High Frequency of non-B Human Immunodeficiency Virus type 1 (HIV-1) subtype Specific Mutations at the Protease Gene among Treatment-naive HIV-1 infected Individuals in Jos, Nigeria.

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ABSTRACT

Aims: To determine the prevalence of non-B HIV-1 subtype specific mutations in the protease gene among antiretroviral drug-naive individuals in Jos, Nigeria.

Study design: This was a cross-sectional study in which randomly selected blood samples of HIV-1 positive anti-retroviral drug-naïve individuals were used for genotyping assay.

Place and Duration of Study: The study was conducted at the adult HIV clinic of the AIDS Prevention Initiative in Nigeria (APIN) programme, Jos University Teaching Hospital (JUTH), Jos, Nigeria between October 2010 and April 2011.

Methodology: Of the one hundred and five plasma samples, 100 samples were successfully reverse transcribed and amplified by nested PCR. The amplicons were directly sequenced on an automated ABI genetic analyzer using BigDye Terminator Cycle Sequencing Kit. Subtyping and phylogenetic analyses were performed using the REGA subtyping tool version 2.0 and MEGA 5.0 software. Both the Stanford HIV database algorithm and IAS-USA 2013 drug resistance update were used for interpretation of drug sensitivity.

Results: The proportion of the non-B HIV-1 subtypes were as follows: CRF02_AG (48%), G (41%), CRF06_cpx (6%), A (5%). Q58E, a major drug resistance mutation to PI, occurred as a low prevalence mutation in subtype G. The most common mutations observed among the subtypes were I13V, K14R, K20I, M36I, R41K, H69K, V82I and L89M.

Conclusion: A non-uniform distribution of non-B HIV-1 subtypes were observed in Jos, Nigeria, with CRF02_AG and G predominating among the antiretroviral drug-naive individuals. Among the different subtypes in circulation, there was a high prevalence of minor mutations and natural polymorphisms associated with the protease gene. Such mutations define the subtype diversity which may impact on virulence and drug 'responses', thus further studies are needed to evaluate the clinical implications of these mutations.

Keywords: Non- B HIV-1 subtypes, protease gene, protease inhibitor mutations, polymorphism, treatment-naïve patients, Nigeria.

1. INTRODUCTION

The current seroprevalence rate of Human Immunodeficiency Virus type 1 (HIV-1) in Nigeria has witnessed a slight decline from 4.6% in 2008 to 4.1% in 2010. Plateau State, Nigeria has a prevalence of 7.6%, which is higher than the average national prevalence of 4.1%. [1]. Nigeria the most populated country in sub-Saharan Africa ranked third among

countries with the highest burden in the world, next to India and South Africa [2]. It has been estimated that 3.5 million people living with HIV (PLHIV) are in Nigeria [3].

HIV-1 mutates rapidly contributing to its high genetic diversity. These variants are continually being introduced into new populations leading to generation of mutants and new recombinant viruses [4, 5]. HIV-1 can be categorized into four groups (M, N, O, and P) with group M being responsible for majority of infections worldwide and it is a major target of drug design strategies [6-9]. The variants are alternative lifelines that HIV-1 uses to evade sustained drug pressure and host immune responses, resulting in resistance to antiretroviral (ARV) drugs [10]. The use of highly active antiretroviral therapy (HAART) has significantly reduced morbidity and mortality from HIV infection globally [11]. Antiretroviral therapy (ART) imposes selective pressure on the virus and this favors emergence of drug-resistant mutants within the HIV infected population [12]. The Nigerian national guidelines for HIV/AIDS treatment and care in adolescent and adults recommended boosted protease inhibitors (PIs); atazanavir/ritonavir, lopinavir/ritonavir or Indinavir/ritonavir for second line plus two nucleoside analogues as ART backbone [13]. ARVs were designed and tested against HIV-1 subtype B isolates that are predominant in the Western world; whereas in Africa the HIV epidemic is driven by non-B subtypes with an increasing prevalence [14]. Resistance to ARVs is one of the major threats to the global control of HIV pandemic as it may impact negatively on clinical outcomes [15, 16]. It is reported that many mutations selected by PI treatment in HIV-1 subtype B patients are now found as natural polymorphisms in wild-type non- B HIV-1 subtypes [17]. This may constitute a major drawback in the use of PIs as salvage therapy. Some studies on HIV-1 genetic diversity have highlighted subtypes A, C, F, and G specific natural polymorphisms to protease inhibitors in subtype B, which were known to contribute to resistance or compensate for viral fitness defects due to primary drug resistance mutations [18, 19]. Although resistance mutation pathways in both subtype B and non-B are similar, differences have been observed in non-B subtypes. However, these pathways have not shown much limitation on the ARV efficacy [20, 21]. The impact of the non-B subtype diversity and associated mutations on treatment outcome is yet to be well understood in developing countries. However, evidence from developed countries has shown that polymorphisms which occur naturally in B subtype do have negative effects on ARV drug resistance and susceptibility [22, 23]. Some earlier studies have documented HIV-1 genetic diversity and associated genotypic profiles of *pol* gene in Nigeria with subtypes CRF02_AG and G being the most common but lack well characterized genotypic patterns [24-27]. Little is known about the prevalence of non-B HIV-1 subtype associated natural polymorphisms to PIs among treatment-naive individuals in Nigeria, particularly now that access to treatment has rapidly expanded. The objective of this study was to determine the frequency of non-B HIV-1 subtype-specific mutations in the protease gene among antiretroviral drug-naive adult individuals in Jos, Nigeria.

