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Detection and circulation of hepatitis B virus immune escape mutants among asymptomatic community dwellers in Ibadan, southwestern Nigeria

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ABSTRACT

Background: In 2012, the first Nigerian Hepatitis B Virus (HBV) immune escape mutant (IEM) case was detected in a pregnant woman in southwestern Nigeria. Consequently, this study was designed to investigate the presence and possible circulation of IEMs amongst asymptomatic community dwellers in southwestern Nigeria.

Methods: Blood specimens collected from 438 asymptomatic community dwellers were screened for HBsAg using ELISA technique. Subsequently, the S-gene was amplified in HBsAg positive samples by a nested PCR protocol, and amplicons sequenced. Isolates were then subtyped by amino acid residues at positions 122, 127, 134 and 160, and genotyped by phylogenetic analysis.

Results: Of the 31 (7.08%) samples positive for HBsAg, the ~408 bp Sgene fragment was successfully amplified and sequenced in 27. Samples obtained from 4 patients could not be amplified due to low titres. Sequence data from only 15 of the isolates could be analysed further as eight of the remaining 12 had multiple peaks while the rest three showed no similarity to any HBV gene when subjected to BLAST analysis. Thirteen of the 15 isolates were identified as genotype E. Eleven of which were subtyped as ayw4 while the remaining two could not be subtyped due to sR122Q/P substitutions. The last two isolates that could not be genotyped and subtyped had other mutations in the "a" determinant associated with IEMs.

Conclusions: This study confirmed presence and circulation of HBV IEM in Nigeria, the country's inclusion in the genotype E crescent, and the value of phylogenetic analysis in HBV identification.

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1. Introduction

Globally, it is estimated that about 360 million people are chronically infected with Hepatitis B Virus (HBV) and over two billion people have serologic evidence of past or present HBV infection.[1] Consequently, HBV ranks as one of the top 10 viral infections.[2] There is evidence that chronic HBV infection could result in cirrhosis and accounts for a significant number of deaths

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from hepatocellular carcinoma.[3] Besides chronic hepatitis B (CHB), HBV infection could also manifest as acute, self-limiting disease.[4]

HBV is a member of the genus *orthohepadnavirus*, family *Hepadnaviridae*. The virion has a host derived lipid envelope and a diameter of ~42 nm. Within the envelope, the partially dsDNA, ~3.2 kb genome is enclosed in a capsid made up the core (C) protein. Based on how different their genomes are, eight genotypes of HBV have been described.[5] Genomes that differ by not more than 8%[6] and 4%[7] are classified as belonging to the same genotype and sub-genotype respectively. The HBV genome has four open reading frames (ORFs) named X, C, P and S with the S ORF overprinted on the P ORF in a different reading frame.

The S ORF codes for the HBV surface antigen (HBsAg) which is the only HBV genome- encoded trans-membrane protein present in the virion membrane.[8,9] HBsAg functions as the virus attachment protein and antibodies elicited against specific epitopes on it have neutralizing activity.[10] In addition, when expressed in eukaryotic cells, HBsAg has the capacity to produce the 22 nm spheres and filaments that are found in HBV infected individuals and account for over 80% of the HBsAg in such people.[9,11]

Initially, these 22 nm spheres and filaments were purified from the serum of HBV infected individuals and used as vaccines.[10,12–14] This has however been replaced by recombinant vaccines produced by expression of HBsAg in eukaryotic cells.[15,16] Furthermore, neutralizing antibodies (HBIg) elicited against specific epitopes on HBsAg have been produced and are available as a form of passive immunization.[3] The availability of an effective and safe HBV vaccine has resulted in a global effort to eradicate HBV. Consequently, in most countries of the world HBV vaccine is administered to at risk individuals and children at birth.[4,17]

Cases in which people with immunological correlates of HBV immunity get re-infected have been described[18–21] and this poses a serious threat to the success of the HBV eradication programme. Isolates recovered from such cases were coined Immune Escape Mutants (IEMs).[18,22,23] First described in 1990,[18] this adaptive response of HBV to selective pressure has been ascribed to the RNA phase in its replication cycle and a polymerase that lacks "proof-reading" ability.[24,25] As a result, mutants develop during replication at almost the same rate as RNA viruses[24,25] and the most fit members of the mutant population can be selected for by anti-HBs and in other cases, nucleoside/nucleotide analogue classes of antiviral drugs.[26]

Though the presence and circulation of IEMs have been reported globally since 1990,[18–21,27] in 2012, the first Nigerian IEM case with mutations in the "a" determinant was detected in a pregnant woman in southwestern Nigeria.[28] Consequently, this study was designed to investigate the possible circulation of IEMs in asymptomatic community dwellers in the region. This study reports the presence and circulation of HBV IEMs in asymptomatic community dwellers in southwestern Nigeria.

