

Utility of a Relatively Affordable In-House HIV-1 Genotyping Assay for Drug Resistance Testing among Non B HIV-1 Infected Drug Naive Patients in Nigeria

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Abstract

Background: The introduction of antiretroviral (ARV) in resource-limited settings has increased life expectancy among non-B HIV-1 infected individuals. We used a validated In-house genotyping assay to characterize non-B HIV-1 and to determine drug resistance mutations among treatment-naive patients. **Methods:** Plasma samples from 105 HIV-1 infected drug-naive adult patients attending a tertiary hospital Jos, Nigeria were subjected to HIV-1 RNA extraction, reverse transcription amplification, and population-based sequencing of the partial *pol* gene on the ABI 3130xl genetic analyzer. Subtyping and phylogenetic analyses were performed by REGA Subtyping Tool v2.0 and MEGA v5.0 respectively. Drug resistance profiles were evaluated according to IAS-USA 2013 drug resistance mutations list. **Result:** One hundred samples (95.2%) were successfully genotyped. The distribution of the non-B HIV-1 subtypes were; CRF02_AG-48%, G-41.0%, CRF06_cpx-6.0%, and A-5.0%. Ten percent of the isolates had at least one major drug resistance mutation in the *pol* gene. The drug-class specific resistance prevalences were 6.0% for NRTIs; M41L-1.0%, K65KR-1.0%, M184IM-1.0%, M184V-2.0%, and T215ADNT-1%, 8.0% for NNRTIs; K103N-2%, 1.0% for K101E, E138A, G190A, P225HP, Y181I, Y188L, Y181C including protease inhibitors' Q58E

(1.0%). **Conclusion:** HIV-1 was heterogeneously distributed; CRF02_AG and G predominate and some known major mutations associated with NRTIs and NNRTIs were determined. The In-house assay is suitable for both characterization of non-B HIV-1 subtypes and detection of drug resistance at a significant lower cost than available commercial genotyping assays. This finding underscores the need to consider use of low-cost In-house genotyping assay as an alternative in resource-limited settings with non-B HIV-1 epidemic.

Keywords

HIV-1, Antiretroviral Therapy, Drug Resistance, Genotyping, Affordable, In-House

1. Introduction

Human Immunodeficiency Virus type 1 (HIV-1) is responsible for chronic infection leading to Acquired Immunodeficiency Syndrome (AIDS) as a result of chronic infection damage [1]. The clinical course of HIV-1 infection is due to a high variability of the viral strain. This heterogeneity is a result of the high frequency of viral replication errors; retroviral reverse transcriptase (RT) lacking proofreading functions. As the virus replicates, genetic variations produced has ability for continued viral survival and growth as well as resistance to antiretroviral drugs (ARVs). However, while ARV is reducing mortality rates from AIDS-related causes, the widespread and long-term usage of drugs raises concerns with regards to emergence of ARV associated resistance mutations in evolving HIV strains. As evidence on the impact of large scale treatment programs in reducing incidence and increasing life expectancy in developing countries continues to accumulate, efforts to increase wider use ARVs will be intensified [2] [3]. The use of potent ARVs can result in a profound and durable suppression of HIV-1 replication resulting in plasma HIV-1 viral load below levels of detection. The presence of some viral mutations introduced into the HIV-1 genome during replication can compromise efficacy, while others display susceptibility of the virus infectivity in combination with ARVs resistance. With this expected long-term and success of ART in developing countries, the coverage and programmatic effort should be complemented by HIV drug resistance (HIVDR) monitoring programs to ensure good clinical outcomes, and to reduce treatment failure and transmission of drug resistance [4]. Thus, there is a need for more and affordable genotyping assays in attempt to effectively monitor and control drug resistance as more individuals are initiated onto ART as recommended by World Health Organization [5]. In-house genotypic drug resistance testing has been successfully used in developed countries; for surveillance and monitoring of drug resistance in HIV infected individuals receiving ART, but has not been integrated into the continuum of care in most settings in Africa. Most international guidelines recommend drug resistance testing for adult or

pediatric patients failing ART-first-line and second-line [6] [7]. However, the affordability due to high-cost of the technology, and infrastructure requirements have limited the implementation of similar approaches to utility of drug resistance monitoring in resource-limited-settings (RLS).

