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Pattern and Prevalence of Hepatitis B virus genotypes Among HBV Discordant Partners in Enugu, Southeastern Nigeria

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Abstract

Hepatitis B virus (HBV) continues to cause significant global health problems despite the presence of a potential vaccine and other clinical approaches. This study was designed to investigate the pattern and spread of HBV genotypes among discordant and concordant partners of hepatitis B virus infection in Enugu State, Nigeria. The study involved a cross-sectional study consisting of 150 subjects (120 discordant and concordant partners of hepatitis B, and 30 controls). HBsAg was screened using a rapid ELISA diagnostic strip and re-screened to reaffirm the result using Ichroma Fluorescence Immunoassay (FIA) analyser. Multiplex nested polymerase chain reaction using type specific primers was used to identify the HBV genotypes. Statistical analysis was performed using Graph Pad Prism. HBV genotype E (75%), A (10%) and a mixed genotype of A and E (mixed infection (15%) were gotten with genotype E being the most predominant. This study has established the high prevalence of genotype E as the most predominant HBV genotype in circulation in Enugu, southeastern Nigeria.

Keywords: Hepatitis B Virus; Genotypes; Mixed-infection; Discordant Partners; Concordant partners; Immunoassay

1. Introduction

Infection with hepatitis B virus (HBV) is a serious global public health problem, with the reported prevalence ranging from 0.1% to 20% [1]. Approximately 350 – 400 million people worldwide are chronically infected and have the high risk for developing cirrhosis, fulminant hepatitis, and end stage liver disease and hepatocellular carcinoma [2]. HBV prevalence is highest in Sub-Saharan Africa and East Asia, where between 5–10% of the adult population are chronically infected accounting for 500,000–1.2 million deaths per year and is the tenth leading cause of mortality worldwide [3]. The clinical presentation and natural history of HBV infection is mediated through complex interactions between the virus and the host immune response.

HBV is differentiated into many genotypes, according to genome sequence, and the sequence is characterized by >8% nucleotide differences for genotypes, and 4-8% nucleotide differences for sub-genotype [4]. Strikingly, the prevalence of different genotypes varies geographically and is strongly associated with ethnicity [5]. Moreover, recent studies elsewhere show unusual HBV mixed genotype infections, suggesting overlapping clinical outcomes [6]. Studies relating

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to the impact of HBV genotype and viral variation on clinical course of the disease have drawn considerable recent attention. Some studies concluded that HBV genotype identification is not only predictive of clinical progression, but also are related to interferon (IFN) treatment response [7]. Detection of hepatitis B virus genotypes are becoming increasingly important in planning of clinical management of Chronic Hepatitis B (CHB) because of its clinical and epidemiological implications and continues to be of great interest [8]. Therefore, this study was designed to screen and identify the current pattern and prevalence of HBV genotypes in Enugu, Nigeria.

2. Material and methods

2.1. Study Area

This study was done at the Enugu State University of Science and Technology Teaching Hospital, (ESUTH) Parklane, Enugu, Nigeria.

2.2. Research Design

This research employed a cross-sectional study, using a simple random sampling technique in sample collection.

2.3. Ethical Consideration

Ethical approval was obtained from ethical committee of ESUT Teaching Hospitals, Parklane, Enugu.

2.4. Specimen Collection

Though a total of 120 subjects were recruited for this study which include 90 discordant and 30 concordant partners of hepatitis B virus infection, only 30 hepatitis B positive sample were genotyped. Blood samples were collected from the subjects in plain tubes after endorsement of consent and were allowed to clot before the extraction of the serum through centrifugation at 3,000 rpm for 5 minutes and temporarily stored in -20^{o}C till the time of analysis.

2.5. Sample Analysis

2.5.1. Serological Screening

The samples were first screened for HBsAg using a rapid diagnostic strip (LabAcon, Biotest Biotech Co. Ltd, China). The test strip is a qualitative solid phase, two site sandwich immunoassay that detects HBsAg in serum or plasma. The tests were done according to the manufacturer's instruction and positive samples were further screened for Hepatitis A, C, and Human immune Virus (HIV), using LabAcon for HCV and hepatitis A; and Determine (Alere Medical Co. Ltd, Japan), and Unigold (Trinity Biotech, Ireland) for HIV. Positive samples of hepatitis A, hepatitis C or HIV were excluded.