2. Methodology

2.1. Study location and Sample collection

This study was carried out in Ibadan, Oyo State, southwestern Nigeria. The study was community based and ethical approval for the study was granted by the Oyo State Ministry of Health (AD3/479/349). Samples analysed in this study were collected from apparently healthy community dwellers between July and September, 2013. During every sample collection visit, before sample collection, consenting participants were educated on HBV infection, its clinical manifestation, prevalence and control. Subsequently, a questionnaire was administered in a bid to retrieve demographic and other relevant information. Afterwards, a blood sample was collected from a total of 438 {median age = 30 years, age range = 1.5 – 87 years (Male = 133; age range = 1.5 – 87 years; Females = 305; age range = 7 – 80 years)} consenting participants.

Using venepuncture, five millilitres of blood was collected from each participant and dispensed into anticoagulant-free, appropriately labelled sterile container. Thereafter, the samples were transported to the laboratory in the Department of Virology, College of Medicine, University of Ibadan, in cold chain. On arrival at the laboratory, the blood sample was centrifuged at 500X*g* for five minutes. The serum was then carefully collected using sterile disposable pipettes and aliquoted into two appropriately labelled cryovials per sample. Subsequently, sera from the blood samples were stored at −20 °C until analysed. Laboratory analysis was carried out in both the Department of Virology, and the Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Nigeria.

2.2. HBsAg ELISA Screening Test

A sandwich enzyme linked immunosorbent assay (ELISA) for detection of HBsAg (Diagnostic Automation/Cortez Diagnostic, California, USA) was used to screen all the 438 sera for the presence of HBsAg. The assay was carried out in accordance with manufacturer's instructions. The Emax endpoint ELISA microplate reader (Molecular Devices, California, USA) was used to determine the optical density after which the result was interpreted in accordance with the manufacturer's instructions.

2.3. DNA extraction and S-gene specific Polymerase Chain Reaction (PCR).

This was done as previously described.[28,29] Briefly, the QIAGEN DNA extraction kit (Qiagen, Hilden, Germany) was used for viral DNA extraction in accordance with the manufacturer's instructions. Afterwards, a ~408 bp stretch within the S ORF of the HBV genome was detected using a nested PCR assay. Primers used were HBV_S1F and HBV_S1R for the first round and HBV_SNF and HBV_SNR for the second round.

Two microlitres of each of the primers (made in 25 μM concentrations) were added to a 50 μL reaction which also contained 10 μL of Red load Taq (Jena Bioscience, Jena, Germany), 4 μL of DNA and 32 μL of RNase free water. Veriti Thermalcycler (Applied Biosystems, California, USA.) was used for thermal cycling as follows; 94 °C for 3 minutes followed by 45 cycles of 94 °C for 30 seconds, 55 °C for 60 seconds and 70 °C for 40 seconds with ramp of 40% from 55 °C to 70 °C. This was then followed by 72 °C for 7 minutes and held at 4 °C till terminated.

Both first and second round PCR reaction conditions were the same except that DNA extract from the sample was used as template for first round while first round PCR product was used as template for second round PCR. All PCR products were resolved on 2% agarose gels stained with ethidium bromide and viewed using a UV transilluminator.

2.4. Amplicon sequencing

Positive PCR reactions were shipped to Macrogen Inc, Seoul, South Korea, for amplicon purification and BigDye chemistry sequencing. Sequencing was done using second round PCR primers.

2.5. Phylogenetic analysis

Determination of HBV serotypes was done using amino acid residues at positions 122, 127, 134 and 160 of the S-gene.[27] Afterwards, partial S-gene sequences generated in this study were aligned alongside S-gene reference sequences downloaded from

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