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Molecular Detection of Hepatitis B Virus Among HBsAg Non-Reactive Blood of Donors

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Abstract

Despite all blood donations being tested routinely for HBsAg as a clinical marker of transmissible HBV, cases of post-transfusion hepatitis B virus infection are still being reported because molecular studies using Polymerase Chain Reaction (PCR) are not routinely available for Transfusion Transmissible Infection (TTIs) testing. In this study, we sought to use the PCR technique to re-screen donated blood that had already been proven to be HBsAg non-reactive with rapid diagnostic testing and ELISA. One hundred and eighty-five samples were obtained from a proportion of the blood deposited at the blood bank of the Federal Medical Center, Birnin-Kebbi, Nigeria. Socio-demographic parameters such as age group, status, ethnicity, occupation, and group PCV were obtained from donors' records. Nested PCR was employed to detect HBV DNA. Furthermore, genotyping was performed to determine HBV genotypes in the positive samples using PCR with genotype-specific primers. Of the 185 donors, it was observed that five (2.7%) of the population were positive for HBV. HBV is more common among people aged 18–30, singles, Hausa, and self-employed. In addition, the five positive samples were of genotype E. This study suggests the need to complement antibody-based tests with DNA testing for effective HBV screening and consequent safe transfusion.

Keywords: *Hepatitis B Virus; HBsAg; PCR; DNA testing*

Introduction

Hepatitis B, otherwise known as serum hepatitis, is a liver illness caused by a double-stranded DNA virus from the Hepadnaviridae family, which is the predominant etiological agent in approximately 75% of chronic liver diseases worldwide (Oladele *et al.,* 2013). In transfusion medicine, hepatitis B virus (HBV) infection via blood transfusion is a significant problem. Blood transfusions could be a major route of infection transmission, especially if donor blood is not checked for HBV DNA (WHO, 2000). Over the last four decades, regular hepatitis B surface antigen (HBsAg) screening of blood donors has lowered the incidence of transfusion-transmitted hepatitis B. (Nna *et al.,* 2014). Even though blood donations are routinely tested for HBsAg, a clinical indication of transmissible HBV,

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molecular investigations employing Polymerase Chain Reaction (PCR) are not commonly available for Transfusion Transmissible Infection (TTIs) testing, cases of post-transfusion hepatitis B virus infection continue to be reported (Adoga *et al.,* 2010; Osuji *et al.,* 2021; Salawu *et al.,* 2010). Furthermore, laboratory testing or methods that might identify hidden hepatitis B virus infection and prevent its transmission from apparently healthy donors to recipients are not included in blood banking practices (Salawu *et al.,* 2010).

Occult (hidden) hepatitis B virus infection (OBI) is defined as the presence of HBV DNA in a patient's serum and liver that is negative or non-reactive to the hepatitis B surface antigen (Amadei *et al.,* 2010). However, studies using nucleic acid testing (NAT) on a large group of blood donors confirmed the phenomenon of OBI and formed the basis for mandatory NAT for transfused blood units in many developed countries; such a testing regimen has not been included in the testing protocol of many laboratories in resource-constrained countries (Osuji *et al.,* 2018). Molecular research has also improved the identification of occult hepatitis B, particularly during the window period (Ifeorah *et al.,* 2017). Thus, in this study, we sought to re-screen donated blood that had already proved to be HBsAg non-reactive with rapid diagnostic testing and ELISA using the PCR technique.

Methodology

Selection of Samples

Blood already donated for transfusion non-reactive for Hepatitis B in the hospital blood bank was selected over two months. Socio-demographic Information of donors was obtained from the blood bank record.

Ethical Consideration

Approval was sought from the Health and Research Ethics Committee of the Federal Medical Center, Birnin Kebbi, with reference number FMC/BK/HP/045/P/517/Vol III.

Sample Collection

Five millilitres were collected from donated blood slated for transfusion previously screened with the LabAcon Rapid Hepatitis Kit and Adaltis p24 ELISA Reagent (Adaltis s.r.l., Rome). All samples collected were centrifuged at 3500 rpm for about 10 min, not more than an hour after collection at room temperature. Sera and plasma were separated. Plasma was stored at -20 ºC in cryovials (Adoga *et al.,* 2010).