The Nigeria HIV treatment and monitoring guidelines do not currently recommend the use of drug resistance testing in clinical choice of ART for individuals failing first-line regimens [8] but are switched based primarily on clinical and virological (HIV-1 viral load) parameters. It is evident that incorporating drug resistance testing into treatment guidelines would be potentially cost effective in the near future. Considering the high cost of alternative second-line ARVs to available first-line, the use of drug resistance testing to identify patients who needs switching will conserve treatment options, minimize economic loss to individuals, program and the nation. Therefore, it is important to reduce the cost of drug resistance monitoring methods by using affordable In-house genotyping assay in order to improve access, quality of care and better treatment outcomes. The available In-house genotyping assay is designed for non-B subtypes with high sensitivity by detecting amino acid substitution at rare positions not commonly found in subtype B specific commercially available assays. The assay which is cost effective and affordable has similar designed protocol to the commercially available assays: HIV Ribonucleic RNA extraction, Reverse Transcription (RT) and nested Polymerase Chain Reaction (PCR) amplification of viral targets, Cycle sequencing using Big-Dye terminator and Bioinformatics methods for analysis of chromatograms, manually check the alignment for misaligned sequences, deletions and insertions and edit accordingly. It also involves the construction of phylogenetic tree using relevant tools to examine and review evolutionary relationships and those with short branches for possible contamination [9] [10].

The In-house HIV-1 genotyping assay is sensitive for detection and sequencing of non-B HIV strain variants specifically and it is CDC-supported Genotyping assays that well popular in East African countries [11] which is currently being deplored to CDC supported Laboratories in Nigeria, though not yet widely in use for clinical decisions. There is the need for Nigeria Government to buy into this program and fund it for effective utilization of the available ARVs to reduce emergence and transmission of drug resistance mutations, and associated economic cost. The utility of this assay will not only support researchers, but also impact positively on the success of HIV treatment populations and stop the spread of HIV and ARV-resistant strains. This study aimed to describe frequency of DRMs in HIV-1 genotypes that circulate in drug naive patients, using a cost-effective in-house HIV-1 GRT suitable for non-B HIV-1 variants.

2. Methods

This study was carried out at the AIDS Prevention Initiative in Nigeria (APIN) CDC supported HIV clinic at the Jos University Teaching Hospital (JUTH), Jos.

The entry point for all patients was either through HIV counseling and testing (HCT) or referrals from other services within Jos metropolis and neighboring states. One hundred and five (105) HIV-1 infected treatment-naïve patients were recruited sequentially after obtaining informed consent between October 2010 and April 2011. The institutional review board at Jos University Teaching Hospital approved the study protocol. A structured questionnaire was used to collect basic demographic data from each participant with no previous ARV exposure and was aged ≥ 18 years. Blood samples were collected in EDTA containers and plasma was extracted and cryopreserved. The samples were shipped in ice-packed containers to the Kenya Medical Research Institute HIV-Resistance Laboratory, Kisian Kisumu, where genotypic testing using affordable In-house genotyping System was done. Of 105 samples tested, 100 successfully amplified for genotypic drug resistance testing.

2.1. Isolation and Amplification of HIV-1 Partial Pol Gene by RT-PCR and Nested PCR

We use two amplification protocols for HIV pol gene as previously described [9], HIV viral RNA were extracted from plasma using the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany) followed by reverse-transcription of the RNA into cDNA using the outer primers Prt-F1-forward (2253 - 2275 nucleotides, nt) and RT-R1 reverse (nt 3370-3348) for RT-PCR. The cDNA were amplified by nested-PCR with primer Prt-F2 (forward, 2265 - 2288 nt) and RT - R2 (reverse, 3326 - 3304 nt). The amplified DNA fragments 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 for expected size by electrophoresis in 1.0% agarose gel stained with 0.5 $\mu\text{g}/\text{ml}$ ethidium bromide and photographed under ultraviolet illumination. The fragments were purified using the QIA quick PCR purification kit (Qiagen, Hilden Germany) in spin columns, and direct population-based sequencing performed on both strands using Big Dye® Terminator v3.1 Cycle Sequencing kit on ABI 3130X1 Genetic Analyzer.

