

# Relevance of serology and molecular methods for detection of hepatitis B virus in Egyptian children with hematological malignancy

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Hepatitis B virus (HBV) infection is an important health problem and the major cause of chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) worldwide. Hepatitis B surface antigen (HBsAg) is the hallmark of HBV infection and is the first serological marker to appear in acute hepatitis B, and persistence of HBsAg for more than 6 months suggests chronic HBV infection. Hepatitis B e antigen (HBeAg) usually indicates active HBV replication and risk of transmission of infection. Recently, occult HBV infection is recognized as the absence of circulating HBsAg in individuals positive for serum or tissue HBV DNA, irrespective of other HBV serological markers.<sup>1</sup>

The reasons for the lack of circulating HBsAg in such patients are unknown. Recent observations have suggested that the lack of HBsAg may be due to rearrangements in the HBV genome that interfere with gene expression or lead to the production of an antigenically modified S protein.<sup>2-4</sup> Despite its potential clinical importance, the prevalence of occult HBV infection in patients with hepatitis C is still undetermined.

Both hepatitis B and C are common in patients requiring multiple blood transfusions, such as those with hematological disorders.

The risk of infection with both hepatitis B virus and hepatitis C virus is well documented in children with hematological disorders, and prevalence rates as high as 50% in leukemia and lymphoma patients have been reported. Many of these children receive multiple transfusions of different blood components, and this could be a potential risk factor for acquiring such infections. Also the children are

highly immunosuppressed, and therefore the manifestations of these infections are mostly subclinical and rarely noticed.<sup>5,6</sup>

This study investigates the prevalence of occult hepatitis B virus (HBV) in children with hematological diseases with or without hepatitis C virus (HCV) infection. Also, to find the most appropriate method for diagnosis of acute hepatitis B in those children.

The study included 67 pediatric patients with hematological disorders. Patients were recruited from Mansoura University Children hospital. Their ages ranged from 9 to 16 years. There were 44 males and 23 females. They were complaining of acute viral hepatitis with jaundice with no previous history of similar condition. All patients were anti-HCV and HBsAg negative before starting their therapies for either haematological diseases or malignancies and have given negative family history of HBV infection. The study was approved by Mansoura Faculty of Medicine ethical committee and informed written consents were obtained from parents of the children.

Blood samples were withdrawn and sera were separated. Complete serological study was performed for hepatitis A by detection of IgM, for hepatitis B virus including HBV s antigen, HBV core IgM and HCV IgG. Positive samples for HCV were tested for HCV RNA by nested RT-PCR. All samples were studied by PCR for HBV DNA.

In addition, hepatic biochemical study was taken for determination of hepatic transaminases (AST and ALT) and for bilirubin.

Viral RNA was extracted from 200 µl of serum using a QIAamp viral RNA kit

(Qiagen GmbH, Hilden, Germany), following the manufacturer's instructions. The extracted RNA was reverse-transcribed to cDNA, HDV-RNA presence was examined by PCR for amplification of the HDV genomic nucleotide positions from 855-1,287 using primers 853P (5'CGG ATG CCC AGG TCG GAC C3') and 1267N (5'GAA GGA AGG CCC TGG AGA ACA AGA3').<sup>2,7</sup>

Viral DNA was extracted from 200 µl of serum using a QIAamp DNA Blood Mini Kit. (Qiagen GmbH, Hilden, Germany), Viral DNA was extracted from 100 µl aliquots of serum by Quigen. Extracted DNA was immediately processed. The S region of the HBV was chosen as a target of PCR amplification. Two overlapping segments spanning the entire sequence of the S gene were amplified by PCR with primer pairs HB1-HB2 and HB3-HB4.<sup>7</sup> The first amplification was performed in a volume of 20 µl containing 5 µl of the extracted DNA, 10X Taq polymerase buffer (500 mM KCl, 100 mM Tris-HCl and 15 mM MgCl<sub>2</sub>), 200 µl each dNTP, 2.5 mM MgCl<sub>2</sub>, 0.1 µl primers and 1.25 U AmpliTaq DNA polymerase (Applied Biosystems, Foster City, California, USA). The mixture was heated to 95°C for 5 min followed by 40 cycles each consisting of 94°C for 30 s, 60°C for 30 s and 72°C for 2 min, with a final extension at 72°C for 7 min. A 2 µl aliquot of the first PCR product was then used in a nested PCR step with the internal primer pairs HB5-HB6 for the HB1-HB2 amplification or HB7-HB8 for the HB3-HB4 amplification. The nested PCR was performed in 40 µl under the same condition as the first reaction, with 1 µl of each primer using 35 cycles. Amplified products were subjected

