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Detection of Anti-HBc and HBV-DNA in Blood Donors Negative for Hepatitis B Virus Surface Antigen in Mosul Central Blood Bank-Iraq

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Abstract: Though sensitive screening assays for detection of hepatitis B virus surface antigen (HBsAg) are available, occasional cases of post-transfusion hepatitis B virus infection (PTH) still occur. The present study was undertaken to assess the prevalence of anti-hepatitis B core (anti-HBc) positivity and presence of HBV-DNA in serum sample of healthy blood donors negative for both HBsAg and anti-HCV antibody in Mosul Central Blood Bank-Iraq. Since anti-HBc detection is mandatory in Iraq. Two thousands serum samples negative for both HBsAg and anti-HCV collected from healthy blood donors were tested for the presence of anti-HBc antibody. All samples positive for anti-HBc antibody were then investigated for determination of anti-HBc titre, anti-HBs titre, HbeAg and anti-HBe antibody by enzyme immunoassay (EIA). Every sample that tested negative for HBsAg but positive for anti-HBc alone or in with other serological markers was also examined for the presence of HBV-DNA by polymerase chain reaction (PCR). Two thousands samples tested, 20 (1%) blood samples were found to be positive for anti-HBc. HBV DNA was detected among 16 of 20 (80%) anti-HBc positive specimens. The mean level of viral load were 3500 copy/ml. Anti-HBsAg (50%), HBeAg (25%), Anti-HBeAg (30% v) were detected among anti-HBc positive samples. The liver function test results were all in normal range except in 4 of 16 HBV-DNA positive subjects. The mean levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in HBV-PCR positive subjects were 14 IU/l and 23.7 IU/l respectively. Anti-HBc antibody should be tested routinely on volunteers blood donors and if the sample found positive regardless of anti-HBs titre, the blood should be discarded.

Key wards: Hepatitis B virus, HBsAg, anti-HBc, ALT.

كشف مضادات دنا HBC و HVB في عينات دم متبرعين غير حاوية على المستضد السطحي لراشح التهاب الكبد B في مصرف الدم المركزي في الموصل

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مصرف الدم المركزي – الموصل

الخلاصة: على الرغم من وجود طرق حساسة للكشف عن المستضدات السطحية لراشح التهاب الكبد B الا انه حصل ان تم تشخيص وجود الراشح بعد التبرع بالدم. الدراسة الحالية هدفت لتقييم ايجابية وجود راشح التهاب الكبد B anti-HBC والمستضد HBV-DNA في حصول متبرعين دم اصحاء سالبين للمستضدات HBsAg و HCV في مصرف الدم المركزي في الموصل. استخدم في هذه الدراسة الفين من عينات المصل المأخوذة من متبرعين دم اصحاء. عزلت العينات الموجبة للمستضد anti-HBC وفحصت من اجل تحديد مستوى المستضدات anti-HBc titer و HBs و HBeAg و anti-HBe باستخدام الطريقة المناعية – الانزيمية (EIA). استخدام تفاعل سلسلة البوليمريز للعينات الموجبة لاي من المستضدات المفحوصة. من جميع العينات المفحوصة وجد بان هناك 20 (1%) فقط موجبة للمستضدات anti-HBc ووجد بان 16 عينة من هذه (80%) كانت موجبة للمستضد anti-HBsAg وقد بلغت نسخ الفايروس في هذه العينات 3500 نسخة/سم3. من ضمن العينات الموجبة للمستضد anti-HBc كان منها 050% موجبة ايضاً للمستضد anti-HBsAg و 25% موجبة للمستضد HBeAg و 30% موجبة للمستضد anti-HBeAg. كما بينت نتائج فحوصات عمل الكبد بانها طبيعية في مجمل الحالات باستثناء 4 عينات من 16 عينة موجبة للمستضد HBv-DNA حيث كان معدل مستوى الانزيم النين امينو ترانسفيريز (ALT) والاسبرتيريت امينو ترانسفيريز (AST) يساوي 14 وحدة عالمية / لتر و 23.7 وحدة عالمية / لتر على التوالي. بينت النتائج بأنه من المفيد استخدام المستضد anti-HBc في فحوصات الدم للمتبرعين من اجل الكشف عن الاصابة براشح التهاب الكبد الفايروسي بالاضافة للمستضدات الاخرى.