2.5.2. HBsAg Assay

An additional test on HBV was done to confirm the positive screened hepatitis B samples using Ichroma Fluorescence Immunoassay (FIA) technique. The test uses a sandwich immuno detection method and dried antibodies once diluted with the diluent, bind with antigens in the sample to form antigen- antibody complex. The complex then migrates through the nitrocellulose matrix and are captured by another set of immobilized antibodies on the test line. The more the antigens HBsAg in the sample serum, the more the antigen- antibody complexes, which leads to stronger fluorescence signal. The signal is then interpreted by a reader and results displayed on the screen. The procedure for the test were followed in accordance with the manufacturer's instruction and results were interpreted as follows (Table 1)

Table 1 Result Interpretation of Ichroma FIA Analyser.

≤0.9	Negative
>0.9, <1.0	Indeterminate
≥1.0	Positive
0–500 miu/ml.	Linearity limitation

2.5.3. Molecular Analysis for Hepatitis B Genotype detection

DNA Extraction from the serum:

Genomic DNA was extracted using Quick-DNA™ Viral Kit (Zymo Research, USA), according to manufacturer's protocol.

Hepatitis B DNA Detection and Genotyping by Nested PCR [9]

This protocol was employed for mere detection of HBV DNA in serum or plasma, and characterization into genotypes. First round primers were used for DNA detection while the protocol proceeds to second round touchdown PCR for the characterization into genotype.

Procedure

Sample: Serum

Kit type- Hot start Premix

Reaction volume - 20µl; Primer sequence (Table 2)

Table 2 Primer sequences for both HBV DNA detection and genotyping by nested PCR [9]

1ST Round	
hepBP1	5' – TCACCATATTCTTGGGAACAAGA–3'
hepBS1 – 2	5' – CGAACCACTGAACAAATGGC–3'
2ND Round	
MIX A	
hepB B2	5' – GGCTCMAGTTCMGGAACAGT–3'
hepB BAIR	5' – CTCGCGGAGATTGACGAGATG–3'
hepB BCIR	5' – GGTCCTAGGAATCCTGATGTTG–3
hepB BBIR	5' – CAGGTTGGTGAGTGACTGGAGA–3'
MIX B	
hepB BE1	5' – CACCAGAAATCCAGATTGGGACCA–3'
hepB B2R	5' – GGAGGCGGATXTGCTGGCAA–3'
hepB BD1	5' – GCCAACAAGGTAGGAGCT–3'
hepB BF1	5' – GYTACGGTCCAGGGTTACCA–3'

First Round PCR – Hepatitis B Virus Detection and Confirmation

The Premix tubes were labelled with the samples identification numbers. The final reaction volume for the first round of the nested PCR were 20µl as describe above. About 2µl of the extracted DNA was added to a master mix 16µl of deionized water, 250 µl of each Dntp, 1x PCR buffer, 15µl of MgCl2 and 1unit of thermostable taq polymerase, and 1µl each of forward and reverse primers.Thermal cycler (PTC- 100TM Programmable Thermal Controller, MJ Research Inc.) was used to perform the PCR, and the reaction conditions were set using this protocol

- Initial activation - 95^{o}C ……. 5 min
- Denaturation - 94^{o}C…….. 20 sec
- Annealing - 55^{o}C …….. 20 sec
- Extension - 72^{o}C …….. 1 min
- Step ii to iv was repeated for 30 cycles

- Final extension - 72^{o}C ……. 5 min.

Second Round PCR: Hepatitis B virus genotypting:

Two different tubes were set for each samples. The first one with the common sense primer (B2), and type specific primers for genotype A, B, and C in Mix A, while the second tube with the common universal anti- sense primer (B2R) and the type specific primers for genotype D, E, F in Mix B, [9]. 17µl of dionionised water was added into each premix tube of A and B and 2µl of the cocktail primers (containing o.5µl each of the 4 primers) were added into the mixtures. 1µl of the first round PCR product were added into each tube of the premix and the PCR conditions were set as follows:

- Activation - 94oC ……... 5 min
- Denaturation - 94oC …….. 20 sec
- Annealing - 59oC …… 20sec
- Extension - 72oC ……30sec
- Go back to ii for 15 cycles
- Activation - 94oC ……... 20sec
- Denaturation - 61oC ……... 20sec
- Annealing - 72oC …….. 30sec
- Go back to vie for 15 sec
- Final extension - 72oC …….. 5min

2.6. Gel Electrophoresis

Twenty microlitres of the genotyped product of the nested PCR were separated by gel electrophoresis and the products: (samples, ladder, negative control) were run on 2% agarose gel (2% w/v in 1x TAE buffer) and electrophoresed for 45 minutes at 100v. The bands were visualized under a gel photodocumentation system (BioRad Cal Doc- XR, USA), and screenshots captured. The size of the separated bands were compared with the GeneRulerTM 100 bp DNA ladder (MB, Fermentas, Life Sciences, Canada).

