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Occult Hepatitis B Virus Infection among Blood Donors at Two Teaching Hospitals in Nigeria: Implications for Blood Transfusion

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Abstract **Background/Objectives:** Occult Hepatitis B virus infection (OBI), characterized by presence of HBV DNA in the absence of HBsAg in individuals has become a challenge to blood safety. The aim of this study is to determine the prevalence rate, HBV serologic markers, viral load and genotypes of occult Hepatitis B Virus infection among blood donors attending Nnamdi Azikiwe University Teaching Hospital and University of Abuja Teaching Hospital Abuja, Nigeria. **Materials/Methods:** Sera from 212 healthy blood donors (108 and 104 from UATH and NAUTH respectively) were retested for HBsAg, HIV, Syphilis antibodies and anti-HCV using 4th generation Enzyme Linked Immunosorbent Assay (ELISA). One hundred samples (50 samples from each study site) that were seronegative were examined for the presence of HBV-DNA by conventional Polymerase Chain Reaction (PCR). Viral load was determined on some positive samples by Real Time PCR. Gene sequencing was done to determine HBV genotypes. HBV serological markers prevalence and pattern was determined using ELISA format and HBV 5 Panel assay. **Results:** Of the 100 seronegative (HBV, HCV, HIV and Syphilis) samples, 14 (14%) were confirmed as OBI. Of the 14 OBI samples, 12 (85.7%) were seropositive for HBV serologic markers and among these, 7 (58.3%) were positive for anti-HBs, 3 (25%) positive for anti-HBc, and 2 (16.7%) positive for both HBsAb and HBcAb ($P<0.0001$). Three (21.4%) out 14 OBI blood donors were positive for anti-HBc IgM $p<0.05$. The mean viral load of OBI blood donors was <100 IU/mL. DNA sequencing and Phylogenetic analysis showed that all OBI isolates belong to Genotype E. **Conclusion/Recommendations:** There is a high prevalence of OBI among blood donors in these two Teaching Hospitals. This study therefore recommends that blood donors in these Hospitals in particular and Nigeria in general be screened for OBI by Nucleic Acid Testing (NAT) and HBV serologic markers.

Keywords: *hepatitis B Virus, OBI, blood donors, HBV Markers, NAT*

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1. Introduction

Hepatitis is an inflammation of the liver. The condition can be self-limiting or can progress to fibrosis (scarring), cirrhosis or liver cancer [1]. Viral hepatitis is a necroinflammatory liver disease of variable severity. It is a systemic infection caused by various hepatitis viruses that primarily infect the hepatocyte cells of the liver [2]. Most liver damage as a result of viral hepatitis is caused by hepatitis B and C viruses [3]. Hepatitis B Virus (HBV) particularly is the major cause of inflammation of the liver and tends to be more serious than other hepatitis viruses. It is also the most common cause of acute hepatitis and most common chronic viral infection worldwide [1]. Hepatitis B Virus is the 9th leading cause of death worldwide. It causes Cirrhosis, liver failure and Hepatocellular Carcinoma (HCC) [4].

Occult hepatitis B virus infection (OBI) is characterized by the presence of HBV DNA in serum and/or in the liver of patients negative for hepatitis B surface antigen [5]. Occult hepatitis B virus infection (OBI) has been described for decades, and Nucleic Acid Testing (NAT) for HBV-DNA detection has confirmed the existence of the OBI, which is defined as the presence of HBV-DNA in the absence of detectable HBsAg with or without anti-HBV antibodies [6]. Occult HBV infection may impact in several different clinical contexts including the risk of HBV transmission with transfusion or transplantation, and endogenous viral reactivation [7]. Studies on a large set of blood donors using NAT confirmed this phenomenon of OBI and formed the basis of mandatory NAT for transfused blood units in many developed countries [8], [9]. Such a testing regimen has not been incorporated into the testing algorithms of many laboratories in developing countries including Nigeria. The frequency of post transfusion HBV infection is apparently due to the fact that HBsAg is in circulation at very low and undetectable level for screening assays. The prevalence of OBI in blood donors has been confirmed from different geographic

areas and ranges from less than 1% to 16% depending on the endemicity of HBV infection [10]. In a general Korean adult population, the prevalence of OBI was 0.7% [11] and the prevalence of OBI was 18% and 8% in resolved HBV infection group and in HBV seronegative individuals (negative for HBsAg, anti-HBs and anti-HBc), respectively [12]. However, the prevalence of OBI was 64% in liver transplanted patients, 62% in HCC patients, 27% in hemodialysis patients and up to 45% in HCV and HIV infected patients [13,14].

Studies on OBI prevalence in Nigeria is unclear, with one study that utilized a smaller sample size (n = 28) reported no prevalence of OBI in healthy subjects [15]. Recent study on OBI by [16] at Abakaliki, south eastern Nigeria observed that 8% of blood donors with HBsAg negative were positive with anti-HBc and HBV-DNA by nested PCR. In another study by [17], they recorded a 17% prevalence of OBI among blood donors in south western Nigeria and genotype E is the most prevalent. However, they did not test for anti-HBc IgM and its involvement in OBI. Recently, [18] reported 5.4% prevalence of OBI among blood donors at Ile Ife, Western Nigeria that were anti-HBc positive. However, they did not determine the anti-HBc IgM status as well as OBI prevalence among seronegative donors and donors that were positive for anti-HBs markers.

Blood transfusion could be an important route for the transmission of infection especially when donated blood is not adequately screened for HBV infection [4]. Screening of donated blood for Hepatitis B surface Antigen (HBsAg) was introduced in the 1970s [19]. This greatly reduced HBV transmission due to blood transfusions as blood found to be HBsAg positive was not transfused. In many developing countries including Nigeria which is highly endemic for HBV infection [20], screening of blood donors or blood donated, for HBsAg alone, is still the only practice on which the prevention of HBV transmission during blood transfusion is based [21]. In addition, blood banking practices do not include laboratory testing/procedures that would identify OBI and prevent transfusion of blood or blood products from apparently healthy donors with OBI to recipients [22].