Inclusion and Exclusion Criteria

Only those whose blood has been non-reactive for Hepatitis B using the conventional method (RDT and ELISA test kits) were included in this study, excluding HBV positive. Those vaccinated against hepatitis B in the previous month were excluded from the study.

Detection of HBV DNA

DNA Extraction

According to the manufacturer's instructions, total DNA was extracted from the plasma using a commercial DNA Extraction Kit from Jena Biosciences (Jena, Germany). A cell lysis solution of 300 µl was pipetted into a clean 2 mL Eppendorf tube. Two hundred micro-litres of the plasma sample were added and mixed thoroughly to lyse the cells. A hundred microliters of protein precipitation solution were added to the cell lysate. It was vortexed vigorously for 20 sec to mix well, then centrifuged with Eppendorf 5920 maximum speed of 2500 rpm for 2 min. The precipitated protein formed a tight, dark pellet.

The supernatant from the step above was added into a clean 2 mL Eppendorf tube containing 300 µl of isopropanol (99%). It was inverted gently and centrifuged with Eppendorf 5920 at a maximum speed of 2500 rpm for 1 min. The DNA was visible as a white pellet. The supernatant was thrown away, and the content in the tube was cleared-out on clean absorbent paper. Three hundred microliters of washing buffer were added, and the tubes were inverted various times to cleanse the DNA pellet and then centrifuged with Eppendorf tub5920 at a maximum speed of 2500 rpm for one minute. The ethanol added was thrown away carefully and dried at room temperature for about 15 min. DNA rehydration was carried out by adding 50 µl of hydration solution. It was vortexed for 5 sec at medium speed to mix. The sample was incubated at 65 ºC for 30 min to accelerate rehydration, and the DNA was stored at 4 ºC.

PCR Amplification of the Pre-S region of the HBV genome

HBV DNA was detected using nested PCR with primers that target 300 base pairs of the Pre-S region of the HBV genome. The reaction was performed in a 25 µl reaction volume tube containing 5 µl of genomic DNA template, 1X complete buffer, and 0.4 pM of each primer pair: MF5'-TCGGATCCGGTATGTTTCCCGTTTGTCC-3', 979-5'-CAAAAGACCCACAATTCTTTGACATACTTTCCAAT-3', SF: 5'-GTGTCTTGGCCAAAATTCGCAGT-3'.

The cycling conditions used a modified method of Singh et al. (2014) and are as follows: initial denaturation step at 95 ºC for 5 min; then denaturation at 95 ºC for 30 sec; then annealing at 62 ºC for 1 min; then elongation at 72 ºC for 90 sec, then final elongation at 72 ºC for 10 mins; hold at 4 ºC.

Nested PCR

Nested PCR was carried out with the modified method of Singh et al. (2014) in which the product of the first-round PCR was diluted 100 times, and 2 µl of the diluted amplicon was used as the template for the second round of PCR in a 25 µl reaction volume containing 2 µl of template, 1X complete buffer, and 0.4 pM of each of primer pair. The cycling conditions are as follows: initial denaturation at 95 ºC for 5 min; then denaturation at 95 ºC for 30 sec; then annealing at 62 ºC for 1 min; then elongation at 72 ºC for 90 sec; final elongation at 72 ºC for 10 min; hold at 4 ºC

Detection of Amplified DNA

After the amplification, 15 µl aliquot of the amplified DNA was subjected to gel electrophoresis (Southern, 1979) on 1.5 % agarose in Tris-borate-EDTA buffer. Ethidium bromide was added to the gel mixture at 0.5 µg/mL concentration. The gel was then visualized using a UV transilluminator (Rajalakshmi, 2017)

DNA Genotyping

The specific genotypes of the detected HBV DNA were determined using PCR with genotype-specific primers. The primers will only amplify specifically targeted genotypes. Thus, primers specific to different genotypes were used. They are: PC1-5′-GGAGACCACCGTGAACGC-3′, RVA-5′-TTCTTCTTCTAGGGGACCTGCCTCAGTCC-3′, RVNONA-5'-TTCTTCTTCTAGGGGACCTGCCTCATCGT-3', FW1865-5'-CAAGCCTCCAAGCTGTGCCTTGGGTGGCTT-3'

Statistical Analyses

Data were analyzed with SPSS (v. 21.0) for windows (Inc. Chicago IL). Variables were compared using the Chi-Square test. Their frequency analyses were described using percentages.