2. MATERIAL AND METHODS

2.1 Settings, Patient Recruitment and Sample Collection

The study was carried out at the adult HIV clinic of the AIDS Prevention Initiative in Nigeria (APIN) programme at the Jos University Teaching Hospital (JUTH), Jos. This clinic located in the city of Jos, Plateau State, Nigeria, provides a comprehensive HIV management services for individuals referred from health facilities within and outside the state. This study was a cross-sectional study from October 2010 to April 2011; in which a total of 105 randomly selected (from computer-generated random numbers) blood samples were used out of 230 blood samples of individuals that were enrolled in a previous study [28]. The socio-demographic data of each participant was collected. Written informed consent was obtained from them for use of their data for this research which was approved by JUTH Ethics Committee. The criteria for inclusion of participants in the study were: age ≥18 years, being HIV-positive and no prior ARV exposure.

Blood samples were collected in ethylenediamine-tetra-acetic acid (EDTA)-lined containers, the extracted plasma was cryopreserved.

The diagnosis of HIV was done using two different rapid HIV tests: Uni-Gold (Trinity Biotech Plc Bray Co Wicklow, Ireland) and Determine HIV-1/2 test (Determine Alere Medical Co., Ltd 357 Matsuhidai, Japan). The $CD4^+$ lymphocyte count was measured on the same day of the blood draw using Partec CyFlow Counter® (Partec GmbH, Munster Germany) according to manufacturer's instructions and as previously described [29]; while HIV-1 RNA viral load was measured using the Roche Cobas Amplicor HIV-1 Monitor, version 1.5 (Roche Diagnostics GmbH, Mannheim, Germany). The selected cryopreserved samples were subsequently shipped in ice-parked containers to the Kenya Medical Research Institute (KEMRI) HIV-Research Laboratory Kisian in Kisumu region of Kenya, where genotypic testing using a validated in-house Genotyping assay was carried out. Of 105 samples shipped and tested, 100 were successfully amplified for genotypic drug resistance testing

