

# Molecular and serological detection of occult hepatitis B virus among healthy hepatitis B surface antigen-negative blood donors in Malaysia.

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## Abstract

**Background:** Occult hepatitis B infections are becoming a major global threat, but the available data on its prevalence in various parts of the world are often divergent.

**Objective:** This study aimed to detect occult hepatitis B virus in hepatitis B surface antigen-negative serum using anti-HBc as a marker of previous infection.

**Patient and methods:** A total of 1000 randomly selected hepatitis B surface antigen-negative sera from blood donors were tested for hepatitis B core antibody and hepatitis B surface antibody using an ELISA and nested polymerase chain reaction was done using primers specific to the surface gene (S-gene).

**Results:** Of the 1000 samples 55 (5.5%) were found to be reactive, of which 87.3% (48/55) were positive for hepatitis B surface antibody, indicating immunity as a result of previous infection however, that does not exclude active infection with escaped mutant HBV. Nested PCR results showed the presence of hepatitis B viral DNA in all the 55 samples that were positive for core protein, which is in agreement with the hepatitis B surface antibody result.

**Conclusion:** This study reveals the 5.5% prevalence of occult hepatitis B among Malaysian blood donors as well as the reliability of using hepatitis B core antibody in screening for occult hepatitis B infection in low endemic, low socioeconomic settings.

**Keywords:** Hepatitis B, surface antigen, core antibody, polymerase chain reaction, occult hepatitis B infection

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## Introduction

Hepatitis B surface antigen (HBsAg) is the main diagnostic marker for hepatitis B infection and for screening of donated blood. Escaped mutants might be as a

result of post transcriptional effect of the mutation on HBsAg expression as described previously<sup>1,2</sup>. In addition, it is a known fact that surface antigen mutation reduced effectiveness of diagnostics and allowed for humoral immune escape thereby reducing vaccination effectiveness<sup>3</sup>. While occult hepatitis B is characterised by undetectable levels of surface antigen, but detectable levels of viral DNA<sup>4</sup>. Occult hepatitis B infection is becoming a major global threat due to: (a) the effect on the health of children born to carrier mothers despite the presence of passive immunoglobulin at birth<sup>5-7</sup>; (b) immune escape of current vaccines<sup>8,9</sup>; and (c) spread through blood and blood products in post transfusion infection, organ dona-

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tion, and sexual transmission<sup>10,11</sup>. There are several concerns surrounding occult hepatitis B infection in clinical settings, including reduced sensitivity to available diagnostic tests, lack of immunity following vaccination with non-mutant HBV variants (vaccine escaped mutant), and failure of passive immunization with HBV immunoglobulin G<sup>12</sup>. The failure of diagnostic assays to detect HBV poses a major risk to recipients of blood transfusions or organ donations<sup>13,14</sup>. Since hepatitis B surface mutants are stable and can be spread through either vertical or horizontal transmission<sup>15</sup>, both a highly sensitive molecular method and an affordable and reliable serological test are required for the diagnosis of occult hepatitis B infection. Therefore, this study aimed at detecting occult hepatitis B among hepatitis B surface antigen negative blood donors using anti-HBc as a marker and compared these results to detection of the hepatitis B S-gene by nested PCR.

## **Methodology**

### **Sample collection**

One thousand serum samples were randomly selected from a pool of hepatitis B surface antigen-negative sera from anonymized leftovers from initial blood testing of blood donors at the National Blood Centre in Kuala Lumpur, Malaysia. Systematic random sampling random selection of first sample systematic selection through a pre-determined interval  $N/n = nth$  term using online sampling software (<https://www.randomizer.org/>)

### **Anti-HBc testing**

Hepatitis B core antibodies were tested for by using a commercially available ELISA kit (DRG International Inc. New Jersey, USA). Serum samples were diluted in wash solution (1:5). Then, 50  $\mu$ l of the diluted serum was added to the micro wells, which are coated with purified recombinant hepatitis B core antigen according to the manufacturer's instructions. The TECAN Magellan ELISA reader version 6.4 was used to measure the absorbance at 450 nm and the results were interpreted based on the optical density (OD).

