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Molecular epidemiology of hepatitis B virus from blood samples obtained from persons in Enugu and Nasarawa States, Nigeria

Kingsley Andrew Egbe[1], Anthony C. Ike[1*], Olusola Anuoluwapo Akanbi[2], Richard Olulowo Ojedele[2], Khadijah Isa Imam[2], Chimaobi Chukwu[2], Friday Egbe[3], Edith Silas Ekwom[4], Nse F. Unam[5] and Ikeata Kenneth Chukwuemeka[2]

Abstract

Hepatitis B virus (HBV) infection remains a major global health concern, with a high burden in countries like Nigeria. This study aimed to investigate the molecular epidemiology of HBV by identifying circulating genotypes and detecting antiviral-resistant mutations in samples from selected communities in Enugu and Nasarawa States, Nigeria. Sample (plasma and serum) quality was evaluated after prolonged storage under freeze-thaw conditions using Qubit 4 fluorometer and Nanodrop One spectrophotometer. High-quality samples underwent molecular characterization and Sanger sequencing for HBV-positive cases. Of the 67 samples analyzed, seven were sequenced: two from Enugu and four from Nasarawa belonged to genotype E, while one from Nasarawa was genotype A. No antiviral resistance mutations were identified. This study provides valuable molecular data contributing to our understanding of HBV epidemiology in Nigeria.

Keywords Hepatitis B virus, Genotype, Antiviral resistance, Molecular epidemiology, Enugu state, Nasarawa state

Introduction

Hepatitis B, a viral infection that targets the liver, can result in both acute and chronic illness [1]. Despite the availability of an affordable, safe, and highly efficient vaccine since 1981, the hepatitis B virus (HBV) remains a global public health challenge of enormous proportion. About 254 million people were living with chronic hepatitis B infection in 2022, with 1.2 million new infections occurring annually, resulting in an estimated 1.1 million deaths, mostly from hepatic cirrhosis and hepatocellular carcinoma [2].

The virus is most commonly transmitted from mother to child during birth and delivery, in early childhood, as well as through contact with blood or other body fluids during sex with an infected partner, unsafe injections, or exposure to sharp instruments [2]. Once in the host, HBV replicates by reverse transcription of the 3.5 kb RNA intermediary known as the pregenomic RNA. Cellular RNA polymerase II transcribes this RNA from the covalently closed circular form of HBV DNA that accumulates in the nucleus of the hepatocyte, and the HBV polymerase (POL) and because POL is incapable of proofreading, the result is sequence heterogeneity, which

*Correspondence:
Anthony C. Ike
anthonyc.ike@unn.edu.ng
[1]Department of Microbiology, Faculty of Biological Sciences, University of Nigeria Nsukka, Nsukka, Enugu State, Nigeria
[2]Genomics Laboratory, Nigerian Centre for Disease Control, National Reference Laboratory, Gaduwa, Abuja, Nigeria
[3]Public Health Department, Federal Capital Territory Administration, Abuja, Nigeria
[4]Department of Microbiology, Faculty of Science, Cross River University of Technology, Calabar, Cross River State, Nigeria
[5]Haematology Unit, Federal Staff Clinic Gwarinpa, Abuja, Nigeria

is a characteristic of HBV [3–4]. This notable generation of enormous genetic variation in the course of an infection can be explained by several factors, including the organization of the viral genome, the high rate of viral turnover (more than 10^{11} virions each day), the virus infectivity, and the recombination events that occur during the virus replication [5].

HBV diagnosis primarily involves detecting HBV antigens (e.g., HBsAg, HBeAg) and antibodies (anti-HBs, anti-HBc) in the blood, alongside viral nucleic acids like HBV DNA [6]. Molecular methods for HBV DNA detection include thermal cycling techniques such as polymerase chain reaction (PCR) and quantitative PCR (qPCR), as well as isothermal methods like nucleic acid sequence-based amplification (NASBA), transcription-mediated amplification (TMA), loop-mediated isothermal amplification (LAMP), and rolling circle amplification (RCA) [6]. Sequencing and phylogenetic analysis, often focusing on the S-region (surface antigen-encoding), are used for genotyping [7].

Grouping of HBV into 4 genotypes based on genome nucleotide variations greater than 8% has been approved by Okamoto et al. [8] in 1988, the complete HBV genome has been additionally classified phylogenetically into at least ten genotypes designated A–J [3, 9]. With an intergroup nucleotide difference of approximately 4% and 8% across the complete genome and good bootstrap support, genotypes A–D, F, H, and I are classified further into at least 35 subgenotypes [1, 3].