Numerous scientific papers have highlighted the presence of HBV infection in some individuals negative for HBsAg but having detectable HBV DNA in the liver or blood and some of these publications have documented HBV transmission resulting from transfusion of blood tested and found to be HBsAg negative [23,24]. It is against this background that this study was embarked on to investigate the prevalence, viral markers and genotype of occult HBV infection among blood donors in these two teaching hospitals (one in the northern Nigeria and the other in the southern Nigeria). This will enable us make evidence-based recommendations for effective screening of blood donors to prevent HBV transmission from donors with OBI to recipients during blood transfusion in Nigeria.

2. Materials and Methods

Study sites: The study sites comprise the blood banks of University of Abuja Teaching Hospital (UATH) and Nnamdi Azikiwe University Teaching Hospital (NAUTH), Nnewi from Northern and Southern Nigeria respectively.

Subjects: These include 212 (108 from UATH and 104 from NAUTH) apparently healthy blood donors who had been screened and found eligible by the respective blood banks for donation were recruited over a period of five months, from June 2016 to October, 2016. Subjects who had hepatitis B vaccination in the previous one month were excluded. Relevant sociodemographic information was obtained from blood donors using structured questionnaire.

Ethical Considerations: Ethical approval was obtained from the ethics committee of both hospitals and informed consent was obtained from the subjects.

Specimens: Ten (10) milliliters of venous blood was collected from each previously screened, eligible blood donor, donating to the blood banks into K^+ EDTA and plain bottles. All samples were centrifuged at room temperature at 3500 rpm for 10 minutes within 1 hour of collection. The plasma and sera were then separated and stored at −70 °C until analyzed.

2.1. Serological Analysis

HBsAg, Anti-HCV and HIV were tested for in all specimens using commercially available 4th generation ELISA kits (Fortress Diagnostics, USA). All seronegative samples were then tested for HBV markers using HBV 5 Panel assay (CTK Biotech USA) and 4th generation ELISA for total anti-HBc, anti-HBC IgM using commercially available Fortress ELISA Kit.

2.2. HBV DNA Studies

One hundred (100) samples (50 samples from each study site) that were seronegative for HBsAg, HIV, HCV and Syphilis were examined for the presence of HBV-DNA by conventional Polymerase Chain Reaction (PCR).

2.3. DNA Extraction from Plasma Samples

DNA extraction was performed according to method of [25] using Zymo extraction kit (Zymo Research Corporation) and marketed by Ingaba Biotech Ltd Pretoria, South Africa. DNA was extracted from HBsAg negative/positive samples and then PCR was used for detection of HBV-DNA.

2.4. PCR Amplification

PCR amplifications were carried out in 20µl reaction volumes with the following PCR components: Master Mix 10ul, forward primer 0.4ul, Reverse primer 0.4ul, DNA Template 2ul and 7.2ul of water making it up to 20ul on an Applied Biosystems GeneAmp PCR System 9700 Thermal cycler. Primer pairs were designed from the highly conserved overlapping regions of the *S* and *P* regions of the HBV genome. A conventional PCR was performed: Forward primer sequence: TCA CCA TAT TCT TGG GAA CAA GA and Reverse Primer sequence: CGA ACC ACT GAA CAA ATG GC. PCR conditions include the following: Thermal cycling parameters were: initial denaturation at 95°C for 5 minutes, followed by 35 cycles of 30sec at 95°C denaturation, 30 seconds at 53°C

annealing temperature, 30 seconds at 72°C extension, followed by a final extension of 2 minutes at 72°C. Thermal cycling parameters remained the same as in the all PCR round done. A positive control (HBV plasmid DNA) and a negative control of the master mix were integrated to each run to validate the PCR products that produce a 340bp fragment. The detection limit of the HBV DNA by conventional PCR is approximately 2.5 copies per reaction (between30-40copies/mL).

2.5. Detection of PCR Product

For analyses of the PCR amplification, 10ul of the amplified samples was electrophoresed on a 2% agarose gel made in Tris acetated EDTA buffer (pH=8.0-8.5) and visualized by UV illumination after ethidium bromide (10 g/ml) staining. Positive and negative controls were also treated as samples.

2.6. HBV DNA Quantification by Real Time PCR

Detection and quantification of HBV DNA was done at Molecular Diagnostic Laboratory, UATH Abuja and all ELISA HBsAg positive and samples positive by conventional PCR were tested for HBV DNA using "COBAS AmpliPrep/COBAS TaqMan HBV Test version 2.0" kits (Roche Molecular Systems Inc. Branchburg, NJ 08876 USA) using the "Cobas Ampliprep instrument and COBAS TaqMan 48 Analyzer" with a limit of detection of less than 20 IU/ml for HBV DNA. All procedures were performed according to manufacturer's instructions. At the end of each run the results were printed out. Positive (HPC and LPC) and negative controls were included in each run.

2.7. Deoxyribonucleic Acid Sequencing and Phylogenetic Analysis

PCR-positive samples (HBV DNA positive) representing five OBI samples and five PCR-positive samples from HBsAg positive carriers totalling ten samples were successfully sequenced. The PCR purified products were directly used for sequencing in the BigDye Terminator cycle sequencing kit and analyzed on a 3510 ABI sequencer by Inqaba Biotechnological, Pretoria South Africa. Sequencing was performed according to manufacturer's instructions.

