Occult hepatitis B viral infection among blood donors in South–Eastern Nigeria

Emmanuel Nna¹, Chinenye Mbamalu², Ifeoma Ekejindu²

¹Safety Molecular Pathology Laboratory, Enugu, Enugu, Nigeria, ²Department of Medical Laboratory Sciences, Nnamdi Azikiwe University, Nnewi, Anambra State, Nigeria

Hepatitis B virus infection is endemic in many parts of sub-Saharan Africa including Nigeria. Occult hepatitis B virus infection (OBI) is a challenging clinical problem characterized by the absence of Hepatitis B surface Antigen (HBsAg) and low viral DNA load. We aimed at determining the prevalence of OBI among repeat blood donors in Abakaliki, south-eastern Nigeria. Of 113 informed consented repeat blood donors enrolled into the study, 12 donors (10.6%) tested positive to both serological HBsAg screening, anti-HBc total and hepatitis B virus (HBV) DNA Nested PCR tests. One donor (0.9%) tested HBsAg positive, anti-HBc positive but Nested PCR negative. All donors were negative for HIV 1 and 2 and HCV infections. Of the 100 HBsAg negative repeat blood donors, 8.0% (eight donors) were HBV DNA positive by nested PCR method and anti-HBc total positive by ELISA. The median viral load, determined by real time PCR-Taqman chemistry, in the OBI blood samples was 51 IU/ml compared to 228 IU/ml of the HBsAg screen positive donors. The observed OBI prevalence of 8.0% corroborated with high endemicity of HBV infection in Abakaliki. We therefore recommend routine HBV DNA testing by real time PCR method on all sero-negative blood donations in Abakaliki and for a similar policy to be evaluated across the sub-Saharan Africa.

Keywords: Occult hepatitis B infection, OBI, Blood donors, Viral load, Nested PCR, Prevalence of OBI, Real time PCR, qPCR

Introduction

Occult hepatitis B virus infection (OBI) is defined as the presence of hepatitis B virus (HBV) DNA in the blood and/or in the liver of individuals testing negative to hepatitis B surface antigen (HBsAg) by currently available assays and with or without antibodies to hepatitis B core antigen (anti-HBc) or antibodies against HBsAg (anti-HBs) in both index and follow-up samples.^{1–3} OBI differs from the window period (WP) of the acute primary infection even though both are characterized by the absence of HBsAg in index blood samples; however follow-up samples after WP will normally test positive for HBsAg but negative for OBI. The main property of window period samples is that they are anti-HBc negative, while most OBI samples are positive. Occult infection may also result from low viral load in circulation (usually <200 IU/ml) due to infection by HBV variants with mutations in the S gene (escape mutants) which produce a modified HBsAg (due to mutations in the major hydrophilic region of the major antigenic determinant of HBsAg) that is not recognizable by some or all commercially available detection assays.^{3–5}

Implementation of hepatitis B surface antigen (HBsAg) test in routine screening of blood donors in the early 1970s has greatly enhanced transfusion safety. The incidence of transfusion-transmitted hepatitis B has been steadily reduced over the last four decades because of routine screening of blood donors for HBsAg.⁴ However, HBV transmission remains the most frequent transfusion-transmitted viral infection.⁶ 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.^{7–9} Carriers of occult infection may be a source of HBV transmission through blood transfusion resulting to classic forms of liver diseases in the recipients.^{10,11} This possible occurrence was first reported in the late 1970s and experimentally confirmed in chimpanzees some years later.^{12,13} At present, post-transfusional hepatitis B infection is a rare event in the western world and occult HBV infection of the donors, perinatal and horizontal transmission appear to be the main factors responsible for the residual cases.¹

Occult HBV is a worldwide spread finding which clinical relevance has not been yet properly established, although its distribution may reflect the general prevalence of HBV infection in various geographic areas and populations. Prevalence of OBI is high in high HBV infection prevalent zones of the world

