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New variant of Hepatitis C Virus genotype 1b in a Human Immunodeficiency Virus (HIV) infected patient in Benin City, Nigeria

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ABSTRACT
Background: Hepatitis C Virus (HCV) disease severity and treatment outcome are affected by circulating genotype. Data on genotypic prevalence of HCV among HIV infected patients in Nigeria is sparse. Against this background this study aimed at determining the genotypic prevalence of HCV among HIV infected patients in Benin City, Nigeria. **Methods:** A cross-sectional study was conducted among 564 HIV infected patients to determine the type and frequency of HCV genotypes among them. Four hundred and thirty-one (431) of these patients were on highly active antiretroviral therapy (HAART). Sera obtained from study participants were used to screen for antibodies to HCV using immuno-chromatographic test kits. Hepatitis C Virus nucleic acid HCV-RNA was extracted from HCV seropositive sera and the 5' untranslated region (5'UTR) of HCV amplified using specific primers, with the resulting amplicons sequenced on an ABI 350xL automated sequencer.
Results: HAART did not significantly affect the sero-prevalence of HCV infection. (HAART naïve vs. HAART exposed) 1.5% vs. 0.9%; OR= 1.630, 95% CI = 0.295, 9.002, P = 0.9344)**.** Of the six patients with HCV antibodies, only 1(16.7%) had detectable HCV-RNA in serum, and the isolate was identified as HCV genotype 1b, which did not cluster around other HCV genotype 1b isolates of Nigerian origin.
Conclusions: This study reports a novel variant of HCV genotype 1b in Nigeria

Key words: Hepatitis C Virus, genotypes, nucleotides, mutation, HIV, Nigeria
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Author's contributions:
This work was conducted and approved in collaboration between all the authors, who takes responsibility for its accuracy and integrity. OBH, EMI, OR, OI, and AOD designed the study, sourced for funding, wrote the protocol, contributed in literature search, did statistical analysis, Contributed in discussions, drafted the manuscript, supervised the study, Wrote the final manuscript, proofread the final manuscript for publication. **Inqaba- South Africa did sequencing.**
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INTRODUCTION

Human immunodeficiency virus (HIV) infection is associated with increased mortality and morbidity worldwide, with prevalence rates that vary from region to region. It is a global health challenge with attendant medical, economic and social implications (1). Sub-Saharan Africa has the highest burden of HIV infection accounting for about two-thirds of all people living with HIV in the world[1]. Only South Africa is reported to have more persons living with HIV than Nigeria in the world (2).

Hepatitis C virus (HCV) infection is a major public health challenge worldwide (3). HCV is the major etiological agent of chronic hepatitis worldwide (4). HCV infection is common among HIV infected patients due to similar route of transmission shared by both viruses (5). Report shows that about 20% of HIV negative patients infected with HCV naturally clear HCV from their system (6). Clearance of the virus occurs in only about 5% of HIV positive patients, (6) making them present with high risk of developing HCV liver related diseases than HCV mono-infected patients.

The standard treatment for HCV is pegylated interferon alfa (PegIFN) plus ribavirin (7). Recently, however, new directly acting antiviral drugs which results in a higher sustained virological response rate have been used in treatment of HCV infected patients (8). Treatment outcomes of HCV infected patients are known to be affected by specific HCV genotypes[9]. Currently, there are eight HCV genotypes designated 1-8[10]. The prevalence rates of the genotypes that differ from region to region (11, 12). HCV genotypes 1 and 4 are generally reported to be more resistant to treatment with pegylated interferon alfa plus Ribivarin than HCV genotype 2 and 3 (13). Also the severity of HCV related liver diseases has been shown to be associated with specific HCV genotypes (14), with HCV genotype 1 incriminated in more a severe form of liver disease than the rest (15).

Although free HIV testing services are provided in many hospitals in Nigeria, routine screening for HCV infection is not done, neither is specific HCV treatment offered to patients (16). This may result in missed diagnosis of HCV infection which over time could progress to other serious medical complications including death of patients. Only one study has focused on the genotypic prevalence of HCV among HIV infected patients in Nigeria (17). To the best of our knowledge, this study is the first study to determine the genotypic prevalence of HCV in the south-south geopolitical region of Nigeria. Against this background, this study aimed at determining the HCV genotypic and subtype prevalence among HIV infected patients in Benin City, Nigeria.