Introduction

The safety of blood products is one of the major issues in the area of transfusion medicine. Transmission of hepatitis B virus (HBV) infection through donated blood is more common than hepatitis C virus (HCV) infection (1:60000 vs. 1:103000)[1]. In spite of availability of sensitive screening assay for detection of hepatitis B virus surface antigen (HBsAg), occasional cases of post-transfusion hepatitis B virus infection (PTH) are common[2]. There are three possible explanations for false negative results in commercial assays. Blood donors infected with HBsAg mutants and those circulating low level of viral protein may escape detection by screening assay and therefore, may affect the safety of blood supply[3]. Another explanation is that virus variants yield sequences that are not recognized by the antibodies employed in the assays[4]. There are variants in other parts of the genome that down regulate the production of HBsAg[5]. Occasionally, a super infection with hepatitis C virus (HCV) may induce clearance of hepatitis B. This could be due to the dominant role of HCV in

eliciting an immune response[6]. Antibodies to hepatitis B core (HBc) antigen are marker of acute, chronic, or resolved HBV infection and remain detectable for life. These can be present in the absence of both HBsAg and anti-HBs antibodies, during the convalescent period following acute hepatitis B before the appearance of anti-HBs antibodies, or in patients who resolved infection but lost detectable anti-HBs antibodies. Anti-HBc is therefore detected in anyone who has been infected with HBV[7].

It has been demonstrated that some HBsAg negative individuals and those positives for anti-HBc continue to replicate HBV[8,9]. These findings suggest that recovery from acute hepatitis B virus infection may not result in complete virus elimination, but rather the immune system keeps the virus at a very low level. A positive correlation has been shown between anti-HBc titer and detection of HBV-DNA in serum samples of HBsAg negative individual[10].

Amis of Study

We undertook this study to assess the anti-HBc positivity and presence of HBV-DNA in serum sample of healthy blood donors negative for both HBsAg and anti-HCV .Since anti-HBc detection is not mandatory in Iraq, we evaluated whether anti-HBc could be adopted as a screening assay for the donated blood.

Materials and Methods

Two thousand blood samples were collected from blood donors negative for HBsAg and Anti-HCV in Mosul Central Blood Bank-Iraq.

Enzyme linked Immuno Sorbent Assay (ELISA)

All serological tests were performed following manufacturers' instructions, HBsAg, anti-HBs, anti-HBc-IgM, HBeAg and anti- HBe antibody were measured using commercially available one step enzyme immunoassay technique (Bio kit, Spin). Anti-HCV (third generation assay) was measured by enzyme immunoassay (EIA) according to manufacture instruction (Bio kit, Spain).

Using Bio kit anti-HBc test, the presence or absence of anti-HBc antibodies was determined by comparing for each sample the recorded absorbance with that of the calculated cut-off value. Samples with an optical density less than the cut-off values were considered to be negative. However, those just below the 10 per cent cut-off value were retested in duplicate according to manufacture instruction. Samples with optical density higher than, or equal to, the cut-off value were considered to be positive and retested in duplicate before the final interpretation.

Bioelisa anti-HBs 3.0 was used for the detection and/or quantitative determination of antibody to hepatitis B surface antigen. A mixture of HBsAg of the ad subtype and ay subtype of human origin has been used for anti-HBs antibody detection.Samples showed an OD below or equal the cut-off value was considered to be negative. For samples showed an OD within the range of the cut-off value,the HBs antibody concentration was determined from the standard curve.

Every sample that tested negative for HBsAg but positive for anti-HBc alone or in combination with other serological markers was also examined for the presence of HBV-DNA assay.

Real time polymerase chain reaction (RT-PCR)

- DNA extraction.
  HBV DNA extraction test were preformed using instant virus DNA kit (analytic jena, Germany).
- 200 µl lysis solution TLS, 200µl of serum and 25µl proteinase k had been add, mix vigorously by pulsed vortexing for 10 second, incubate at 50°C for 15 minutes.
- 400µl binding solution TBS had been add to the lysed sample, mix by vortexing several times.
- Apply the sample to the spin filter located in a 2.0µl receiver tube. Close the cap and centrifuge at 10.000xg for 1 minute. Discard the receiver tube with the filtrate, place the spin filter into a new 2.0 ml receiver tube.
- Open the spin filter and add 500 µl washing solution HS, close the cap and centrifuge at 10,000xg for I minute, discard the receiver tube with filtrate. Place the spin filter in to a new 2.0ml reciver tube.
- Open the spin filter and add 650µl washing solution MS, close the cap and centrifuge at 10.000xg for 1 minute. Discard the receiver tube with the filtrate. Place the spin filter in to a new 20.ml receiver tube.
- Centrifuge at max speed for 2 minutes to remove all traces of ethanol. Discard the 2.0 ml receiver tube.
- Place the spin filter into a 1.5 ml elution tube. And open the cap of the spin filter and add 100 µl pre-heat elution buffer (50°C).
- Incubate at room temperature for 2 minutes. Centrifuge at 6,000xg for 1 minute.

