学位論文

Doctoral Thesis

Circulating pre-treatment and acquired HIV drug resistance Mutations in Dar es Salaam, Tanzania

（タンザニアで流行する薬剤耐性 HIV-1 変異に関する研究）

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1. ABSTRACT

Objectives

We investigated the prevalence and patterns of pre-treatment and acquired HIV-drug resistance mutations (DRM) in Tanzania as "treat all" strategy, virological monitoring and progressive increase in usage of tenofovir are being implemented in HIV treatment program.

Methods

Viral RNAs were isolated from plasma of 60 antiretroviral therapy (ART)-naïve and 166 treated but viremic (>400 copies/ml) HIV-1-infected adults attending care and treatment clinic at Muhimbili national hospital, Dar es Salaam, Tanzania, between June and October 2017. Viral genes encoding protease and reverse transcriptase and integrase were PCR amplified and directly sequenced.

Results

Viral genotyping of successfully amplified samples revealed pre-treatment DRM in 14/47 (29.8%) of ART-naïve subjects. Of these, 7/47 (14.9%) harboured mutations that confer high-level resistance to at least one drug of the default first-line regimen. In treated but viremic subjects, DRM were found in 100/111 (90%), where, DRM against NNRTI, NRTI and PI were observed in 95/100 (95%), 92/100 (92%) and 13/100 (13%), respectively. Tenofovir-resistance mutations K65R, K70G/E or ≥3 thymidine analogue resistance mutations including M41L and L210W were found in 18/36 (50%) of subjects on tenofovir containing regimen at failure. Four patients harboured multiple DRM, which can confer resistance to all available ART regimens in Tanzania. In contrast we did not detect any major integrase resistance mutation, accessory resistance mutations ware present in 8/158 (5.1%) of all integrase sequences.

Conclusions

Taken together, pre-treatment and acquired DRM were highly prevalent which represented a major risk for the efficacy of ART program in Tanzania. Availability of newer generation of antiretroviral drugs with higher genetic barrier to resistance and robust treatment monitoring is warranted for effective and sustainable HIV treatment.

2. PUBLICATION LIST

I. Reference publication

Godfrey Barabona, Macdonald Mahiti, Salim Masoud, Peter Mbelele, Amina Shaban Mgunya, Lilian Minja, Bruno Sunguya, Urara Shigemi, Masakazu Matsuda, Atsuko Hachiya, Yasumasa Iwatani, Eligius Lyamuya, Takamasa Ueno, Pre-treatment and acquired HIV drug resistance in Dar es Salaam, Tanzania in the era of tenofovir and routine viral load monitoring, *Journal of Antimicrobial Chemotherapy*, Volume 74, Issue 10, October 2019

II. Other Publication

Salim Masoud, Doreen Kamori, **Godfrey Barabona**, Macdonald Mahiti, Bruno Sunguya, Eligius Lyamuya, Takamasa Ueno Circulating HIV1 integrase genotypes in Tanzania: Implication on the introduction of Integrase Inhibitors ‑ based ART regimen. *AIDS Research and Human Retroviruses.* March 2020

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4. ABBREVIATION

AIDS	Acquired immunodeficiency syndrome
ART	Anti-retroviral therapy
CCR5	C-C chemokine receptor -5
CD4	Cluster of deferentiation-4
CTC	Care and treatment clinic
CRF	Circulating recombinant form
DNA	Deoxyribonucleic acid
DRM	Drug resistance mutation
GSS	Genotypic susceptibility score
HIV	Human Immunodeficiency Virus
HLA	Human Leukocyte Antigen
IQR	Interquartile range
INSTI	Integrase strand transfer inhibitor
MNH	Muhimbili National Hospital
MUHAS	Muhimbili University of Health and Allied Sciences
NIMR	National Institute for Medical Research
NNRTI	Non-nucleoside reverse transcriptase inhibitors
NRTI	Nucleos(t)ide reverse transcriptase inhibitors
PCR	Polymerase Chain Reaction
PI	Protease inhibitors
PLHIV	People living with HIV
PR	Protease

RNA	Ribose Nucleic acid
RT	Reverse transcriptase
TAM	Thymidine analogue mutation
THIS	Tanzania HIV impact survey
WHO	World Health Organization

5. BACKGROUNG AND AIM

5.1 HIV-1/ AIDS epidemic

The first documented illness that was linked to HIV infection appeared in early 1980's in the United States. From early on it was established that an infectious agent was causing a form of secondary immune deficiency (AIDS) where, sexual contact and use of drugs was identified as risk of acquiring the infection.[1] Since then over 75 million people have been infected with the virus and about 32 million have succumbed due to HIV infection. At the end of 2018, about 37.9 million (32.7–44.0 million) people were living with HIV. The distribution of the burden of HIV infections shows a marked variation across geographical regions, where, sub Saharan Africa (SSA) is most severely affected with over two third of global population living HIV. Similarly, some subpopulation are most severely affects than the other including men who have sex with men, female sex workers and intravenous drug users.[2]

5.2 HIV epidemic in Tanzania

Tanzania is among top 15 countries in the world with high number of people living with HIV. The first official case was reported in 1983 and by 1987 all regions in the country had reported at least one case of HIV infection.[3] By year 2003/2004, national HIV prevalence was 7.2% and since then there have been steady but slow decline of the severity of the epidemic due to medical and community interventions that were put in place. By 2017, HIV prevalence was 4.6% among adult of 15-49 years equivalent to 1.4 million people living with HIV. On the other hand

HIV prevalence among children aged 0-14 years who predominantly acquired the infection during perinatal period was 0.4%.[3] Heterosexual contact accounts for the majority of new infection in Tanzania where it is estimated that 72,000 individuals acquires HIV every year. Women are disproportionately affected compared to men where in 2017, women comprised of about 58.7% (880,000) people living with HIV (PLHIV) aged above 15 years in Tanzania. Wide inter regional variation of severity of HIV epidemic exist, with some regional prevalence of over 10% versus less than 1% in other regions. (Figure1)