These genotypes display significant regional distribution patterns that are not only simply a reflection of the differences in epidemiology among different nations but also include ethnic heterogeneity and transmission mechanisms [10–11]. For example, the global distribution of genotype A, which comprises eight subgenotypes (A1–A8), is pluralistic [12]. Genotypes B and C are more widespread in Asia, while Genotype D has a global distribution. Genotypes A and E are common in sub-Saharan and Western Africa, in Northern and Eastern Europe [13–14]. Genotype F has a worldwide distribution but is mainly endemic in Central and Southern America [15]. Genotype G has been documented in France, Germany, and the USA. Genotype H is frequently encountered in Central and South America, while Genotype I has been noted in Vietnam and Laos [16]. Genotype J has been documented in the Ryukyu Islands of Japan [16]. In central, eastern, and southern Africa, Genotype A is the most common, while in North Africa, Genotype D is the most prevalent, in western Africa, Genotype E is primarily reported [17]. In addition to being geographically distinct, these genotypes have been shown to have a clinical influence on therapy response and the course of the disease [13]. The rate at which HBeAg and HBcAg seroconvert, levels of viremia, immune escape, the emergence of mutants, the pathogenesis of liver disease, the response and resistance to antiviral agents, and the effectiveness of vaccination against the virus have all been found to be influenced by specific HBV genotypes, sub-genotypes, and mutations in specific regions of the HBV genome [18].

By permanently suppressing HBV replication, antiviral treatment using nucleos(t)ide analogs (NA) that target the HBV polymerase (P) can stop the progression of the disease. However, the development of drug-resistant mutations and insufficient medication adherence can cause first generation NA therapy to fail [19], and other studies have recently documented the incidence of drug-resistant HBV mutations including rtM204I/V/S, and rtL180M + rtM204I/V mutations [20].

Currently, antiviral treatment is not reliant on a particular HBV genotype or subgenotype, even though these factors can affect the natural history of HBV infection [21]. However, the molecular epidemiology of HBV is essential for tracking transmission dynamics and directing public health initiatives [22]. In the study area, there are, however, relatively few available descriptions of HBV genotype in dispersion. Therefore, the serum and plasma samples from participants in the two investigations in Nigeria's Enugu and Nasarawa states that were previously published were sequenced and presented in this report.

Methods

Samples

We included 67 HBV positive plasma and serums samples obtained from a screening of 1000 persons in communities and towns in two different States in Nigeria (500 hundred persons from each States), published in two studies [23–24] and stored for further analysis with consent of the participants. Briefly, in Enugu, the participants included 205 males and 295 females (ratio 1:1.4), with 26.0% aged 35–44, 19.0% aged 25–34, 3.2% aged 0–14, and 3.4% aged 65+; education levels were 39.0% secondary, 24.6% tertiary, 18.6% primary, 11.4% non-formal, and 6.4% postgraduate. In Nasarawa, participants comprised 161 males and 339 females (ratio 1:2.1), with 41.4% aged 25–34, 10.0% aged 0–14, and 0.6% aged 65–74; education distribution was 33.0% secondary, 28.6% each for primary and tertiary, 7.2% non-formal, and 2.6% postgraduate. A total of 67 HBV-positive cases were recorded: 14 in Enugu (9 males, 5 females; 6 singles, 7 married, 1 widow; 7 primary, 2 secondary, 3 tertiary, 2 postgraduate; 6 aged 15–24, 2 aged 25–34, 3 aged 35–44, 3 aged 45–54; 1 farmer, 2 government workers, 1 health worker, 2 traders, 8 students) and 53 in Nasarawa (19 males, 34 females; 26 singles, 24 married, 1 divorced, 2 widows; 3 non-formal, 15 primary, 19 secondary, 16 tertiary; 4 aged 0–14, 15 aged 15–24, 28 aged 25–34, 5 aged 35–44, 1 aged 45–54;

4 farmers, 4 government workers, 19 traders, 9 students, 13 unemployed, 4 public servants).

After prolonged storage, seven HBV-positive samples were selected for sequencing: 2 from Enugu (1 male, 1 female, both aged 15–24) and 5 from Nasarawa (4 females, 1 male; 2 aged 15–24, 3 aged 25–34).

