International Journal of Infectious Diseases 39 (2015) 102–109

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

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

Temitope Oluwasegun Cephas Faleye [a,g], Olubusuyi Moses Adewumi [a,*], Ijeoma Maryjoy Ifeorah [b], Adegboyega Akere [c], Adeleye Solomon Bakarey [d], Ewean Chukwuma Omoruyi [e], Kemi Oketunde [h], Oluwajumoke Bosede Awonusi [h], Modupe Racheal Ajayi [g], Johnson Adekunle Adeniji [a,f]

[a] Department of Virology, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria
[b] Department of Surgery, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria
[c] Department of Medicine, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria
[d] Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria
[e] Institute of Child Health, College of Medicine, University of Ibadan, Ibadan, Oyo State, Nigeria
[f] WHO National Polio Laboratories, University of Ibadan, Ibadan, Oyo State, Nigeria
[g] Department of Microbiology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti State, Nigeria
[h] Department of Science Laboratory Technology, Faculty of Science, Ekiti State University, Ado Ekiti, Ekiti, State, Nigeria

ARTICLE INFO

Article history:
Received 5 March 2015
Received in revised form 25 July 2015
Accepted 9 August 2015

Corresponding Editor: Eskild Petersen, Aarhus, Denmark

Keywords:
Hepatitis B virus
HBV
Immune escape mutant
IEM
Ibadan
Nigeria

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.

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

* Corresponding author.
E-mail addresses: faleyetemitope@gmail.com (T.O.C. Faleye), adewumi1@hotmail.com (O.M. Adewumi), maryjoy1023@yahoo.ca (I.M. Ifeorah), adeakere@yahoo.co.uk (A. Akere), drbakarey@yahoo.com (A.S. Bakarey), omochukwu2001@yahoo.com (E.C. Omoruyi), oluwakemisolavera@gmail.com (K. Oketunde), b_jummy@yahoo.com (O.B. Awonusi), ajayimodupe@yahoo.com (M.R. Ajayi), adek1808@yahoo.com (J.A. Adeniji).

http://dx.doi.org/10.1016/j.ijid.2015.08.008

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

the HBV database (http://hbvdb.ibcp.fr/HBVdb/). This was done using the CLUSTAL W program in MEGA 5 software with default settings.[30] Subsequently, using the MEGA 5 software, Kimura-2 parameter model and 1,000 bootstrap replicates, a neighbour-joining tree was constructed and used to determine the genotypes of all isolates. In addition, S-gene pairwise distance was estimated using MEGA 5 software with Kimura-2 parameter model.[31]

2.6. SimPlot Analysis

SimPlot version 3.5[32] was used to assess similarity to reference sequences of sequences that could not be typed using the above methods. It was also used to assess the probability that such sequences were of recombinant origin. To ensure recombinant sequences will be detected, two genotype D/E recombinant sequences (gnl|hbvcds|AB033559 and gnl|hbvcds|AB048702) were downloaded from the HBV database (http://hbvdb.ibcp.fr/HBVdb/) and included in multiple sequence alignment of reference and query sequences used for this analysis.

Similarity plots were generated using the Kimura (2-parameter) distance model, a window size of 200 bp moving in steps of 20 bp. Subsequently, using the Kimura (2-parameter) distance model, a neighbour-joining tree model and 100 bootstrap replicates, bootscan analysis was run with a window size of 200 bp moving in steps of 20 bp in a bid to detect any recombination event.

2.7. Nucleotide sequence accession numbers

Accession numbers of S-gene sequences retrieved from the HBV database are indicated in the sequence names on the phylograms. The S-gene sequences reported in this study have been submitted to GenBank under "the accession numbers KP780133 to KP780147".

3. Results

3.1. HBsAg ELISA Screening Test

HBsAg was detected in 31 (7.08%) of the 438 samples screened. The remaining 407 (92.92%) samples were negative for HBsAg. HBsAg was detected in samples from subjects within the ages of 18 – 67 years. Among the males, 17 (12.78%) of the 133 samples collected were positive for HBsAg. This represents 54.84% (17/31) of the total number of HBsAg positive samples recorded in this study. Among the females, 14 (4.59%) of the 305 samples collected were positive for HBsAg. This represents 45.16% (14/31) of the total number of HBsAg samples detected in this study. (Table 1).

3.2. DNA extraction, S-gene specific Nested Polymerase Chain Reaction (nPCR) and Sequence analysis

All the 31 HBsAg positive samples were subjected to DNA extraction and HBV *S*-gene specific nPCR. Only 27 (87.1%) of the samples yielded the expected ~408 bp amplicon. The remaining four (12.9%) samples yielded no amplicon despite repeated attempts using varied annealing and extension temperatures.

All the 27 amplicons were sequenced. However, 9 (33.33%) were not exploitable due to the presence of multiple peaks. The sequence data of the exploitable 18 isolates were then subjected to further analysis.

3.3. Phylogenetic analysis

After the forward and reverse sequencing results per isolate were stitched into contigs, each was subjected to a BLASTn search on the NCBI BLAST webpage. Fifteen of the isolates showed significant similarity to HBV *S*-gene. The remaining three isolates were similar to a human gene and showed no similarity to the HBV *S*-gene. Consequently, these three isolates were stepped down from further analysis.