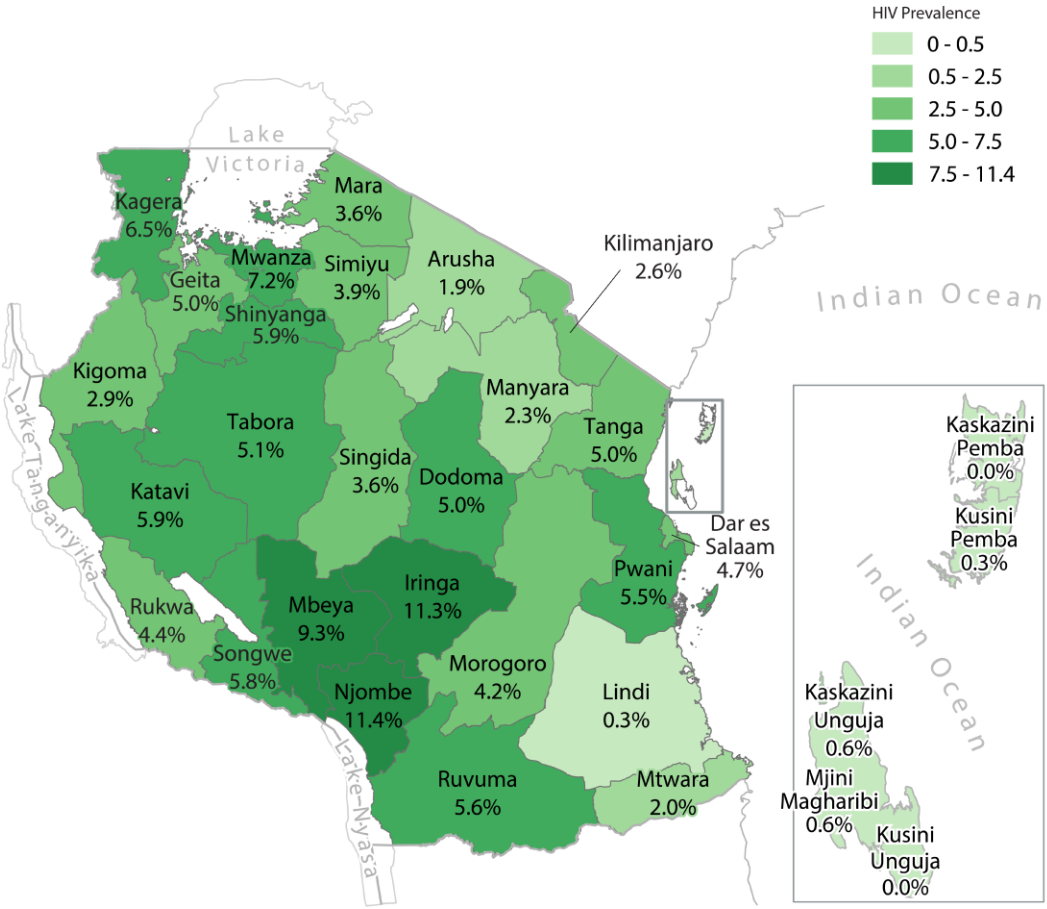

Figure 1. ***HIV prevalence among adults aged 15 years and older, by region.*** *Figure adapted from the Tanzania HIV impact survey (THIS) report 2016-2017*[3]

5.3 Biology of Human Immunodeficiency Virus.

HIV is an enveloped virus with single stranded pair of positive sense RNA genome that specifically infect human and compromising the immune system leading to development of AIDS.[45] HIV belongs to the group of primate lentiviruses that evolved from several transfers of nonhuman primate immunodeficiency viruses including chimpanzees, gorillas and sooty mangabeys into human.[6] Within the host, HIV primary target are cells expressing CD4 molecules on their surfaces in which the virus utilizes the molecule as a receptor for entry into the cell.

The virus replication cycle involves receptor-mediated fusion of viral envelope followed by delivery of the core into the cytoplasm. Reverse transcription of viral genome by the viral RNA dependent DNA polymerase (reverse transcriptase enzyme) is followed by integration of the provirus to the host cell genome within few hours of infection. Transcription and then translation of viral protein from the integrated provirus usually follows. Assembly of viral protein and precursor protein into viral particles is then followed by budding off the cell membrane the process of which gives the immature viral particle an envelope. (Figure 2) Further maturation to infectious viral particle continues after budding of the virus by cleavage of polyprotein precursors by HIV protease enzymes.[7]

HIV replication is responsible for the cytopathic effects seen in infected cells, contributing to declining of CD4+ T cells population during HIV infection.[8] The HIV genome also codes for accessory proteins that are essential in evading various host viral restriction factors and immune surveillance.[910] In addition the error prone reverse transcriptase enzyme allow for rapid evolution of the virus to adapt the new host environment and thus sustain the infection.[11] In addition, the ability of the virus to establish latent infection in the form of non-replicative integrated proviruses

that can spontaneously reactivate makes HIV infection very difficult to clear once established in a host.[12]