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**Table 1.** Hepatitis viruses in patients (n = 67)

No.	%	
27	39.1	HBV by any methods
10	14.5	HCV
6	8.7	Combined HBV and HCV

**Table 2.** Hepatitis B markers by various methods (n = 67)

No.	%	
20	29.0	HBVcoreIgM
8	11.6	HB S antigen
20	29.0	HBV DNA

**Table 3.** Correlations of different hepatitis B markers to each other

p	r	
-0.14	0.3	HBV S antigen versus HB core IgM
0.46	0.0001	HBV S antigen versus HBV DNA
0.43	0.0001	HBV core IgM versus HBV DNA

**Table 4.** Univariate Analysis for Hepatitis B infection

Odds ratio	p	
p = 0.0001	3.5	HCV

to 1.5% agarose gel electrophoresis in 1X Tris borate-EDTA buffer, stained with ethidium bromide, and visualized by UV transillumination. A 100 bp ladder (Gibco BRL, Grand Island, NY) was used as a molecular size marker.

Statistical analysis was performed by SPSS 10 package (Chicago). Dichotomous variables were compared by chi-squared or Fisher's exact tests. Continuous variables were compared by the Student's t-test or Kruskal-Wallis test. Differences in levels of exposure were compared by the chi-squared test. All p values were based on two-sided tests and considered positive if  $p < 0.05$ . Mutual relations between sensitivity and specificity of the methods used were presented according to the receiver operator characteristic (ROC) curve concept.<sup>8</sup>

Between June 2008 and March 2009, 67 pediatrics patients with hematological disorders as 43 acute leukemia and 24 acute lymphoma were included in the present study. Patients were under chemotherapy. There were 44 males and 23 females with mean age  $\pm$  SD  $11 \pm 2.0$  years. The mean SD of alanine aminotransferase was

$213 \pm 23.5$  Iu/L, and for aspartate aminotransferase was  $170 \pm 15$  Iu/L, data not shown.

Hepatitis B virus was the most prevalent virus associated with acute hepatitis (29.0%) as diagnosed by any positive markers for HBV followed by hepatitis C virus (14.5%) and compound hepatitis B and C were found in 8.7% of patients, **Table 1**. None of patients had positive IgM for HAV or positive D virus antigen.

The most frequent hepatitis B marker was positive HBV core IgM and HBV DNA (29.0%) followed by positive HBV s antigen, **Table 2**.

There was positive significant correlation between HBV s antigen detection and HBV DNA ( $p = 0.0001$ ) and HBV core IgM and HBV DNA ( $p = 0.0001$ ). Nevertheless there was insignificant negative correlation between HBV s antigen and HBV core IgM ( $p = 0.3$ ), **Table 3**.

There were eight patients with isolated HBV core IgM (11.9%) and seven patients with isolated HBV-DNA (10.49%), (data not shown).

By univariate analysis for HBV infection, the presence of HCV was the most

significant associated factor ( $p = 0.0001$ ) (**Table 4**).

Interesting finding in the present study was found in ROC for methods used in diagnosis of HBV as HBV DNA and core IgM had the best sensitivities and specificities in defining acute HBV infection 74% and 73% respectively for sensitivity and 97% for each specificity (**Fig. 1**).

In the present study we elucidated several important findings. First was the presence of hepatitis B virus as a common viral hepatitis in our patients followed by hepatitis C infection.