METHODS

Study Area

This study was carried out in the University of Benin Teaching Hospital, a public tertiary health institution with referral status located in Edo State, south-south geographical zone of Nigeria. Edo state lies between longitude 5º35 and 5º44 East and Latitude 6 º44 and 6 º21 North.

Study population

A total of 564 HIV infected patients attending HIV clinics at the University of Benin Teaching Hospital were recruited using a random sampling technique for this study. Of this number, 431 were on HAART. Verbal informed consent was obtained from all patients prior to specimen collection. Consent was obtained from parents or guardians of minors. A questionnaire was used to obtain relevant information from study participants. Approval for study was gotten on the 10th day of April 2014 from the Ethics and Research Committee of the University of Benin Teaching Hospital, Benin

City. Protocol number: ADM/E 22/A/VOL. VII/1014.

Specimen collection and processing

Five milliliters of blood was collected from all participants and dispensed into plain containers. All subjects were screened for the presence of antibodies to HCV using an immunochromatographic test strips – (Clinotech Diagnostics, Richmond, Canada) following the manufacturer's instruction. Two milliliters of all HCV seropositive sera was placed into cryo-vials and stored at – 80 ^{O}C in the Medical Microbiology Laboratory Unit of University of Benin Teaching Hospital for molecular analysis.

Detection of HCV-RNA

HCV-RNA was extracted from 140µl of patient's sample with the use of Zymo ZR Viral DNA/RNA kit (USA). The RNA was eluted in 50 µl DNase/RNase free water and subsequently used for genome amplification of the 5` untranslated region (UTR) region of HCV by Reverse Transcription Polymerase Chain Reaction (RT-PCR) using One Step RT-PCR kit (New England Bio Labs). The reaction mixture contained 12.5µl of One Taq one-step reaction mix (2x), 1µl of One Taq one step enzyme mix, 1µl of forward primer 5` CTGTGAGGAACTACTGTCTT-3`, 1µl of reverse primer 5`ATACTCGAGGTGCACGGTCTACGAGAC CT-3`, 2.5µl of RNA extract and 7µl of nuclease free water to give a final volume of 25µl. Amplification was initiated with the Eppendorf Thermocycler. The cycling conditions for the RT-PCR were as follows: Initial cycles at 50°C for 30 min and 95°C for 15 min; followed by 45 cycles at 95°C for 30 s, 50°C for 30 s and 72°C for 1 min; and a final extension at 72°C for 10 min. The amplified products were visualized on ethiduim bromide stained 1.5% agarose gel following electrophoresis with Wealth Dolphin Doc UV trans-illuminator and photographed.

Sequencing was done using ABI 350XL sequencer. Sequencing of the HCV amplicon (HCV-RNA Positive) was done at Inqaba Biotec South Africa.

The PCR product was purified using the ExoSAP protocol. Firstly, the exoSAP master mix preparation was done as follows; A 50 µl volume of Exonuclease I (NEB M0293) 20U/ ul was placed into a 0.6ml micro-centrifuge tube. 200 µl of Shrimp Alkaline Phosphatase (NEB M0371) 1U/ ul was then added to the tube. The mixture was agitated gently and kept aside for use as exo/SAP master mix.

A 10.0 µl volume of PCR product was added to2.5 µl of prepared Exo/SAP Mix. The mixture was agitated and incubated at 37OC for 30 minutes. The reaction was stopped by heating the mixture at 95OC for 5 minutes. The labelled product was then cleaned with the ZymoSeq clean-up kit.

Sequencing was then done with the ABI V3.1 Big dye Kit (Applied Bio-system) on an ABI3500XL automated sequencer (Applied Bio-system) with a 50cm array using POP7. True HCV infection in this study was defined as the presence of HCV-RNA in patients' sera (18).

Characterization of HCV isolate

Sequence output of ABI350XL analyser (chromatogram) was opened to reveal nucleotide sequences (Fig 1), using the software FinchTV available at http://www.geospiza.com/ftvdlinfo.html. The identity of aligned HCV sequence was confirmed using the BASIC LOCAL ALIGNMENT SEARCH TOOL (BLAST) available at the National Center for Biotechnology information (NCBI). Confirmed HCV isolate was compared with known genotypes in GenBank database and HCV subtyping was performed using the Oxford HCV automated subtyping tool version 2.0 (available at: http://dbpartners.stanford.edu/RegaSubtyping/html/subtypinghcvSUB.html). Phylogenetic tree of study isolate and 44 other HCV genotype 1b

isolates from Genbank database was created as previously described (19, 20). Pair–wise alignment of the 5'UTR nucleotide sequences of study and HCV reference strains (Genbank Accession number: NC_004102) was done using EMBOSS- Needle software

Data Analysis

Analysis of data was carried as appropriate using the statistical software INSTAT® (Graphpad Software Inc., La Jolla, CA, USA). $P < 0.05$ was considered significant.