Expected amplicon size

- Mix A:
- Type A – 68bp
- Type B - 281bp
- Type C - 122bp
- Mix B:
- Type D -119bp
- Type E -169bp
- Type F -97bp

3. Interpretation

The HBV genotype was detected as presented above. Each genotype A- F has different expected amplicon size and were identified based on their size.

3.1. Data Analysis

Statistical analysis was performed using Graph Pad Prism version 6.0 software (GraphPad Software, Inc. San Diego, California, USA). Frequency and percentages of the identified genotypes were presented in tables.

4. Results

Out of 75 positive hepatitis B subjects gotten from 60 discordant and 30 concordant partners from General out Patient Department (GOPD) and Antenatal Clinic (ANC), 30 samples were randomly selected for hepatitis B genotyping, using multiplex nested polymerase chain reaction with type specific primers. Fig 1 show the genotype image of gel electrophoresis of HBV surface antigen positive DNA samples. Electrophoregram of samples from HBsAg DNA positive study participants show bands representing identified genotype. Bands from Mix “A” of the primer setup were shown at the upper part of the gel, while bands from Mix “B” were shown at the lower part of the gel. From fig 1, ten samples

were setup together with molecular marker "M" and negative control "-ve" and the result showed that lane 5, 6 and 11 had 68 bp after separation given rise to genotype A in mix "A" gel, while mix "B" yielded 169 bp in lane 6, 7, and 9 to give genotype E. Lane 6 gave a mixed genotype of genotype A and E.

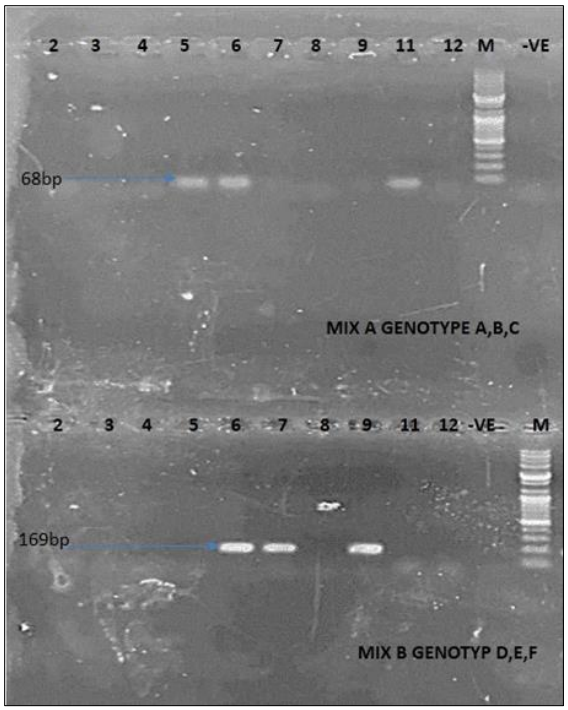

Figure 1 Agarose gel electrophoregram of HBV surface antigen of sample 2 to 12 for mix A and B preparations for HBV genotypes, M is the molecular ladder, while –ve is the negative control.

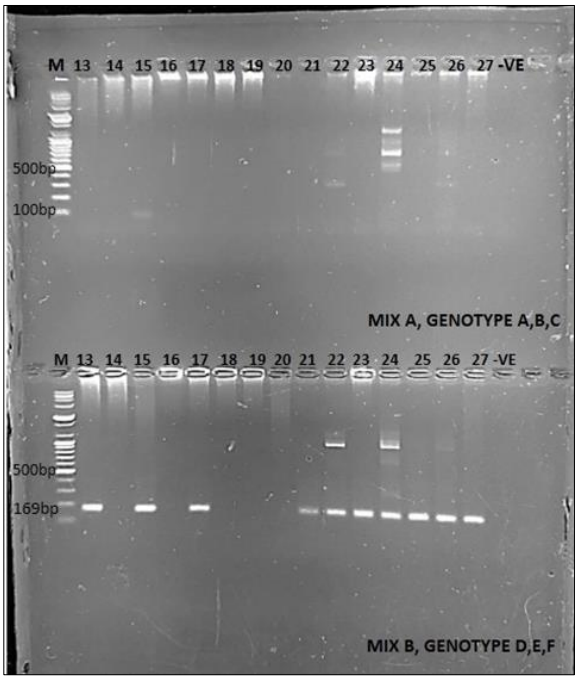

Figure 2 Agarose gel electrophoregram of HBV surface antigen of sample 13 to 27 in Mix A and B preparations for HBV genotypes, M represents the molecular marker, while –ve represents the negative control