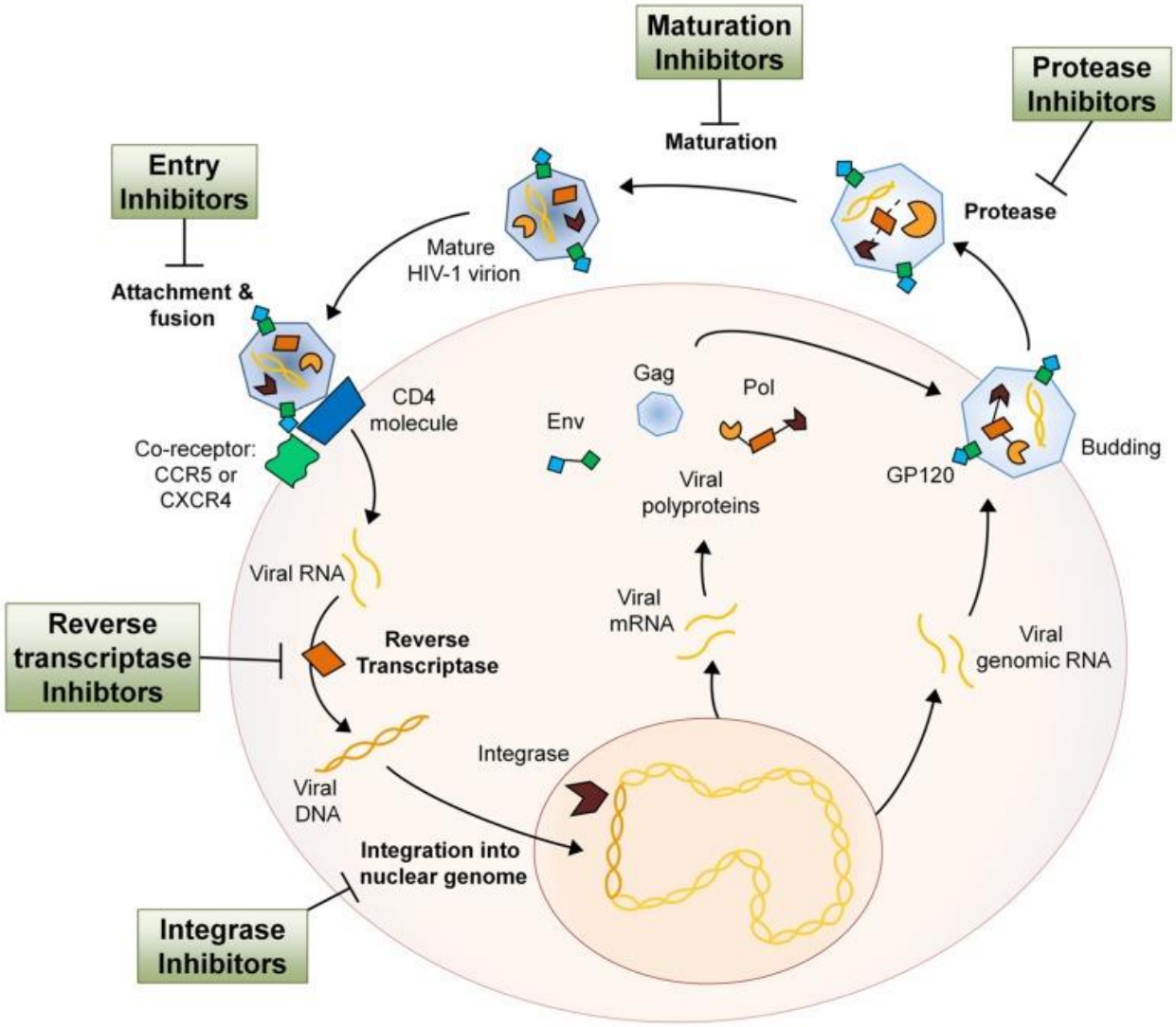

Figure 2. ***Life cycle of HIV and targets steps of antiretroviral drugs.*** *Figure adapted from the Reuben et al frontier in genetics 2013*[7].

5.4 Antiretroviral treatment

Antiretroviral therapy can inhibit HIV replication by targeting crucial steps during viral replication. (Figure 2) In clinical settings antiretroviral treatment lead to undetectable levels of plasma viremia, immunological recovery and thus, halt progression to AIDS.[13,14] Treatment of HIV infected individuals has led to a significant decline in incidence of AIDS and related mortality worldwide. In addition, use of ART have impact in reduction of transmission of HIV because low-level viremia lowers the risk of transmission from individuals who are on ART. Recently the prophylactic benefits of Antiretroviral has been realized and are now used for prevention of acquisition of HIV before exposure (pre exposure prophylaxis) and after exposure (post exposure prophylaxis).

To date, over 28 antiretroviral drugs have been approved for use in HIV treatment that include fusion inhibitors, entry inhibitors, reverse transcriptase inhibitors (nucleos(t)ide and non-nucleoside reverse transcriptase inhibitors), integrase inhibitors and protease inhibitors.[15] enfuvirtide is the only approved fusion inhibitor. It works by mimicking the heptad repeat region of HIV envelope gp 41 subunit and prevent formation of post fusion structure during HIV entry.[16] Maraviroc is an entry inhibitor, which works as a CCR5 antagonist that bind to the hydrophobic pockets of CCR5 and stabilizes its conformation making it unrecognizable by HIV envelope.[17] Nicleos(t)ide reverse transcriptase inhibitors (NRTI) causes chain termination in the growing DNA during RNA dependent or DNA depended DNA synthesis by the viral reverse transcriptase enzyme due to their lack of 3'-hydroxil group on their sugar moiety. NRTI were the first drugs to be approved for HIV treatment and currently there are nine approved NRTIs: namely; abacavir, didanosine, emtricitabine, lamivudine, stavudine, zalcitabine, zidovudine, tenofovir disoprovil fumarate and tenofovir alafenamide.[15] In contrast to NRTI, non-nucleoside reverse transcriptase

(NNRTI) inhibit the action of HIV reverse transcriptase by binding to a hydrophobic pocket proximal to the active site which induces conformation changes that affect negatively the binding of substrate.[18] Four NNRTIs have been approved namely etravirine, delavirdine, efavirenz, and nevirapine.[15] Integrase inhibitors target the strand transfer process by specifically binding to complex between integrase and viral DNA and interact with both DNA and the two essential Mg^{2+} cofactors in the integrase active site.[19] Integrase inhibitors are relatively new antiretroviral class with three drugs currently approved; raltegravir, dolutegravir and elvitegravir. Protease inhibitors are peptide analogs that specifically bind within the active site of HIV protease, and thus prevent the active site from acting on the long precursor HIV protein produced during viral infection[20] Over ten protease inhibitors have been approved; amprenavir, atazanavir, darunavir, fosamprenavir, indinavir, lopinavir, nelfinavir, ritonavir , saquinavir and tipranavir.[15]

5.6 HIV Drug resistance

HIV drug resistance is caused by mutation(s) in the genetic structure of HIV that affects the ability of a specific drug or combination of drugs to block replication of the virus. Mutations arise from error prone HIV reverse transcriptase enzyme that can introduce a mutation in every 10^3 to 10^4 bases synthesized.[11] This ability of HIV to rapidly generate genetic diversity is responsible for emergence of drug resistance variants within short time. As the result, early approaches to treat HIV infection using a single antiretroviral drug did not lead to substantially suppression of viral replication. Currently a combination of at least three drugs is used to produce a durable inhibition of HIV replication.

