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HEPATITIS B VIRUS CORE GENE MUTATIONS IN APPARENTLY HEALTHY INDIVIDUALS IN SOUTHWEST NIGERIA

Adesina O. A.[1*], Akanbi O. A[2] ,Oluyege A[3]

[1]Department of Microbiology, Obafemi Awolowo University, Ile-Ife, Nigeria
[2]Nigeria Centre for Disease Control and Prevention, Abuja, Nigeria
[3]Department of Microbiology, Ekiti State University, Ado-Ekiti, Nigeria

***Author for Correspondence: :** adesinaf@oauife.edu.ng;

ABSTRACT

The hepatitis B virus has four open reading frames whose products have definite roles relating to the diagnosis, pathogenesis, and by extension the management of hepatitis B. While studies have been done on the S gene in Africa, there is a paucity of data on other genes, including the HBc gene. This study was designed to determine the prevalence and types of HBc gene mutations in southwest Nigeria. After serological screening of 223 apparently healthy and febrile individuals, one hundred and ninety-nine (199) samples were positive for HBsAg, out of which thirty-five (35) had the HBc gene amplified and sequenced. Whereas only six (17.1 %) of the 35 HBV C gene positive reported to the medical facility for being sick, the 29 others (82.9 %), who are likely chronic carriers of the virus, were apparently healthy. This study reveals the occurrence of cS83P (18; 51.4 %) mutation which has been associated with hepatocellular carcinoma and cR147C (18; 51.4 %) which has been reported to influence antigenicity and stability of HBV and may create escape mutants. More studies need to be carried out on HBV genes to aid its control in West Africa.

Keywords: Hepatitis, Core gene, mutations, escape

INTRODUCTION

Despite the intervention of vaccines, infection as a result of Hepatitis B virus (HBV) remains a global public health issue . Approximately twenty-five percent of the global population has been exposed to HBV and the annual mortality rate as a result of progressive liver diseases such as cirrhosis and hepatocellular carcinoma (HCC) sums up to about 1 million . The hepatitis B virus has a genome of about 3200 base pairs that form a relaxed circular DNA (rcDNA) with a complete negative strand and an incomplete positive strand .

Of note is the fact that the HBV evolution rate ranges from about 2.2×10^{-6} to 7.7×10^{-4} nucleotide substitutions/site/year. As a result of the uniqueness of the HBV life cycle where an error-inclined reverse transcriptase is used for genome replication, HBV portrays a large genetic variability, resulting in 10 genotypes (A–J), approximately 40 sub-genotypes, various recombinants, clades, and quasispecies. The genome of HBV comprises four overlapping open reading frames (ORFs) namely P, preS1/S2/S, preC/C, and X that encode major products. A polymerase protein (P) contains the domains of DNA polymerase, reverse transcriptase, and RNase H, The preS/S region codes for the three forms of surface proteins which are L/preS1, M/preS2, and S (HBsAg), HBV C gene is divided into preC and a C region that is responsible for encoding HBcAg and HBeAg proteins, and X ORF encodes HBx, which has been reported to regulate viral transcription and oncogenic activity' .

The HBV core promoter and precore region regulate the production of core proteins which make it a trouble spot for mutation. Noteworthy is

the close relationship between these mutations and the severity of Chronic Hepatitis B (CHB) viral infection . The HBx gene's C-terminal coding sequence and the HBV core promoter region overlap, and mutations in this area may enhance carcinogenesis by altering the biological properties of HBx . Different types of preC/C mutants have been reported such as G1896A (nt), C1766T (nt), T1768A (nt), A1726T (nt), G1764A (nt), and T1753V (nt). The level of significance/impact of these mutants includes prevention of the expression of HBeAg protein, increase in the HCC tumour growth in vivo via enhancing HBV replication and activating the ERK/ MAPK pathways, enhanced viral replication and viral infectivity. The combination of C1766T/T1768A mutants has been associated with higher HBV-DNA levels and amplified liver disease. The major target of the host immune system is the HBcAg, and significantly, mutations in the preC/C region causing immune escape could induce persistent HBV infection .