Real time RCR tests

- Quantitative of HBV DNA.
- HBV DNA quantitation test were performed using HBV DNA (primer design kit) and used real times PCR detection (9 tower for analytic gene company, Germany).
- Prepare a reaction mix according to the table below.

Component	Volume
2x precision TM Master Mix	5 µl
pathogen primes probe Mix	0.5 µl
External extraction control primer/probe Mix	0.5 µl
RNAse/DNAse free water	1.5 µl
Final volume	7.5 µl

- Pipette 7.5 µl of this mix into each well.
- Pipette 2.5 µl of DNA template and standard in to each well and negative control for negative control wells use 2.5 µl of RNA se/DNAse free water.
- Preparation of standard curve dilution series.
- Pipette 900 µl of RNA se/DNAse free water into 5 tube and label 2-6.
- Pipette 100 µl of positive control template in to tube 2.
- Vortex thoroughly.
- Chang pipette tip and pipette 100 µl from tube 2 into tube 3.
- Vortex thoroughly
- Repeat steps 4 and 5 to complete the dilution series.

Amplification protocol

	Step	Time	Tempreture
	Enzyme activation	10 mints	95 °C
50 cycles	Denaturation	5 sc	95°C
	Data Collection	25 sc	60°C

Interpretation results according the instruction manual

Biochemical Tests

For all the PCR positive samples biochemical factors including aspartate aminotransferase (AST), alanin aminotransferase(ALT), alkaline phosphatase and total bilirubin werealso performed using standard methods.

Results

Serological results

The cut-off points between positive and negative for detection of Anti-HBc antibody in serum samples was found to be OD450/620(nm) 0.62. Twenty out of 2000 (1%) of the HBsAg negative blood samples were found to be positive for Anti-HBc antibody was shone in (table 1).

Table (1): Percent of Anti-HBcIgM& IgG among negative HBsAg blood donors

Total No. blood donors	Anti-HBcIgM&IgG	
	Positive%	Negative%
2000	20 (1%)	1980(99%)

The cut-off points between positive and negative for detection of anti-HBs antibody in serum samples was found to be OD450/620(nm) 0.20. Overall, 10 out of 20 (50%),5 out of 20(25%) ,6 out of 20(30%) anti-HBc antibody positive subjects were found positive for anti-HBs antibody (>10 mlU/ml), HBeAg , Anti-HBeAg respectively was shown in (table 2).

Table (2): Percent of Hepatitis B serology marker among anti-HBc positive blood donors

Total No. of Anti-HBc Positive	HBV marker		
	Anti-HBsAg	HBeAg	Anti-HBeAg
20	10(50%)	5(25%)	6(30%)

Molecular findings

HBV DNA was detected among 16 out of 20 (80%) anti-HBc positive specimens was shown in (table 3).

Table (3): Percent of HBV DNA positive among anti-HBc positive blood donors

Total No. of Anti-HBc Positive	HBV DNA	
	Positive%	Negative%
20	16 (80%)	4(20%)
Mean viral load	More than 3500 copy/ml	NOT detected

Biochemical Markers

Table (4) was shown the liver function tests (LFT) aspartate aminotransferase (AST), alanineaminotransferase (ALT), bilirubin, and alkaline phosphatase, were performed on HBV-PCR positive samples. LFT results were in normal range in all except in 4 in whom AST was more than normal (i.e., >33 IU/l). The mean levels of ALT and AST in HBV-PCR positive subjects were 14 ± 5.0 IU/l and 23.7 ± 9.9 IU/l respectively. Because our anti-HBc positive blood donors had normal ALT levels.