HIV have been demonstrated to acquire drug resistance mutations (DRM) to all current antiretroviral drugs leading to partly or fully inactive drugs. Mechanism of drug resistance vary

between drug class as well as mutations involved. Even though cross-resistance between drugs is common, selection of DRM, tend to be drug specific. In NRTI resistance, two mechanisms are involved; 1) ATP-dependent pyrophosporolysis and 2) increased discrimination between the native deoxyribonucleotide substrate and the drug. Thymidine analogue mutations (TAMs); M41L, D67N, K70R L210W, T215T/F and 219E/Q promote pyrophosphorolysis and are involved in excision of zidovudine and stavudine. On the other hand, K65R and M184V/I mediates preferential selection of native deoxyribonucleotide over the drug.[21–23] NNRTI resistance mutations occurs in the NNRTI binding pocket and hence prevent the drug from binding to the reverse transcriptase enzyme. This include substitution in amino acid position L100, K101, K103, E138, V179, Y181, and Y188.[21–23] Mutations that mediates integrase resistance usually occur in the integrase active site near the amino acid residue that coordinate the magnesium cofactor. Selection of integrase inhibitors resistance mutation usually follow a specific pathway involving substitution at Y143, N155 or Q148 followed by secondary mutations including L74M, E92Q, T97A, E138K, G140S/A, V151L and G163R.[21,23,24] Over 20 substitution are known to confer resistance to protease inhibitors, these mutations occurs near the active site of the enzyme at positions located at the substrate/inhibitor binding site. They include substitution at D30, V32, L33, M46, I47, G48, I50, I54, L76, V82, I84, N88 and L90.[2123]

5.7 Epidemiology of HIV drug resistance.

WHO commonly classify HIV DRM into three main categories; 1) resistance that develop due to viral replication in presence of ARV drugs - acquired HIV drug resistance 2) resistance due to infection with resistance virus to uninfected individuals - transmitted HIV drug resistance 3)

resistance detected in individuals initiating or reinitiating treatment either acquired or transmitted –pretreatment drug resistance.[25] In low and middle-income countries, HIV treatment follows a public health approach and routine HIV drug resistance test prior to treatment initiation is generally not performed.[26] In these countries, pretreatment resistance to first-line regimen can pause a major risk in effectiveness of ART programs. Prevalence of pre-treatment HIV drug resistance has been increasing as the global access to ART expanded in the recent years. In recent WHO standard surveys conducted in 18 low and middle-income countries, prevalence of pre-treatment resistance to efavirenz and niverapine (drug of choice for first line regimen) was exceeding 10% in 12 out of 18 countries. Further, prevalence was as twice in women compared to men and exposure to ARV drugs prior to treatment initiation was common, ranging from 1.2% to 26.3%.[25] Due to high levels of NNRTIs resistance, WHO is currently recommending integrase inhibitor-dolutegravir that has a higher genetic barrier to resistance to substitute NNRTIs in first-line regimen in countries where pretreatment resistance exceed 10%.[25]

High prevalence of acquired HIV drug resistance in patients with viral non-suppression implicate the role of drug resistance in treatment response. In middle and lower income countries, 60-90% of PLHIV failing treatment harbours drug resistance mutations against routinely used NNRTIs and NRTIs.[27] On the other hand, resistance to protease inhibitors is relatively uncommon. Pattern of DRM selection show some variation between geographical regions however in countries where HIV treatment follow public health approach, substitution K103NS that confer high-level resistance to efavirenz and niverapine tend to be the most frequently selected NNRTI DRM. On the other hand substitution M184VI selected by lamivudine and emtricibine tend to be the most frequently selected for NRTI DRM.[2528] Tenofovir has increasingly been used as NRTI backbone for first and second line regimen in low and middle-income countries replacing didanosine and

stavidune due to its better virological, pharmacological and toxicity profile.[29] Since its use tenofovir resistance has been reported to be over 50% in patients failing tenofovir based first line regimen in sub-Saharan Africa and between 20-40% in North America, Asia and Europe.[28] Even though a combination of TAM can confer several fold resistance to tenofovir, substitution K65R and K70EG are preferentially selected and constitute the important cause of resistance to tenofovir worldwide.[28]

Left unchecked, HIV drug resistance could hamper the achieved success in the fight against HIV. For instance, pretreatment resistance to NNRTI in sub Saharan Africa is estimated to results to additional 135,000 AIDS related death and 105,000 new infections plus additional cost of 650 million dollar between 2017 and 2022 if NNRTI are to continue to be used when resistance exceed 10%.[30] Thus HIV drug resistance is one of the priority in the fight against HIV.

