High frequency of non-B HIV-1 subtypes specific mutations at the protease gene among treatment-naïve HIV-1 infected individuals in Jos, Nigeria

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ABSTRACT

Aim: To determine the prevalence of non-B HIV-1 subtypes specific mutations in the protease gene among antiretroviral drug-naïve adult patients in Jos, Nigeria.

Design: The study prospectively recruited HIV-1 positive drug-naïve patients for genotyping assay.

Place and duration: The study was conducted **at the Jos University Teaching Hospital , 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 from Stanford HIV drug resistance database and MEGA software for the unrooted tree estimating the evolutionary distances between the sequence isolates. 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.

Conclusions: 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-naïve patients. Among the different subtypes in circulation, there is a high prevalence of minor mutations and natural polymorphisms associated with the protease gene. Such mutations define the subtype diversity which may dictate virulence and drug responses, thus further studies are needed to evaluate clinical implications of these mutations.

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

1. INTRODUCTION

Human immunodeficiency virus (HIV) type 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 [1, 2]. HIV-1 can be categorized into four groups (M, N, O, and P) with group M being responsible for majority of infections worldwide, including Sub Saharan Africa (SSA) and the major target of drug resistance and design strategies [3-6]. The variants are alternative lifelines HIV-1 uses to evade sustained drug pressure and host immune responses eventually resulting in resistance to antiretroviral drugs [7]. The use of highly active antiretroviral therapy (HAART) has remarkably reduced the morbidity and mortality caused by HIV and AIDS globally [8]. Antiretroviral therapy (ART) imposes selective pressure and this favor emergence of drug-resistant mutants within the HIV infected population [9]. Protease inhibitors (PIs) are one of the recommended second-line antiretroviral (ARV) drugs in Nigeria. They 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 increasing prevalence [10]. Resistance to antiretroviral (ARV) drugs is one of the major threats to the global control of HIV pandemic; as it may impact on clinical outcomes [11, 12]. It is being 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 [13]. This may constitute a major drawback in the use of PIs. Several studies on HIV-1 protease gene genetic diversity have highlighted that subtypes A, C, F, and G have natural polymorphisms that are associated with protease therapy in subtype B. These mutations are known to contribute to resistance or compensate for viral fitness defects due to primary drug resistance mutations in subtype B [14,15]. Although resistance mutation pathways in both subtype B and non-B are similar, several other different pathways have been observed in non-B subtypes but have not shown much limitations on the efficacy of ARVs [16, 17]. The impact of the non-B subtype diversity and mutations on treatment outcome is yet to be well understood especially in developing countries. However, evidence from developed countries has shown that polymorphisms which occur naturally in B subtype impacts on antiretroviral drug resistance and susceptibility [18, 19]. Some earlier studies have documented HIV-1 genetic diversity and associated genotypic profiles of *pol* gene in Nigeria [20-23], a region with the second highest proportion of people living with HIV. Subtypes CRF02_AG and G are the most common in Nigeria, but lack well characterized epidemic trends [22]. Also, little is known about the prevalence of non-B HIV-1 subtype associated natural polymorphisms to PIs among treatment-naïve 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 subtypes specific mutations in the protease gene among antiretroviral drug-naïve adult patients in Jos, Nigeria.

2. MATERIAL AND METHODS

2.1 Settings, Patient Recruitment and Sample Collection

Plasma samples were collected from HIV-1 seropositive individuals attending a reference HIV treatment center at the Jos University Teaching Hospital (JUTH) in Jos, Nigeria. The entry point for all patients was either through HIV counseling and testing (HCT) at the center or referral from other services within the hospital, the community or neighboring states. A total of 230 HIV-1 infected treatment-naive patients were recruited consecutively after obtaining informed consent between October 2010 and April 2011. The JUTH Ethics Committee approved the study protocol and consent form.

Questionnaires were used to collect demographic data from study participants. The criteria for inclusion were patients who were 18 years or older, HIV-positive and had no prior ARV exposure. Blood samples were collected in ethylenediamine-tetra-acetic acid (EDTA)-lined containers and plasma extracted and cryopreserved.