RESULTS

The sero-prevalence of HCV in this study was 1.1%, and did not differ significantly with respect to HAART status of HIV infected patients (P = 0.9344). (Table 1). Of the 6 HIV/HCV sero-positive patients found in this study, only one had detectable HCV-RNA (Table 2). Generally, HAART-naïve HIV infected patients had a 9 times higher risk (HAART-naïve vs. HAART exposed: 50.0% vs. 0.0%; OR = 9.000, 95% CI = 0.2233, 362.81.P = 0.333) of acquiring true HCV infection than their HAART-exposed counterparts (Table 2).

Molecular characterization of the HCV isolate showed that it was HCV genotype 1b. Phylogenetic analysis revealed that study strain did not cluster around any previously identified HCV genotype 1b isolate of Nigeria origin. (Figures 2 and 3).

Compared to the reference strain (NC_004102) there were nucleotide deletions on positions 1-132 of study isolate. Nucleotide substitutions were observed in20 different positions as follows G143T, G146T, C151T, C156T, G163T, A167C, C168T, A172T, T191C, A204C, A205G, A206G, G224T, G231T, C232G, A243C, A244C, G253C, G28A and T162G. Insertions in our HCV strain were observed beyond positions 331 of reference strain (Figure 4).

Table 1: Sero-prevalence of HCV infection among study population

HIV INFECTED	N	No. HCV SEROPOSITIVE (%)	OR	95% CI	P value
HAART NAÏVE	133	2 (1.5)	1.630	0.295, 9.002	0.9344
HAART EXPSOED	431	4 (0.9)			
TOTAL	564	6 (1.1)			

N- number of patients examined, **HCV -** hepatitis c virus, **OR**- odd ratio, **CI**- confidence interval

Table 2: Prevalence of true HCV infection among study population

HCV SERO-POSITIVE	N	No. HCV-RNA POSITIVE (%)	OR	95% CI	P value
HAART STATUS					
HAART NAÏVE	2	1(50.0)	9.000	0.2233, 362.81	0.333
HAART EXPSOED	4	0 (0.0)			
GENDER					
Male	1	1 (100.0)	33.000	0.440, 2478.2	0.1667
Female	5	0 (0.0)			

N- number of patients examined, **HCV-RNA -** hepatitis c virus ribonucleic acid, **OR**- odd ratio, **CI**- confidence interval