### **DNA extraction**

HBV DNA was extracted from 200  $\mu$ l of serum using a QIAampBlood mini kit (Qiagen, Hilden, Germany) according to the manufacturer's instruction. Briefly, 20  $\mu$ l of protease was added to the serum in a 1.5 ml tube. Then, 200  $\mu$ l of cell lysis solution (AL buffer) was added,

vortexed, and incubated for 10 min at 56°C. The DNA was eluted using 50  $\mu$ l of elution buffer and stored at -20°C until further analysis.

### **Nested Polymerase Chain Reaction (PCR)**

Two sets of published primers were used to amplify the hepatitis B S-gene from the DNA extracted from the serum samples<sup>16</sup> with the first round of PCR using the outer primers, which amplify a 916 bp segment which includes the S-gene, under the following conditions: 30 cycles of 94°C for 5 min, 94°C for 30 sec, 63.8°C for 30 sec, and 72°C for 60 sec, followed by a final extension at 72°C for 10 min. The second round of PCR was performed using the inner primers, which amplify the 656 bp S-gene amplicon of the surface antigen under the following conditions: 30 cycles of 94°C for 5 min, 94°C for 30 sec, 63.8°C for 30 sec, and 72°C for 60 sec, with a final extension at 72°C for 8 min. A 50  $\mu$ l reaction mixture containing 1  $\mu$ l of DNA sample, 25  $\mu$ l PCR pre mixed solution (Promega, USA), 1.25  $\mu$ l of forward and reverse primers (final concentration 0.5  $\mu$ M), and 21.5  $\mu$ l of nuclease free water. This reaction was used for both the first and second rounds of PCR amplification using a thermal cycler (Bio-Rad) as described previously<sup>16</sup>. Genomic hepatitis B DNA and no DNA template control were used as positive and negative controls, respectively. The PCR products were analysed by gel electrophoresis using 1.5% (w/v) agarose gel (Seakem LE, USA). PCR products were visualised with UV illuminator and the product size was determined by comparison to the DNA molecular marker (GeneDireX). Sample bands corresponded to the size of the positive control bands. To ensure that the primers used in this study specifically amplified the S-gene of HBV, they were tested against a DNA sample from an HBV-positive serum that is positive for anti-Hbc, anti-HBe, and negative anti-HBs. The absolute detection limit of the nested PCR was determined by a 10-fold dilution of the standard HBV-positive serum and the detection limit was found to 5 copies per  $\mu$ L.

### **Data analysis**

The data from the findings was analyzed using SPSS statistical version 20 (Chicago, Illinois). The percentage of occult hepatitis B infection prevalence was then calculated.

### **Ethical approval**

Ethical approval to carry out this study was obtained

from the Institute for Medical Research (IMR) in Malaysia with reference No. NMRR-12-469-11762 and the Faculty of Medicine and Health Sciences Ethical Committee, Universiti Putra, Malaysia (Ref: UPM/.FPSK/100-9/2-MJKEtikaPen)..

### Results

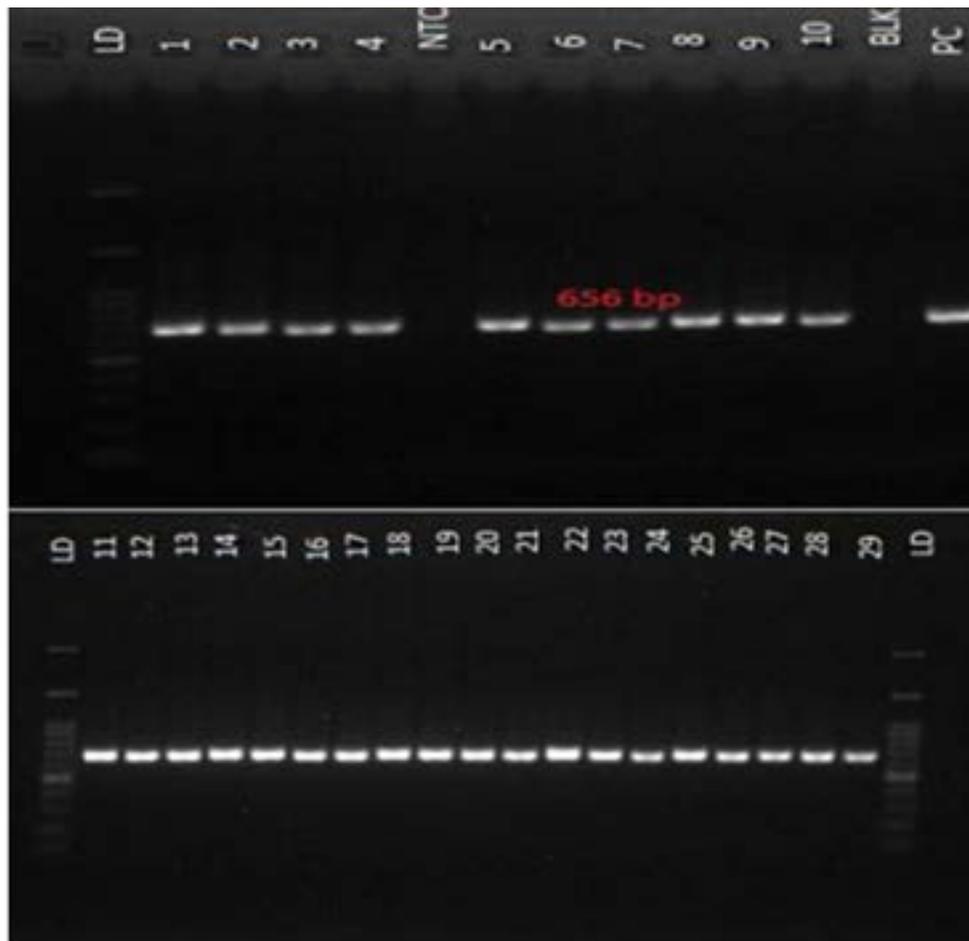
Of the 1000 samples that were screened, 5.5% (55/1000) were found to be anti-HBc-positive, all of which were also positive for HBV DNA. Additionally, 84.8% (848/1000) of the samples were anti-HBs-positive (Table 1).