Using amino acid residues at positions s122, s127, s134 and s160,[27,28] eleven of the 15 isolates were subtyped as ayw4 (Table 2). As a result of substitutions at positions s122 (Table 1, Figure 1), the remaining four isolates (NGR IBGP 20 C4 HBsAg, NGR IBGP 73 D6 HBsAg, NGR IBGP 121 E1 HBsAg and NGR IBGP 408 H2 HBsAg) could not be subtyped.

In a bid to genotype the isolates, representative HBV *S*-gene sequences of genotypes A – H were downloaded from the HBV database (http://hbvdb.ibcp.fr/HBVdb/). These reference sequences alongside HBV *S*-gene sequences previously described in the region[28] were used to construct a phylogram. The phylogram showed that all the eleven isolates previously subtyped as ayw4 were genotype E. In addition, NGR IBGP 121 E1 HBsAg and NGR IBGP 408 H2 HBsAg which could not be subtyped because of sR122Q/P substitutions also belonged to genotype E (Figure 2). In fact, these two isolates were only 1.42% and 1.7% divergent (Table 3) from a reference genotype E isolate (AB091255).

The remaining two isolates, NGR IBGP 20 C4 HBsAg and NGR IBGP 73 D6 HBsAg were recovered from a 30 year old male and an 18 year old female respectively. These isolates did not belong to genotype E as they were more than 11% divergent from the reference genotype E sequence (Table 3). The phylogram (Figure 2) however showed both isolates clustering with an isolate (NGRUC-12-054 A8 HBsAg) previously described as an Immune Escape Mutant (IEM).[28] Subsequently, isolates NGR IBGP 20 C4 HBsAg and NGR IBGP 73 D6 HBsAg were shown to be more than 11% divergent from genotypes A – H, 3.46% divergent from each other, and 3.47%

Table 1
Age and gender distribution of HBsAg prevalence amongst asymptomatic community dwellers in Ibadan, Oyo State, Nigeria.

S/N	Age Group (Years)	MALE			FEMALE			TOTAL		
		Frequency	HBsAg Positive (%)	HBsAg Negative	Frequency	HBsAg Positive (%)	HBsAg Negative	Frequency	HBsAg Positive (%)	HBsAg Negative
1	<18	15	0 (0.0%)	15	11	0 (0.0%)	11	26	0 (0.0%)	26
2	18–27	38	5 (13.16%)	33	142	7 (4.93%)	135	180	12 (6.67%)	168
3	28–37	28	5 (17.86%)	23	40	3 (7.50%)	37	68	8 (11.76%)	60
4	38–47	22	3 (13.64%)	19	43	3 (6.98%)	40	65	6 (9.23%)	59
5	48–57	15	3 (20.00%)	12	45	1 (2.22%)	44	60	4 (6.67%)	56
6	58–67	7	1 (14.29%)	6	14	0 (0.0%)	14	21	1 (4.76%)	20
7	>67	8	0 (0.0%)	8	10	0 (0.0%)	10	18	0 (0.0%)	18
	TOTAL	**133**	**17 (12.78%)**	**116**	**305**	**14 (4.59%)**	**291**	**438**	**31 (7.08%)**	**407**

Table 2
Serotype of sequenced HBV isolates

S/N	Sample ID	Amino acid residues at positions within the surface antigen for serotype determination				Serotype/ Subtype
		s122	s127	s134	s160	
1	NGR IBGP 20 C4 HBsAg	Q	L	F	K	Untypable
2	NGR IBGP 22 C5 HBsAg	R	L	F	K	ayw4
3	NGR IBGP 30 C6 HBsAg	R	L	F	K	ayw4
4	NGR IBGP 32 C7 HBsAg	R	L	F	K	ayw4
5	NGR IBGP 34 C8 HBsAg	R	L	F	K	ayw4
6	NGR IBGP 43 D1 HBsAg	R	L	F	K	ayw4
7	NGR IBGP 53 D2 HBsAg	R	L	F	K	ayw4
8	NGR IBGP 71 D5 HBsAg	R	L	F	K	ayw4
9	NGR IBGP 73 D6 HBsAg	Q	L	F	K	Untypable
10	NGR IBGP 121 E1 HBsAg	Q	L	F	K	Untypable
11	NGR IBGP 137 E2 HBsAg	R	L	F	K	ayw4
12	NGR IBGP 296 G3 HBsAg	R	L	F	K	ayw4
13	NGR IBGP 393 G8 HBsAg	R	L	F	K	ayw4
14	NGR IBGP 402 H1 HBsAg	R	L	F	K	ayw4
15	NGR IBGP 408 H2 HBsAg	P	L	F	K	Untypable

and 4.06% divergent from the previously described IEM (NGRUC-12-054 A8 HBsAg) (Table 4). The similarity of isolates NGR IBGP 20 C4 HBsAg and NGR IBGP 73 D6 HBsAg to NGRUC-12-054 A8 HBsAg is further demonstrated in the conservation of almost the same amino acid substitutions in the three isolates (Figure 1). The Major Hydrophilic Region (MHR; s122 – s160) of isolate NGR IBGP 20 C4 HBsAg and NGR IBGP 73 D6 HBsAg had the following amino acid substitutions which they shared with NGRUC-12-054 A8 HBsAg; sR122Q, sC124Y, sA128T, sG130K, sC138Y, sG145K, sC147Y, sA157T and sG159K (Figure 1). In addition, NGR IBGP 20 C4 HBsAg also had sD144N.