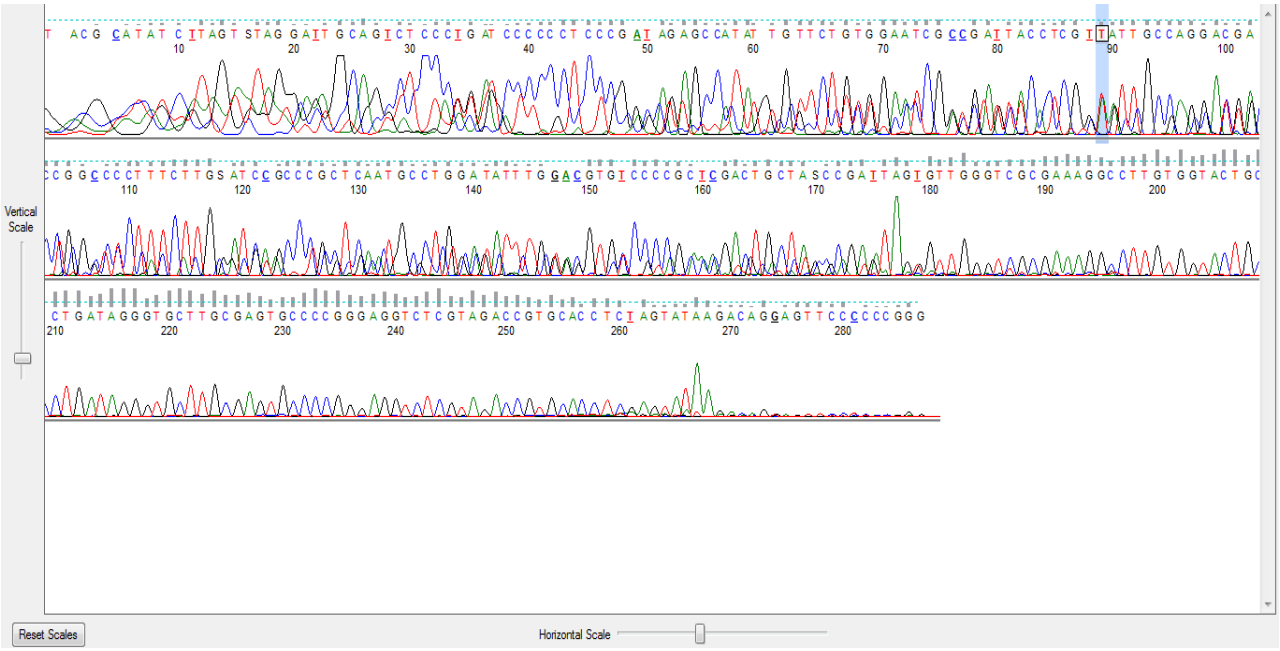

Figure 1: Chromatogram for HCV sequence

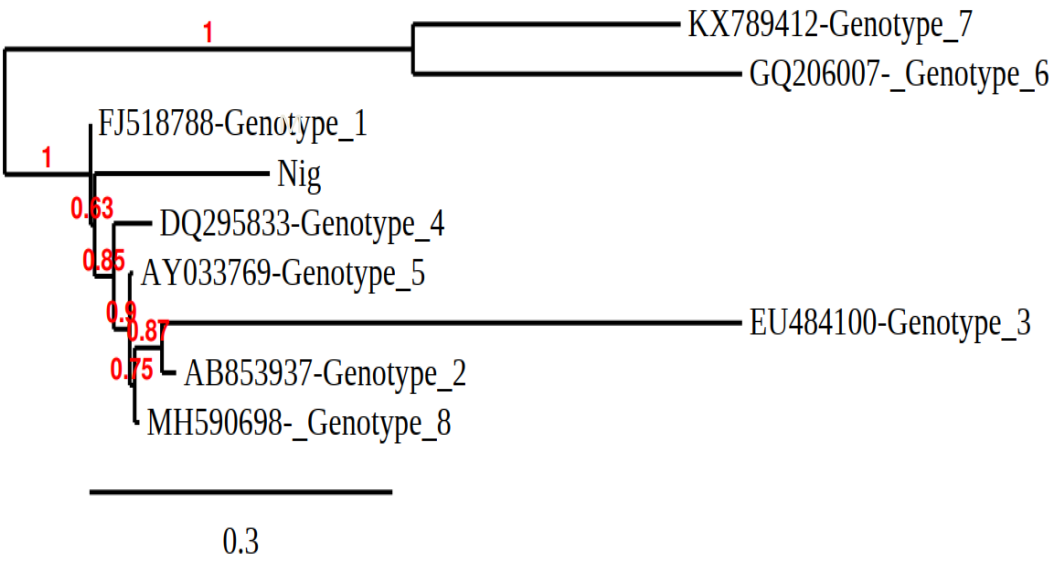

Figure 2: Genotypic characterization of Hepatitis C virus isolate from study

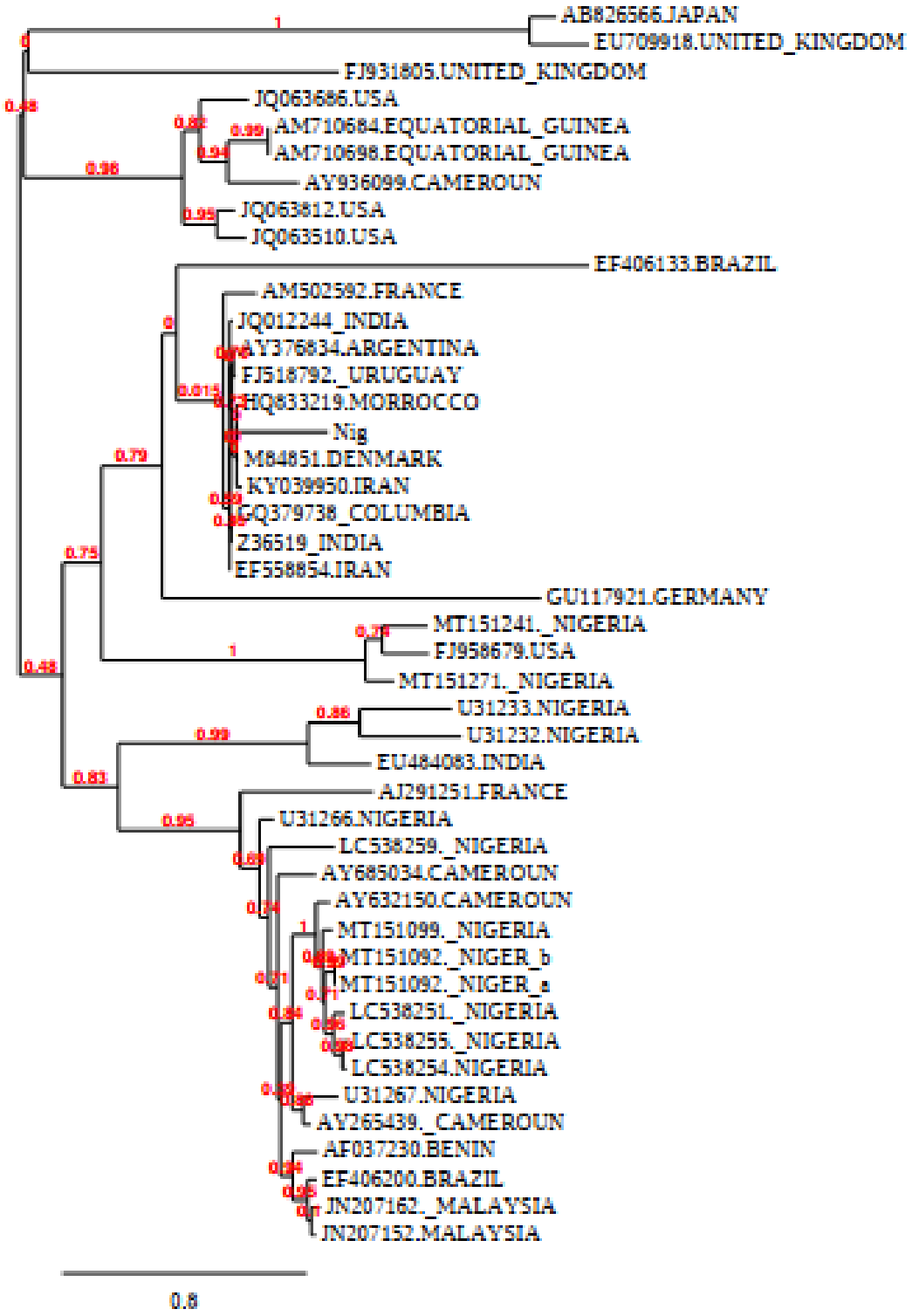

Figure 3: Phylogenetic analysis of study HCV isolate and HCV/1b isolates retrieved from Genbank

```
NC_004102          1 GCCAGCCCCCTGATGGGGGCGACACTCCACCATGAATCACTCCCCTGTGA     50

Nig                1 --------------------------------------------------      0

NC_004102         51 GGAACTACTGTCTTCACGCAGAAAGCGTCTAGCCATGGCGTTAGTATGAG    100

Nig                1 --------------------------------------------------      0

NC_004102        101 TGTCGTGCAGCCTCCAGGACCCCCCCTCCCGGGAGAGCCATAGTGGTCTG    150
                                             ||||||||||.||.||||
Nig                1 --------------------------------GAGAGCCATATTGTTCTG     18

NC_004102        151 CGGAACCGGTGAGTACACCGGAATTGCCAGGACGACCGGGTCCTTTCTTG    200
                     .||||.||||||.||||..|||.||||||||||||||||.|||||||||
Nig               19 TGGAATCGGTGATTACCTCGGTATTGCCAGGACGACCGGGCCCTTTCTTG     68

NC_004102        201 GATAAACCCGCTCAATGCCTGGAGATTTGGGCGTGCCCCCGCAAGACTGC    250
                     |||...|||||||||||||||.|||||..||||||||||..||||||
Nig               69 GATCGGCCCGCTCAATGCCTGGATATTTGGTGGTGCCCCCGCCCGACTGC    118

NC_004102        251 TAGCCGAGTAGTGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG    300
                     ||.||||.|||.||||||||||||||||||||||||||||||||||||||
Nig              119 TACCCGAATAGGGTTGGGTCGCGAAAGGCCTTGTGGTACTGCCTGATAGG    168

NC_004102        301 GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACC---------    341
                     |||||||||||||||||||||||||||||||||||||||||
Nig              169 GTGCTTGCGAGTGCCCCGGGAGGTCTCGTAGACCGTGCACCTCCAGTATA    218

NC_004102        342 ----------------------------------------    341

Nig              219 AGACAGGTTTGCAAGGATCACCGCCTTATCCTCACGGATTTT    260
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Figure 4: Alignment of nucleotide sequence of the Nigeria genotype 1b variant (Nig) and the reference HCV genotype 1b (NC_004102)

DISCUSSION