Correspondence to: Emmanuel Nna, Safety Molecular Pathology Laboratory, Plot 44, Rangers Avenue, Enugu, Enugu 400001, Nigeria. Email: e.nna@safetybiomedical.org

and low in low HBV infection prevalent zones of the world.¹⁴ Prevalence rates of 0.17, 4.0 and 6.4% were observed for OBI among blood donors in Nanjing, China,¹⁵ in Taiwan¹⁶ and in Mexico,¹⁷ respectively. A prevalence rate of 9.8% was found among accepted blood donors in Hunan province, China.¹⁸ Hepatitis B virus infection is highly endemic in Nigeria and it is estimated that about 12% of the total population of Nigerians are chronic carriers of Hepatitis B surface Antigen (HBsAg).¹⁹ About 75% of the Nigerian population is reportedly likely to have been exposed to HBV at one time or the other in their life.²⁰ This is possible because of a high level of occurrence of blood transfusion demanding health conditions in Nigeria due to increase in road accidents, pregnancy-related hemorrhage, armed robbery attacks, and violent ethnic/tribal wars. These may increase the possibility of transmission of HBV (and other blood-borne pathogens) through contaminated blood as reported by United Nations System in Nigeria.²¹ Sero-prevalence rates of HBV, detected by HBsAg testing, among blood donors in Nigeria were 14.3% in Jos,²² 2.4% in Yola,²³ 15% in Zaria,²⁴ and 8.0% in Abakaliki.²⁵ However, there is no information on occult HBV infection among blood donors in Nigeria due to lack of molecular testing facility. It is possible that the high sero-prevalence of HBV infection in Nigeria may be sustained by undetected OBI among blood donors.

However, current opinion is still that transmission of HBV in Africa occurs mainly during childhood and transfusion is only one of several routes.

Although HBsAg is a common diagnostic marker of HBV infection, it does not provide information about low levels of viral replication. Therefore, the gold standard for testing OBI is the analysis of DNA extracts from liver or blood samples performed with a nested PCR and/or with a real time PCR (qPCR). In nested PCR, specific primers are used to amplify three different regions of HBV in two rounds of PCR per region and visualized by agarose gel electrophoresis. This improves both sensitivity and specificity of detecting low copy number targets of HBV. With this approach, only the cases in which HBV DNA is detected using at least two different sets of primers may be considered positive for the cryptic infection.²⁴ With qPCR, using TaqMan chemistry, ultra-sensitivity, reliability and quantitation of viral load are achieved rapidly at lower cost.²⁶

Materials and Methods

Ethical approval and informed consent

An ethical approval was obtained from the Nnamdi Azikiwe University Teaching Hospital Health Research Ethics Committee. Written informed consent was obtained from all the participants who completed 'blood donors questionnaire' adapted from the National Blood

Transfusion Service. The study was conducted in line with the World Medical Assembly declaration of Helsinki as revised in Seoul 2008.

Subjects

The subjects were repeat blood donors between 18 and 65 years of age. One hundred and thirteen (113) subjects who had given informed consent were enrolled in the study. Based on the answers provided by the subjects in the blood donors' questionnaire, only those (113 participants) who met the criteria for blood donation by the standards of the National Blood Transfusion Service were included in the study. All the participants had donated blood at one time or the other in the past and were willing to donate blood in the future if required.

Sample collection

A sample of 5 ml whole blood was collected into commercially prepared EDTA tubes and mixed by inversion. Samples were centrifuged at 3000 rpm for 5 minutes within 24 hours of collection and plasma harvested and stored in aliquots into sterile 1.5 ml tubes. Plasma samples were frozen at -20°C until required for analysis.

Serological HBsAg testing

The enrolled repeat blood donors were screened for HBsAg using rapid test kit (ACON Laboratories, Inc. San Diego, CA, USA). The analytical sensitivity, estimated by the manufacturer of the kit, was 1.0 ng/ml. The rapid test is an immunochromatographic strip immobilized with antibodies that are specific to the hepatitis surface antigen. Internal control is also included in the strip. Positive samples would show two red lines.