Results

Figure 1 shows agarose gel electrophoresis of the amplified HBV gene of donors infected with HBV (lanes 1, 3, 6, 7, and 8 had the expected band size of 300 bp, which was positive for HBV DNA). Among the 185 screened samples, 180 (97.3%) were negative, and 5 (2.7%) were positive for HBV DNA (Figure 2).

Five (5) samples in the age group of 18 to 30 years were HBV positive (1.6%), followed by those within the age ranges of 31–40 years and 41–50 years (1 each, 0.5%) respectively). However, those within the 51–60year age range were negative for the HBV DNA test. There is no significant relationship ($p > 0.05$) between HBV DNA and the age of the donors (Table 1). When their marital status was compared, it was found that singles were more infected with HBV (1.6%) than married people (1.1%). There is no relationship between HBV status and the marital status of the donors ($p > 0.05$). Among the 185 blood samples used in the study, 5 (2.7%) were HBV DNA positive, of which 4 (2.2%) were found to be among the Hausa ethnic group, and 1 (0.5%) was found to be among the Yoruba ethnic group. Igbo and other ethnic groups showed no positive HBV DNA. There was no significant association between tribe ($p > 0.05$) and HBV DNA infection ($p = 0.813 > 0.05$). When the occupation of donors positive for HBV DNA was studied, it was found that the infection was more common in civil servants than in self-employed people. No HBV infection was found among students. HBV infection was prevalent among self-employed (1.6%) and civil servants (1.1%) but not

among students. Figure 3 shows agarose gel electrophoresis after PCR reveals that all HBV obtained from the samples belongs to genotype E after amplifying the 360 bp core gene. NC, NP, and L are negative control, positive control, and ladder, respectively..

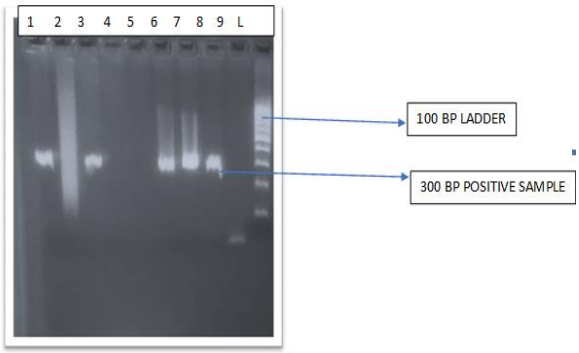

Figure 1: PCR Amplification of the Pre-S region of the HBV Genome

Five sample lanes (1, 3, 6, 7, and 8) were positive for HBV DNA. They all have the expected band size of 300 bp.