2.2 HIV-1 RNA Extraction, Amplification and Detection

HIV-1 RNA was isolated using the Viral RNA Mini Kit (Qiagen, Hilden, Germany) and amplified immediately or stored at -80 ^{o}C until amplified. The primer design and modifications for amplification of all HIV-1 group M subtypes and circulating recombinant forms (CRFs) of the *pol* (protease and partial reverse transcriptase) gene region associated with resistance was performed as previously described [30]. Two amplification protocols for HIV polymerase gene were used; the outer primers for a one-step reverse transcription RT-PCR were Prt-F1 (forward, 5'-CCTCAAATCACTCTTTGGCARCG-3', nucleotides (nt) length of 2253-2275 based on HIV-1 HXB2) and RT-R1 (reverse, 5'-ATCCCTGCATAAATCTGACTTGC-3', nt 3370-3348). The reaction conditions of the RT-PCR in the ABI GeneAmp 9700 thermo Cycler were 65 ºC for 10 min, 50 ºC for 45min, 40 cycles at 94 ºC for 2 min, 94 ºC for 15 sec, 50 ºC for 20 sec and 72 ºC for 2min, 72 ºC for 10min and 4 ºC until removal. The nested PCR also had two primers; Prt-F2 (forward, 5'-CTTTGGCAACGACCCCTYGTCWCA-3', nt length 2265 - 2288) and RT-R2 (reverse, 5'-CTTCTGTATGTCATTGACAGTCC-3', nt 3326 -3304) both at 4µM concentration. For RT-PCR mixture contained primers (Prt-M-F1 and , RT-R1 8µM each, RT-PCR mixture DEPC-treated water and the SuperScript III one-step RT-PCR system with Platinum *Taq* DNA polymerase high fidelity, following the manufacturer's protocol (Invitrogen, Carlsbad, CA). For nested PCR, the product of RT-PCR was added to primers (Prt-F2 and RT-R2, each 8 µM), dNTPs, GeneAmp gold buffer II, 2 mM MgCl2, AmpliTaq gold LD DNA polymerase mixture (Applied Biosystems, Foster City, CA) DEPC-treated water. After initial denaturation at 94°C for 4 min, 40 cycles of PCR were performed in a GeneAmp 9700 thermal cycler with PCR conditions of 94 °C for 15 sec, and 55 °C for 20 sec and an extension at 72 °C for 2 min, followed by further extension at 72 °C for 10 mins and hold at 4 °C for ∞. The products (final reaction volume-50ul) from nested PCR were verified for desired size and gene target specificity by visually comparing the intensity of each sample's band with reference to standard low-molecular weight markers, with concentrations of at least 20ng, and molecular weights ranging 200-2000bp (Invitrogen Corporation, CA, USA). This was performed by running a 1% agarose gel electrophoresis stained with 0.5µg/ml ethidium bromide and photographed under ultraviolet illumination using GelDoc-It imaging system. The amplicons were purified using the QIAquick PCR Purification Kit (Qiagen, Hilden Germany) in QIAquick spin columns, and were directly sequenced using six customized primers and BigDye Terminator v3.1 Cycle Sequencing Ready Reaction Kit with buffered AmpliTaq DNA polymerase on an automated ABI Prism 3130*xl* Genetic Analyzer [27].

2.3 Sequence Interpretation

The obtained nucleotide sequences were edited using Sequencer software v5.0 that assembles overlapping sequence segments from the primers to form a contiguous sequence from which consensus sequences are generated. The quality of generated sequences was checked using Sequence Quality Assessment Tool. For preliminary genetic subtype determination and phylogenetic analyses, population-based genotyping was performed using the REGA subtyping tool version 2.0 from Stanford HIV drug resistance database (http://hivdb.stanford.edu/), a worldwide subtype references were obtained from the Los Alamos database (http://hiv-web.lanl.gov), and sequences were aligned against the known reference strains based on maximum composite likelihood neighbor-joining. Further phylogenetic analysis was done on MEGA 5.0 using Maximum Likelihood (ML) statistical method with a bootstrap of 1000 replicates for assessment of the strength of the phylogenetic tree and values above 70% were considered significant. An out-group HIV-1 subtype O was used to root the tree and Hasegawa-Kishino-Yano model (+G+I) selected for the analysis based on the Bayesian Information Criterion (BIC) scores of 24 different nucleotide substitution models. Nearest-Neighbor-Interchange (NNI) method was used as the ML Heuristic option for tree inference [31].. Sequences were submitted to Stanford University HIV data base and mutations obtained were classified and analyzed according to the International AIDS Society (IAS)-USA 2013 guidelines [32].The presence of mutations not associated with high-level drug resistance were defined as those that occurred in more than 5% of sequences, while subtype-difference mutations were those more prevalent in a given subtype [33].

2.4 Statistical Analysis

The data for all 100 participants were entered into Microsoft office excel work sheet (Microsoft office system, 2007) and then exported into the Stata software version 10.0 (Stata Corporation, College Station, Texas, USA) for analysis. Categorical variables (sex, marital status and mode of transmission) were summarized as percentages; while continuous variables (age, CD4+ T cell count and viral load) which were not normally distributed had their medians (IQR) determined. The graphs were plotted using Microsoft offices excel.