Similarity plots (Figure 3A & B) showed the three isolates to be very similar to one another but different from the other reference sequences, the recombinant sequences inclusive. Furthermore, bootscan analysis didn't detect any evidence of these three sequences being of recombinant origin (Figure 3C) despite being able to detect reference sequences of recombinant origin as such (Figure 3D).

4. Discussion

4.1. HBV IEMs

Two (NGR IBGP 20 C4 HBsAg and NGR IBGP 73 D6 HBsAg) of the HBV isolates described in this study have the sG145K substitution which alongside the sG145R substitution have been repeatedly documented and associated with HBV IEMs.[18,22,33–35] In fact, the IEM status of these two isolates was confirmed by the geno2pheno software (http://hbv.geno2pheno.org/) (data not shown). The results of this study therefore confirm the findings of Faleye et al.,[28] that HBV IEMs are present in Ibadan, southwestern, Nigeria. It further shows that IEM is present in asymptomatic community dwellers in the region.

In the isolates NGR IBGP 20 C4 HBsAg and NGR IBGP 73 D6 HBsAg, the cysteine residues at positions sC124Y, sC138Y and sC147Y have been substituted with tyrosine (Figure 1). These substitutions will abolish the formation of disulphide bonds that have been predicted to stabilize the double-loop structure of the HBV MHR.[36] The consequent conformational alteration of the MHR might further enhance the predicted IEM phenotype as previously suggested.[28]

The HBV IEMs described in this study share substitutions in the "a" determinant between themselves and with the HBV IEM recently described in a pregnant woman in the same geographical region (Figure 1).[28] For instance, between amino acids 122 to 160, these three isolates share nine substitutions together. This level of conservation confirms the results of the phylogram (Figure 2) which suggests that these isolates share a common

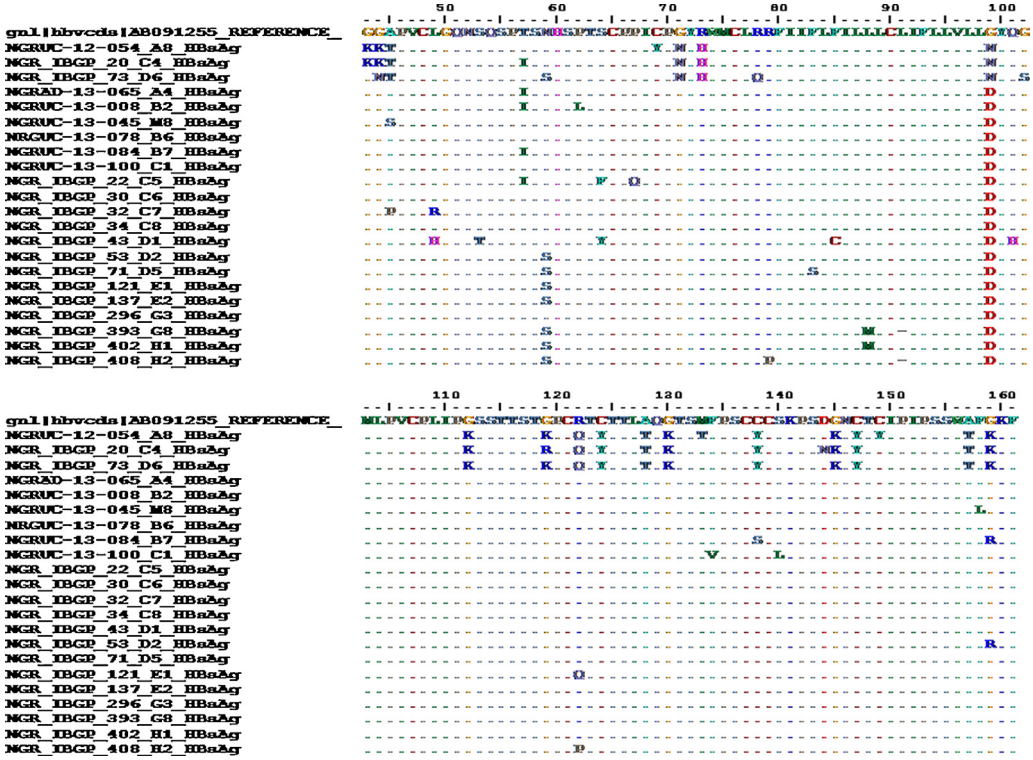

Figure 1. Alignment of amino acid residues of the fifteen isolates sequenced in this study and the seven sequenced in [28] against a reference genotype E strain. Of particular interest are isolatesNGRUC-12-054-A8-HBsAg, NGRUC-13-084-B7-HBsAg and NGRUC- 13-100-C1-HBsAg with mutations in the MHR (s122 to s16.