2.8. Statistical and Phylogenetic Analysis

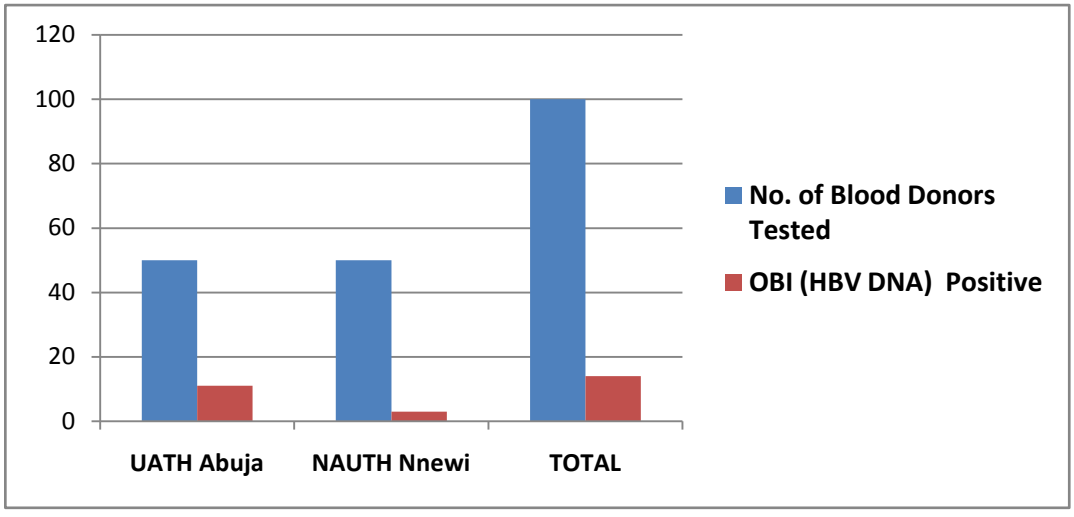
Data processing and statistical analyses were performed using Epi Info 7 Software by CDC. Descriptive statistics were calculated and reported for sociodemographic characteristics. Percentages were used to describe frequency analyses of categorical variables. Chi-squared test was used to compare categorical variables. A p value <0.05 was considered to indicate statistical significance.

Obtained sequences were edited using the bioinformatics algorithm Trace edit, similar sequences were downloaded from the National Centre for Biotechnology Information (NCBI) data base using BLASTN. These sequences were aligned using ClustalX. The evolutionary history was inferred using the Neighbor-Joining method in MEGA 6.0 [26]. The bootstrap consensus tree inferred from 500 replicates [27] is taken to represent the evolutionary history of the taxa analysed. The evolutionary distances were computed using the Jukes-Cantor method [28].

3. Results

The overall prevalence of OBI among blood donors at two Teaching Hospitals under study stood at 14%. This is shown in Figure 1. There is statistically significant difference in the prevalence between the two study sites (p=0.002).

Table 1: presents the prevalence of HBV serologic markers among blood donors with occult HBV infection. The result shows that out of 14 donors with OBI, 5 (35.7%), 3 (21.4%) and 9 (64.3%) were positive for Anti-HBc total, Anti-HBc IgM and Anti-HBs respectively. This is statistically significant. We recorded 0% prevalence for HBeAg and anti-HBe serologic markers among blood donors with OBI.



Statistically Significant (alpha< 0.05) Chi-Square = 5.3; p-value = 0.02

Figure 1. Prevalence of Occult HBV Infection among Blood Donors at UATH, Abuja and NAUTH, Nnewi

Table 1. Prevalence of HBV Serologic Markers among Blood Donors with Occult HBV Infection

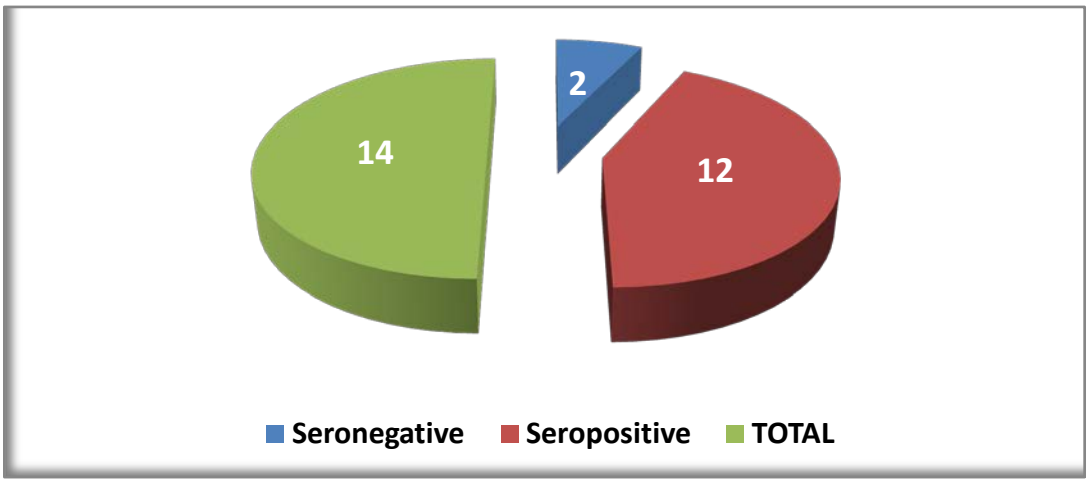
HBV Serologic Markers	No of OBI Blood Donors	No. (%) Positive	Chi-Square (p-value)
Anti-HBc Total	14	5 (35.7%)	*8.4 (0.003)*
Anti-HBc IgM	14	3 (21.4%)	*6.2 (0.04)*
Anti-HBs	14	9 (64.3%)	*18.2 (0.015)*
Anti-HBe	14	0 (0%)	*2.517 (0.4721)*
HBeAg	14	0 (0%)	*2.517 (0.4721)*

Statistically significant ($p<0.05$).

Figure 2: shows the classification of OBI into Serological groups. Out of 14 OBI isolated in this study, 12 were seropositive OBI representing 85.7% while the remaining 2 blood donors were seronegative OBI representing 14.3% prevalence.