3. RESULTS AND DISCUSSION

The median age of the 100 study subjects was 35.5 years with the majority of them being females (56%). Majorities (68%) of the participants were married and the most common mode of HIV transmission was by the heterosexual route (98%). The median $CD4^+$ T-cell count of the patients at baseline was low - 141 cells/mm^3 while their viral load ranged from 22, 202 to 153,725 copies/ml (Table 1).

3.1 HIV-1 *pol* Subtyping

One of the purposes of this study was to describe the prevalence of non-B HIV-1 subtype among the individuals attending the APIN clinic of JUTH, Jos, Nigeria. Phylogenetic analyses of the partial *pol* gene revealed heterogeneous distribution of four non B HIV-1 strains: CRF02_AG (48%), G (41%), CRF06_cpx (6%) and A (5%). HIV-1 subtypes CRF02_AG and G accounted for majority of the infections (89%).

3.2 Frequency of HIV-1 Subtype CRF02_AG Specific Protease Inhibitor Mutations

The most frequent mutations identified in non-B HIV-1 subtype CRF02_AG were; I13V (48%), M36I (45%), H69K (45%), L89M (44%), R41K (41%), K20I (39%) and K14R (33%). The least frequent mutations were at amino acid substitutions L10I (1%), V11LV (1%), K20R (1%), E34A (1%), L38I (1%), P39G (1%), K45R (1%), K61N (1%), I62V (1%), L63H (1%), C67GS (1%), H69Q (1%), I72IM (1%), I72AEKT (1%), and the presence of an unusual mutation at position L90V (1%); where V (valine) is observed instead of the M (methionine) non-B HIV-1 polymorphisms (Figure 1).

3.3 Frequency of HIV-1 Subtype G Specific Protease Inhibitor Mutations

One sample from non-B subtype G harbored a major drug resistance mutation (Q58E) to protease inhibitors. High rates of naturally occurring mutations in the protease gene were detected among the subtype G in the following proportions; L89M (41%), I13V (40%), M36I (38%), R41K (35%), V82I (34%), H69K (32%), K20I (31%), K14R (29%) and C67E (24%). It was observed that minor mutations of interest with low-level resistance to some PIs were identified in the following decreasing proportions; M36I (38%), V82I (34%), H69K (32%), K20I (31%), L63P (12%), K20IM (8%), L10I (5%), L10LV (4%), G16E (3%), I62V (2%), H69KR (2%), L33F (1%). The proportion of V82I mutation was higher in subtype G compared to subtypes A, CRF06_cpx and CRF02_AG. The observed mutation at position L10M was unusual and rare with the frequency of 1% (Figure 2).

3.4 Frequency of HIV-1 Subtype A Specific Protease Inhibitor Mutations

The most common mutations were observed at positions; I13V (5%), M36I (5%), R41K (5%), and L89M (5%), and the least were found at positions; L10LV (1%), T12K (1%), I15V (1%), K20R (1%), N37DN (1%), P39S (1%), P39G (1%), R57EK (1%), C67GS (1%), H69Q (1%), K70R (1%), and I72VT (1%) (Figure 3).

3.5 Frequency of HIV-1 Subtype CRF06_cpx Specific Protease Inhibitor Mutations

The highest proportions of mutations associated with non-B Subtype CRF06_cpx were at positions; I13V (6%), H69K (6%), L89M (6%), M36I (5%), and R41K (5%) among a total of 20 mutations observed. The low frequency of some minor mutations linked to low-level resistance to some PIs were; K20I (4%), K20IM (2%), L63P (2%), V82I (2%) and L10LV (1%) (Figure 4).

The observed minor mutations such as L10LV and V82I are frequently selected by almost all PIs except darunavir, while K20I/M/R mutation is selected by atazanavir, indinavir and lopinavir; L33F is selected by atazanavir, darunavir, lopinavir and tipranavir. Mutation M36I is selected by atazanavir, indinavir, nelfinavir and tipranavir; I62V is selected by atazanavir and saquinavir; L63P is selected by lopinavir. Mutation V77IV is selected by indinavir, nelfinavir and saquinavir; while H69K and L89M are selected by tipranavir.

Table 1 . Baseline characteristics of HIV-1 infected antiretroviral treatment-naïve individuals.

