patient was also found by Sybr green real time PCR technique compared to (44.1%) among anti-HBcore negative patients. however both results have not reached the level of significance.

The reasons for persistence of low levels of HBV-DNA in the absence of detectable HBsAg remain largely undefined, but it is supposed that both host and viral factors are important in suppressing viral replication and keeping the infection under control (Hollinger, 2008; Pollicino and Raimondo, 2014).

Low levels of viral replicative activity may result from the presence of defective interfering particles or to mutations in transcription control regions or the polymerase domain leading to inefficient replication in conjunction with the discordant release of HBsAg by the hepatocytes. This may explain the lower rate of amplification of *pol* gene (10%) by conventional PCR compared to amplification of s and /or core gene (53.3%) in the present study (Pollicino and Raimondo, 2014).

Additional mechanisms responsible for HBsAg negativity in OBI include co infection with hepatitis C virus (HCV) that results in down regulation of HBV replication and a reduction in antigen synthesis (Emara *et al.,* 2010).

There is general agreement that patients infected with HCV should be considered as a category of individuals with high prevalence of occult hepatitis B. The incidence of OBI in HCV patients varies greatly, ranging from 0%–52% (Emara *et al.,* 2010; Levast *et al.,* 2010). It was shown that HCV core protein inhibits HBV replication and gene expression (Pasquinelli *et al.,* 1997), but this observation has

recently been challenged by Bellecave *et al.,* (Bellecave *et al.,* 2009) who observed that HBV and HCV can replicate in the same cell line without evidence for direct interference in vitro. In the present study HBV-DNA was detected in 50% of anti-HCV positive compared to 58% of anti-HCV negative, this was not statistically significant. The clinical significance of OBI remains largely unknown (Squadrito *et al.,* 2014). OBI was initially detected in patients with HCC or unclassified chronic liver diseases. There is therefore a clear association between OBI and liver diseases.

In conclusion, it seems that one of the main mechanisms for OBI transmission is most likely through infected blood and its components and evaluation of the prevalence of OBI in donors and patients, especially those with thalassemia and other haematological diseases should be considered. With the use of HBsAg as the sole detection marker for HBV, there is a danger of HBV transmission through blood transfusion. Hence, more sensitive screening tests should be added to the routine blood donor screening test to eliminate most of the risk of unsafe blood donation.

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