Qubit 4 and nanodrop one analysis

After prolonged storage based on circumstance beyond our control, HBV DNA concentration and contamination level in our samples was measured using Invitrogen-Thermo Fisher Scientific Qubit ™ 4 Fluorometer (Life Technologies Holdings Pte Ltd, Singapore) and NanoDrop one spectrophotometer; which can measure the level of contamination in a sample. Briefly, 1 µl of serum or plasma was mixed with 199 µl of Qubit buffer and loaded into the device; the result was read within 3 s according to the manufacturer's protocol. Seven samples without contamination and with significant quality of DNA were selected for further analysis.

HBV DNA Preparation and amplification

Following the manufacturer's protocol, we extracted HBV-DNA from 200 µl serum and plasma samples using QIA amp DNA Blood Mini Kits (Qiagen, Hilden, Germany).

Nested PCR and gel electrophoresis

For PCR, 4.25 µl of the extracted DNA was amplified in a nested PCR targeting the S-gene and P-gene regions of the HBV genome using two sets of primers (primer 18 & primer 16) which were synthesized by Inqaba Biotech (Pretoria, South Africa) as presented in Table 1.

Briefly, for each PCR mix tube (containing Taq DNA Polymerase, dNTPs, MgCl2, and 1 x PCR buffer), 6.25 µl of master mix was added. This was followed by the addition of 0.5 µl each of the forward and reverse primers (10 pmol), 0.5 µl of RNAse-free water and 4.25 µl of the extracted DNA to make 12 µl total volume. Positive and negative control tubes were prepared by adding the same 6.25 µl of the master mix into each tube. To the positive control tube, 4.25 µl of a known DNA (genotype E) sample was added, while 4.25 µl of deionized water was added to the negative control tube, making 11.5 µl total volume. The content of the tube was centrifuged for 30 s using a micro-centrifuge, and the tubes were loaded into the programmable thermal controller ProFlex™ PCR System (Thermo Fisher Scientific, USA). The first round PCR was programmed to first incubate the samples for 5 min at 95 °C, followed by 35 cycles consisting of 98 °C for 10 s, 98 °C for 10 s, 56 °C for 30 s, 72 °C for 1 min 90 s, and 72 °C for 10 min.

Table 1 Primers and their sequences used for HBV detection and sequencing

Primer name	Direction	Sequence	Reference
Primer 1	Forward	5'CTGCTGGTGGCTCCAGTTCAGGA3'	[25–26]
Primer 17	Reverse	5'GGGGTTGCGTCAGCAAACACT3'	
Primer 16	Forward	5'GCAGTATGGATCGGCAGAGGA3'	[27–28]
Primer 18	Reverse	5'CTGTATCTTCCTGCTGGTGGCT 3'	

After the 1st PCR the amplicons (1179 bp) were taken for gel electrophoresis using 2% agarose gel.

(Nusieve GTG, USA) dissolved in 1X TAE buffer (Sigma Eldrich, Germany) and 3 µl of SYBR Green (Invitrogen-USA). Briefly, 2 µl of loading dye was mixed with 5 µl of the amplicon and carefully transferred into the well on the gel, and this was repeated for all the samples. The loaded gel was then transferred to electrophoresis gel tank (Advanced Mupid-One, Japan) set at 135 Volts, for 30 min. Axygen gel documentation system (Corning, USA) was used for visualization of the amplified DNA depending on their availability. Samples with Positive bands were selected for the second round of PCR. In the second round, 1 µl of all 1st round PCR positive amplicons were subjected to the second stage amplification that was similar to the 1st PCR using a different set of primers (1 and 17) as shown in Table 1. Briefly a PCR master mix of 6.25 ul per tube was prepared and the 2nd PCR profile was similar to the one used in the 1st PCR.

Clean up, library preparation, and Sanger sequencing

The methodology for Sanger sequencing using the BigDye™ Terminator v3.1 Cycle Sequencing Kit was conducted following the manufacturer's guidelines (Thermo Fisher Scientific Inc., USA).

Microcentrifuge tubes were labeled "forward" and "reverse", and the sequencing reaction components were added according to the following composition: 2 µl of BigDye™ Terminator 3.1 Ready Reaction Mix, 1 µl of BigDye™ Terminator v1.1 & v3.1 5X Sequencing Buffer, 1 µl (10 pmol) of Forward primer (primer 1), 1 µl of Reverse primer (primer 17), 4 µl of deionized water (RNase/DNase-free) and 1 µl of DNA template. The plate was sealed with MicroAmp™ Clear Adhesive Film and vortexed for 2 to 3 s before brief centrifugation (10 s) in a swinging bucket centrifuge at 1,000 x g.