Characteristics of patients	All Patients (n=100)
Sex	
Male	44 (46%)
Female	56 (56%)
Median Age (years**) (IQR)**	35.5 (31 - 42)
Marital status	
Single	19 (19%)
Widowed/ Divorced/ Separated	13 (13%)
Married	68 (68%)
Mode of transmission	
Heterosexual intercourse	98 (98%)
Blood transfusion	2 (2%)
Median $CD4^+$ T-cell count (cells/mm^3) (**IQR**)	141 (68 - 263)
Median HIV-1 RNA (copies/ml**) (IQR)**	65,218 (22,202 - 153,725)
Median HIV-1 RNA Log viral load (copies/ml) (**IQR**)	11.08 (10.01-11.94)

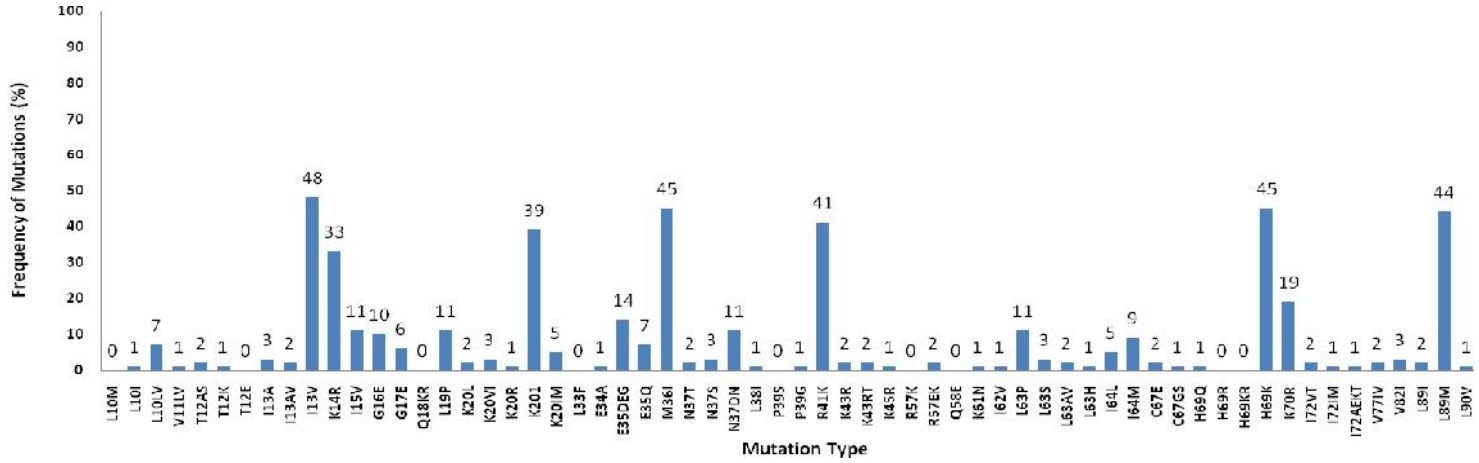

Figure 1. Frequency of protease mutations in HIV-1 subtype CRF02_AG isolates among antiretroviral treatment-naïve individuals.