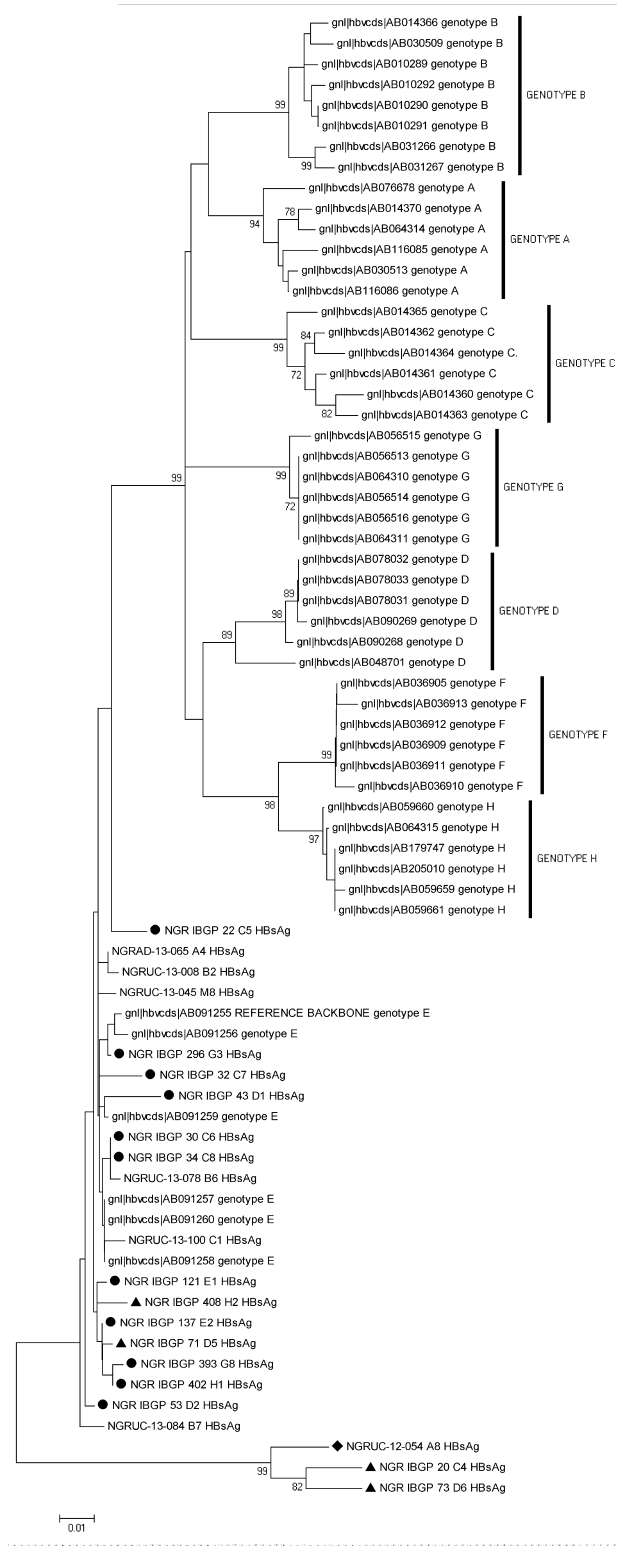

Figure 2. Phylogenetic relationship of HBV isolates. The phylogram is based on an alignment of the partial HBsAg sequences. The newly sequenced strains are highlighted with black circle and triangle. Specifically, the isolates that could not be typed using amino acid residues at positions s122, s127, s134 and s160 are indicated with black triangles. On the other hand, those that were typable by the amino acid residue algorithm are indicated with black circles. The HBV IEMs previously described [28] is indicated with a black diamond. The accession numbers of the strains are indicated in the phylogram. Bootstrap values are indicated if ≥70%.

ancestor and are likely to be strains of a circulating IEM that emerged at one point in time. To further buttress the evidence for circulation is the fact that the 18 year old female from whom NGR IBGP 73 D6 HBsAg was recovered also had HBcIgM (unpublished data). This suggests she was sampled within the first six months of infection. The results of this study therefore show that HBV IEMs are not just present but also circulating in asymptomatic community dwellers in Ibadan, southwestern, Nigeria. It is not clear when this isolate emerged and how long it has been in circulation. However, what is obvious is that none of the studies done in the region before 2012 ever reported detection of HBV IEMs with this combination of mutations in the 'a' determinant.[29,37] To the best of the authors' knowledge, the only report from Nigeria, prior to our discovery of this strain in 2012,[28] describing mutations in HBV suggestive of immune escape phenotype,[37] found deletions in the pre-S2 region. The HBV IEM strains described in this study and Faleye et al., 2015[26] might therefore be a recent development.

Despite the fact that the IEMs detected in this study and that are described in Faleye et al.,[28] were >11% divergent from all reference HBV genotypes A – H isolates used in this study and were were not of recombinant origin (Figure 3), these isolates are within the 8% genotype threshold[6] amidst themselves (Table 4). In fact, going by the 4% sub-genotype threshold[7] they all appear to belong to the same sub-genotype because NGR IBGP 20 C4 HBsAg is 3.46% and 3.47% divergent from NGR IBGP 73 D6 HBsAg and NGRUC-12-054 A8 HBsAg respectively. It has therefore not escaped our notice that these three isolates might represent members of a new genotype of HBV that have gone undetected till recently. However, more studies are required to test the validity of this hypothesis.