The sealed plate(s) were then placed in a thermal cycler, and the volume and cycle conditions were set as follows: an initial incubation at 96 °C for 1 min, followed by 25 cycles of denaturation at 96 °C for 10 s, annealing at 50 °C for 5 s, extension at 60 °C for 4 min, and a final hold at 4 °C until purification.

Following cycle sequencing, the reaction plate was briefly vortexed using VWR shakers and movers (Reax

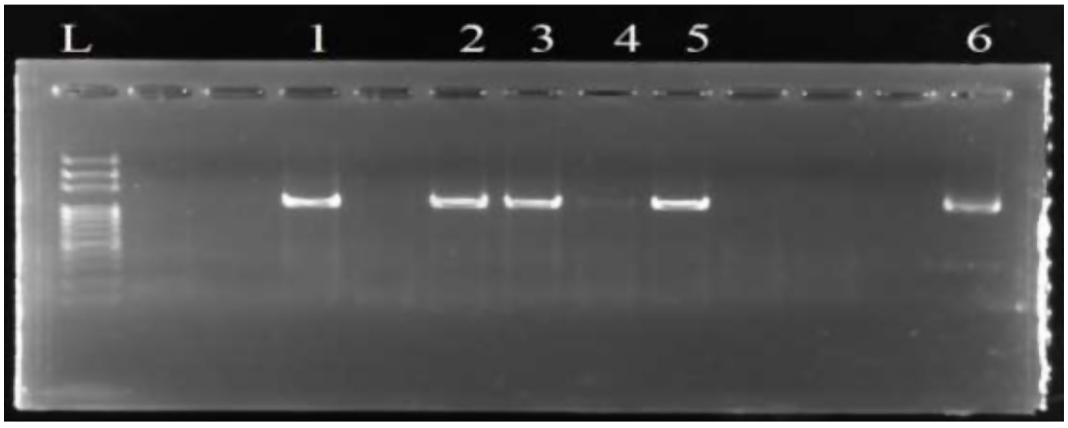

Fig. 1 Agarose gel electrophoresis micrograph depicting DNA bands (1179 bp) from selected HBV samples in comparison to a 100 bp molecular ladder. The letter 'L' denotes the molecular ladder, while numbers 1–6 indicate only samples displaying positive samples

top WVR, UK) and centrifuged for 30 s at 1,000 g using Ohaus Frontier™ 5816 (Ohaus corporation, NJ, USA). Subsequently, 40 µl of XTerminator™ Solution, which scavenges unincorporated dye terminators and free salts, and 10 µl of SAM™ Solution, which enhances the XTerminator™ Solution's performance and stabilizes post-purification reactions, were pipetted into each well. 1 µL of the sample was then extracted and inserted into a Qubit tube and mixed with 199 µl of Qubit buffer, and the tube was loaded into the Qubit chamber for reading. Samples were then pushed for sequencing. Finally, the purified plate was sealed using a heat seal or Clear Adhesive Film, vortexed for 30 min, and then briefly centrifuged. The prepared reaction plate was then loaded and analyzed using the Applied Biosystems DNA analyzer.

Phylogenetic analysis and assessment of hepatitis B virus drug resistance mutations

The evolutionary history was inferred by using the Maximum Likelihood method and Tamura-Nei model [29]. Evolutionary analyses were conducted using PhyML in GeneiousPrime. The overall mean (average) and maximum distance were used as proxies for genetic diversity. After being converted to protein sequences, the overlapping surface (S) and pol gene sequences were recovered and matched with the references in GeneiousPrime. Geno2pheno (HBV) version 2.0 (https://hbv.geno2pheno.org/index.php), a program that looks for homology between the input sequence and other DNA sequences in the existing stored database for drug resistance and surface gene mutations, was used to perform escape mutant analysis and anti-viral drug resistance analysis. The HBV Drug Resistance Database's GenBank BLAST and HBVseq tools were used to confirm genotype designations.

The sequences from this study have been deposited in the GenBank of the NCBI database with accession numbers PQ041721-PQ041722, and PQ045400-PQ045404.

Table 2 Molecular classification of HBV genotypes

Sample ID	Post cleanup Qubit values	Genotype
UNN/EN/HBV/21/004	1.65	E
UNN/EN/HBV/21/005	3.94	E
UNN/NAS/HBV/21/009	4.88	E
UNN/NAS/HBV/22/006	4.42	E
UNN/NAS/HBV/22/007	5.88	A
UNN/NAS/HBV/22/038	6.68	E
UNN/NAS/HBV/22/051	10.32	E

Results

The amplification reaction successfully amplified seven HBV samples, as evidenced by the gel electrophoresis results in Fig. 1. Six samples displayed clear DNA bands at approximately 1179 bp, compared to a 100 bp molecular ladder.