2.2. Sequence Analyses, Determination of Drug Resistance Mutation and Subtyping and Phylogenetic Analyses

The generated nucleotide sequences were viewed using the Sequence Analysis Software v3.7, and aligned and edited using Sequencher version 5.0, which assembles the six overlapping sequence segments for the six primers to form a contiguous sequence. Sequences with frame shifts or stop codons were excluded from analysis. The quality of the generated sequences was checked using Sequence Quality Assessment Tool (SQUAT). The sequences in fasta format were then subjected to the Stanford HIVdb algorithm (<http://hivdb.stanford.edu/>) for subtyping and determination of mutations conferring various antiretroviral drugs resistances. Mutations in the sequences were defined as differences from the consensus B reference sequence and were further characterized as RTI and

PI associated resistance mutations. HIV-1 subtyping was performed using REGA HIV-1 subtyping tool V2.0 from Stanford HIV drug resistance database (<http://hivdb.stanford.edu/>), a worldwide subtype references were obtained from Los Alamos database (<http://hiv-web.lanl.gov>), and sequences were aligned against the known reference strains. Phylogenetic analysis was performed by the neighbor-joining (NJ) method as implemented in MEGA V.0. The bootscanning method was used to detect and study recombination, as implemented in the SIMPLOT software v.2.5). The recombination was further confirmed with Recombination Identification Program (RIP) v.3.0 available online (<http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html>). Subtypes assignments by this method are shown to the right of the tree with isolates clustered around the various references. Analyze the sequence using the HIVDB program at <http://hivdb.stanford.edu>.

3. Result

Of 105 samples, the proportion of female and male was 55% and 45% respectively. Mode of transmission; 98% were heterosexual and 2% blood transfusion, marital status; 69% married, 19% single (never married), while 8% were separated/divorced and 5% were widowed. The median baseline CD4 cell count was 186 cells/mm³ (IQR: 12 - 737). WHO disease staging of the subjects was: stage I (24%) stage II (21%), stage III (36%) and stage IV (19%). 50% of the spouses of the participants were HIV positive, while 50% were HIV negative. The participants whose spouses were on ARV were 14%, and those whose spouses were not on ARV were 86%.

3.1. Subtyping and Phylogenetic Analyses

Subtyping and phylogenetic analyses were performed by REGA Subtyping Tool v2.0 and MEGA v6.0 respectively. **Figure 1** showed the distribution relationship of different genetic subtypes in the pol region using 21 references from Los Alamos HIV Sequence database. The subtypes AG and G clustered around references from Nigeria and other West and Central African neighboring regions. Of 105 samples, 100 (95.2%) were successfully amplified and Sequenced. The distribution of the non-B HIV-1 subtypes were; CRF02_AG (48%), G (41.0%), CRF06_cpx (6.0%), and A1 (5.0%). Ten percent (10.0%) of the isolates had at least one major drug resistance mutation in the pol gene. We observed more major mutation in patients with subtype AG (4/10), G (4/10), A1 (1/10) and cpx06 (1/10). Drug resistance profiles were evaluated according to IAS-USA 2017 update of drug resistance mutations list [12].

3.2. Detection of Major Drug Resistance, Accessory Mutations and Natural Polymorphisms

The In-house genotyping assay results from the samples of all treatment naive revealed some major IAS-defined major DRM in the viral pol gene [12]. The