Table (4): Percent of HBV DNA positive blood donors were altitude height liver function test

Total No. of HBV DNA Positive	Liver Function test			
	ALT N.R (Up to12 U/l)	AST N.R (Up to12 U/l)	ALP (30-85 U/l)	Bilirubin (7-15 mmol/l)
16	0%	25%	0%	0%

Discussion

The prevalence of anti-HBc positivity and presence of HBV DNA in sera of healthy blooddonors negative for both HBsAg and anti-HCV antibody was investigated. At present, HBsAg detection is the major and only diagnostic screeningtest for HBV infection in blood transfusion centers in Iraq. We found about one per cent of HBsAg negative donated blood positive for anti-HBcAg, which was lower than that reported by (17)in SaudiArabia (16.4%). They had however, not mentionedwhat percentage of the donated blood also waspositive for anti-HCV antibody.

Most studies on occult HBV infection have reported higher rates of HBV-DNA detection in liver or peripheral mononuclear cells compared with serum or plasma(18). In our study population the overall prevalence of occult HBV infection (DNA in serumsample) in healthy blood donors was one per cent ,among anti-HBc positive individuals.

Nevertheless, no association was found between the presence of anti-HBc and positivity of HBV-DNA.The frequency of post-transfusion hepatitis (PTH)is apparently due to the fact that HBsAg is circulating at undetectable levels for current screening assays.However, screening test for anti-HBc antibody can eliminate some of these donor units2. HBV is nothighly endemic in Iraq and it may be practical to introduce anti-HBc screening in blood banks. Sincewe have not tested anti-HBc negative samples for the presence of HBV DNA, whether removal of anti-HBc positive units would lead to elimination of PTHB cannot be ascertained.

According to hypothesis offered by (19)we divided our blood donor population into two specific groups: HBsAg positive and HBsAg negative individuals. Sero negative subjects were further divided into two sub-groups: anti-HBc positive and anti-HBc negative individuals. The anti-HBc positive subgroup which consisted of one per cent of blooddonor population, can be further divided into two groups, with anti-HBsAg(50%) and without anti-HBs (50%)individuals, respectively. It has been reported thatHBV-DNA is found in HBsAg negative, anti-HBc positive and anti-HBs positive donors(20,21).

In our study the highest rate of HBV-DNA was detected in individuals positive for anti-HBc but negative for anti-HBs antibody (50%). These individuals may have recovered from previous infection but have persistent low level of HBV.Symptomatic hepatitis B has never been observed inimmunized persons who develop anti-HBs titer more than 10 IU/ml. Some vaccine recipients may develop e anti-HBc, which is indicative of HBV infection; butthey usually do so in the absence of disease11. Theprotective anti-HBs antibody is normally directedagainst the "a" determinant of HBsAg. In some cases,the antibodies are directed against one of thedeterminants other than "a" and are unable to neutralize the circulating virion. These cases shouldtherefore be regarded as chronic infection (22). Detection of HBV-DNA in anti-HBc and anti-HB s positive individuals may be due to chronic persistentHBV infection.

The exclusion of anti-HBc positive donors is impractical in countries where HBV infection isprevalent and more than 20 per cent of the populations anti-HBc positive(23). However, in our study onlyoneper cent of blood donor population was found to be positive for anti-HBc. In organ transplant, anti-HBc test can be used as an indicator for HBV infection. In case of positive results, sera should besubjected to PCR to detect HBV DNA. In case ofpositive results, organ should not be used, orrestricted to emergencies only.

In conclusion, anti-HBc antibody should be testedroutinely on blood donor volunteers and if the foundpositive regardless of anti-HBs titer, the blood should be discarded. Further testing for HBV-DNA wouldbe beneficial to follow up the blood donor for HBV infection.

References

1. Schreiber GB, Busch MP, Klein man SH, Korelitz JJ. Therisk of transfusion-transmitted viral infections. The Retrovirus Epidemiology Donor Study.N Engl J Med 2007;334: 1685-90.

2. Saraswat S, Banerjee K, Chaudhury N, Mahant T,Khandekar P, Gupta RK, et al. Post-transfusion hepatitis type B following multiple transfusions of HBsAg-negative blood.J Hepatol2006; 25: 639-43.

3. Jongerius JM, Wester M, Cuypers HT, van Oostendorp WR,Lelie PN, van der Poel CL, et al. New hepatitis B virusmutant form in a blood donor that is undetectable in severalhepatitis B surface antigen screening assays. Transfusion 2008; 38 : 56-9.

4. Carman WF. The clinical significance of surfaceantigen variants of hepatitis B virus.J Viral Hepat2009;4(Suppl 1) : 11-20.