This study was designed to determine the frequency of and the types of mutations in the C gene of HBV in patients and apparently healthy individuals in Southwest, Nigeria.

MATERIALS AND METHODS

Study Location and Population

Serum samples were obtained from about 3ml of venous blood collected from consenting apparently healthy persons, pregnant women, and sick individuals presenting with fever, and abdominal disturbance from Ekiti, Lagos, Ondo, Osun, and Oyo States, southwest Nigeria. The participants were drawn from Private hospitals, Primary Healthcare Centres (PHC), and Secondary Healthcare facilities in the states. Excluded from this study were those with known hepatitis conditions and those already presenting with complications of hepatitis such as cirrhosis and hepatocellular carcinoma (HCC). A structured questionnaire was administered to the participants to gather demographic information, health conditions, and travel history, among others, before blood sample collection.

Ethical Considerations

Ethical approval and/or permission were obtained, as appropriate, from the Research and Ethical Committees of the health facilities where samples were collected.

Serology

The serological screening for hepatitis B surface antigen (HBsAg) was done using a WANTAI ELISA kit (Beijing WANTAI Biological Pharmacy Enterprises Co. Ltd., China) for the identification of likely carriers of the virus. The procedures were carried out following the manufacturer's instructions . All HBsAg positive and some randomly selected negative samples were prepared for viral DNA extraction for further molecular analysis.

Nucleic acid extraction

This was carried out with 135 µl of each serum sample using the QIAampMinElute Virus Spin Kit (Qiagen, Hilden, Germany). Qiacube robot (Qiagen, Hilden, Germany) was used for the automated extraction process which produced 60 µl of purified viral nucleic acids eluted in the provided buffer for each sample and stored at -20^{0}C until used.

Nucleic acid detection by quantitative real-time PCR (qRT-PCR)

The extracted nucleic acids were subjected to quantitative real-time PCR (qRT-PCR) to detect the presence or otherwise of hepatitis B virus using Superscript III/Platinum one-step RT-PCR Kit (Invitrogen, Germany) and a Lightcycler 480 for the run using appropriate probes, primers and Taq polymerase and specified protocol.

HBc gene amplification and sequencing

The HBc gene was detected and amplified in a 12.5 µl reaction including 1 µl of the template, 4.25 µl of PCR grade water, 6.25 µl of HotStart master mix (Qiagen, Hilden, Germany), and 0.5 µl each of primer sets HBV-46 and HBV-68 for first round of PCR conditioned at 10 mins at 95°C, [30 secs at 94°C; 30 secs at 54°C; 45 secs at 72°C] 40 cycles, 10 mins for 72°C and holding at 8°C. For the second PCR, 6.25 µl of HotStart master mix, 4.75 µl of PCR grade water, and 0.25 µl each of the primers HBV-46 and HBV-61 and 1 µl of the first-round amplicon were used. The conditions for the amplification are 10 mins at 95°C, [30 secs at 94°C; 30 secs at 54°C; 45 secs at 72°C] 40 cycles, 10 mins at 72°C, and holding at 8°C. The expected band size for the second reaction is 650 bp.

The amplified PCR products were visualized using 1.5% agarose gel electrophoresis stained

with gel red. The power system was set at 100V, 100mA for 30-60 minutes while visualization under the UV light source was done using a Biometra Analytic image viewer (Jena, Germany). After cleaning, the products of all the samples positive for the HBc gene in the second PCR run were sequenced using the Sanger method.

Phylogeny reconstruction was done using the neighbour-joining method on the tree explorer of MEGA 11 with HBV standard references and out groups. Mutation analysis was done using BioEdit version 7.2.6, MEGA 11, and geno2pheno software. Genomic sequences obtained for the HBc gene were subjected to phylogenetic analysis and then translated into amino acid (aa) sequences to compare them with HBV reference sequences on the NCBI database.

RESULTS

Out of the one hundred and ninety-nine (199) samples positive by HBV serological screening, thirty-five of all the samples screened were found to have the HBc gene amplified and sequenced. The sequences were genotyped, and all belonged to genotype E. The gel image and the phylogenetic tree are shown in Plate 1 and Figure 1, respectively.