The pattern of HBV serologic markers among blood donors with OBI is presented in Figure 3. Out of 12 seropositive OBI blood donors identified, 7 blood donors have Anti- HBs marker representing 58.3%, 3 blood donors have Anti-HBc marker representing 25% and 2 blood donors with OBI have a combination of Anti-HBc and Anti-HBs markers representing16.7%.

Table 2 presented HBV DNA viral load among blood donors with occult HBV infection compared with individuals with overt HBV infection. The result shows that most of the OBI isolates tested were unquantifiable indicating that the viral load is below 20 IU/mL, the LLOD of COBAS Roche Real Time PCR used. However the average HBV DNA for OBI blood donors that were quantifiable stood at 93 IU/mL with a range of < 20-128 IU/mL. It was also observed that the average HBV DNA (viral load) for blood donors positive for HBsAg was 33176108 IU/mL with a range of 924-99520111IU/mL.



Statistically significant ($p<0.05$) p-value: 0.9965

Figure 2. Serological Classification of OBI among Blood Donor Participants

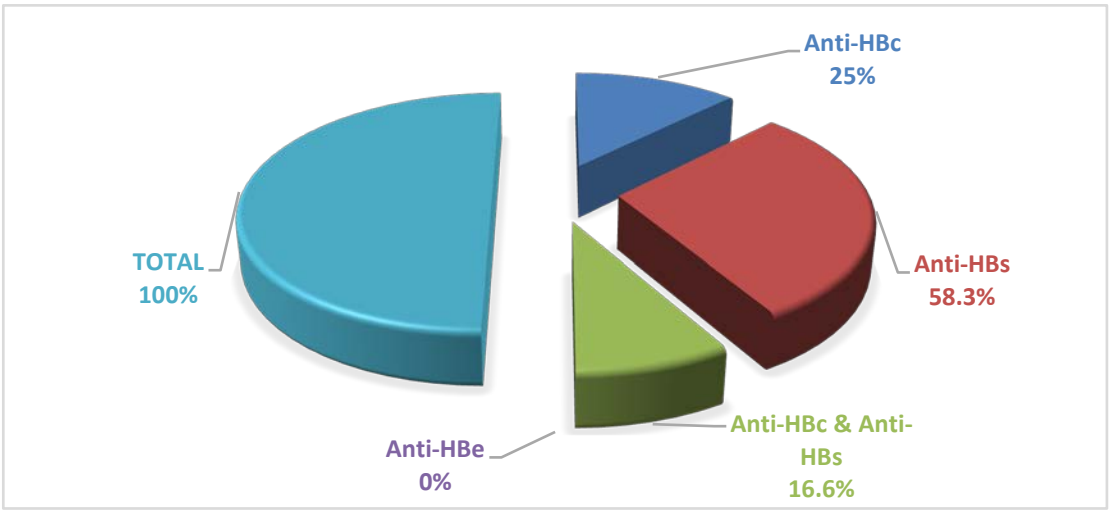


Figure 3. Pattern of HBV Serologic markers among blood donors with occult HBV Infection

Table 2. HBV DNA (Viral load) among Blood Donors with Occult HBV Infection Compared with HBsAg Positive Samples

HBV DNA(Viral Load) Status of Blood Donors	No. of Samples Tested	Average (Mean) in copies/mL	Range in copies/mL
OBI Positive Blood Donors	4	93 IU/mL	<20-128 IU/mL
HBsAg Positive Blood Donors	3	33,176,108 IU/mL	924-99520111IU/mL

3.1. Gene Sequencing Results and Phylogenetic Analysis

The sequences were analyzed by using BioEdit 9.7 and Codon-code Aligner 4.0 software. Sequence analysis and comparison were conducted by using molecular programs deposited in the web site of the National Centre for Biotechnology Information (NCBI). The sequences were compared with the same region of HBV sequences from different genotypes found in the genotyping reference set available on the NCBI website. The phylogenetic tree was constructed according to previous methods [29]. The HBV gene sequence result shows that all HBV isolates belong to Genotype E. Sequences were deposited in Gen Bank, accession numbers from NCBI Genbank: MG 562502 – MG562503.