HCV disease severity is affected by circulating genotypes and subtypes (9). There is a dearth of knowledge on the prevalence of HCV genotypes among Nigerian patients. There is paucity of data on the genotypic prevalence of HCV among HIV infected patients in Nigeria. Against this background this study was conducted.

In this study, HIV infected patients were found to have an HCV sero-prevalence of 1.1%. This is lower than values reported in some studies (21, 22), but higher than another reported elsewhere[16]. The sero-prevalence of HCV is known to differ from place to place even within the same country (23). This may account for the variation in sero-prevalence of HCV in these studies. Although, HAART naïve group of patients were observed to have a higher seroprevalence of HCV than their HAART exposed counterparts, the difference failed to reach statistically significant proportion. This is consistent with findings from an earlier study (24).

Study participants who were HCV seropositive and not on HAART were found to have a 9 times higher risk of having true HCV infection than their HAART exposed counterparts. A healthy CD4+ cell response is central to the control of HCV infection (25). It is therefore possible that the use of HAART among HCV seropositive patients may have resulted in a drop in HIV viremia and increase in CD4+ count, factors that have been reported to be associated with reduction in HCV-RNA levels in a previous study (26). The gender-wise prevalence of true HCV infection showed that males had about 33 times higher risk (OR= 33.00) of acquiring true HCV infection than females. HCV clearance among women is reported to be associated with high level of estrogen (27). This may explain the observed lower prevalence of true HCV infection among the female group of our study population.

Genotypic characterization of HCV isolate in this study revealed that it was genotype 1. Other studies have reported HCV genotype 1 as the predominant genotype in Nigeria (28, 29). With respect to subtype identity, the HCV genotype 1 isolate was found to belong to subtype class b. It is important to note that past studies have reported HCV genotype 1b among Nigerian populations (28, 29). One French study reported an association between HCV genotype 1b and transfusion of blood and blood products (30). Indeed, the patient with HCV genotype 1b in this study claimed to have been transfused with blood in the past.