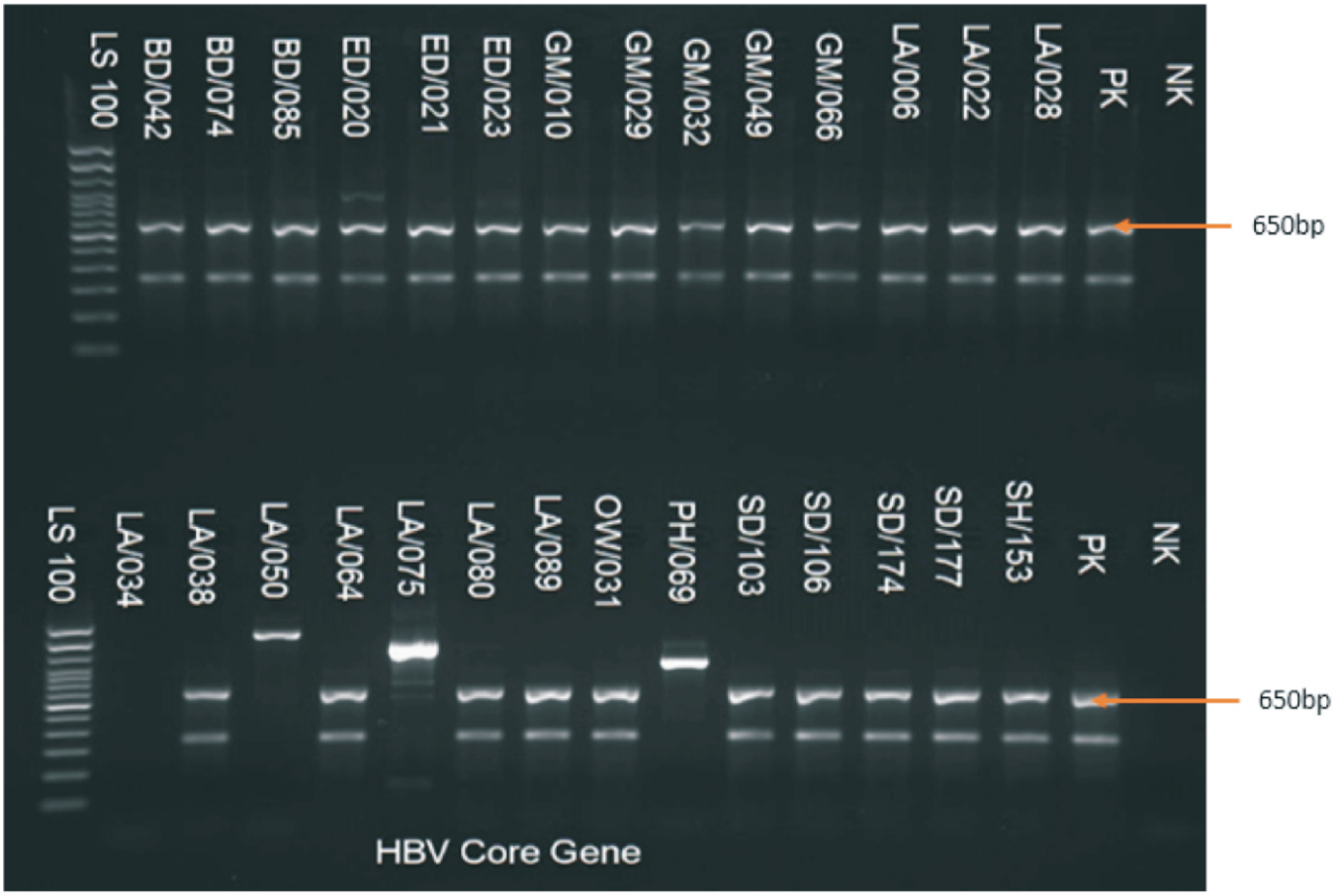

Plate 1. A representative HBV C gene gel picture after nested PCR at 650bp using a 100bp marker.
Note: NK – Negative control; PK – Positive control; LS 100 – 100 base pair Ladder/Marker. LA/xxx, OW/xxx, SD/xxx, PH/xxx, SH/xxx, BD/xxx and ED/xxx – codes representing the samples from this study.