Serological tests for HIV 1 and 2, anti-HCV and anti-HBc total

All samples were tested for HIV infection using HIV 1 and 2 antigen/antibody 4th Generation ELISA test (Diagnostic Automation, USA). All samples were tested for anti-HCV using anti-HCV ELISA (Diagnostic Automation, USA) while anti-HBc total was tested for using HBcAb ELISA (Diagnostic Automation, USA). The wells were read at 450 nm using DAR 800 Micro-Well Plate Reader (Diagnostic Automation, USA). All the ELISA results were reported as index values or optical density ratios.

Viral DNA isolation

Viral DNA was extracted from plasma using the phenol-chloroform method. Briefly, 250 μl of plasma was treated with 20 μl of Proteinase K (Qiagen, UK) and incubated at 56°C for 10 minutes in a Techne Heating block. The treated plasma was transferred into 750 μl of TRIzol reagent (Thermofisher, UK). The DNA was precipitated with absolute alcohol, washed twice with 0.1M sodium citrate in 10% ethanol, rinsed

in 75% ethanol and air-dried in heating block at 37°C. Finally, 60 µl of 10 mM Tris-EDTA (TE) buffer, pH 8.0 was added to each sample to dissolve the DNA. Quality and quantity of viral DNA yield was checked on Eppendorf Biophotometer Plus reading at A260/280 ratio. The viral DNA extracted by the phenol-chloroform method was used for the nested PCR. For real time PCR, viral DNA was extracted by Spin Column method using the QIAamp DNA Blood Mini Column kit from Qiagen UK. Briefly, 200 µl of plasma was treated with 20 µl of Proteinase K and 200 µl of AL Buffer in 1.5 ml tube, incubated at 56°C for 10 minutes. Then 200 µl of molecular grade ethanol (VWR, UK) was added to the sample, mixed and pulse-vortexed for 15 seconds. The tube was micro-centrifuged for 15 seconds. The sample mixture was transferred into the QIAamp mini column carefully without wetting the rim and viral DNA extraction was carried out according to the Manufacturer's instructions. The viral DNA was eluted with 60 µl of AE buffer. The quality was checked in Eppendorf Biophotometer at A260/280 nm.

Detection of HBV DNA by nested PCR

The HBV DNA was detected by nested PCR using specific primers, shown in Table 1, which were designed to amplify regions coding for 's antigen', 'c (core) antigen' and the pre-core 'p region' respectively.¹⁵ All primers, HPLC grade, were purchased from Integrated DNA technologies Europe. The first round PCR of all the nested PCR was carried out in a final volume of 25 µl, containing 5 µl of DNA template in 0.2 ml thin walled PCR tube. The thermal profile was 35 cycles (20 seconds at 94°C, 30 seconds at 55°C, 45 seconds at 72°C) after initial denaturation for 5 min at 95°C. A final extension step was performed for 1 minute at 72°C using the ABI 2730 thermal cycler (Applied Biosystems, UK). A commercially prepared 2 × hot start Rovlab PCR master (VhBio, Newcastle, UK) was used for all

the first round PCR. The second round PCR was carried out using 5 µl of the first round PCR product as the template under the same conditions as the first round PCR but for 40 cycles. The PCR products were electrophoresed on 1.5% agarose gel, stained with SYBR safe (Invitrogen, UK), and then photographed with Genome mini Gel documentation system (VWR, UK). To prevent cross-contamination, DNA extraction, 1st round and 2nd PCR preparation were done in different rooms separate from where the thermal cyclers were kept. The WHO standards for hepatitis B virus DNA, 97/750 and 99/732 containing 10⁶ and 10³ IU/ml were obtained from the National Institute for Biological standards and controls (Hertfordshire, UK) and were used as HBV DNA positive plasma controls during testing. DNase/RNase free water was used as a negative control. The dynamic range of the nested PCR has been previously reported¹⁵ that it can detect as low as 20 copies of HBV/ml of plasma.