All laboratory tests were done according to the treatment program guidelines; no additional laboratory assessments were done except for the purposes of the analyses described here. $CD4^+$ lymphocyte count was measured same day of the blood draw using Partec CyFlow Counter® (Partec GmbH, Munster Germany) according to manufacturer's instructions and as previously described [24] and HIV-1 RNA viral load was measured using the Roche Cobas Amplicor HIV-1 Monitor, version 1.5 (Roche Diagnostics GmbH, Mannheim, Germany). Due to the high cost of HIV-1 genotyping, 105 randomly selected (from computer-generated random numbers) samples out of 230 were assayed. The 105 samples were subsequently shipped in ice-parked containers to the Kenya Medical Research Institute (KEMRI) HIV-R Laboratory Kisian, Kisumu Kenya, where genotypic testing using in-house Genotyping assay. 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 reverse transcriptase) gene region associated with resistance was as previously described [25]. 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) 2253-2275 based on HIV-1 HXB2) and RT-R1 (reverse, 5'-ATCCCTGCATAAATCTGACTTGC-3', nt 3370-3348); the reaction conditions in the ABI GeneAmp 9700 thermoCycler included 65 ºC for 10 min, 50 ºC for 45min, 94 ºC for 2 min, 94 ºC for 15 sec, 50 ºC for 20 sec, 72 ºC for 2min, 72 ºC for 10min and 4 ºC until removal, and the nested PCR primers were Prt-F2 (forward, 5'-CTTTGGCAACGACCCCTYGTCWCA-3', nt 2265 - 2288) and RT-R2 (reverse, 5'-CTTCTGTATGTCATTGACAGTCC-3', nt 3326 -3304) both at 4µM concentration. For RT-PCR mixture (primers Prt-M-F1, RT-R1 8µM each, RT-PCR mixture 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). 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 15sec, and 55 °C for 20sec following an extension at 72 °C for 2 min, 72 °C for 10 mins and 4 °C for ∞. The products from nested PCR were verified by visually comparing the intensity of each sample's band to that of the DNA mass ladder's bands of known DNA quantity. This was performed on 1% agarose gel electrophoresis stained with 0.5µg/ml ethidium bromide and photographed under ultraviolet illumination. 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 AmpliTaq DNA polymerase on an automated ABI Prism 3130*xl* Genetic Analyzer [23].

2.3 Sequence Interpretation

The generated nucleotide sequences were aligned and edited using Sequencer v5.0 (Gene Codes Corporation) that assembles overlapping sequence segments from the primers used to form a contiguous sequence from which a consensus sequence can be generated. Sequences with frame shifts or stop codons were excluded from analysis, and the quality of the generated sequences checked using Sequence Quality Assessment Tool (SQUAT). For 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. The Kimura two-parameter model using the software MEGA 4.0 for the unrooted tree estimated the evolutionary distances between the sequence isolates [26]. The strength of the neighbor-joining method was assessed by bootstrap (1000 replicates), and values above 70% were considered significant. The submitted sequences to Stanford University HIV drug resistance database obtained mutations that were classified and analyzed according to the International Antiviral Society (IAS)-USA 2013 guidelines. These guidelines recommend the following list of mutations as minor in relation to PI drug-specifics: atazanavir/ritonavir (L10I/F/V/C, G16E, K20R/M/I/T/V, L24I, V32I, L33I/F/V, F34Q, M36I/L/V, M46I/L, G48V, F53L/Y, I54L/V/M/T/A, D60E, I62V, I64I/M/V, A71V/I/T/L, G73C/S/T/A, V82A/T/F/I, I85V, L90M, and I93L/M); darunavir/ritonavir (V11I, V32I, L33F, T74P, and I89V); fosamprenavir/ritonavir (L10F/I/R/V, V32I, M46I/L, I47V, I54L/V/M, G73S, L76V, V82A/F/S/T, and L90M); indinavir/ritonavir (L10I/R/R/V, K20M/R, L24I, V32I, M36I, I54V, A71V/T, G73S/A, L76V, V77I, and L90M); lopinavir/ritonavir (L10F/I/R/V, K20M/R, L24I, L33F, M46I/L, I50V, F53L, I54V/L/A/M/T/S, L63P, A71V/T, G73S, I84V, and L90M); nelfinavir (L10F/I, M36I, M46I/L, A71V/T, V77I, V82A/F/T/S, I84V, and N88D/S); saquinavir/ritonavir (L10I/R/V, L24I, I54V/L, A71V/T, G73S, V77I, V82A/F/T/S, and I84V); tipranavir/ritonavir (L10V, L33F, M36I/L/V, K43T, M46L, I54A/M/V, H69K/R, and L89I/M/V). Major mutations were defined as atazanavir /ritonavir (I50L, I84V, and N88S); darunavir/ritonavir (I47V, I50V, I54M/L, L76V, and I84V); fosamprenavir/ritonavir (I50V and I84V); indinavir/ritonavir (M46I/L, V82A/F/T, and I84V); lopinavir/ritonavir (V32I, I47V/A, I76V, and V82A/F/T/S), nelfinavir (D30N and L90M); saquinavir/ritonavir (G48V and L90M); tipranavir/ritonavir (Q58E, T74P, V82L/T, N83D, and I84V) [27]. 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 mutations that were more prevalent in a given subtype.