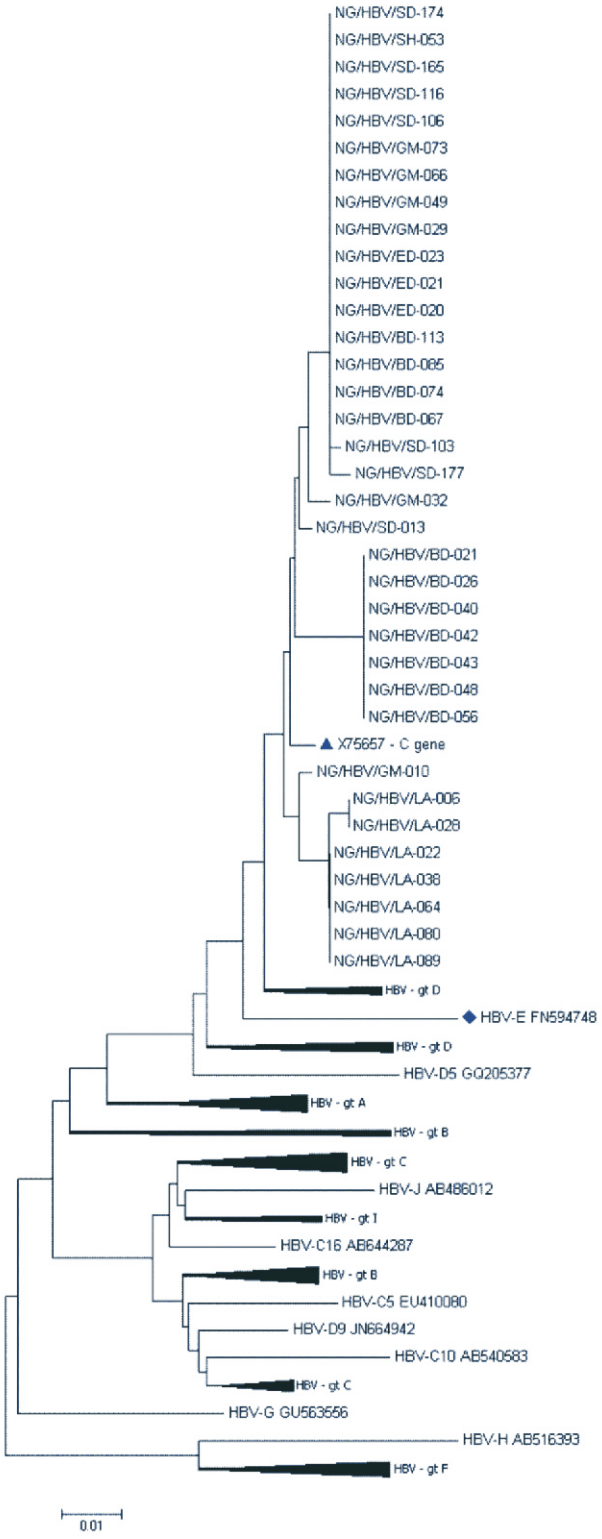

Table 1: Combinations and frequency of mutations observed on the C ORF

Combination of mutations	Number of samples (%) (N=35)
Q2H + L55F + S59G + V70L + K60* + V70L + V76I + C86G + P116S + S117G	7 [a] (20)
L55F + H85Y	8 (22.9)
S83P + C86G	18 (51.4)
C86G + K199Q	1 (2.9)
T97P + D99E	1 (2.9)
P116S + P127T + R147C	1 (2.9)
P127H + R147C + F213I	1 (2.9)
P127T + H137Y + F154I	6 [b] (17.1)
R147C	16 (47.1)
P127T + H137Y + F154I + F213I	1 (2.9)

Key: a - All vaccine-escape mutants; b - All from Lagos.