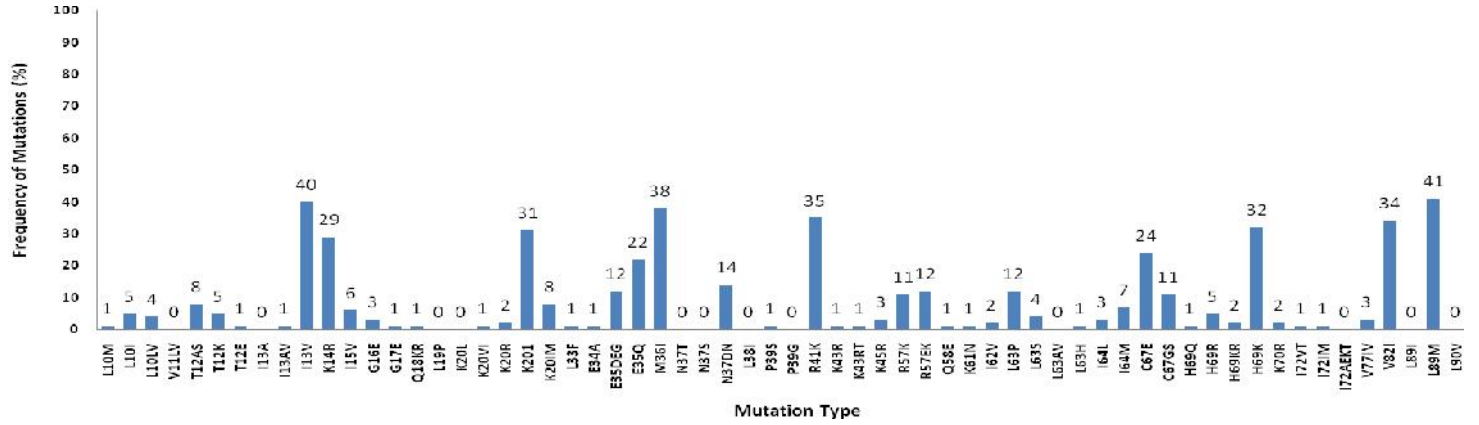

Figure 2. Frequency of protease mutations in HIV-1 subtype G isolates among antiretroviral treatment-naïve individuals.

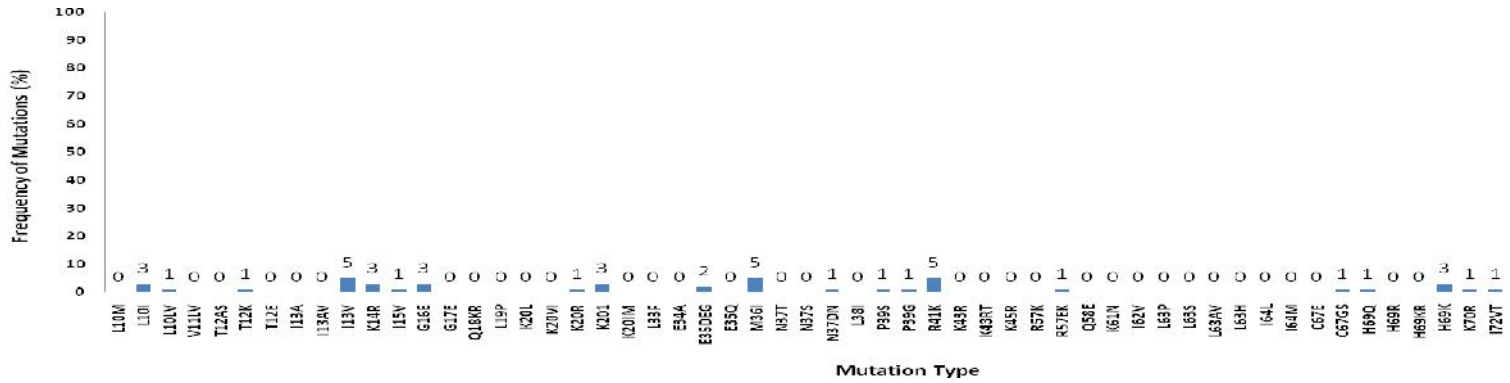

Figure 3. Frequency of protease mutations in HIV-1 subtype A isolates among antiretroviral treatment-naïve individuals.