4.2. The Genotype E Crescent

Thirteen of the 15 HBV isolates detected in this study were identified as belonging to genotype E, thus further confirming the inclusion of Nigeria in the genotype E crescent.[28,29,37–40] If only subtyping was done, using amino acids at positions s122, s127, s134 and s160, for isolate classification, two of the thirteen typed isolates would have remained untypable. Hence, the findings of this study emphasize the need to use more than one algorithm when identifying HBV isolates in a bid to reduce the occurrence of untypable isolates.[28] However, the impact of the sR122Q/P substitution on the immunological properties and the consequent subtype of these two isolates remain to be determined.

4.3. HBV DNA Detection

Of the 31 HBsAg samples detected in this study, the HBV DNA detection assay produced the expected band size in only 27. The inability to detect the expected band size in the remaining four samples might be an indication of low viral load in the samples. In addition, it might even be suggestive of mutated or recombinant origin of the isolates in these samples and the resultant inability of the primers to recognize the target site. Furthermore, the possibility exists that these four individuals are inactive carriers.[4] However, considering that these samples are yet to be fully profiled for all HBV serological markers, the carrier state of these four individuals is at best speculative. Effort is however ongoing to better resolve the status of these cases.

There were nine samples whose sequence data were unexploitable due to the presence of multiple peaks. This might be suggestive of multiple isolates or genotypes in these individuals. In our experience, focused group discussions with such cases revealed that a good number were involved in practices that

Table 3
Comparison of reference sequence gnl|hbvcds|AB091255 genotype E to all isolates recovered in this study

S/N	ISOLATE ID	GENOTYPE E REFERENCE	Dist%	Siml%
1	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	**11.99**	**88.01**
2	NGR IBGP 22 C5 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	1.99	98.01
3	NGR IBGP 30 C6 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	0.85	99.15
4	NGR IBGP 32 C7 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	1.70	98.30
5	NGR IBGP 34 C8 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	0.85	99.15
6	NGR IBGP 43 D1 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	2.28	97.72
7	NGR IBGP 53 D2 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	1.13	98.87
8	NGR IBGP 71 D5 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	1.13	98.87
9	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	**11.95**	**88.05**
10	NGR IBGP 121 E1 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	1.42	98.58
11	NGR IBGP 137 E2 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	0.85	99.15
12	NGR IBGP 296 G3 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	0.28	99.72
13	NGR IBGP 393 G8 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	1.42	98.58
14	NGR IBGP 402 H1 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	1.13	98.87
15	NGR IBGP 408 H2 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	1.70	98.30

could result in multiple exposures like unprotected sexual intercourse with multiple sexual partners (unpublished).

Three isolates yielded exploitable data that were most similar to a human gene and totally unrelated to HBsAg (data not shown). This raises concerns about the nPCR assay used in this study. Specifically, the assay should not be used in isolation for HBV diagnosis, especially in the investigation of "occult" HBV infection. This is because the detection of the expected band size without subsequently sequencing the amplicon does not confirm the presence of HBV DNA.

4.4. Prevalence of HBsAg

HBsAg prevalence in Nigeria has been estimated to vary between 2.5% to about 40% depending on the studied population.[29,41] Consequently, Nigeria is considered to be an HBV endemic country.[29] However, the results of this study show HBsAg prevalence of 7.08% in asymptomatic community dwellers in Ibadan southwest Nigeria. This falls within the range defined by previous studies[29,41] and is in perfect agreement with the findings of Okonko et al.,.[42]

Table 4
Comparison of isolates NGR IBGP 20 C4 HBsAg and NGR IBGP 73 D6 HBsAg to reference sequences from genotypes A to H, NGRUC-12-054 A8 HBsAg (previously described IEM) and each other.

S/N	Species 1	Species 2	Dist%	Siml%
1	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB014370 genotype A	17.30	82.70
2	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB014370 genotype A	17.27	82.73
3	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB030513 genotype A	17.27	82.73
4	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB030513 genotype A	17.23	82.77
5	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB010289 genotype B	15.88	84.12
6	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB010289 genotype B	15.84	84.16
7	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB010290 genotype B	15.88	84.12
8	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB010290 genotype B	15.84	84.16
9	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB014360 genotype C	19.29	80.71
10	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB014360 genotype C	19.24	80.76
11	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB014361 genotype C	18.16	81.84
12	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB014361 genotype C	18.11	81.89
13	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB048701 genotype D	16.75	83.25
14	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB048701 genotype D	16.71	83.29
15	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB078031 genotype D	17.77	82.23
16	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB078031 genotype D	17.73	82.27
17	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB091256 genotype E	11.89	88.11
18	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB091256 genotype E	11.86	88.14
19	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	11.89	88.11
20	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB091255 REFERENCE BACKBONE genotype E	11.86	88.14
21	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB036905 genotype F	18.07	81.93
22	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB036905 genotype F	18.03	81.97
23	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB036909 genotype F	18.07	81.93
24	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB036909 genotype F	18.03	81.97
25	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB056513 genotype G	17.73	82.27
26	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB056513 genotype G	17.69	82.31
27	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB056514 genotype G	17.73	82.27
28	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB056514 genotype G	17.69	82.31
29	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB059659 genotype H	16.85	83.15
30	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB059659 genotype H	18.33	81.67
31	NGR IBGP 20 C4 HBsAg	gnl|hbvcds|AB059660 genotype H	16.85	83.15
32	NGR IBGP 73 D6 HBsAg	gnl|hbvcds|AB059660 genotype H	17.57	82.43
33	NGR IBGP 20 C4 HBsAg	NGRUC-12-054 A8 HBsAg	3.47	96.53
34	NGR IBGP 73 D6 HBsAg	NGRUC-12-054 A8 HBsAg	4.06	95.94
35	NGR IBGP 20 C4 HBsAg	NGR IBGP 73 D6 HBsAg	3.46	96.54