Phylogenetic analysis of HCV isolate obtained in this study with 44 other HCV genotype 1b reference strains showed that the HCV isolate from this study did not cluster around HCV genotype Ib isolates of Nigeria origin, indicating that it is a new variant of HCV genotype 1b circulating in Nigeria. This suggests that our study strain may have been imported into the country, although we did take note of the travel history of the subject. To our knowledge, only one study which was conducted in South-Western Nigeria, (17) has documented the genotypic prevalence of HCV among HIV infected patients. This is the first study to report the HCV genotypic prevalence among HIV infected patients in south-south geopolitical zone of Nigeria.

Pairwise alignment of nucleotide sequences of study and reference strains (NC_004102), showed high mutations in the former, with nucleotide deletions, substitutions and insertions observed on several positions of study isolate in relation to reference strain. This may have grave implication for the therapeutic efficacy of existing anti-HCV drugs, as such modifications are capable of leading to poor drug performance. This finding reinforces the need for drug manufacturers to constantly take into account HCV sequence diversities from around the world in order to produce drugs with

global potency. The sequence of HCV isolate from this study has been deposited in Genbank database with the accession number KY852360.

CONCLUSIONS

A seroprevalence of 1.1% was recorded among HIV infected patients in this study. The only HIV infected patient with true HCV infection in this study was found to harbor a novel variant of HCV genotype 1b, which did not cluster among other HCV genotype Ib isolates of Nigeria origin. This study underscores the presence of a new variant of HCV genotype 1b in Nigeria.

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CONFLICT OF INTEREST: None

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