2.4 Statistical Analysis

The data for all 100 patients were entered into Microsoft office excel work sheet (Microsoft office system, 2007) 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 office excel

3. RESULTS AND DISCUSSION

The median age of the 100 study patients was 35.5 years with the majority of them being females (56%). Majority (68%) of the patients were married and the commonest 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 with their viral load ranging 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 subtypes among the individuals attending JUTH reference Centre, Jos, Nigeria. Phylogenetic analyses of the partial *pol* gene revealed heterogeneous distribution of four non B HIV-1 strains at different prevalence: CRF02_AG (48%), G (41%), CRF06_cpx (6%) and A (5%). HIV-1 subtypes CRF02_AG and G accounted for majority of the infections(89.0) .

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 samples from non-B subtype G harbored a major drug resistance mutation (Q56E) 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%). V82I mutation was highly associated with this subtype than with the other three subtypes. The observed mutation at position L10M is unusual. It occurred at a 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-naïve patients.

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)

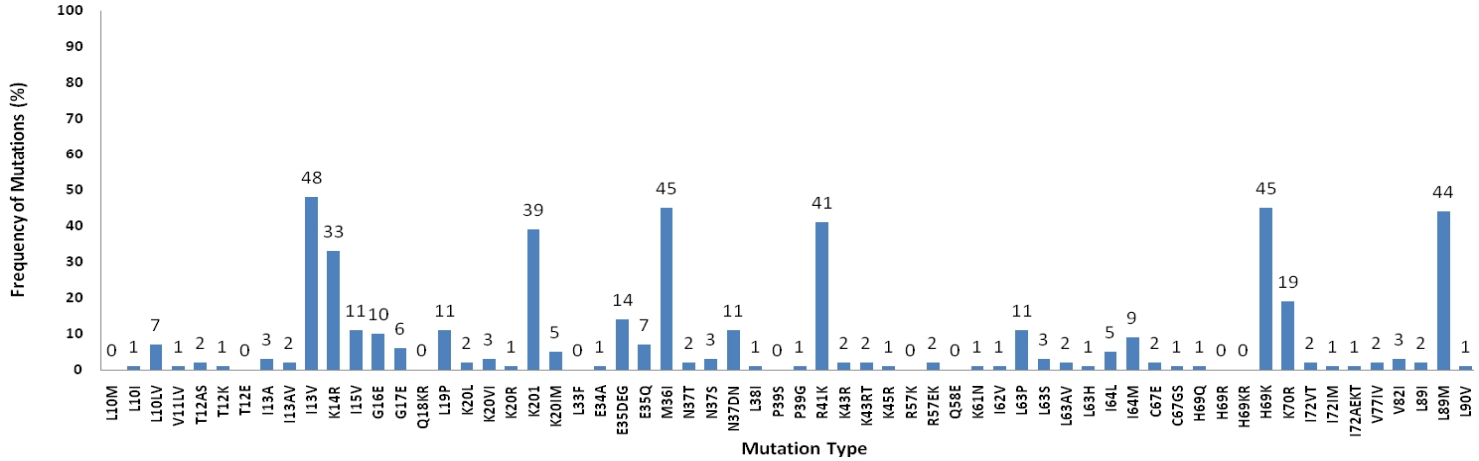

Figure 1. Frequency of protease mutations in HIV-1 subtype CRF02_AG isolates among antiretroviral treatment-naïve patients.