**Table 1 Serological markers and molecular detection of HBV DNA.**

	<b>HBsAg</b>	<b>Anti-HBs</b>	<b>Anti-HBc</b>	<b>Nucleic Acid Amplification Test</b>	<b>Polymerase Chain Reaction (PCR)</b>
Positive	0	848	55	0	55
Negative	1000	152	945	1000	945
<b>Total</b>	<b>1000</b>	<b>1000</b>	<b>1000</b>	<b>1000</b>	<b>1000</b>

The results of the nested PCR show the presence of a 656 bp fragment, indicating a positive result with the no template and blank showing no band as such excluding chances of contaminant from the PCR reagents. Positive control also showed a specific band that is similar in size with the positive samples confirming its positivity (Figure 1).

Of the 5.5% (55/1000) of the samples that were positive for occult hepatitis B, 87.3% (48/55) were also hepatitis B surface antibody-positive, indicating immunity as a result of previous infection<sup>17</sup>, while 12.7% (7/55) were negative for the presence of the antibody. However, none of these samples were hepatitis B surface antigen-positive.



**Figure 1: Positive nested PCR result from hepatitis B surface-negative, core-positive serum samples of healthy volunteers. A 656 bp DNA product was amplified using primers specific for hepatitis B S-gene. All lanes show a PCR product of the expected size. LD: 100 bp molecular weight marker; PC: Positive control; and NTC: Non-template control (NTC). 160x161mm (72 x 72 DPI)**

### Discussion

Hepatitis B is of global health importance with about 350 million chronic carriers worldwide, constituting a major global threat. According to World Health Organization (WHO), Western Pacific region accounts for about 50% of the world's chronic hepatitis B infection<sup>18</sup>. Likewise, in Malaysia the prevalence of HBV among children decreased to 0.4% from 3.0% prior to introduction universal infant hepatitis B vaccination<sup>19</sup>. However, the prevalence in general population ranges from 1.5 to 9.8% but reported to be lower (0.4%) among repeated blood donors<sup>20</sup>. In this study HBV DNA was detected in HBsAg-negative serum using nested PCR, which is in line with previous studies that demonstrated the same result in peripheral blood mononuclear cells, serum, and liver samples<sup>21-24</sup>.

Nested PCR approach improves the sensitivity of the PCR assay by avoiding artefacts and non-specific binding associated with the conventional PCR method as shown in this study. In serum, HBV DNA levels are less than 104 copies/mL<sup>25</sup>, which is considerably lower than those that are HBsAg-positive<sup>22,23</sup>. The use of NAT in detecting HBV has been a debatable issue, since the viral load in occult infection is usually very low, thereby reducing the effectiveness of NAT testing as shown in this study where NAT and HBsAg negative sera of blood donors were found to be HBV DNA positive by nested PCR (Table 1). Therefore, there is a need for a more sensitive HBsAg system that is capable of detecting a very low HBsAg level and S gene mutants in order to reduce the risk of transfusion and organ transplant associated hepatitis B infection.