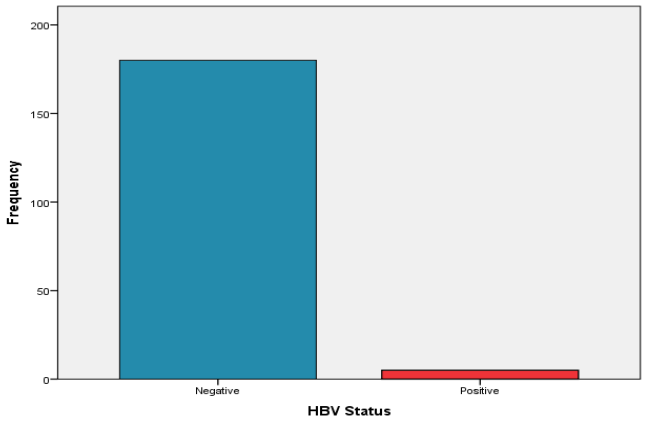

Figure 2: The HBV status of donors negative for HBsAg

Table 1: Donors' socio-demographic characteristics and distribution of HBV DNA

Variables	**HBV DNA**		**Test of Statistics Significance**		
	Negative (%)	Positive (%)	*Chi-square* (x^2)	Df	*p*-value
Age group			**0.903**	**3**	**0.825**
18-30	75 (40.5)	3 (1.6)			
31-40	66 (35.7)	1 (0.5)			
41- 50	34 (18.4)	1 (0.5)			
51-60	5 (2.7)	0 (0.0)			
Marital Status			**1.00**	**1**	**1.00**
Single	108 (58.4)	3 (1.6)			
Married	72 (38.9)	2 (1.1)			
Tribe			**0.950**	**3**	**0.813**
Hausa	126 (68.1)	4 (2.2)			
Yoruba	26 (14.1)	1 (0.5)			
Igbo	19 (10.3)	0 (0.0)			
Others	9 (4.9)	0 (0.0)			
Occupation			**1.297**	**2**	**0.523**
Civil Servant	54 (29.2)	2 (1.1)			
Self-employed	89 (48.1)	3 (1.6)			
Student	37 (20.0)	0 (0.0)			

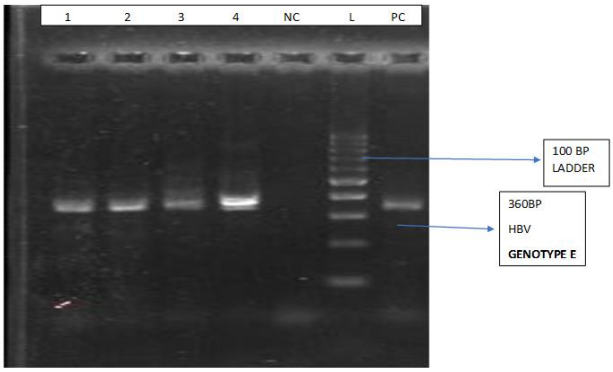

Figure 3: Genotyping of HBV in blood samples

Agarose gel electrophoresis after PCR reveals that all HBV obtained from the samples belongs to genotype E following amplification of the 360 bp core gene. NC, NP, and L are negative control, positive control, and ladder, respectively.

Discussion

Screening blood donors for HBV DNA can minimize the risk of HBV infection via blood transfusion (El-Zayadi *et al.,* 2008). This study shows the prevalence of pre-screened donors being HBV DNA positive at 2.7%, which is lower than a study on pre-screened anti-HBC eighty donated blood samples in Egypt. It was revealed that 6.25% of the samples tested positive for HBV DNA (Antar *et al.,* 2010). The prevalence found in our study is also comparable to a study conducted by Adekeye et al. (2013), where HBV DNA was found in only 20.8 % of anti-HBc positive and HBsAg negative blood of blood donors. The reason could be due to the insufficient effectiveness of HBsAg screening.

The prevalence of OBI presented in this study is quite similar to that reported in Spain by Di Lello et al. (2012), France by Neau et al. (2005), and Taiwan by Liang et al. (2010) (0.7%, 0.6%, and 2.3% respectively), but lower than that reported in Sicily by Tramuto et al. (2013) and European countries such as the Netherlands (Cohen *et al.,* 2009). Also, among a large sample size of 493 344 blood donors in Canada reported by O'Brien et al. (2007), 29 (0.52%) were HBsAg negative but HBV DNA-positive. With a larger sample size in Northeast China, the prevalence was up to 10.6% among less than 400 HBsAg-negative healthy individuals (Fang *et al.,* 2012). In a study conducted in Iran on 14 HBsAg negative samples by Jafarzadeh *et al.* (2008), 28.56 % were positive for HBV DNA. This sample size is small compared with the 185 employed in our study.