The effectiveness of the prophylactic strategy that was conducted by the Ministry of Health in Egypt against viral hepatitis was established for HBV in previous study carried out in Delta region, the same area of our study. Nevertheless, the significant decrease of prevalence of anti-HBc was not associated with a similar decline of HBs Ag trend even among the vaccinated group as would be expected, which needs confirmation among larger group.<sup>9</sup> The age of our patients is within the age group of children to be vaccinated, however there was delay in the vaccination due to their clinical condition and administration of chemotherapy. Moreover, the high prevalence of HBV in patients can be attributed to blood transfusion. While the practice of screening of blood donors for HCV appears adequate, the screening for HBV by HBV s antigen need to be revised.

Infection with escape mutant variants, can lead to misdiagnose silent cases of HBV among blood donors which need to be checked.<sup>10</sup>

An important issue in the present study was the finding of both isolated HBV core IgM and isolated patients with HBV DNA. One study found that 10% of Taiwanese children with HBV infection did not develop anti-HBc on 5 years follow-up.<sup>11</sup> Occult HBV was reported among anti-HBc and or anti-HBs-positive individuals.<sup>12</sup>

Most studies suggested that detection of "anti-HBc alone" could reflect unrecognized occult HBV infection. Nevertheless, the interesting finding of this study is that occult HBV infection among some children had no anti-HBc. A similar result reported that 8.1% (33/407)

of HBV (HBsAg, anti-HBs and anti-HBc) seronegative subjects were HBV DNA seropositive in a North American community-based population.<sup>13</sup> Recently, Zerbini et al. characterized two different profiles of HBV-specific T cell responses in occult HBV infection.<sup>14</sup> Anti-HBc positive patients showed a T-cell response typical of protective memory. In contrast, HBV-specific T cells in anti-HBc seronegative occult patients did not readily expand and produce interferon- $\gamma$  in vitro. They proposed that these distinct behaviors of cell-mediated immune responses may reflect different modalities of HBV transmission. As shown for the woodchuck HBV, exposure to low woodchuck HB doses ( $<10^3$  virions) may get a persistent infection without serum markers.<sup>15</sup>

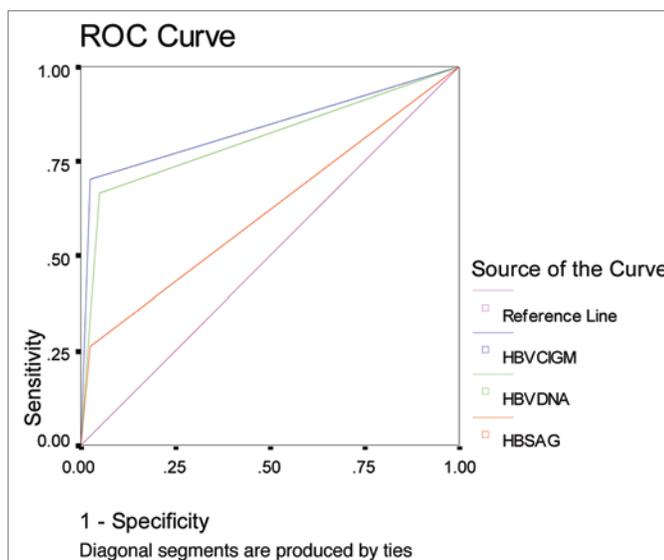
Therefore anti-HBc negative in our children may result from a low-dose infection, which is insufficient to allow maturation of protective memory. Or as in our patients they are immuno compromised patients with defect in immune response. Other explaining theory is reactivation of hepatitis B virus by chemotherapy. Patients with an occult hepatitis B virus (HBV) infection undergoing deep immunosuppression are potentially at risk of HBV reactivation.<sup>16</sup>

The clinical consequence of occult HBV infection remains unclear; several studies have demonstrated that carriers of occult infection may be reactivated under conditions of immunosuppression and leads to development of a severe hepatitis B and sometimes even fulminant hepatitis. Occult HBV is also a risk factor for HCC development and might favor the progression of the liver disease to cirrhosis.<sup>12,13</sup> For this reason, the issue and consequences of occult HBV infection should not be neglected and must be followed up.

An important finding of the present study was the high specificity and sensitivity for HBV DNA detection as found by ROC curve. Nevertheless, there were missed cases when each of the HBV markers was evaluated in separate manner.

This finding highlights the importance of combination of both serological and virological markers for diagnosis of acute hepatitis B viral infection.