In fig 2, only one genotype A of lane 15 was gotten in mix "A", while lane 13, 15, 17 21, 22, 23, 24, 25, 26, 27 yielded 169 bp to give rise to genotype E. Also, there is one mix A and E genotype in lane 15. Molecular marker "M" of 100 base pair plus (100 bp+) served as the marker for identifying the genotypes of the HBV. Figure 3 showed the results of genotype of 28 to 32 samples. Lane 28 and 29 gave genotype A, and lane 28, 29, 30, 31, and 32 produced 169 bp of genotype E, while lane 28 and 29 were equally a mixed genotype of A and E. Table 2 shows the distribution of hepatitis B genotypes in Enugu south eastern Nigeria. The result showed that genotype A got (2)10%, genotype E (15)75%, while mix infection of genotype A and E yielded (3) 15%. There was no detection of genotype B, C, D and F

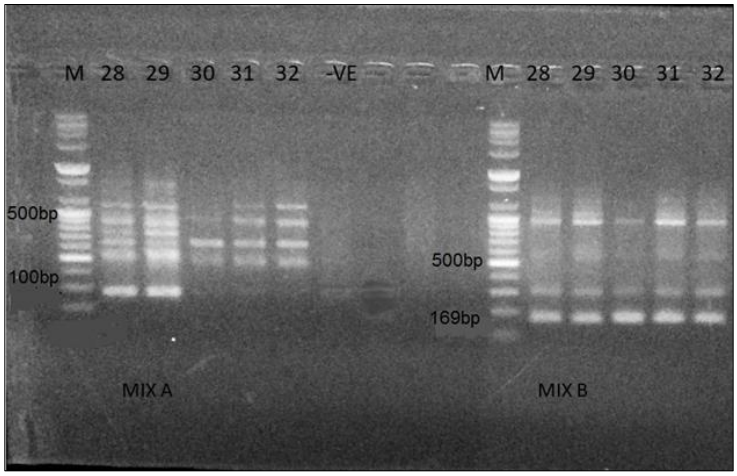

Figure 3 Agarose gel electrophoregram of HBV surface antigen of sample 28 to 32 in Mix A and B preparations for HBV genotypes, M represents the molecular marker, while –ve represents the negative control

Table 3 HBV genotype distribution

Genotype	No	%
A	2	10
B	-	-
C	-	-
D	-	-
E	15	75
F	-	-
Mixed A/E	3	15

5. Discussion

HBV infection is an important global health problem that places a continuously increasing burden on developing countries of Africa especially Nigeria. About 400 million people worldwide are chronic carriers of the virus [10]. The risk of contracting HBV in Nigeria is substantial, not only due to low vaccination rates but also given that as many as 75% of the population will be exposed [11]. In Enugu state, southeastern part of Nigeria, most of the studies conducted on HBV infections were restricted mainly to the prevalence of the virus in different population subgroups such as blood donors, pregnant women and children, [12, 13, 14], and some focused on a meta-analysis which pooled the different subgroups to arrive at some figures, [15]. Although HBV infection is widely reported to be endemic in Nigeria [16, 17, 18], the molecular epidemiology of the virus is poorly understood across the country with only limited available data. This present study among other things attempts to unveil the genotypic prevalence of HBV infection in Enugu, southeastern Nigeria. Our study was able to detect and genotype the infecting HBV in 66.7% of the tested samples. This was consistent with the findings in Egypt, [6] using innogenetics line probe assay (INNO-LiPA) which is based on the reversed hybridisation principle; in the United Arab Emirates using the nucleic acid testing (NAT), [19], in India using the NAT, [20], and in Wasit province of Iraq using nested PCR, [21] where they were able to detect and genotype 71.4%, 95%, 38.7%, 69.7% and 60.5%, respectively. Doumbia in Cote d'Ivoire using nested PCR got 38.8% lower than this study

[22], while Ferreira et al, working on hepatitis B virus infection profile in hemodialysis patients in central Brazil got a detection rate of 65.4% [23]. Though different methodologies may be used in HBV genotype determination, but they all maintained the variability in HBV-DNA isolation in relation to HBsAg positivity, in that not all HBsAg-positive samples yielded positive results for HBV-DNA detection, irrespective of the method of detection. Being a non-encapsulated virus, HBV-DNA tends to rapidly degrade, whereas in the absence of or with inadequate anti-HBs, viral surface antigen may remain in circulation for a prolonged periods of time, [24]. This could depend on the stage of infection such as in patients who are chronic carriers with inactive infection. It could also be explained by intermittent viraemia or an extremely low and undetectable levels of HBV-DNA either due to prior treatment or natural clearance, [25].