Figure 1: Phylogenetic tree showing the HBV C gene isolates from SW Nigeria and other HBV C gene reference strains. The tree was constructed using the neighbour-joining method in MEGA. Reference sequences were obtained from the GenBank database. The bar indicates the variation scale.

The sequences were aligned with a reference HBc gene sequence - X75657 to identify points of mutation after translation. A representation of the sequence alignment is shown in Figure 2.

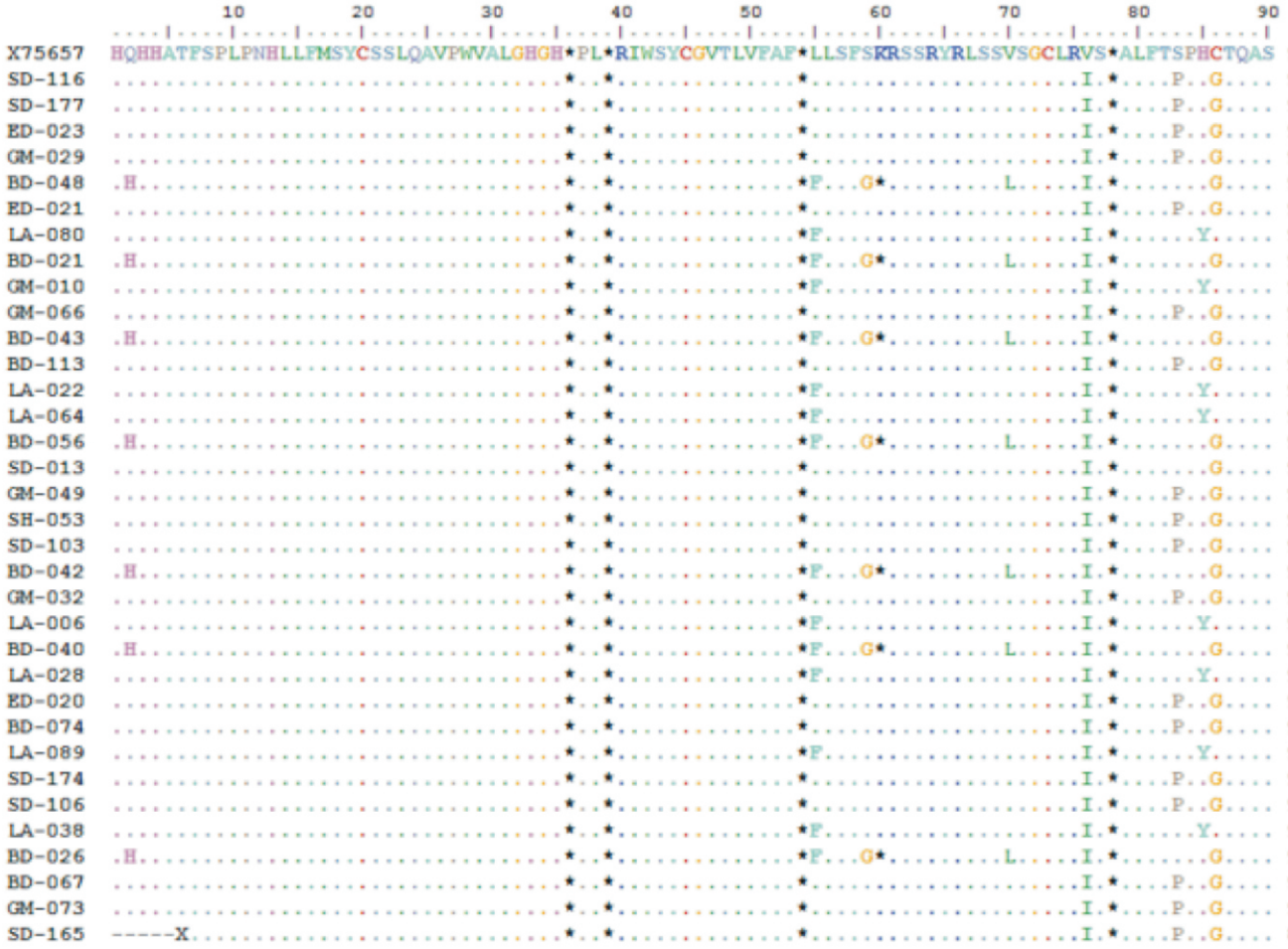

Figure 2: Representative HBc ORF from the study aligned with a reference C gene amino-acid sequence - X75657 showing points of mutation on the C gene
Note: Not all the observed mutations are shown