5. Carman WF, Mimms LT. Pre-S/S gene variants of hepatitisB virus. In: Rizzetto M, Purcell RH, Gerin JL, Verne G,editors. Viral hepatitis and liver disease. Torino, Italy:Edizioni Minerva Medica; 2009 p. 108-15.

6. Tsai S, Liaw Y, Yeh C, Chu CM, Kuo GC. Cellular immuneresponses in patients with dual infection of hepatitis

B andC viruses: dominant role of hepatitis C virus. Hepatology2009; 21 : 908-12.

7. Lee WM. Hepatitis B virus infection. N Engl J Med 2010;337: 1733-45.

8. Ben Ayed M, Triki H, Cointe D, Karray H, Masmoudi MA,Dellagi K, et al. The isolated presence of anti-HBcantibodies: prevalence and interpretation based on the resultsof viral DNA research and anti-HBs antibodies measurementafter vaccination. Ann BiolClin (Paris) 2010; 59 : 53-60.

9. Gomes SA, Yoshida CF, Niel C. Detection of hepatitis Bvirus DNA in hepatitis B surface antigen-negative serum bypolymerase chain reaction: evaluation of different primerpairs and conditions. ActaVirol2010; 40 : 133-8.

10. Iizuka H, Ohmura K, Ishijima A, Satoh K, Tanaka T,Tsuda F, et al. Correlation between anti-HBc titers and HBVDNA in blood units without detectable HBsAg. Vox Sang2009; 63 : 107-11.

11. Zervou EK, Dalekos GN, Boumba DS, Tsianos EV.Value of anti-HBc screening of blood donors for preventionof HBV infection: results of a 3-year prospective study inNorthwestern Greece. Transfusion 2001; 41 : 652-8.

12. Malekzadeh R, Khatibian M, Rezvan H. Viral hepatitis inthe world and Iran. J Iranian Med Council 2007; 15 :183-200.

13. Merat SH, Malekzadeh R, Rezvan H, Khatibian M.Hepatitis B in Iran. Arch Iranian Med 2008; 3 : 192-201.

14. Amini S, Mahmoodi MF, Andalibi S, Solati AA .Seroepidemiology of hepatitis B, delta and humanimmunodeficiency virus infections in Hamadan province,Iran: a population based study. J Trop Med Hyg2009; 96 :277-87.

15. Kwok S, Higuchi R. Avoiding false positives with PCR.Nature 2009; 339 : 237-8.

16. Gish RG, Lau JY, Brooks L, Fang JW, Steady SL,Imperial JC, et al. Ganciclovir treatment of hepatitis B virusinfection in liver transplant recipients. Hepatology2006;23: 1-7.

17. Bernvil SS, Andrews V, Kuhns MC, McNamara AL. HepatitisB core antigen antibody as an indicator of a low grade carrierstate for hepatitis B virus in a Saudi Arabian blood donorpopulation. TransfusSci2007; 18 : 49-53.

18. Torii N, Hasegawa K, Joh R, Hayashi N. Configuration andreplication competence of hepatitis B virus DNA inperipheral blood mononuclear cells from chronic hepatitisB patients and patients who have recovered from acute selflimitedhepatitis. Hepatol Res 2007; 25 : 234-43.

19. Brechot C, Thiers V, Kremsdorf D, Nalpas B, Pol S,Paterlini-Brechot P. Persistent hepatitis B virus infection insubjects without hepatitis B surface antigen: clinicallysignificant or purely "occult"? Hepatology2010; 34 :194-203.

20. Hennig H, Puchta I, Luhm J, Schlenke P, Goerg S,Kirchner H. Frequency and load of hepatitis B virus DNAin first-time blood donors with antibodies to hepatitis B coreantigen. Blood 2009; 100 : 2637-41.

21. Roche B, Feray C, Gigou M, Roque-Afonso AM,Arulnaden JL, Delvart V, et al. HBV DNA persistence 10years after liver transplantation despite successful anti-HBSpassive immunoprophylaxis. Hepatology2008; 38 : 86-95.

22. Seddigh-Tonekaboni S, Waters JA, Jeffers S, Gehrke R,Ofenloch B, Horsch A, et al. Effect of variation in thecommon "a" determinant on the antigenicity of hepatitis Bsurface antigen. J Med Virol2008; 60 : 113-21.

23. Nandi J, Benerjee K. Detection of hepatitis B virus DNAin donor blood by the polymerase chain reaction.Natl Med J India2008;5:5-7.