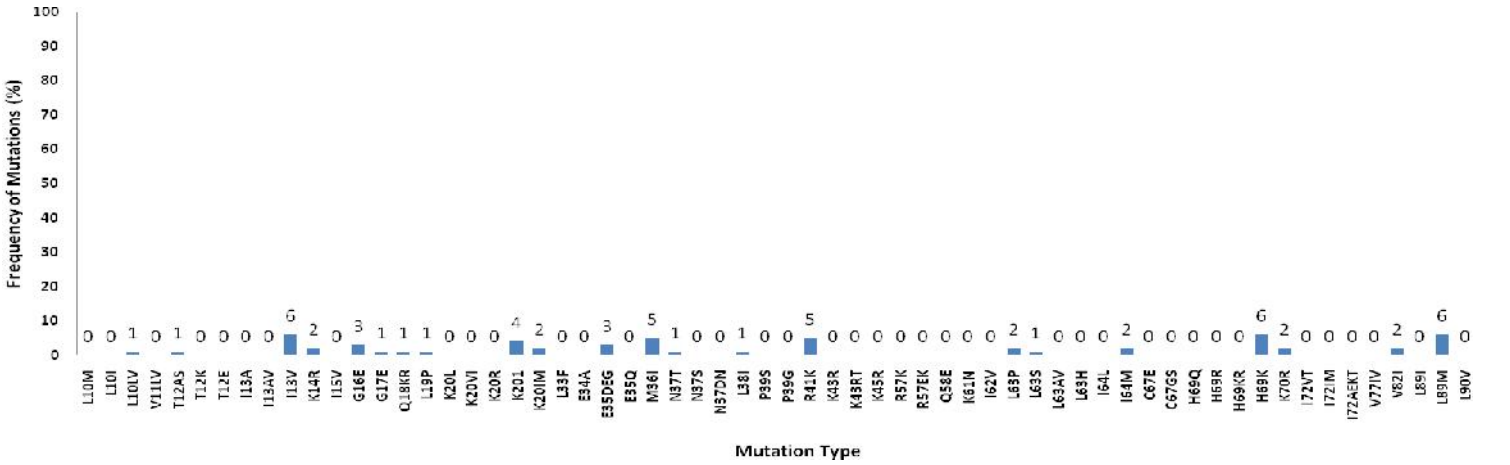

Figure 4. Frequency of protease mutations in HIV-1 subtype CRF06_cpx isolates among antiretroviral treatment-naïve individuals.

With the scale-up of ART in Nigeria, the knowledge of the prevalence of non-B HIV-1 subtypes and PIs resistance mutations will be a useful tool in optimizing drug selection options. Non-B HIV-1 subtypes may exhibit different differential drug responses [34] due to dissimilar fashions for developing drug resistance [35] since genetic diversity may contribute to differences in phenotypic and clinical properties [26]. For example, some subtype G viruses have been shown to be less susceptible to ARVs [34]. As evidenced in our study, most of the patients (98%) acquired HIV infection through heterosexual mode of transmission, with women being the majority (56%) (Table I). In Africa, this is the most common transmission route. The phylogenetic analyses showed that the patients were largely infected with subtype CRF02_AG (48%) and G (41%), and this is consistent with earlier studies done in Jos, Nigeria [34, 35]. Though the impact of non-B subtype variations on disease progression was not established, it could suggest HIV-1 rates of replication and transmission ability of the viral strains in circulation. The immigration from neighboring Cameroon and other West and

Central African countries where these subtypes predominate may partly explain the increasing prevalence of CRF02_AG in Nigeria.

A total of 62 mutations were observed, with subtype CRF02_AG having the highest proportions of mutations. Most of these mutations were listed by the international AIDS society (IAS)-USA as minor mutations with low-level resistance to some available protease inhibitors. It was found that among the minor mutations listed by IAS-USA, some of the observed mutations at positions 12, 13, 14, 15, 17, 19, 35, 37, 38, 39, 41, 45, 57, 67, 70 and 72 were not listed. Interestingly, an overall high prevalence of these mutations was observed; 13V, 14R, 20I, 36I, 41K, 69K and 89M. This finding contrasts with the report of other researchers who had observed a lower prevalence of the mutations in both treatment-experienced and naive-individuals [36, 37]. This however, suggests that the identified mutations could be natural polymorphisms associated with the Nigerian isolates. The distribution of amino acid substitutions in non-B subtypes differs from B subtypes; the prevalence of amino acid at positions 13V, 20I, 36I and 69K [38] is higher in non-B subtypes, while in B subtype the prevalence was also found to be relatively high at positions 63P, 64V, 62V and 77I [39-41] which is consistent with our findings. The observed minor mutations at positions 10, 20, 33, 36, 77, 82 confer low-level resistance in subtype B viruses, but were found as natural polymorphisms in non-B subtypes. Recent studies have demonstrated that these mutations in non-B subtypes confer no resistance to protease inhibitors among treatment- naïve individuals but confer fitness and hyper susceptibility to some viral strains [42].

However, mutations K20R/M/I/T/V are among the known selected mutations by some protease inhibitors. Interestingly, our study observed that K20I was found to be more common in all the non-B subtypes identified. Mutation K20I was known to be a consensus amino acid substitution for non-B subtypes CRF02_AG and G [42], but its high prevalence could suggest that it is a natural polymorphism associated with the Nigerian isolates.