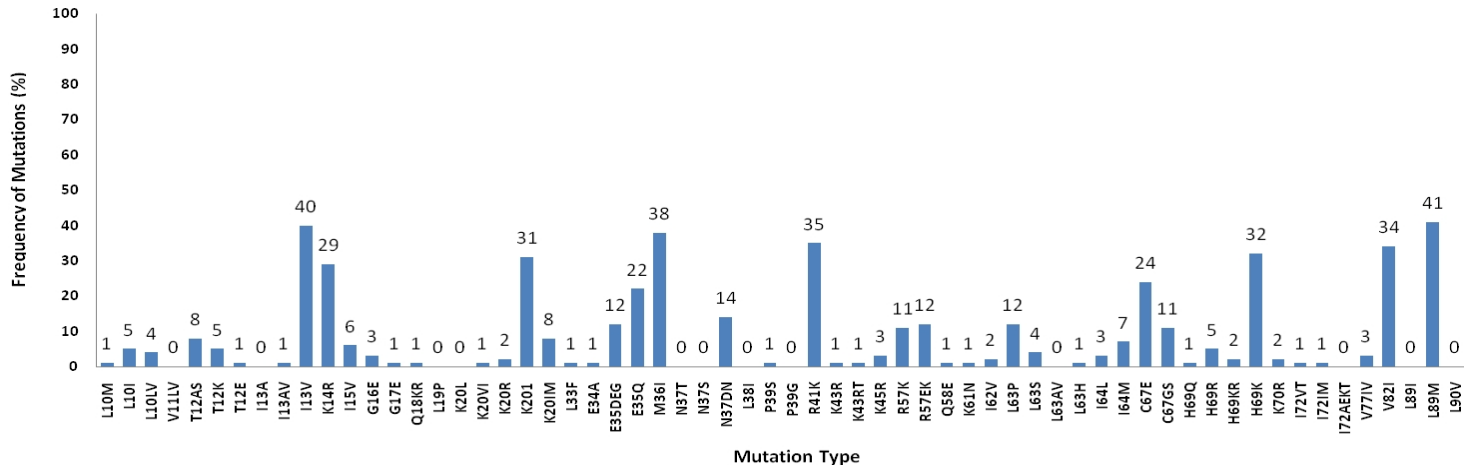

Figure 2. Frequency of protease mutations in HIV-1 subtype G isolates among antiretroviral treatment-naïve patients.

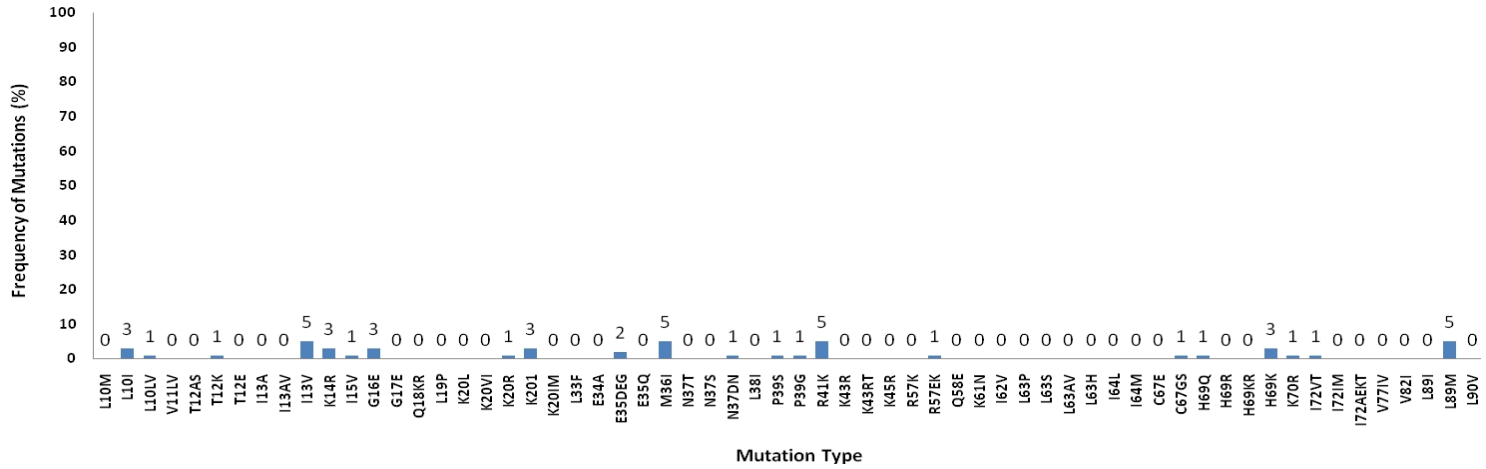

Figure 3. Frequency of protease mutations in HIV-1 subtype A isolates among antiretroviral treatment-naïve patients.