5.8 HIV drug resistance in Tanzania

In Tanzania, like in most SSA countries, national wide ART program employs NNRTI-based first-line and Protease inhibitor (PI)-based second-line regimen in treatment of adult PLHIV.[31,32] By 2016, about two thirds of 1.3 million adult PLHIV in Tanzania were on ART treatment;[33] viral suppression was achieved in 84% and 89.2% of males and females, respectively,[34] falling short of the 90% UNAIDS viral suppression goal. It is estimated that 13% to 19% of HIV, infected individuals initiated to ART will fail treatment in the first three years of first line regimen in Tanzania.[35,36] Typically, at failure on non-nucleoside reverse transcriptase inhibitors (NNRTI) based first-line regimen, most of individuals display resistance genotype to both NRTI and NNRTIs. In studies conducted in Tanzania from samples collected between 2006 and 2013

revealed that 33% to 86% and 50% to 86% of individual failing first line regimen, harbored genotypic resistance to at least one NRTI and NNRTI respectively.[36–39]

In the recent 5 years, Tanzania have been implementing to its national ART program, WHO recommendations that involved; 1) use tenofovir-based default first-line regimen, 2) treatment of all newly HIV-diagnosed individuals regardless of CD4+ cell count, and 3) use of routine viral load test for monitoring treatment.[31,40] These changes intend to expand access and exploit the benefits of early initiation of ART.[41,42] In addition, viral load monitoring ensures early detection of treatment failure and thus, prompt switching of regimen to avoid the consequences of delayed switch that includes accumulation of HIV DRM.

5.9 Aim of the study

Pre-treatment and acquired DRM could pose a barrier towards achieving the viral suppression goal. Thus understanding the prevalence and patterns of circulating drug resistance is crucial to inform ART programs. However, data from Tanzania are limited and available ones, involve studies that based their analysis on samples collected between 2003 and 2013, which effectively leaves a data gap on the current drug resistance state. Importantly, since 2013, Tanzania has been adopting major changes in HIV treatment program ("treat all strategy", expand use of tenofovir and routine viral load monitoring) which could potentially have an impact in pattern and prevalence of circulating DRM. In addition, since the programmatic introduction of tenofovir in 2009, substitution of thymidine analogues with tenofovir is common, a practice that is becoming increasingly associated with acquisition of resistance to both drugs in SSA countries.[43] The magnitude of tenofovir resistance and how it is influenced by thymidine analogue exposure in Tanzanian setting is largely uncharacterized.

Moreover, pre-treatment DRM is expected to become increasingly prevalent in SSA as the "treat all" strategy among other factors, is being implemented in this region.[44] Therefore, current data for pre-treatment DRM prevalence and patterns well as predicted efficacy to the currently WHO recommended first-line regimen, tenofovir + emtricitabine + efavirenz,[31] in Tanzania is vital. To address this data gap, we sought to determine currently circulating HIV DRM and predict phenotypic resistance profiles, by genetically analysing plasma viral RNA that were recently obtained from ART naïve and viremic subjects failing first or second-line regimen in Dar es Salaam, Tanzania.

6. MATERIALS AND METHODS

6.1 Study participants and Setting

Participants for this cross-sectional study were recruited between June 2017 and October 2017 at Care and Treatment Clinic (CTC) in Muhimbili National Hospital in Dar es Salaam, Tanzania. HIV-infected adults who were viremic (plasma viral load >400 copies/ml within 3 months of sample collection) on first or second-line regimen for more than one year were enrolled to the study during their routine visit to the CTC. Also, recruited to the study are ART-naïve individuals as they enrolled to CTC for treatment initiation during the study period. At the end of the study period, 226 consenting subjects were enrolled. Participants' demographics and clinical information were retrieved from clients' medical records and through interviews.

Ethical approval was obtained from Muhimbili University of health and Allied Sciences Senate Research and Publications Committee, National Institute for Medical Research (NIMR) and Muhimbili National Hospital administration in Tanzania. Written informed consent was obtained from each study participant. Genotypic drug resistance profile of successfully genotyped subjects was made available to the clinicians for treatment decisions according to the Tanzania HIV treatment guideline.

6.2 Sample collection and viral genotype analyses

Whole blood was collected from each study participant in EDTA coated tubes. Plasma separation by centrifugation was performed within 2 hours of sample collection and then

immediately stored at -80°C. Portions of plasma samples from each subject were transported to Kumamoto University, Japan for viral genotype analyses.

Viral RNA was extracted from 140 μL of plasma using QIAamp Viral RNA Mini kit, (QIAGEN) as per manufacturer's protocol. Up to 5 μL of eluted RNA was used in one-step RT-PCR to amplify HIV-1 *pol* gene. Two microliters of first PCR products was used for nested PCR. About 1017 bp of *pol* gene, encompassing protease (PR) and a part of reverse transcriptase (RT)-encoding region, (Table 1) and 864bp encompassing *integrase* region was sequenced using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA). (Figure 1) Automated sequencer (3500/3500XL genetic analyser; Applied Biosystems) was employed for sequence determination. (Figure 3) Sequence assembly was performed in Seqscape software version 2.7, and each sequence was manually scrutinized to ensure sequence quality. HIV subtype assignment was performed using phylogenetic analysis (MEGA v.6.0) and REGA HIV subtyping tool v.2.0 (http://www.bioafrica.net/subtypetool/html/subtypinghiv.html). Unassigned sequences to a defined subtype were regarded as recombinants.

6.3 Assignment of Drug resistance mutations

Presence of DRM was assessed in the viral gene encoding PR and the first 240 codons of RT using the Stanford University HIV Drug Resistance Database, HIVdb Program version 8.6.1 (https://hivdb.stanford.edu/hivdb). Each subjects' protease and RT sequences was used for prediction of susceptibility to antiretroviral drugs by REGA algorithm v10.0.0 (https://rega.kuleuven.be/cev/avd/software/). Genotypic susceptibility score (GSS) was defined as 1, 0.5, and 0 for fully susceptible, intermediate resistant and resistant, respectively, to NNRTI and

NRTI; and GSS of 1.5, 0.75 and 0 was defined as fully susceptible and intermediate resistant, and resistant, respectively, to PI.

Table 1. Primer selection for HIV drug resistance genotyping.