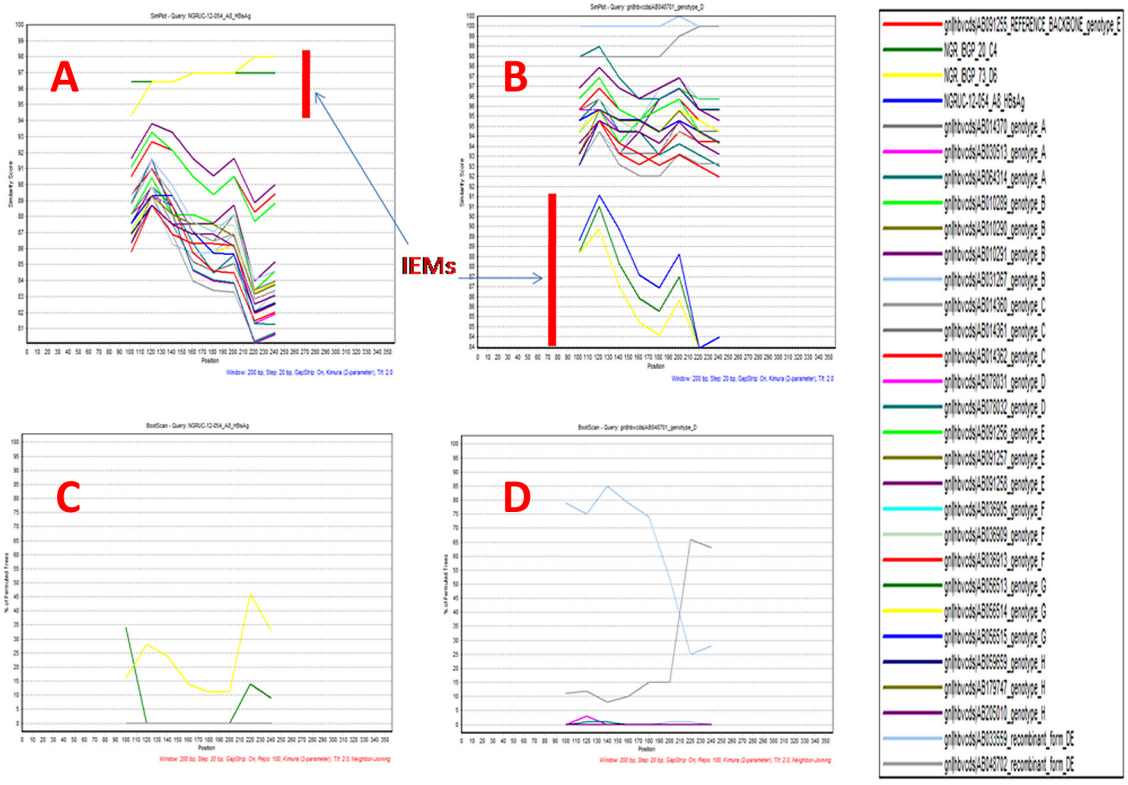

Figure 3. Similarity Plot and Bootscan analysis of the suspected Immune Escape Mutants and reference sequences. Figures 3A and 3B show similarity plots while Figures 3C and 3D show bootscan analysis results. NGRUC-12-054-A8-HBsAg is the query sequence in Figures 3A and 3C while gnl|hbvcds|AB048701_genotype_D is the query sequence in Figures 3B and 3D.

Studies have shown that HBsAg prevalence is usually higher in males than in females.[41,42] The findings of this study are in no wise different with HBsAg prevalence of 12.78% and 4.59% in males and females respectively. Reasons for this gender dependent difference have been suggested elsewhere.[41,42]

5. Conclusions

The results of this study confirmed that HBV IEM is present in Ibadan, Southwest Nigeria. Furthermore, it shows for the first time that HBV IEMs are circulating in the region. It also confirms the inclusion of Nigeria in the genotype E crescent and reaffirms the value of phylogenetic analysis in HBV identification. Finally, it raises concerns about the use of HBV DNA detection in isolation (i.e without sequencing and sequence analysis) for HBV diagnosis, because the detection of the expected band size without confirmation by subsequently sequencing the amplicon might not confirm the presence of HBV DNA.