Phylogenetic analysis revealed that both samples from Enugu State were classified as genotype E. Of the five samples from Nasarawa State, four were genotype E, and one was genotype A. (Table 2).

From the results, no known antiviral resistance mutations to any known hepatitis B treatment drug were detected.

We conducted a phylogenetic analysis of HBV strains, comparing our results with reference sequences as shown in Fig. 2.

Discussion

Our findings confirm that genotype E is the predominant HBV genotype in circulation, accounting for 85.7% of the samples, while genotype A was detected only in Nasarawa State. This aligns with the established geographical distribution of HBV genotypes [13–14]. In Nigeria, genotype E is the most prevalent, notably dominant in Plateau State [30], Enugu in Southeast Nigeria [31], the Niger Delta [32], and Southwest Nigeria [33]. Phylogenetic and phylogeographic studies suggest Nigeria as the likely origin of genotype E [34]. In Nasarawa State, genotype E is

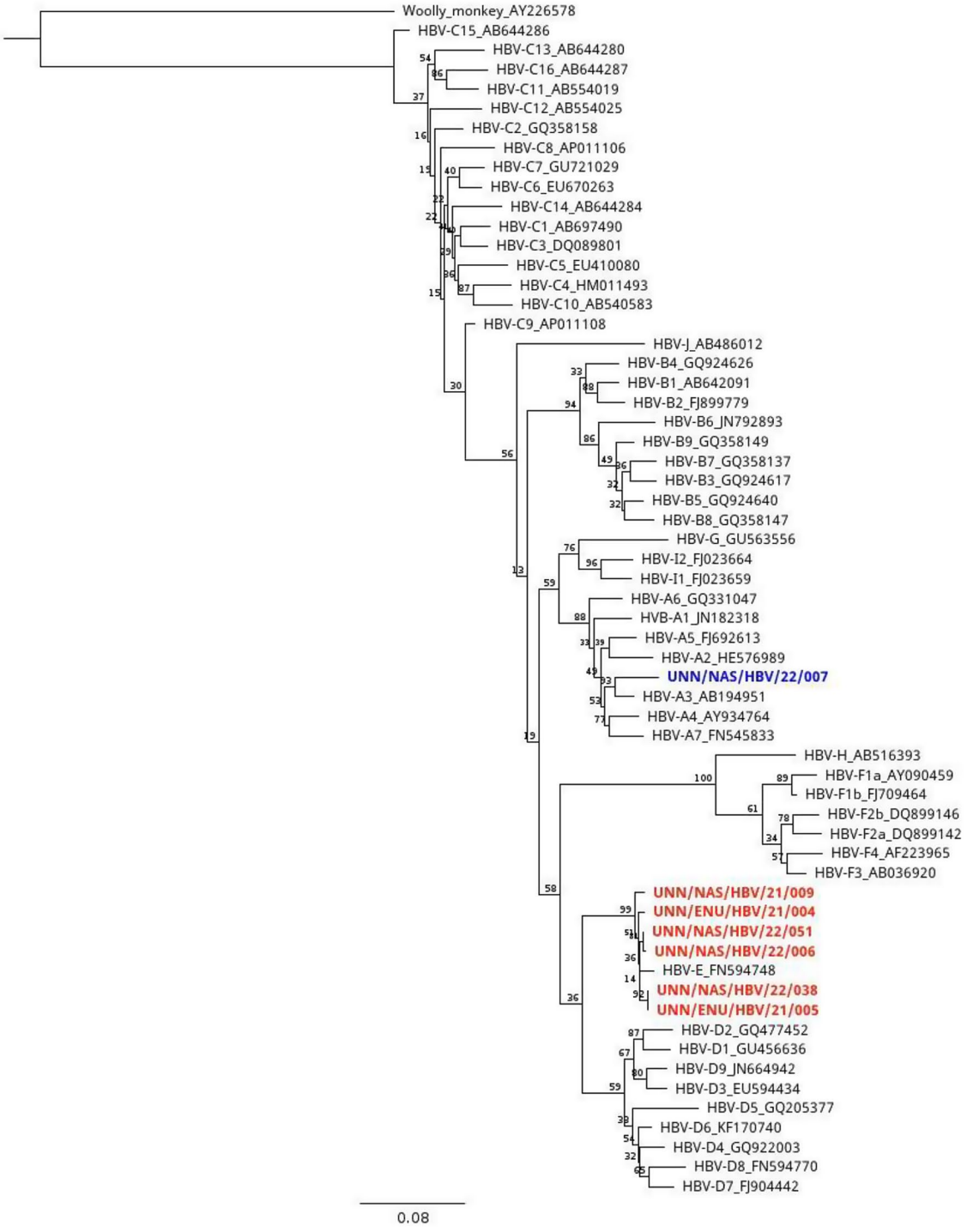

Fig. 2 Maximum likelihood tree of the partial sequence of the HBV polymerase (p) gene. Highlighted red or blue are query sequences from this study, in black are reference. HBV p-gene sequences with the genotypes and accession number shown

more common than genotype A [35]. Outside Nigeria, genotype A predominates in Cameroon [36] and has been reported in Oman [37].

Data on the clinical and virological characteristics, evolution of disease, and outcomes and treatment efficacy in patients infected with genotype E are scarce [38]. However, compared to other genotypes, HBV genotype E is known to exhibit greater rates of chronicity, a high viral load, and a high rate of HBeAg positive [39]. It has been hypothesized that HBV genotype E was introduced into the African population only within the past 200 years, probably through cross-species transmission [40].

Hepatitis B virus (HBV) genotype E almost exclusively occurs in African people, and its presence is more commonly associated with the development of chronic HBV (CHB) infection [34]. Additionally, an epidemiological correlation has been discovered between the prevalence of HBV genotype E infection and high hepatocellular carcinoma incidence in African nations, and it appears to possess inherent carcinogenic potential [40, 41].

Analysis of some of the reference sequences revealed that various genomic fragments clustered with different genotypes on phylogenetic trees, suggesting potential recombination events. Although our genotype E samples matched some of these sequences, indicating the possibility of recombination, our analysis did not extend to confirming recombination events in these strains.