The study also observed that 68-100% of mutations at positions I13V, K14R, M36I, R41K and L89M were found in all the identified subtypes. This finding confirmed earlier reported prevalence of these mutations in the Nigerian epidemic among PI-naive individuals [26, 36]. Among the mutations at position 10, 10I/V appears to be commonly selected by PIs and our study observed the high proportion of L10LV (7%) while V11I (1%) was selected by darunavir. The observed mutation G16E was selected by atazanavir. K20R/M/I/V substitutions were selected by atazanavir, indinavir and lopinavir based regimen. It was observed that point mutations (10LV, G16E and K20I) were at highest proportions in non B subtype CRF02_AG. The single mutation V11LV was observed in one isolate of subtype CRF02_AG. L10M is a rare single mutation identified in one HIV-1 infected individual harboring subtype G isolate.

The study observed that Q58E mutation was recently described as a major mutation that causes resistance to tipranavir (TPV) in B subtypes, although the mutation was once reported to be a minor (non-polymorphic) mutation [12], but current IAS-USA update [31] on resistance linked the mutation as major to TPV resistance. Our finding corroborates this report as observed in the non-B subtype G isolates. Consistent with our findings, was the reported low prevalence of Q58E mutation in non-B HIV-1 infected drug-naive individuals [44]. Accepting that these patients are ARV treatment-naïve, the mutation may have been acquired from transmitted virus. Thus the presence of Q58E is not a rare event but may not be retained under drug pressure, and this suggests that with the increased use of PI-based regimens in Africa, it is important to have studies done on the clinical implications of these mutations in non-B subtypes.

This study also revealed the presence of single mutation L33F in subtype G and this mutation has been reported to be the most common lopinavir/ritonavir resistance-related mutation that confers cross resistance to darunavir, which was known to be a salvage regimen in individuals failing lopinavir/ritonavir [45]. Recent studies showed that L33F was implicated in

patients with decreased susceptibility to ritonavir-boosted tipranavir [46, 47]. Although our study did not assess the clinical impact of L33F, but having this mutation prior to treatment may suggest caution with the use of PIs

A recent study has demonstrated that mutations G17E/I64M increases viral fitness and hypersensitivity in subtype CRF02_AG, which is known to delay the emergence of drug resistance mutation. The appearance of these mutations in subtype CRF02_AG and G suggests that the use of PIs could be beneficial in treatment-naive individuals. The frequency of naturally occurring mutations varies greatly and is dependent on the subtype. It has been reported that subtype C, G and CRF02_AG are more susceptible to indinavir than HIV-1 subtype B isolates [19]. Studies have also shown that minor mutations and polymorphisms occurring in non-B HIV-1 subtype among treatment-naïve individuals impact on drug resistance and susceptibility. Amino acid substitution at positions 10I/V, 20R/M, 33F, 36I, 63P and 89M have been found to be associated with low-level resistance to some PIs in subtype B isolates which were also identified in non-B subtypes [48, 49], and this suggests differences in the drug susceptibility in relation to subtype-specific mutations.

4. CONCLUSION

Although our study was a cross-sectional study, the heterogeneous genotypes derived from HIV-infected individuals in Nigeria are representative of viruses and associated mutations in our geographic region. The study indicates for the first time subtype-specific mutations in Nigeria, although at a low frequency for one major mutation (Q58E) associated with subtype G isolate. There were high frequencies of minority mutations associated with the non-B HIV-1 subtypes identified. The identification and description of non-B subtype-specific mutations in protease gene is necessary for monitoring the emergence and transmission of resistance mutations, for treatment initiation strategies, and for interpreting genetic resistance in patients who experience failure of second line treatment regimens. Further larger studies are needed to evaluate the clinical implications of these mutations to protease inhibitors susceptibility in order to optimize available treatment regimen options.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

AUTHORS' CONTRIBUTIONS

Joseph A. Anejo-Okopi: conception, design, experiments, results, manuscript writing and manuscript review
Kennedy Were, Harris Onywera, Newton Otecko and Preston Owiti: protocol and experiments, manuscript writing and manuscript review, Augustine O. Ebonyi: data analysis/ results, tables/ graphs, manuscript writing and manuscript review
Lohya Nimzing, Oche O. Agbaji and Ameh James: manuscript writing and manuscript review, Patricia A. Agaba, Samson E. Isa, Solomon A. Sagay, Stephen Oguche, David E. Jatau, Steve O. Olonitola and John A. Idoko: manuscript review

CONSENT

An informed consent was obtained from all the recruited individuals.

ETHICAL APPROVAL

Jos University Teaching Hospital Ethics Committee approved the study protocol

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