We can conclude from the present study that hepatitis B virus infection is



**Figure 1.** Receiver Operative Curve for various markers of Hepatitis infection compared to identification of positive cases.

common in children with hematological malignancies. Efficient vaccination program should be adapted for those patients. Both serological and molecular methods are required for proper laboratory diagnosis for screening of patients with hematological malignancy to detect hepatitis B virus.

#### References

1. Kao JH. Diagnosis of hepatitis B virus infection through serological and virological markers. *Expert Rev Gastroenterol Hepatol* 2008; 2:553-62.
2. Blum HE, Galun E, Liang TJ, von Weizsacker F, Wands JR. Naturally occurring missense mutation in the polymerase gene terminating hepatitis B virus replication. *J Virol* 1991; 65:1836-42.
3. Yamamoto K, Horikita M, Tsuda F, et al. Naturally occurring escape mutants of hepatitis B virus with various mutations in the S gene in carriers seropositive for antibody to hepatitis B surface antigen. *J Virol* 1994; 68:2671-6.
4. Carman WF, Van Deursen FJ, Mimms LT, et al. The prevalence of surface antigen variants of hepatitis B virus in Papua New Guinea, South Africa and Sardinia. *Hepatology* 1997; 26:1658-66.
5. López-Jiménez J, Cancelas JA, García-Laraña J, Sastre JL, Cervero C, Zamora C, et al. Post transfusion hepatitis after induction chemotherapy in acute non lymphoblastic leukemia: implications for long-term management and outcome. *Transfusion* 1995; 35:313-8.
6. Myers B, Irving W, Hollingsworth R, Readett D, Lilleyman JS, Dolan G. Hepatitis C virus infection in multi-transfused children with haematological malignancy. *Br J Haematol* 1995; 91:480-2.
7. Koibushi T, Nakamura T, Nojiri N, Nakajima K, Juji T, Iwamoto A. Shifting in genotype trends of hepatitis B virus in Japan. Abstracts of the IDSA 38<sup>th</sup> Annual Meeting. New Orleans 2000; 14.
8. Luna-Harera J, Martinez-Cabrera G, Parra-Maldonado R, et al. Use of receiver operating characteristic curves to assess the performance of a microdilution assay for determination of drug susceptibility of clinical isolates of *Mycobacterium tuberculosis*. *Eur J Clin Microbiol Infect Dis* 2003; 22:21-7.
9. Sherbini El A, Mohsen SA, Dip ZS, Ghany Al A, Moneib A, Abaza CA. Hepatitis B virus among schoolchildren in an endemic area in Egypt over a decade: Impact of hepatitis B vaccine. *Am J of infect Cont* 2006; 34:600-2.
10. Banatvala JE, Damme PV. Hepatitis B vaccine: do we need boosters? *J Viral Hepat* 2003; 10:1-6.
11. Lee SD, Lo KJ, Tsai YT, Wu JC, Wu TC. HBsAg carrier infants with serum anti-HBc negativity. *Hepatology* 1989; 9:102-4.
12. Raimondo G, Pollicino T, Squadrito G. Clinical virology of hepatitis B virus infection. *J Hepatol* 2003; 39:26-30.
13. Minuk GY, Sun DF, Uhanova J, Zhang M, Caouette S, Nicolle LE, et al. Occult hepatitis B virus infection in a North American community-based population. *J Hepatol* 2005; 42:480-5.
14. Zerbini A, Pilli M, Boni C, Fiscaro P, Penna A, Di Vincenzo P, et al. The characteristics of the cell-mediated immune response identify different profiles of occult hepatitis B virus infection. *Gastroenterology* 2008; 134:1470-81.
15. Michalak TI, Mulrooney PM, Coffin CS. Low doses of hepadnavirus induce infection of the lymphatic system that does not engage the liver. *J Virol* 2004; 78:1730-8.
16. Ferraro D, Pizzillo P, Di Marco V, Vultaggio A, Iannitto E, Venezia G, et al. Evaluating the risk of hepatitis B reactivation in patients with haematological malignancies: is the serum hepatitis B virus profile reliable? *Liver Int* 2009; 29:1171-7.