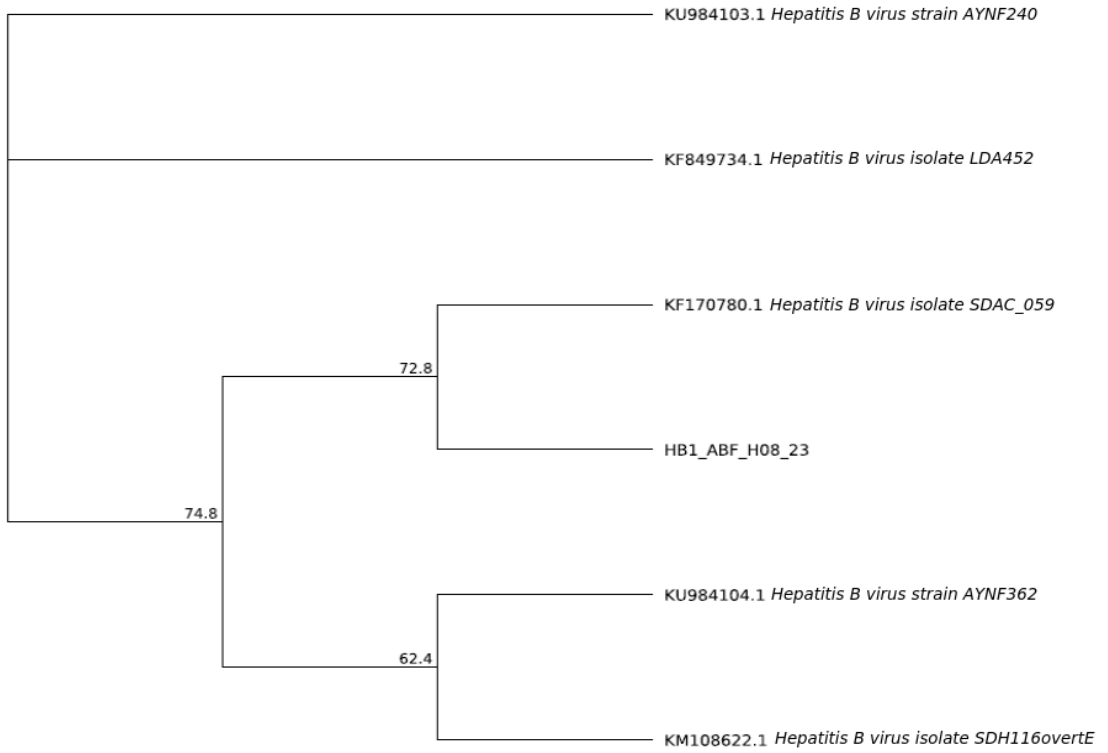


Figure 4. Phylogenetic Tree showing Relationship between HB1 Isolated from Blood Donor with OBI and other Hepatitis B viruses

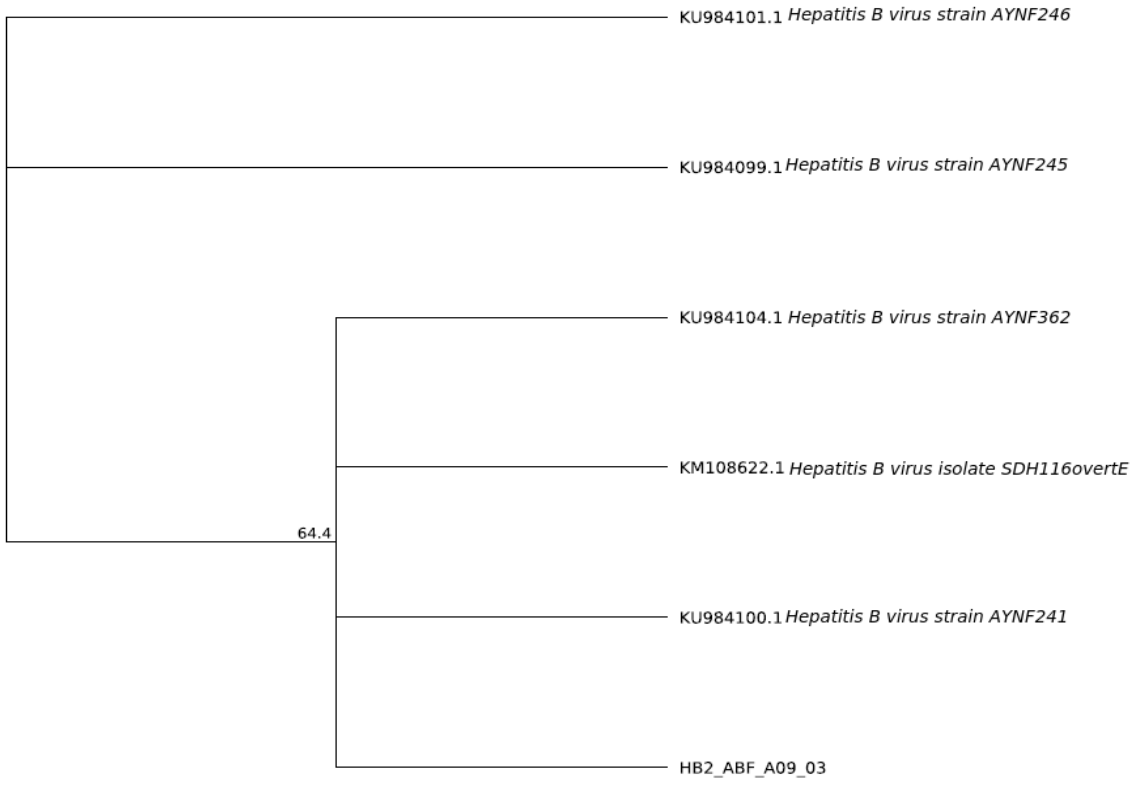


Figure 5. Phylogenetic Tree showing Relationship between HB2 Isolated from Blood Donor with OBI and other Hepatitis B viruses