The sequences have been deposited in the GenBank with accession numbers MT570112 – MT570146. Different kinds of mutations observed are listed in Table 1. Only six (17.1 %) of the 35 HBc gene-positive reported being sick, while 29 (82.9 %) were apparently healthy asymptomatic HBV carriers.

DISCUSSION

HBV precore/core ORF encoding proteins, hepatitis B e antigen (HBeAg), and core antigen (HBcAg), are two indicators of active viral replication. This suggests the reason why this gene needs to be studied so that false negative results will not be reported in cases of active hepatitis B virus infection, which can enhance its further spread and pathogenesis. The double mutation, A to T transversion at 1762 and G to A transition at 1764 is often present in patients with chronic hepatitis, hepatocellular carcinoma, and fulminant hepatitis, and less often in asymptomatic carriers, and in carriers without HBV markers (Kramvis and Kew, 1998) were not recorded in this study.

Most of the mutations reported in this study have not been reported or previously studied to have known effects on the pathogenesis of HBV infection. This study was faced with the challenge of the paucity of related genotype E data to compare with, however, two of the previously reported HBV C gene mutations cS83P and cP116s were observed across the five states. To buttress the importance of HBV precore/core mutations, point mutations between aa 74–89 may reduce both HBe and HBc antigenicity (Gunther *et al.,* 1998) while the cellular and humoral immunity are affected by mutation at codon 130 (P to T/S) as this codon is part of domain recognized by B cells and T cells

(Sallberg *et al*., 2012).

Despite the availability of an effective vaccine which was introduced into Nigeria's National Programme of Immunization (NPI) about twenty years ago, HBV infection remains a major public health concern, particularly in endemic areas and hyperendemic areas like Nigeria. Basal core promoter/precore mutations have been said to have clinical implications for HBeAg-negative hepatitis (Lazarevic, 2017). In this study, we observed Immune-escape mutations in the M2RR of HBcAg, particularly in the hotspot region comprising amino acid residues 81-105 in agreement with the report of Ferrari and colleagues(Ferrari *et al*., 1991)with the occurrence of cS83P (18; 51.4 % - 9 from Osun, 6 from Oyo and 3 from Ekiti states), this mutation is also reported to be implicated in HCC. Furthermore, cR147C (18; 51.4 %), a mutation that has been reported to influence antigenicity and stability of HBV, which may also create escape mutants leading to persistent and severe liver disease among others was also observed in the same proportion with cS83P. This report calls for a more in-depth study on the escape mutations observable in HBV to aid our understanding of the evolution of the virus and hence its control and management.

While V76I and R96G mutations were observed in all the samples studied, P127T, H137Y and F154I were observed in all the samples from Lagos state only, this could be one of the reasons why they cluster together distinctly on the phylogenetic tree. Although the effect of these mutations may not be related to escape mutation or tumorigenesis, yet they need to be further investigated for their roles in other aspects of the virus and host infection.

Molecular epidemiologic studies of diseases have the purpose of improving our understanding of the pathogenesis of diseases by identifying specific pathways, molecules, and genes that influence the risk of developing the disease. It also reveals how the genetic composition of the pathogen, and the environmental factors affect or aid the production and perpetuation of a disease. This has been hampered by a lot of factors ranging from poverty, high illiteracy levels in some areas, inadequate research funding, and attitude among others.

Most of the studies on the mutations observable in HBV ORFs in Nigeria have been limited to sequence analysis however, the phenotypic effects of such mutational changes have not been investigated. Such studies would give more insight into the effects of the changes on pathogenesis, diagnosis, persistence, and prevention of the disease. Such studies will probably provide some answers to the peculiarity of HBV being geographically distributed and possibly the effect of the vaccines available.

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