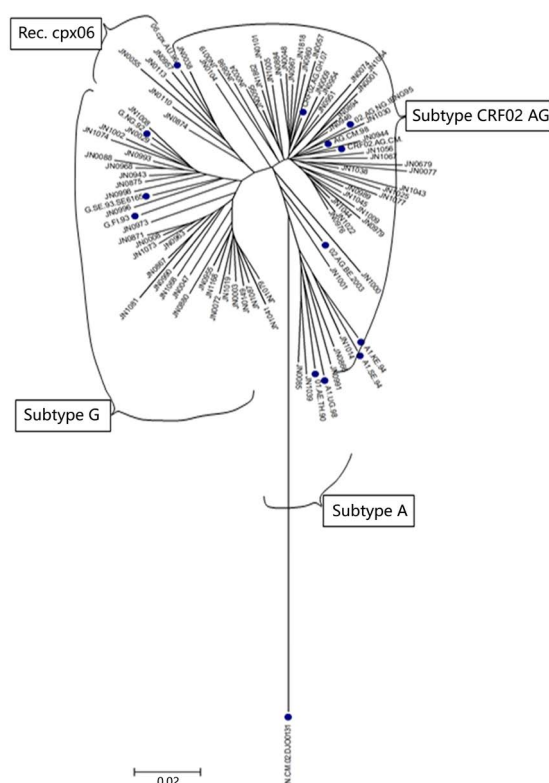


Figure 1. Phylogenetic Analysis of Isolates among the ARV Treatment naive Patients Jos, Nigeria.

frequency of the major DRMs for NRTIs, nNRTIs, and PIs among treatment-naive patients samples of subtype non-B are presented in **Table 1**. In the RT region, thymidine analogue mutations (TAMs) identified was M41L which could confer reduced susceptibility to currently approved nNRTIs (AZT, d4T, ddI, ABC and TDF), however, the degree of cross-resistance depends on the number and presence of some accessory mutations. NRTIs (5.0%), M41L (1.0%), M184I (1.0%), T215ADNT (1%), K65KR-1.0%, M184V (2.0%). The presence of M184V prevent emergence of and accumulation TAMS of and other mutations. We observed nNRTIs (9.0%), K103N (2%), G190A (1.0%), Y181I (1.0%), Y181C (1.0%), K101E (1.0%), P225HP (1.0%), Y188L (1.0%), E138A (1.0%), and PI major mutation was Q58E (1.0%). Mutations K101E and E138A which occur as natural polymorphism and have been shown have reduced susceptibility to rilpivirine. The TAMM41L was prevalent in patients with subtype AG (1.0%). We identified T215 revertants in one patient in subtype A1 which may increase risk of developing virological failure on AZT- or d4T-containing first line ARVs because in contrast to the wild type threonine (T), most revertants require just a single base-pair change to develop T215Y/F and because the presence of a revertant by standard sequencing may indicate the presence of T215Y/F as a minority variant [12]. K65R (1.0%) was detected in one patient who also had nNRTI along Y181C. The frequency of the accessory mutations in the RT region were; R211K, V179IE, G196EG, V118I, V106I, A98SG, T69N, L228HL, and E44DE,

Table 1. Prevalence of major ARV Resistance Mutations among the ARV Treatment-naïve Patients.

PTID	Age	Sex	CD4+Cell count	HIV-1 RNA VL	Duration HIV-1 Infection(AI)	HIV-1 Subtypes	Spouse on ART	Major RT mutations NRT In NRTI		Major PR
JN1073	34	F	600	10,946	Recent	G	No	-	-	Q58E
JN1041	36	F	18	23,050	LI	G	Yes	M184I	K101E, Y181I	-
JN0055	31	F	31	99,892	Recent	CRF06_cpx	No	-	Y188L	-
JN0679	34	M	93	216,034	LI	CRF02_AG	No	K65R	Y181C	-
JN0866	27	F	21	635,542	LI	A1	Yes	T215ADNT	-	-
JN0990	39	F	272	22,193	LI	G	Yes	M184V	K103N, P225HP	-
JN0088	45	F	389	5788	LI	G	Yes	M184V	Y181C, G190A	-
JN0110	29	F	237	99,898	LI	CRF02_AG	No	M41L	-	-
JN0114	43	F	21	19,331	LI	CRF02_AG	No	-	K103N	-
JN1054	55	M	259	3758	LI	CRF02_AG	No	-	E138A	-

V90I, and L210M. We also observed diverse natural polymorphisms which frequent in the protease gene as earlier reported L10I/V, V11LV, T12K, I13V, K14R, I15V, K20I/R/M, L33F, E34A, M36I, N37DN, L38I, P39G/S, R41K, K45R, R57E/K, K61N, I62V, L63H, C67/G/S, H69K/Q/R, I72AEK/M/V/T, V82I, L89M,, 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 with various proportions [9].