Quantitation of HBV load

Plasma HBV DNA in nested PCR positive samples was quantified by qPCR, based on a previously described Taqman chemistry method²⁶ using a set of primers and a TaqMan MGB probe shown in Table 1. The ABI One Step Plus real time PCR system and 2 × TaqMan Universal Master Mix with Rox as a passive reference (Applied Biosystems, UK) were used with a universal thermal profile: 50°C for 2 minutes, 95°C for 15 minutes and 45 cycles of 95°C for 15 seconds and 60°C for 1 minute (data was captured at the 60°C for 2 minute stage). A volume of 5 µl DNA elute was used in setting up a final reaction volume of 25 µl in a 48 well plate and all samples were tested in duplicates. The dynamic range and performance characteristics of the real time PCR assay have been previously shown to detect an order of 10¹ IU/ml,²⁶ which is suitable for clinical monitoring of HBV treatment. The TaqMan chemistry method has also been shown to correlate very positively

Table 1 Sequences of primers and probe

Primers for nested PCR ¹⁵				
Region	Name	Sequence 5'–3'	Amplicon size In bp/PCR round	Final concentration (nM)
S	1stS192F	TCGTGTTACAGGCGGGGTTT	513	250
	1stS704R	CGAACCACTGAACAAATGGC	1st round PCR	250
Pre C	2ndS455F	CAAGGTATGTTGCCCGTTTG	233	250
	2ndS687R	GGCACTAGTAACTGAGCCA	2nd round PCR	250
	1st 2023F	GCCTTAGAGTCTCCTGAGCA	442	250
	1st 2464R	GTCCAAGGAATACTAAC	1st round PCR	300
C	2nd2046F	CCTCACCATACTGCACTCA	340	300
	2nd2385R	GAGGGAGTTCTTCTTAGG	2nd round PCR	300
	1stC1869F	ACTGTTCAAGCCTCCAAGCT	600	250
	1stC2468R	GGAATACTAACATTGAGATTCGAG	1st round PCR	300
	2ndC2015F	TGCTCTGTATCGGGAGGC	280	300
	2ndC2294R	AGTGCGAATCCACACTC	2nd round PCR	
Real time PCR primers and probe ²⁶				
S	HBV116 F	CCT GGY TAT CGY TGG ATG TGT	116 bp	200
	HBV 116R	GGA CAV ACG KGC AAC ATA CCT T		200
	HBV 116P	FAM-CTA TGC CTC ATC TTC TT-MGB-NFQ		100

with existing commercial nucleic acid testing kits.²⁶ Criteria for accepting valid qPCR results were set as follows: the slope of the standard for each run should be between -3.30 and -3.60 , y-intercept should be ≤ 40 , correlation coefficient R^2 between log of starting quantity and cycle of quantitation (Cq also known as Ct, that is cycle of threshold) should be ≥ 0.98 , the difference in Cq (ΔCq) value between duplicates ≤ 1.5 ; the threshold was set at 0.01 with auto-baseline. The maximum plateau of the normalized relative fluorescence (Rn) should be 1.0

Statistical analysis

Generated data were analyzed in GraphPad Prism version 5.02 (GraphPad, USA). The D'Agostino and Pearson Omnibus normality test was performed on viral load data sets to check for Gaussian distribution before choosing Mann-Whitney test for analysis. Sensitivity, specificity and predictive values were tested for in evaluating HBsAg serology. Fisher's exact test was used to compare the result of the serologic HBsAg test to a similar study conducted in the same locale²⁵.

Results

Serological testing for HIV, HCV, HbsAg and anti-HBc total

Of the 113 repeat blood donors, 13 (11.5%) were positive for HBsAg serologic test. All the samples were negative for HIV and HCV. Twenty-one samples (19%) tested positive for anti-HBc total, of which all the 13 HbsAg positive samples were also anti-HBc positive. Eight of the anti-HBc positive samples were HbsAg negative. The 13 HbsAg positive repeat donors were then excluded from further testing except that their viral load and nested PCR were determined. They were also excluded from any donation. The eight HbsAg negative, anti-HBc positive were also tested for both nested PCR and viral load quantitation.