Amplification primers

	Primer name	Direction	Position	Sequence
pol	K	F	2020-2039	AAGGGCTGTTGGAAATGTGG
	DRIN02	R	5243-5264	CCTGTATGCAGACCCCAATATG
PR-RT	K4	F	2039-2059	GAAAGGAAGGACACCAAATGA
	U12	R	3599- 3622	CTCATTCTTGCATATTTTCCTGTT
IN	DRIN05	F	4146-4168	CTGGCATGGGTACCAGCACACAA
	DRIN04	R	5195-5217	TAGTGGGATGTGTACTTCTGAAC

Sequencing primers

	Primer name	Direction	Position	Sequence
PR-RT	T	F	2664-2683	ACAGAAATGGAAAAGGAAGG
	A2	F	2583-2601	TTAAAGCCAGGAATGGATG
	A3	F	2929-2947	ATACTGCATTTACCATACC
	PRO4	F	2260-2279	TCACTCTTTGGCAACGACCC
	U12	R	3600-3622	CTCATTCTTGCATATTTTCCTGTT
	L	R	3000-3017	TGATCCTTTCCATCCCTG
	F	R	2703-2723	AGTATTGTATGGATTTTCAGGC
	DRPRO4(B3)	R	2572-2592	CTGGCTTTAATTTTACTGGTA
IN	DRIN07	F	4150-4170	CATGGGTACCAGCACACAAAG
	DRIN11	F	4377-4396	ATGCATGGACAAGTAGACTG
	DRIN08	R	5192-5214	TGGGATGTGTACTTCTGAACTTA
	DRIN14	R	4750-4770	TGAATACTGCCATTTGTACTG

IN, Integrase; PR_RT, protease-reverse transcriptase: F, forward: R, reverse

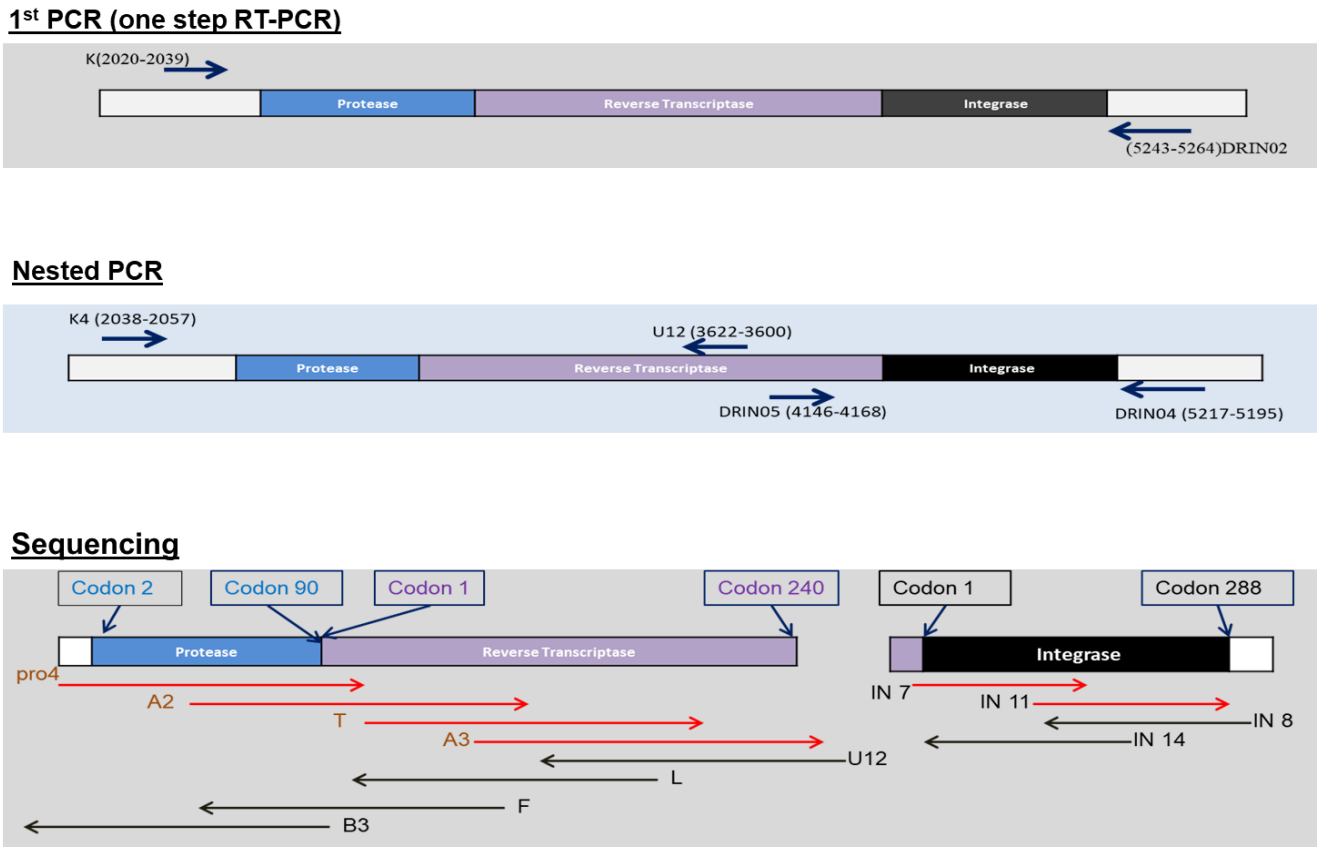

Figure 3. ***Drug resistance genotyping approach.*** *Relative primer positions for nested amplification system and sequencing are indicated. Protease and part of reverse transcriptase region was sequenced separately with integrase region for each sample.*

6.4 Statistical analysis

The nonparametric Kruskal-Wallis test was used to compare median age and viral loads between three treatment groups. For comparison of treatment duration between the groups, Mann Whitney U test was employed. To assess variation in the distribution of marital status and HIV subtypes between different treatment groups, χ^2 test was employed. A P value of < 0.05 was considered significant. All statistical analyses were performed using GraphPad Prism v.6.0b (GraphPad Software, La Jolla, CA)

7. RESULTS

7.1 Subjects characteristics

During the study period, 166 treated but viremic subjects and 60 ART-naïve subjects were enrolled (Table 1). Of 166 treated viremic subjects, 136 and 30 subjects were on the first-line regimen and the PI-containing second-line regimen, respectively, during sample collection. In first-line regimen, tenofovir was more commonly co-administered in combination with lamivudine (36/55; 65.5%) compared to emtricitabine (19/55; 34.5%) (P = 0.0215), even though the former combination is reported to be associated with higher prevalence of tenofovir resistance than the latter. [45] This is because the Tanzania's National HIV treatment guideline, which recommended tenofovir + lamividune + efavirenz as the default first-line regimen. In the case of the second-line regimen, combination of tenofovir and emtricitabine plus either of PI (ritonavir boosted lopinavir or atazanavir) was the commonest (Table 1).