Acknowledgement

The authors thank the participants for their cooperation during the period of the study. We also express our profound gratitude to Professor O. G. Ademowo of the Institute for Advanced Medical Research and Training, University of Ibadan, for providing Laboratory space and equipment used at some point during the execution of this study.

This study was carried out by the Evolutionary Dynamics of Hepatitis in Nigeria (EDHIN) research group. This study was not funded by any organization.

Competing interests: The authors declare that they have no competing interest.

Author Contributions:

1. Study Design (FTOC, AMO, IIM, OEC and BSA)
2. Sample Collection (AMO, IIM, OEC and AA)
3. Acquisition of reagents, laboratory and data analysis (All Authors)
4. Wrote the first draft of the Manuscript (FTOC)
5. Revised the Manuscript (All Authors)
6. Read and Approved the Final Draft (All Authors)

References

1. World Health Organization. Hepatitis B vaccines. *Weekly epidemiological record* 2009;20–405.
2. Perz JF, Armstrong GL, Farrington LA, Hutin YJ, Bell BP. The contribution of hepatitis B virus and hepatitis C virus infections to cirrhosis and primary liver cancer worldwide. *J Hepatol* 2006;**45**:529–38.
3. Beasley RP, Hwang LY, Lin CC, Stevens CE, Wang KY, Sun TS, et al. Hepatitis B immune globulin (HBIG) efficacy in the interruption of perinatal transmission of hepatitis B virus carrier state. Initial report of a randomised double-blind placebo-controlled trial. *Lancet* 1981;**2**(8243):388–93.
4. Gerlich WH. Medical Virology of Hepatitis B: how it began and where we are now. *Virol J* 2013;**10**:239.
5. Schaefer S. Hepatitis B, virus taxonomy and hepatitis B virus genotypes. *World J Gastroenterol* 2007;**13**:14–21.
6. Okamoto H, Tsuda F, Sakugawa H, Sastrosoewignjo RI, Imai M, Miyakawa Y, et al. Typing hepatitis B virus by homology in nucleotide sequence: comparison of surface antigen subtypes. *J Gen Virol* 1988;**69**:2575–83.
7. Norder H, Courouce AM, Coursaget P, Echevarria JM, Lee SD, Mushahwar IK, et al. Genetic diversity of hepatitis B virus strains derived worldwide: genotypes, subgenotypes, and HBsAg subtypes. *Intervirology* 2004;**47**:289–309.
8. Prince AM. Relation of Australia and SH antigens. *Lancet* 1968;**2**(7565):462–3.
9. Dane DS, Cameron CH, Briggs M. Virus-like particles in serum of patients with Austrailian-antigen-associated hepatitis. *Lancet* 1970;695–8.
10. Szmuness W, Stevens CE, Harley EJ, Zang EA, Oleszko WR, William DC, et al. Hepatitis B vaccine: demonstration of efficacy in a controlled clinical trial in a high-risk population in the United States. *N Engl J Med* 1980;**303**:833–41.