Molecular findings

The average A260/280 ratio of the DNA samples was 1.86 (range: 1.54–1.98).

Of the remaining 100 samples HBV DNA was detected in 8 (8.0%) that were sero-negative for HBsAg

testing. The PCR positivity was considered as 2/3 or 3/3 of S, P and C regions in nested PCR. Figure 1A and B shows the agarose gel pictures for the S and P regions. One of three (1/3) PCR positivity was considered negative. Four (4%) donors tested positive for S region only (1/3) and were still considered negative. Of the eight positive cases (OBI), only two donors tested positive for the C region; most of the positive cases were S and P regions together. On the basis of the results obtained, the positive and negative predictive values of the HBsAg tests in relation to HBV DNA detection were 58 and 99% respectively. Table 2 shows the sensitivity, specificity, positive and negative predictive values of the HBsAg tests. The median viral load for the HbsAg negative, anti-HBc positive and nested PCR positive (PCR+) donors was 51 IU/ml (range 30–80 IU/ml). The HBsAg-, anti-HBc positive and PCR+ group was regarded as the OBI group. The median viral load for the 13 samples that were HBsAg+ and anti-HBc positive, regarded as HBV infection group was 228 IU/ml (range: 98–896 IU/ml). The median viral load between the OBI and HBV infection groups was significantly different (Mann-Whitney test, $P = 0.0002$). Figure 1C shows box plots of viral load for both OBI and HBV infection groups (screen positive).

Discussion

The observed sero-positivity of HbsAg and anti-HBc in this study did not differ significantly from the previously reported sero-prevalence of HBsAg,²⁵ $P = 0.6359$ (Fisher's exact test). Both findings indicated high endemicity of HBV sero-prevalence. The average sero-prevalence of HBsAg in Nigeria is still above 7%, which defines endemicity.²⁷ The observed OBI prevalence did not differ significantly from the reported sero-prevalence ($P = 0.8115$, Fisher's exact test). Our study showed, for the first time in Abakaliki, south eastern Nigeria, a prevalent occult hepatitis B infection rate of 8.0% amongst repeat blood donors which corroborated with high HBV infection endemicity.¹⁴ It also highlighted the need for quality controlled HbsAg testing as 13 repeat donors were found positive for

Table 2 Sensitivity, specificity and predictive values of HBsAg testing

Data analyzed	HBsAg+ (Screen positive)	HBsAg- (Screen negative)	Total
PCR positive (true positive)	12	8	20
PCR negative (true negative)	1	92	93
Total	13	100	113
Sensitivity and specificity			
Sensitivity	0.9167		
95% confidence interval	0.6152 to 0.9979		
Specificity	0.9208		
95% confidence interval	0.8499 to 0.9652		
Positive predictive value	0.5789		
95% confidence interval	0.3350 to 0.7975		
Negative predictive value	0.9894		
95% confidence interval	0.9421 to 0.9997		
Likelihood ratio	11.57

Figure 1 Results of agarose gel electrophoresis and viral loads. (A) From left to right is 50 bp DNA ladder L, followed by a negative control, two positive samples and positive control. Amplicon size for S region is 233 bp. (B) From left to right is 50 bp DNA ladder, followed a negative sample, three positive samples and a positive control. Amplicon size for the P region is 340 bp. A carrier over amplicon of 442 bp from first round PCR appears in the final detection of the P region. (C) Box plot of HBV load in HBsAg screen positive and nested PCR positive HBsAg screen negatives.