The prevalence of 2.7% found in this study is smaller than that of the 14 % reported by Osuji et al. (2018), a study carried out in Nigeria with a sample size of a hundred seronegative blood donors assayed for HBV DNA. Nna et al. (2014) found a prevalence of 8 % in Abakaliki, South-Eastern Nigeria, among a hundred donors. Opaleye et al. (2014) discovered a very high prevalence of 36% among four hundred and twenty-nine donors, while Oluyinka et al. (2015) obtained a prevalence of 17 % in Southwestern Nigeria among four hundred and twenty-nine donors. These prevalences are all higher than those found in this study. In a study conducted earlier in Kebbi State (Yakubu *et al.,* 2016), a prevalence of 16.6% hepatitis B infection was reported using the rapid test kit. Our study and that of Yakubu et al. (2016) could reflect the burden of HBV infection in Kebbi State even though the latter screened only with the rapid test kit.

In our study, HBV has been molecularly detected the age range of 18–30 years. At $p > 0.05$, no significant relationship exists between age group and prevalence. This finding agrees with a study (Olotu *et al.,* 2016) that reported that most positive HBV DNA samples were found to be in the age range of fewer than 36 years. This author also suggested that many donors be included in the study to have reliable statistical significance. This finding in our study also agrees with a study in Bahrain, where the highest prevalence of HBV infection was reported for the age group of 21–30. Concerning marital status, the virus is more common among single (1.6%) than married (1.1%). This finding agrees with Eke et al. (2011), in which no significant relationship exists between HBV DNA and marital status. The reason could be that singles have a high rate of denial of sexual relationships and surreptitious use of contraception for protection (Huang *et al.,* 2016)**.** This finding is in disagreement with Okonko et al. (2010), who indicated a prevalence of 8.0% and 14.4% among singles and married people, respectively, with a highly significant association between marital status and HBV infection ($p < 0.05$). Concerning tribe, it was observed that 4 (2.2%) of the HBV-

positive were Hausas, and there was no association between the tribe and HBV infection at p (0.813) > 0.05. This finding may be because the study population is predominantly Hausa.

We also noticed that all five HBV were found to be in the Genotype E subtype. This observation agrees with Odemuyiwa et al. (2001), who reported HBV genotype E as the most common in Nigeria. It was also found that HBV Genotype E has been most prevalent in southwest Nigeria (Osuji *et al.,* 2018). In a study conducted in Zaria, Nigeria, by Ahmad et al. (2019), genotype E was identified as having the highest prevalence. A study by Faleye et al. (2015) also confirmed the indigeneity of HBV, the risk of mother-to-child transmission and the transmission of the genotype E subtype in Nigeria. This endemicity could be because genotype E is endemic in sub-Saharan Africa and exhibits low genetic diversity (Odemuyiwa *et al.,* 2001). In a study conducted in Ghana, genotype E was the most predominant (Dongdem *et al.,* 2016). As observed in a study conducted by Elmaghloub et al. (2017), Egyptians have a significantly high-rate detection of genotype E. This observation contradicts previous studies and implies that the HBV subtype A3 and the recombination between HBV subtypes A and E are frequently observed in Cameroon, West Africa (Kurbanov *et al.,* 2010). Likewise, in Sudan, samples typed as genotype A have the highest percentage (Salih *et al.,* 2018). This high percentage could result from previous and ongoing migration waves from surrounding territories. In South Africa, genotype A circulates predominantly and is related to severe liver complications and rapid degeneration to hepatocellular carcinoma (Matlou *et al.,* 2019).

Conclusion and Recommendations

Nest PCR revealed a 2.7% prevalence of HBV in a sample population of 185 previously confirmed and presumed safe for transfusion using Rapid Test Kit and ELISA, implying the possibility of the infection still being transmitted. It is logical to conclude that the PCR-based technique may be more sensitive and robust than other methods in screening donated blood for transfusion medicine. This study recommends using PCR as the most reliable and accurate test for detecting HBV. The government should fund blood bank units for capacities in PCR-based techniques and make them affordable for patients.

Conflict of Interest

No conflict of interest arose regarding this article.

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