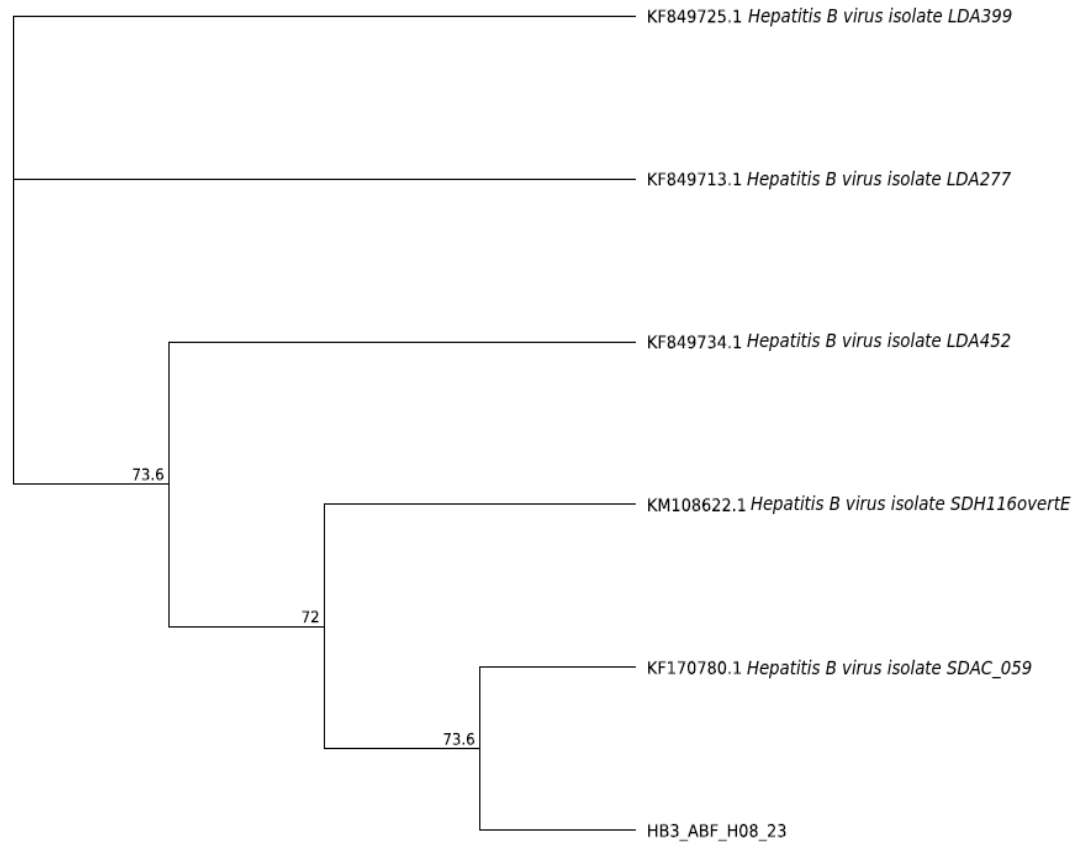


Figure 6. Phylogenetic Tree showing Relationship between HB3 Isolated from Blood Donor with OBI and other Hepatitis B viruses

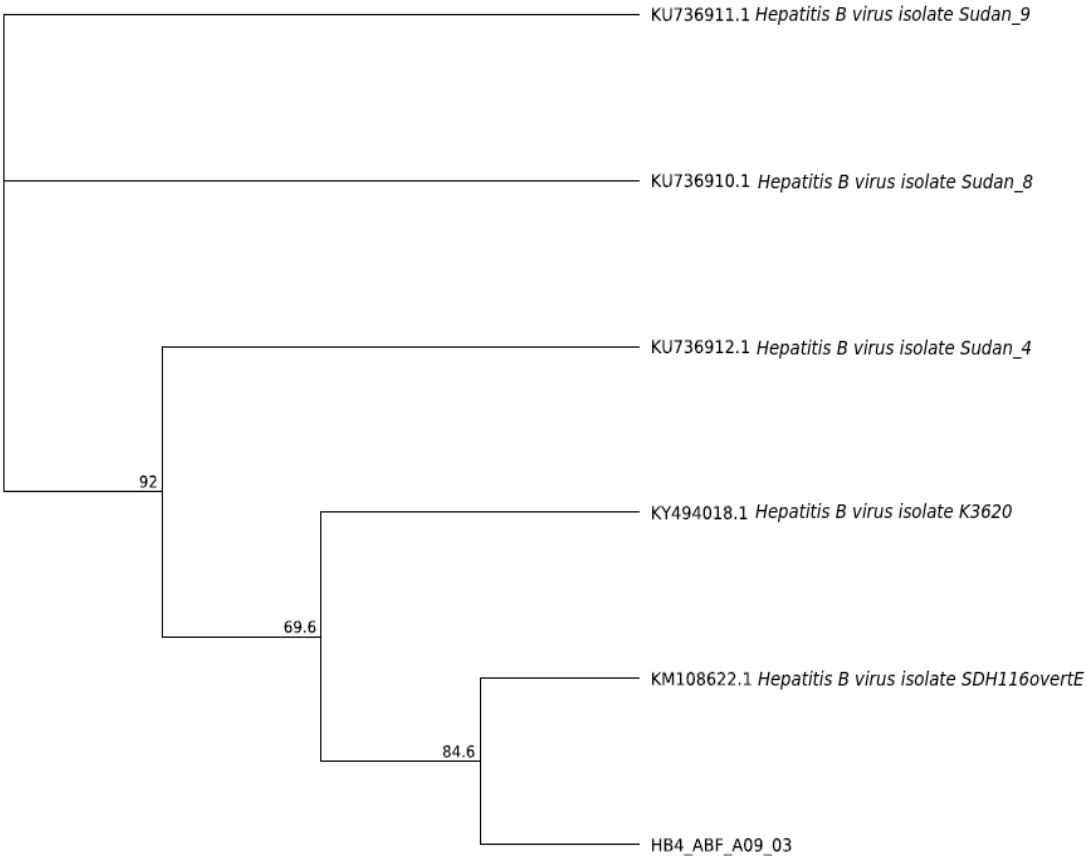


Figure 7. Phylogenetic Tree showing Relationship between HB4 from Blood Donor with OBI and other Hepatitis B viruses