11. Blumberg BS. Hepatitis B – The hunt for a killer virus. Princeton: Princeton University Press; 2002.
12. Krugman S, Giles JP, Hammond J. Viral hepatitis, type B (MS-2 strain), Studies on active immunization. *JAMA* 1971;**217**:41–5.
13. Purcell RH, Gerin JL. Hepatitis B subunit vaccine: a preliminary report of safety and efficacy tests in chimpanzees. *Am J Med Sci* 1975;**270**:395–9.
14. Maupas P, Goudeau A, Coursaget P, Drucker J, Bagros P. Immunisation against hepatitis B in man. *Lancet* 1976;**1**(7974):1367–70.
15. Valenzuela P, Medina A, Rutter WJ, Ammerer G, Hall BD. Synthesis and assembly of hepatitis B virus surface antigen particles in yeast. *Nature* 1982;**298**:347–50.
16. Harford N, Cabezon T, Crabeel M, Simoen E, Rutgers A, De Wilde M. Expression of hepatitis B surface antigen in yeast. *Dev Biol Stand* 1983;**54**:125–30.
17. Lavanchy D. Viral hepatitis: global goals for vaccination. *J Clin Virol* 2012;**55**:296–302.
18. Carman WF, Zanetti AR, Karayiannis P, Walters J, Manzillo G, Tanzi E, et al. Vaccine-induced escape mutant of hepatitis B virus. *Lancet* 1990;**336**:325–9.
19. Harrison TJ, Hopes EA, Oon CJ, Zanetti AR, Zuckerman AJ. Independent emergence of a vaccine-induced escape mutant of hepatitis B virus. *J Hepatol* 1991;**13**:105–7.
20. Fujii JI, Moriyama K, Sakamoo N, Kondo T, Yasuda K, Hiraizumi Y, et al. Gly 145 to Arg substitution in HBs antigen of immune escape mutant of hepatitis B virus. *Biochem Biophys Res Commun* 1992;**184**:1152–7.
21. Theamboonlers A, Chongsrisawat V, Jantaradsamee P, Poovorawan Y. Variants within the "a" determinant of HBsAg in children and adolescents with and without hepatitis B vaccination as part of Thailand's Expanded Program on Immunization (EPI). *Tohoku J Exp Med* 2001;**193**:197–205.
22. Lada O, Benhamou Y, Poynard T, Thibault V. Coexistence of Hepatitis B Surface Antigen (HBs Ag) and Anti-HBs Antibodies in Chronic Hepatitis B Virus Carriers: Influence of "a" Determinant Variants. *J Virol* 2006;**80**(6):2968–75.
23. Ramezani F, Norouzi M, Sarizade GR, Poortahmasebi V, Kalantar E, Magnius L, et al. Mutation Hot Spots in Hepatitis B Surface Antigen in Chronic Carriers from Khoozestan Province, Southern of Iran. *Iran J Allergy Asthma Immunol* 2013;**12**(3):269–75.
24. Orito E, Mizokami M, Ina Y, Moriyama EN, Kameshima N, Yamamoto M, et al. Host-independent evolution and a genetic classification of the hepadnavirus family based on nucleotide sequences. *Proc Natl Acad Sci USA* 1989;**86**:7059–62.
25. Mizokami M, Orito E. Molecular evolution of hepatitis viruses. *Intervirology* 1999;**42**:159–65.
26. Cento V, Mirabelli C, Dimonte S, Salpini R, Han Y, Trimoulet P, et al. Overlapping structure of hepatitis B virus (HBV) genome and immune selection pressure are critical forces modulating HBV evolution. *J Gen Virol* 2013;**94**:143–9.
27. Forbi JC, Ben-Ayed Y, Xia G, Vaughan G, Drobeniuc J, Switzer WM, et al. Disparate distribution of hepatitis B virus genotypes in four sub-Saharan African countries. *J Clin Virol* 2013;**58**:59–66.
28. Faleye TOC, Adewumi MO, Ifeorah IM, Omoruyi EC, Bakarey SA, Akere A, et al. Detection of hepatits B virus isolates with mutations associated with immune escape mutants among pregnant women in Ibadan, southwestern Nigeria. *Springeropen* 2015. http://dx.doi.org/10.1186/s40064-015-0813-1.
29. Forbi JC, Vaughan G, Purdy MA, Campo DS, Xia G-l, Ganova-Raeva LM, et al. Epidemic History and Evolutionary Dynamics of Hepatitis B Virus Infection in Two Remote Communities in Rural Nigeria. *PLoS ONE* 2011;**5**(7.):e11615. http://dx.doi.org/10.1371/journal.pone.0011615.
30. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;**28**:2731–9.
31. Kimura M. A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *J Mol Evol* 1980;**16**(2):111–20.
32. Lole KS, Bollinger RC, Paranjape RS, Gadkari D, Kulkarni SS, Novak NG, et al. Full-length human immunodeficiency virus type 1 genomes from subtype C-infected seroconverters in India, with evidence of intersubtype recombination. *J Virol* 1999;**73**:152–60.
33. Carman WF, Korula J, Wallace L, MacPhee R, Mimms L, Decker R. Fulminant reactivation of hepatitis B due to envelope protein mutant that escaped detection by monoclonal HBsAg ELISA. *Lancet* 1995;**345**:1406–7.
34. Grethe S, Monazahian M, Bohme I, Thomssen R. Characterization of unusual escape variants of hepatitis B virus isolated from a hepatitis B surface antigen negative subject. *J Virol* 1998;**72**:7692–6.
35. Karthigesu VD, Allison LM, Fortuin M, Mendy M, Whittle HC, Howard CR. A novel hepatitis B virus variant in the sera of immunized children. *J Gen Virol* 1994;**75**(Pt2):443–8.
36. Prange R, Streeck RE. Novel transmembrane topology of the hepatitis B virus envelope proteins. *EMBO J* 1995;**14**(2):247–56.
37. Odemuyiwa SO, Mulders MN, Oyedele OI, Ola SO, Odaibo GN, Olaleye DO, et al. Phylogenetic analysis of new hepatitis B virus isolates from Nigeria supports endemicity of genotype E in West Africa. *J Med Virol* 2001;**65**:463–9.
38. Mulders MN, Venard V, Njayou M, Edorh AP, Bola Oyefolu AO, et al. Low genetic diversity despite hyperendemicity of hepatitis B virus genotype E throughout West Africa. *J Infect Dis* 2004;**190**:400–8.
39. Olinger CM, Venard V, Njayou M, Oyefolu AO, Maiga I, Kemp AJ, et al. Phylogenetic analysis of the precore/core gene of hepatitis B virus genotypes E and A in West Africa: new subtypes, mixed infections and recombinations. *J Gen Virol* 2006;**87**:1163–73.
40. Andernach IE, Hubschen JM, Muller CP. Hepatitis B virus: the genotype E puzzle. *Rev Med Virol* 2009;**19**:231–40.
41. Okonko IO, Okerentugba PO, Akinpelu AO. Prevalence of HBsAg among attendees of ARFH centre in Ibadan, Southwestern Nigeria. *American-Eurasian Journal of Scientific Research* 2012;**7**(3):100–5.
42. Okonko IO, Okerentugba PO, Adeniji FO, Anugweje KC. Detection of Hepatitis B surface antigen (HBsAg) among intending apparently healthy blood donors. *Nature and Science* 2012;**10**(4):69–75.