A few studies had reported the predominance of genotypes A, B, and E in different population groups across Nigeria [26, 27]. In this current study, genotype E, A, and mixed infection of E and A were gotten in Enugu, southeastern Nigeria. Genotype E was found to be the predominant genotype with a prevalence of 75%, genotype A 10%, while mixed infection of genotype A and E stood at 15%. The finding of genotype E as the most predominant in Enugu is in consonance with an earlier work that investigated genotype distribution of hepatitis B virus in a subset of infected young people in central Nigeria [27] with the highest prevalence of genotype E at 44.8% . Regardless of occurrence in the form of mixed or mono-infection, a study in Zaria [28], northern Nigeria, identified genotype E to be with the highest prevalence, followed by genotype B, then genotypes A, then C and genotype D with the least prevalence. In Benin City of mid- western Nigeria, another study got all their 26 HBV co-infected with HIV to be from genotype E [29], and also in southwestern Nigeria, all 17 cases of HBV in a study were genotype E [30], while in Port Harcourt, Nigeria, a preponderance of genotype E was reported [31].

Genotype E is predominant in the sub-Saharan Africa, and has been discovered in Central African Republic, Senegal, Namibia, and in East Africa [32]. However, HBV genotype E has been reported to account for a majority of HBV infection in West Africa, [33, 34]. Reports show that HBV genotype E is essentially not found outside of Africa, even among African-American populations, many of whom are of West African origin, which suggests that it is probably a recently evolved genotype, [33]. The identification of HBV genotype E in our study therefore is in line with the finding and in consonance with the suggestion that genotype E is indigenous to West Africa and Nigeria in particular [26]. Genotype E is very difficult to treat requiring a longer duration [31]. Furthermore, the mean decrease in the concentrations of the hepatitis B surface antigen was lowest among patients infected by genotype E when compared with B and D, whereas, HBsAg relapse was documented for individuals with genotypes B and E [35].

Considering mono- infection only, genotype A with a prevalence of 10% was gotten in this study and is far lower in prevalence when compared to genotype E. The finding of high prevalence of genotype E in association with low prevalence of genotype A in this study is in consonance with other previous works both in Nigeria and across West African sub region, [32, 36, 37, 38]. While all the previous work across Nigeria confirm the preponderance of genotype E, there was difference in other accompanying genotypes of mono- infections across different regions. Also a study in central Nigeria got 13.8% of genotype A, genotype B (34.5%), genotype E (44.8%) and a mix genotype B/E (6.9%) and made remark that it was the first time genotype B was reported in Nigeria [27].

Mixed genotype infection of A and E has a prevalence of 15% in this study. This equally agrees with previous data that showed different degrees of co-infections of different HBV genotypes. While Pennap et al., got 6.9% prevalence of mixed HBV genotypes, Ahmad et al., got a very high prevalence of 82.6% of mixed infections [27, 28]. The mixed-infection recorded in this study or elsewhere could be as a result of possible recombination between genotypes as noted in earlier findings, [39,40], or it could also be as a result of migrations and long-term travels for overseas studies, businesses, peacekeeping missions or other reasons for economic sustenance.

6. Conclusion

We have reported HBV genotype for the first time in Enugu State, with genotype E (75%), genotype A (10%), and mixed genotype A and E (15%) being in circulation. This finding will go a long way for better epidemiological and clinical management of HBV infection in the eastern region of Nigeria.

Compliance with ethical standards

Acknowledgments

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Disclosure of conflict of interest

All authors declare that they have no competing interests.

Statement of ethical approval

Ethical approval was obtained from the ethical committee of ESUT Teaching Hospitals, Parklane, Enugu, and following the code of ethics for biomedical research involving human subjects.

Statement of informed consent

Informed consent was obtained from each participating individual and a questionnaire was administered to all the participating subjects.

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