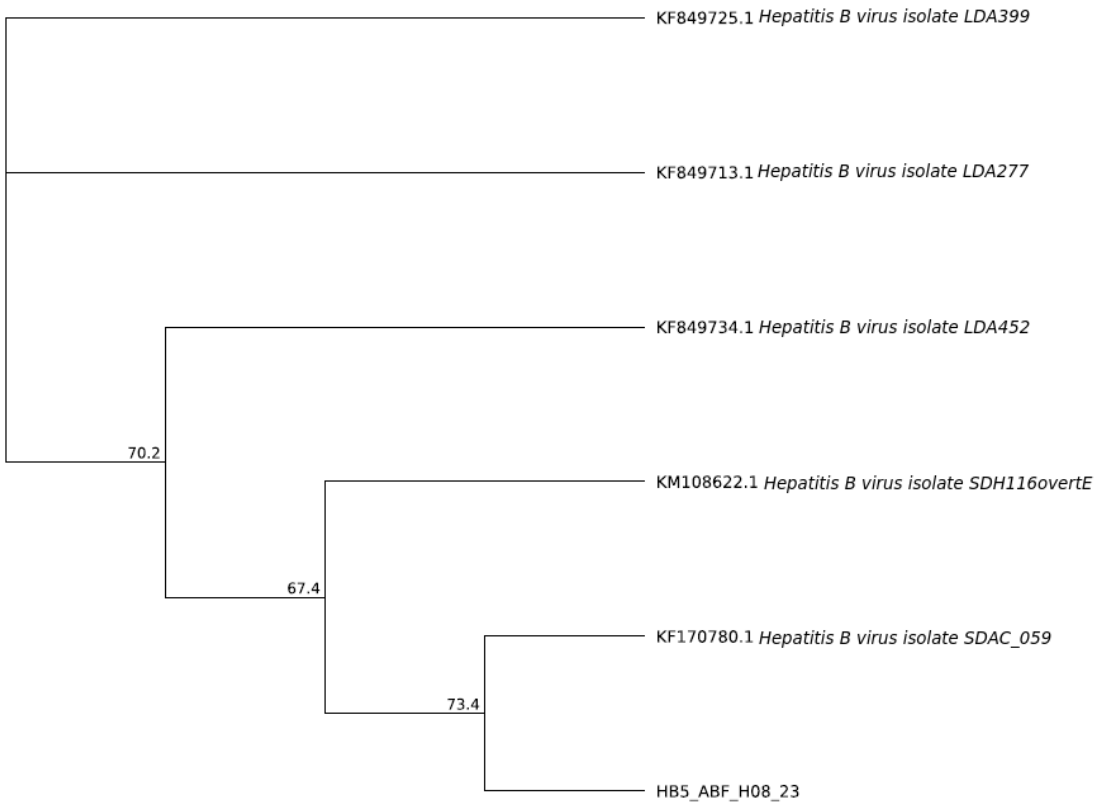


Figure 8. Phylogenetic Tree showing Relationship between HB5 from Blood Donor with OBI and other Hepatitis B viruses

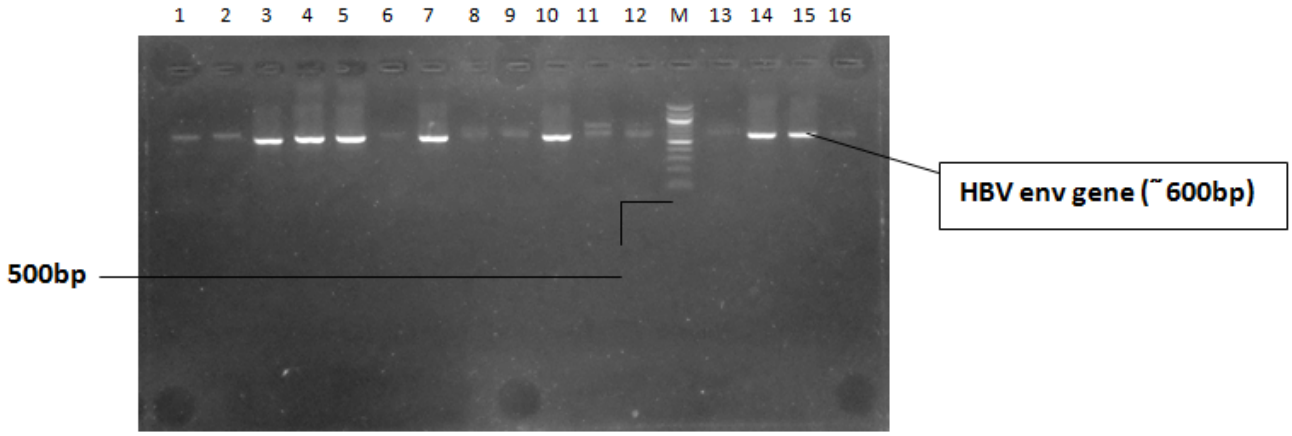


Plate 1. Agarose Gel Electrophoresis Showing Amplified HBV Gene of Blood Donors with Occult HBV Infection (Lanes 1,2,3,4,5,6,7, 10, 11 13 14, 15 showing successful amplification, Lane M represents a 100bp ladder.)

4. Discussion

The result of this study shows an overall prevalence of occult HBV infection stood at 14% with 22% and 6% prevalence rates at UATH, Abuja and NAUTH Nnewi respectively. This is statistically significant $p<0.05$. A study on Occult Hepatitis B virus infection (OBI) in Nigeria has reported a prevalence rate of 8% among 100 blood donors in Abakaliki, South-Eastern Nigeria [16]. However, the finding from this study is comparable to that of [17] that reported 17% prevalence among 429 blood donors in South Western Nigeria. In another study, [30] found a very high prevalence of 36 % among 429 blood donors. These prevalence rates observed from different areas is not surprising as there may be differences in the prevalence of OBI among blood donors from one part of the country to another, reflecting differences in the prevalence of overt HBV infection which exist from one part of the country to the other [31]. Occult Hepatitis B Virus infection (OBI) is unexpectedly high in Nigeria taking into account that the seroprevalence of HBV infection in Nigeria is from 9%-39% [32,33]. Other studies on OBI in Nigeria have been in other subject populations such as [15] who found OBI in 2 of 28 chronic hepatitis C patients in Ibadan, [34] also in Nigeria found a prevalence of 11.2 % of OBI in HIV positive patients using archived specimens from Ikole Ekiti, however these subjects were not likely to qualify as blood donors and as such no risk of their blood being transfused. The prevalence of 14% recorded in this study was higher than the 1.7 % found in Ghana by [35]. In Brazil, [36] found 3.3 % but used a PCR assay with a Low Limit of Detection (LLOD) of about 200 IU/mL, which is less sensitive than the real-time PCR assay used in this study

with LLOD of about 20 IU/mL. This could have resulted in a lower detection of OBI. The prevalence found in this study is much higher than what has been found in the USA and some other western countries where only 0.1-2.4 % of HBsAg negative, anti-HBc positive blood donors were found to have HBV DNA [37]. This is not surprising because only about 5 % of the population have come in contact with HBV in those regions unlike in Nigeria where over 70 % of the population have at some time in their lives been exposed to or infected with HBV [38], [18].