HIV genotyping was successful in 47/60 (78.3%) ART-naïve and 111/166 (66.9%) treated but viremic subjects. Amplification failure was at least in part due to insufficient viral RNA in the reaction because samples whose HIV RNA failed to be amplified had significantly lower viral load (median 3.67 log copies/ml) compared to those successfully amplified (median 4.59 log copies/ml) (P < 0.0001). HIV subtypes were A (41.8%), C (33.5%), D (8.9%) and recombinant forms between A, C, D and G (15.8%). Only two out of 25 recombinant forms were linked to the known circulating recombinant forms (*i.e.*, CRF 02_AG and 10_CD). We found no significant difference in HIV-1 subtype distribution between ART naïve and treated but viremic groups (P =0.75) (Table 2).

Table 2. Demographic and clinical characteristics of recruited subjects

	ART naïve (n=60)	Treated but viremic		P value
		On first-line regimens (n=136)	On second-line regimens (n=30)	
Demographic data				
Median age in years (IQR)	38.5 (32- 48)	43.5 (32-52)	45 (30-50)	0.2628[a]
Gender -female (%)	40 (66.7)	90 (66.2)	18 (60)	0.7926[b]
Marital Status (%)				
Single	9 (15)	28 (20.6)	8 (26.7)	
Married/cohabiting	31 (51.7)	58 (42.6)	10 (33.3)	0.5008[b]
Separated/Divorced/Widowed	20 (33.3)	50 (36.8)	12 (40)	
Clinical data				
Median Years on treatment (IQR)	NA	7.5 (4-10) (20 missing data)	6.5 (3.8-11) (4 missing data)	0.773[c]
Median viral load, log copies/ml (IQR)	5.15 (4.66-5.66)	4.42 (3.67-4.99)	4.06 (3.16-4.47)	<0.0001[a]
Current ART regimen (%)				
First-line regimen				
AZT + 3TC +EFV		27 (19.6)		
AZT + 3TC +NVP		44 (32.4)		
TDF + FTC +EFV		19 (14)		
TDF + 3TC +EFV		36 (26.5)		
ABC + 3TC+ EFV		4 (2.9)		
Missing data		5 (3.6)		
Second-line regimen				

TDF+ FTC + LPV/r			6 (20)	
ABC+ 3TC+ ATV/r			4 (13.3)	
ABC+3TC + LPV/r			4 (13.3)	
TDF + FTC+ATV/r			11 (36.7)	
TDF + 3TC + ATV/r			1 (3.3)	
AZT+ 3TC + ATV/r			4 (13.3)	
Genotyping data				
HIV subtype (%)				
Analyzed sequences	n=47	n= 89	n =22	
A	16 (34)	42 (47.2)	8 (36.4)	
C	19 (40.4)	27 (30.3)	7 (31.8)	0.7509[b]
D	5 (10.6)	7 (7.9)	2 (9.1)	
Recombinants between A,C,D and G	7 (14.9)	13 (14.6)	5 (22.7)	

[a] Kruskal–Wallis test by rank; [b] χ^2 test; [c] Mann–Whitney U test

NA, not applicable; IQR, interquartile range; ATV/r, atazanavir boosted with ritonavir; LPV/r, lopinavir boosted with ritonavir; EFV, efavirenz; NVP, niverapine; TDF, tenofovir; 3TC, lamivudine; FTC, emtricitabine; AZT, zidovudine; ABC, abacavir; ART, antiretroviral therapy

7.2 Pre-treatment drug resistance mutations

We first determined the burden of pre-treatment DRM in 47 sequences from 60 subjects who presented to care for ART initiation during the study period and then predicted the antiviral susceptibility to WHO recommended first-line regimen (tenofovir+lamivudine+efavirenz). Pre-treatment DRM was detected in 14/47 (29.8%) subjects; where, 12/47 (25.6%) subjects had NNRTI resistance mutations and 4/47 (8.5%) subjects had at least one NRTI mutation whereas no subject had PI-resistance mutation (Table 3). K103N, G190A and/or V106M that confer high-level resistance to NNRTI, efavirenz and niverapine, were observed in 7/47 (14.9%) subjects rendering

the recommended first-line regimen only partially active (GSS < 3) in these subjects. In particular, we wondered that the sequences from one subject (NV-053) had ≥5 NNRTI and NRTI DRM, where all three drugs of first-line regimen were predicted to be resistant/intermediate resistant (GSS <1). This subject exhibited K70E and M184V (Table 3), the mutations that are very rarely observed in combination in treatment-naïve subjects (<0.2% in a total of 65,026 sequences) in the Stanford HIV database, and therefore NV-053 may have an undisclosed previous ART exposure. In addition, E138A that confers resistance to a second generation NNRTI, rilpirivine, was identified in 4/47 (8.5%) subjects infected with subtype A, C and D. This mutation has been reported naturally occurring and overrepresented in individuals expressing *human leukocyte antigen (hla)-B*18*, which is prevalent in SSA including Tanzania.[46] However, the association between E138A and HLA allele was not tested here because of unavailability of the HLA type of the subjects. Taken together, these results suggest that, about 30% of PLHIV in Dar es Salaam who presented to care for treatment initiation harbour at least one DRM and nearly 15% were initiated to a partially active regimen.