HBsAg. The OBI finding may become an important baseline for evaluating OBI in Nigeria. It underpins a risk of increasing transmissibility of hepatitis B Virus infection through blood transfusion in Nigeria. A previous study reported anti-HBs and anti-HBc IgM prevalence of 15.2 and 13.0% respectively among HBsAg negative blood donors in Nigeria.²⁸ The low prevalence of anti-HBs in Nigeria makes it very unlikely that occult hepatitis infection is not a key driving force that sustains HBV endemicity in Nigeria. Although infectivity of OBI donations per HBV genome is much lower than that of window period infection,²⁹ OBI prevalence in Nigeria is rarely reported. Therefore there is a need for a better screening strategy in blood donation and transfusion services. Sero-negative blood donations (screened by HbsAg and anti-HBc IgM ELISA) should be further screened using HBV DNA testing methods. The real time PCR (TaqMan chemistry) is sensitive, less laborious, less contamination affected and quantitative. It is more dependable and cost effective.

The number of samples per minipool should be determined empirically depending on the analytical sensitivity of the HBV DNA PCR test. One major limitation in this study is that the analytical sensitivity of Acon HBsAg rapid test does not compare well with some other commercial HBsAg ELISA tests. This may affect the detection rate of HBsAg marker in the repeat donors thereby affecting the reported OBI prevalence. Countries with low HBV prevalence such as the USA and some European countries carry out minipool nucleic acid testing (NAT) as a routine test in blood donors.^{3,30} This approach may help to reduce the HBV infection burden and related viral hepatitis complications in the region. In the recent past, there has been cases of violent inter-tribal wars in Abakaliki region, a mainland city in south-east Nigeria. This resulted in

many casualties who might have received ‘occult infected’ blood products.

In addition, there is a need for more investigations into the clinical implications of ‘S region only’ positivity. The four cases that tested positive for S region but negative for P and C regions in nested PCR were also quantifiable in the real time PCR system because the primers and probe were designed to amplify the S region of all HBV genotypes. It is possible that the primers for P and C regions are not sensitive and optimal in detecting those regions compared to that of S region. There are suggestions that these ‘S genes only’ are escape mutants, HBV variants with mutations in the promoter and/or S regions of the virus.² Those mutations cause low viral load and such strains are known to lack infectivity and replication competence. It is not known if the ‘S region only’ cases will lead to chronic carrier state. However, S mutants escaping neutralization by antibodies especially in vaccine induced immunity or immunotherapy with human specific immunoglobulin have been shown to be infective and can replicate in the liver causing acute hepatitis.³ Therefore caution should be exercised; the ‘S region only’ cases should also be excluded from blood transfusion uses. More investigations are required to determine if such cases would require treatment. The diagnostic workup requires a nested PCR first to enable detection of other regions of the HBV, for example, the Pre C, C, X and pol genes/regions. The qPCR chemistries target different genes but the most dependable of them all rely on the s gene. A combination of both nested PCR and qPCR is necessary to ensure maximum sensitivity and specificity in HBV DNA testing and to reduce false positives.

In conclusion, the reduction of HBV residual risk and occult infection depends on the implementation of effective quality controlled HBsAg screening and

HBV NAT for donor blood screening, either in minipools or more efficiently in individual samples. The risk of HBV transmission by blood transfusion in Nigeria is relatively high based on this study and previous findings from other studies. It is recommended that NAT for HBV DNA should be evaluated in routine screening in many Centres across the country. A diagnostic workup comprising nested PCR and real time PCR may be evaluated for reliable detection of HBV infections especially in the window period and occult infections.

Disclaimer Statements

Contributors Emmanuel Okechukwu Nna and Ifeoma Ekejindu designed the project, Emmanuel Okechukwu Nna and Chinenye Mbamalu carried out the lab work, Emmanuel Okechukwu Nna did the statistical analysis and re-writing of manuscript. Chinenye Mbamalu drafted the early manuscript and EN developed it fully. Emmanuel Okechukwu Nna, Chinenye Mbamalu and Ifeoma Ekejindu reviewed the manuscripts critically. Emmanuel Okechukwu Nna revised the manuscript.

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