Our finding revealed 5.5% prevalence of anti-HBc among healthy blood donors, which is higher than what was reported in Italy<sup>26</sup>, Egypt<sup>27</sup>, Germany, UK<sup>28</sup> and USA<sup>29</sup>, but less than what was reported in Korea<sup>30</sup> and Greece<sup>31</sup>. This study revealed a 100% prevalence of occult hepatitis in hepatitis B core antibody-positive healthy individuals. This is similar to the frequency reported in China<sup>32</sup>. Additionally, other studies have reported finding occult hepatitis B virus more frequently in individuals with anti-HBc-positive serology than in those with anti-HBc-negative serology<sup>33,34</sup>. On the other hand, a prevalence of 33.3% was reported in North East China<sup>32</sup>, as compared to 0.11% in Taiwan<sup>35</sup>. The risk transmission depends on presence of anti-HBs and viral dose; viral load: copies/ml x vol. of plasma, the higher the viral load, and the higher the chance of transmission. Anti-HBs; in donor or passive also influence the rate of transmission. Immune status of the recipient is also an important determinant of its transmission<sup>36</sup>. In a look back data from Japan and Hong Kong: transmission of HBV from donors with OHBI was approximately 3%<sup>36</sup>. In Malaysia NAT has been implemented in the National Blood Centre since 5th November 2007 and was used to test the donated blood from others catchment hospitals such as Klang, Kajang, Kuala Kubu Baru, Termerloh, Bentong, Raub, Kuala Lipis and Seremban. There was sufficient evidence to support that the effectiveness of using NAT blood screening test to detect HBV infection in donated blood. Hence, reducing the risk of transfusion associated HBV infection. However, it is not cost-effective to implement it in the low endemic setting as such anti-HBc can be considered in such setting.

The risk of transmitting occult hepatitis B infection depends on presence of copies of HBV DNA in the plasma and the volume of the plasma transfused therefore, the higher the viral load in the transfused blood the higher the chance of infectivity<sup>37</sup>. The presence of high anti-HBs in the donors blood also influence the rate of infectivity<sup>38</sup>. Immune status of the recipient and donors also play an important role in determining infectivity<sup>36</sup>. Recent infectivity data indicate transmission rate 3.8%<sup>38</sup>, but the rate is higher in unvaccinated recipients of occult hepatitis B blood or blood products. Blood products from donors with occult hepatitis B carry a high risk of HBV transmission by transfusion. This may justify safety measures such as anti-HBc and HBV nucleic acid test screening depending on epidemiology. Anti-HBc may be

used in < 2% to 4% prevalence, while, HBV-NAT in high endemic areas<sup>37</sup>. Occult hepatitis B infections have significant clinical importance since they can become reactivated when the immune system is suppressed and can be transmitted through blood or blood product transfusion, organ transplant, and sexual intercourse. It may also enhance the progression of liver fibrosis and, subsequently, hepatocellular carcinoma.

## Conclusion

This study revealed a 5.5 % prevalence of occult hepatitis in asymptomatic, healthy blood donors. We found that 100% of samples that were positive for hepatitis B core antibodies were also positive for HBV DNA, thus indicating the reliability of hepatitis B core antibody in screening for occult hepatitis B infection, especially in low socio-economic, low endemic settings. Therefore, complimenting screening methods like anti-HBc and NAT based on epidemiology may be of important benefit in decreasing the risk associated with post transfusion HBV infection as such anti-HBc may be used in < 2% to 4% prevalence, while, HBV-NAT in high endemic areas. The detection of hepatitis B viral DNA in both the so-called “immune as a result of natural infection” and the isolated anti-core group indicates the need for further study and reclassification.

## Authors' contributions

SA Hudu and Z Sekawi conceived the idea for this project. R Hassan and SA Hudu collected serum sample for this study. SA Hudu and M I Saeed performed the experiments. SA Hudu wrote the first draft of the manuscript, which was substantially revised by Z Sekawi, YA Malik, NM Taib, NS Harma, MI Saeed, AS Alshrari and R Hassan. All authors read and approved the final manuscript.

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## Competing interests

We declare no competing interests.

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