Although HBV possesses a DNA genome, its replication involves an unstable RNA intermediate, during which its error-prone reverse transcriptase lacks proofreading capability. This deficiency leads to the continuous accumulation of mutations, giving HBV an unusually high mutation rate compared to other DNA viruses. Mutations in HBV linked to vaccine resistance and immune evasion have been widely reported across multiple countries, posing significant challenges to disease control and surveillance efforts. No antiviral resistance mutations were detected in any of the samples from this study, in contrast to reports from Saudi Arabia, where multiple mutations in the RT gene linked to antiviral resistance and mutations both within and outside the major hydrophilic region of the S gene associated with immunogenicity and potential immune escape were identified in nearly all patients [42]. Similarly, a study by Adesina et al. [43] in Nigeria reported immune escape mutations (IEMs) in 18.2% (8/44) of HBV-positive individuals with available sequence data, but no drug resistance mutations (DRMs), aligning with our findings. This study therefore provides valuable genotype data and confirms the absence of antiviral resistance mutations.

Conclusion

From our results, HBV genotype E is the predominant genotype in circulation in the study area, without any escape and antiviral mutations.

Abbreviations

HCV	Hepatitis C Virus
HBV	Hepatitis B virus HBcAb: Antibodies against hepatitis B core antigen
HBsAb	Antibodies against hepatitis B surface antigen HBV Hepatitis B virus
PCR	Polymerase chain reaction
DNA	Deoxy Ribonucleic Acid

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Author contributions

KAE conceptualized and designed the study and drafted the manuscript. FE, NFU, and ESE assisted in sample collection. ROO, KII, CC, and IKC: contributed to DNA extraction and sequencing. OAA conducted sequencing and genomic data analysis. ACI Supervised the study and critically revised the manuscript. All authors read and approved the final version of the manuscript and agree to be accountable for all aspects of the work, ensuring its accuracy and integrity.

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Data availability

The sequences generated in this study are publicly available on the NCBI database (accession numbers PQ041721, PQ041722, and PQ045400, PQ045401, PQ045402, PQ045403, and PQ045404).

Declarations

Ethics approval and consent to participate

This study was carried out to the ethical principles of the Declaration of Helsinki. The study received ethical approval from the Research Ethics Committees of both the Enugu State Ministry of Health Nigeria (MH/MSD/REC20/135) and the Nasarawa State Ministry of Health Nigeria (NHREC Protocol NO: 18/06/2017). Informed consent was actively sought from all participants and obtained before the commencement of sample collection.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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