This prevalence of 14% obtained in this study is much smaller than the 38 % that was reported by [39] in Japan but the sample size was just 50 blood donors which is small compared to this study with sample size of 100 seronegative blood donors that was assayed for HBV DNA. This may also be the reason why [40] found 28.56 % prevalence in Iran as they only assayed 14 HBsAg negative, anti-HBc positive samples for HBV DNA and found four samples positive. The prevalence of OBI varies to a great extent in different countries, depending on a number of factors that includes HBV endemicity, liver disease, HBV screening method, sample size and primers employed for NAT [6]. In Northeast China, the prevalence of OBI was observed up to 10.6% among 359 HBsAg-negative healthy individuals [41]. In other studies, only 0.1% of OBI was detected among 10,727 seronegative blood donors from Taiwan [42] and 3% in an Italian migrant population [43]. In Laos a HBV endemic region, reported high OBI prevalence (10.9%) among blood donors who were HBsAg-negative, anti-HBc and/or anti-HBs-positive [44]. In North Africa, a study conducted among 1026 Egyptian blood donor samples revealed that 8% were reactive to anti-HBc and 0.5% was positive for HBV-DNA [45]. The data on OBI prevalence is limited in sub-Saharan Africa. Nevertheless, studies in patients with HIV infection from Ivory Coast and Sudan have shown that OBI prevalence was 10% and 15%, respectively [46,47].

This study demonstrates that the OBI samples had significantly lower HBV-DNA copies compared to HBsAg positive blood donors. The average viral load for OBI samples tested stood at 93 IU/mL while for overt HBV infection is over 33,000,000 (33 Million) IU/mL. This is in concordance with other studies done recently in Nigeria [17,18]. Both of these studies obtained HBV DNA load less than 100 IU/mL among blood donors with occult HBV infection. The low level of viral load inferred in this current study showed almost all OBI cases are infected with replication incompetent HBV, revealing a strong suppression of replication activity and gene expression, thereby resulting in a reduced viral load [48]. This study also observed that all OBI blood donors as well as those with overt HBV infection belong to HBV Genotype E. This finding is in accord with the report of [17] that found HBV Genotype E been most prevalent in South west, Nigeria. This finding contradicts previous studies and indicates that the HBV subgenotype A3 and the recombination between HBV genotypes A and E were observed frequently in West Africa [49], [50]. HBV genotype E is the most prevalent in Nigeria [51]. HBV genotype E is endemic in West Africa and also exhibits low genetic diversity [52]. In this current study, we could genetically distinguish between the HBV genotypes E from other HBV genotypes by phylogenetic analysis of the *PreS/S* and *PreC/C* regions. Therefore, based on the phylogenetic analyses of the *PreS/S* regions, it was assumed that all the OBI isolates from this study belong to HBV genotype E. The phylogenetic analysis as seen in phylogenetic trees developed showed that most of our HBV isolates have high relationship with HBV isolates from Sudan [53]. This could be as result of intra border transfer of these viruses from one country to another. Moreover the HBV isolates from UATH Abuja and NAUTH Nnewi are similar. This could be as result of people moving from state to another part of Nigeria and in the process spread this virus from one person to another as HBV can be transmitted through various modes. This include through sex, needle prick injuries and visiting commercial barbing saloon to mention but a few [54].

The prevalence and pattern of HBV serologic markers associated with blood donors with OBI in this study showed that most OBI isolates are seropositive for HBV serologic markers. Of the 14 OBI isolated in this study, 12 were seropositive representing 84.7% of OBI population while only 2 (14.3%) are seronegative. The pattern of seropositivty shows that anti-HBs have the highest prevalence among all the serologic markers detected. Seven (7) out of 12 seropositive OBI blood donors identified were anti-HBs seropositive representing 58.3% while 3 (25%) were anti-HBc seropositive. Two OBI blood donors have a combination of anti-HBs and anti-HBc seropositivity representing 16.7% prevalence. None of OBI blood donors has HBeAg and anti-HBe markers. This prevalence and pattern disagrees with what [17] obtained. Their study recorded 62% seropositivity of anti-HBc, 35% of anti-HBs and 3% of HBeAg. However, our study corroborates with theirs because none of OBI donor has more than two HBV markers positive. This signifies the usefulness of testing for HBV markers in screening blood donors before donation. The presence of anti-HBs or/and anti-HBc is a suspect that the blood unit could be infected with HBV and hence should be confirmed using PCR. Moreover, this study correlates with the result of [55] that obtained 88% of OBI blood donors were seropositive and 12% were seronegative.

5. Conclusion/Recommendations

Hepatitis B virus infection still continues to be a menace to the society because the prevalence of the disease is still very high in the general population. Occult hepatitis B virus infection (OBI), characterized by detection of HBV DNA in the serum or tissues of subjects who tested negative for HBsAg has become a challenge to blood safety. This study has established 14% prevalence of occult HBV Infection among blood donors at our study population. Moreover, most of the OBI isolates were seropositive with anti-HBc and anti-HBs seropositivity in high prevalence. The incorporation of HBV markers particularly anti-HBc and anti-HBs in blood donor screening will reduce HBV transmission risk. The present study also showed that HBV is highly endemic in Nigeria, with a predominance of HBV genotype E in blood donors with occult HBV infection at UATH Abuja and NAUTH,

Nnewi. Nucleic acid tests (NATs) should be introduced for routine screening of blood donors in blood transfusion as it is highly sensitive and specific.

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Conflicting Interests

The authors declare that they have no conflicting interests.

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