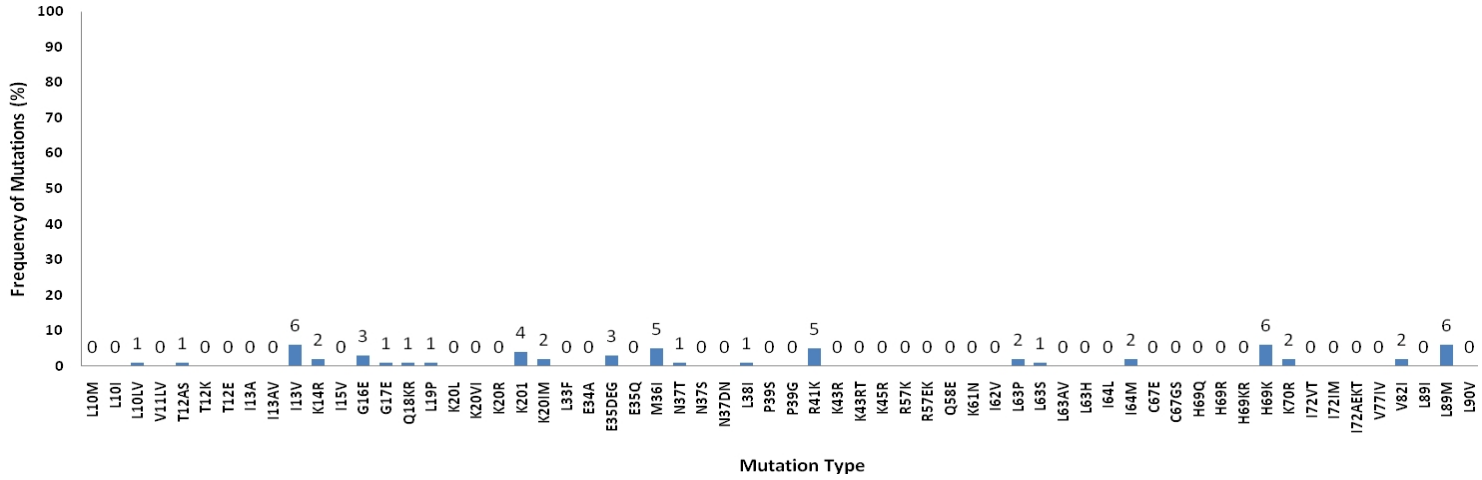

Figure 4. Frequency of protease mutations in HIV-1 subtype CRF06_cpx isolates among antiretroviral treatment-naïve patients.

With the scale-up of antiretroviral therapy 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 [28] due to dissimilar fashions for developing drug resistance [29] since genetic diversity may contribute to differences in phenotypic and clinical properties [22]. For example, some subtype G viruses have been shown to be less susceptible to ARVs [28]. As evidenced in our study, most of the patients (98%) acquired HIV infection through heterosexual mode of transmission, with women being the majority (55%) (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 [28, 29]. Though the impact of non-B subtypes 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 interntional 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 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 on the overall, high prevalence of these mutations were observed: 13V, 14R, 20I, 36I, 41K, 69K and 89M. This finding corroborates the report of other researchers who had observed a lower prevalence of the mutations in both treatment-experienced and naïve-patients [30, 31]. 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 [32] is higher in non-B subtypes while in B subtype the prevalence is high at positions 63P, 64V, 62V and 77I [33-35] 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 patients but confer fitness and hyper susceptibility to some viral strains [36].

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 [37] 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 patients [22, 29]. 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%) is selected by darunavir. The observed mutation G16E is selected by atazanavir. K20R/M/I/V mutations are selected by atazanavir, indinavir and lopinavir based regimen. It was observed that these mutations (10LV, G16E and K20I) are of highest proportions in non B subtype CRF02_AG. The single mutation V11LV was observed in one patient of subtype CRF02_AG. L10M is a rare single mutation identified with one patient in subtype G.

We 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 thought to be a minor (non-polymorphic) mutation, but IAS-USA 2013 update on resistance linked the mutation to TPV resistance. Our finding corroborates this report as observed in the non-B subtype G isolates. Consistent with our findings, the low prevalence of Q58E mutation has also been documented in non-B HIV-1 infected drug-naïve patients [38]. 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 on the clinical implications of these mutations on 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 patients failing lopinavir/ritonavir [39]. Recent studies showed that L33F was implicated in patients with decreased susceptibility to ritonavir-boosted tipranavir [40, 41]. Although our study did not assess the clinical impact of L33F, having this mutation prior to treatment may suggest caution with PI use.

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-naïve 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 [16]. Studies have 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 found in non-B subtypes [42, 43], and this suggests differences in the drug susceptibility in relation to subtype-specific mutations.

4. CONCLUSION

Although our study is a cross-sectional study, the heterogeneous genotypes derived from the patients in Nigeria are representative of viruses and associated mutations in our geographic region. There is high frequency of minority mutations associated with the non-B HIV-1 subtypes identified. Further studies are needed to evaluate the role of these mutations in the emergence of drug resistance, clinical implications and PIs susceptibility in order to enhance understanding.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

CONSENT

An informed consent was obtained from all the recruited patients.

ETHICAL APPROVAL

Jos University Teaching Hospital Ethics Committee approved the study protocol.

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