4. Discussion

With the widespread availability of HAART in RLSs, the demand for sensitive and affordable monitoring of patients on ART is becoming a high priority. However, a better access to ARVs without adequate monitoring might result in a widespread transmission of drug resistant strains which will result to therapeutic failure. The current epidemic of drug-resistant HIV strains in RLSs can only be reduced by deliberate interventional strategies that would guarantee effective patients' regimen options, enhance correct intake of the medication, support uninterrupted drug supply, ensure the drug pharmaco-vigilance, treatment outcomes and provide useful therapeutic guidelines in case of treatment failures. The access to commercial genotypic assays and viral load monitoring in Africa is still limited and beyond the reach of the average patients due to high cost. Although these approved assays has demonstrated good performance on both subtype B non-B HIV-1, the high cost implication hinder their routine application in RLSs [13].

This study used a modified In-house GRT which had improved primer binding quality suitable for testing, the increased sensitivity, and found high degree of HIV-1 subtypes with the detection of diverse and rare mutations. The sub-

types and drug resistance mutations identified corroborates with earlier reported findings in Africa including Nigeria [4] [14] [15] [16] [17] [18]. However, our finding of accessory mutations and polymorphisms both at RT and PR were more diverse and this could be due to the broad sensitivity of the in-house genotyping assay used. Though the procedure without doubt still requires a well-equipped laboratory, but the assay can be performed for reagents cost of about 100 USD, which is significantly lower than the FDA approved available systems. Studies have reported high sensitivity of RT-PCR step with a detection limit of <500 copies/ml which permits its use as a screening test in patients under treatment [19] [20]. The use of this method in the follow-up of patients under treatment will allow the fast detection of emerging drug resistance at a more affordable cost than the commercial tests. The in-house assay is broadly sensitive in the detection of drug resistance genotyping of HIV-1 group M viral strains and has proved to be more sensitive than the commercially available assays in detecting mixed viral populations (Zhou *et al.* 2011). The substantial reagent cost saving and broad sensitivity make this assay more accessible for RLS where HIV drug resistance surveillance is strongly recommended to minimize the development and transmission of HIV drug resistant mutants [21] [22] [23] [24].

Although recombinant subtype AG is still the most prevalent, it is responsible for only less than half of all the infections in Nigeria [9]. Studies have shown the extensive genetic diversity of HIV-1 strains from West Africa including Nigeria and Cameroon with significant representations of the recombinant form CRF02_AG, subtypes G, J, and F [25]. Although the performance of the two available genotyping assays can be considered as appropriate, but problems with non-B subtype strains do occur occasionally [21].

The main objective of this report was to present the utility of relatively affordable in-house HIV genotyping system for the monitoring of HIV-infected patients under treatment and surveillance. The method identified diverse major, accessory mutations and polymorphism which could be used as a guide for careful treatment options and further research into drug resistance surveillance, and the test can be performed at a lower cost compared to commercially available genotyping assays. Although, one is not ignorant of the need for expensive instrumentation, well trained personnel, the laboratory equipment requirement, however, the equipped laboratory can also be used for other applications including research. Now that the therapy has become widely available, affordable in-house genotyping for monitoring of patients on ARVs can be adopted and if properly utilized will avoid emergence of drug resistance in the population of HIV-1 strains.

5. Conclusion

HIV-1 was heterogeneously distributed; CRF02_AG and G predominate and some known major mutations associated with NRTIs, NNRTIs, diverse accessory, and natural polymorphism in the pol gene including one major mutation

to PIs(Q58E) were determined. The In-house HIV-1 genotyping assay is suitable for both characterization of non-B HIV-1 subtypes and detection of drug resistance at a significant lower cost than available commercial genotyping assays. This finding underscores the need to consider use of low-cost In-house genotyping assay as an alternative in resource-limited settings with non-B HIV-1 epidemic, and if well utilized, has potential to guide treatment options thereby keep the emergence of viral resistant strains at an acceptable level. In Nigeria, the burden of costs for treating HIV-infection is high, not only for the average citizens especially with the introduction of some laboratory investigation fees, but also the country's healthcare systems. Therefore the low cost and yet effective genotyping assay for non-B subtypes is a viable and practicable solution to expensive genotyping platforms.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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