Table 3. List of detected pre-treatment drug resistance mutations in 14 of the 47 antiretroviral naïve subjects

Subject ID	HIV Subtype	Viral load (log copies/mL)	PI resistance mutation	NRTI resistance mutations	NNRTI resistance mutations
NV-003	C	5.08	-	-	G190A
NV-004	C	4.39	-	-	G190A
NV-006	A1	5.16	-	-	K103N, E138A
NV-008	D	4.68	-	-	E138A
NV-018	AC	5.61	-	-	H221HY
NV-019	C	5.23	-	-	E138A
NV-021	C	5.43	-	-	K103N, V106M
NV-025	AC	5.85	-	D67G, K70R, T215I, K219E	K101E, K103N, Y181C, G190A
NV-026	A1	4.87	-	-	V179T
NV-027	C	6.21	-	-	K103KN
NV-046	A1	5.07	-	E44ED	-
NV-048	A1	4.72	-	-	-
NV-049	A1	5.67	-	-	E138A
NV-051	C	3.95	-	D67G	-
NV-053	C	5.48	-	K70E, M184V, K219R	K103N, P225H

"-" Denoted no DRM detected

Abbreviations: NRTI, nucleoside reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor

7.3 Drug resistance mutations in subject on first or second-line regimen experiencing viremia

We next analysed 111 sequences from treated viremic subjects failing first or second-line regimen. Subjects failing treatment (defined as >400 copies/ml) were identified through routine 12-monthly viral load testing for enrolment to this study. Median viral loads in subjects failing first and second-line regime were substantially high, with 4.42 (IQR: 3.67-4.99) and 4.06 log copies/mL (IQR: 3.16-4.47), respectively, but significantly lower compared to median viral load of ART naïve subjects, with 5.15 log copies/mL (IQR: 4.66-5.66) (P <0.0001) (Table 2). Sequencing analyses revealed high burden of drug resistance, where 100/111 (90%) of the sequences harboured at least one DRM. DRM against NNRTI, NRTI and PI were found in 95/100 (95%), 92/100 (92%) and 13/100 (13%), respectively (Figure 4). In contrast, DRM to any drug

class was not detected in only 11/111 (9.9%) viremic subjects' sequences, suggesting that DRM is a major factor of viremia in subjects failing treatment.

M184I/V, which is selected by lamivudine and emtricitabine, was the most common NRTI DRM, observed in 93/100 (93%) of subjects with at least one DRM. Lamivudine or emtricitabine was a part of treatment regimen in all of our study subjects. On the other hand, K103N/S in RT and M46I in PR were the commonest NNRTI and PI DRM observed in 59/100 (59%) and 7/100 (7%) of subjects, respectively (Figure 4). Of note, NNRTI DRM pattern involving K101E/P, Y181C/V and/or G190A was eventually observed in 9/100 (9%) subjects. When combined, these are known to become high level phenotypic resistance to a newer generation NNRTI etravirine,[47] which is proposed for third line regimen in Tanzania.

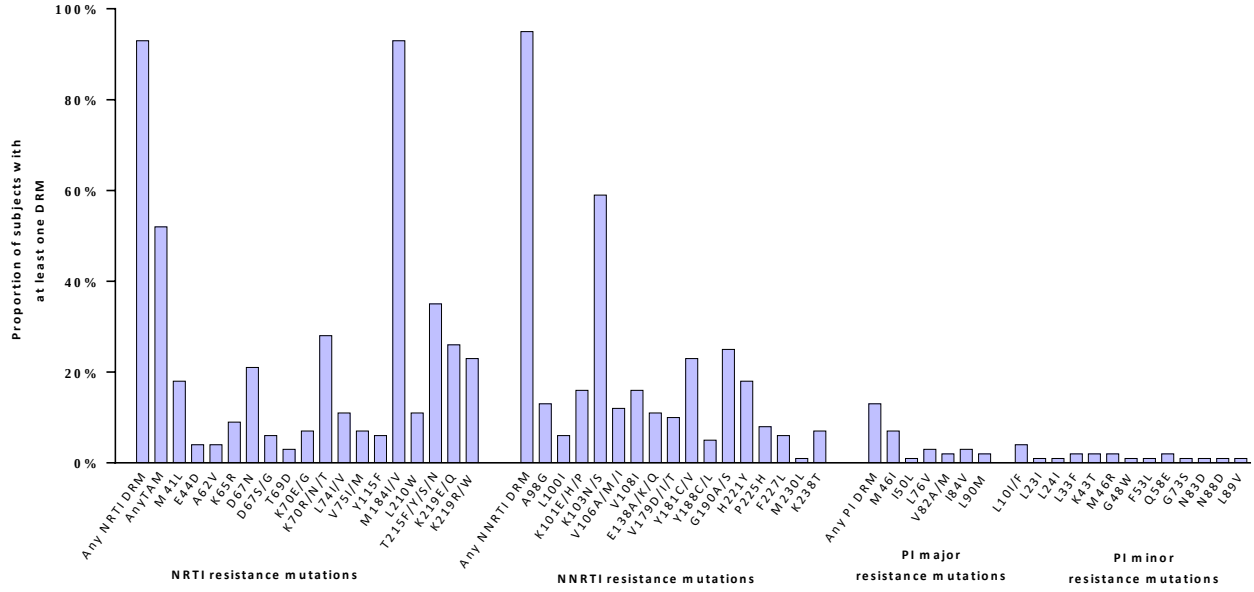

Figure 4. ***Distribution of Drug resistance mutations among 100 viremic subjects failing first or second-line regimen with at least one DRM***

Each bar represents frequency of DRM class and mutations on individual codons in 100 subjects with at least one DRM failing first or second-line regimen. Frequency of DRM occurring on same codon but attri

buted to phenotypic resistance to different drugs are represented in separate bars. Abbreviations; NRTI, nucleos(t)ide reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.

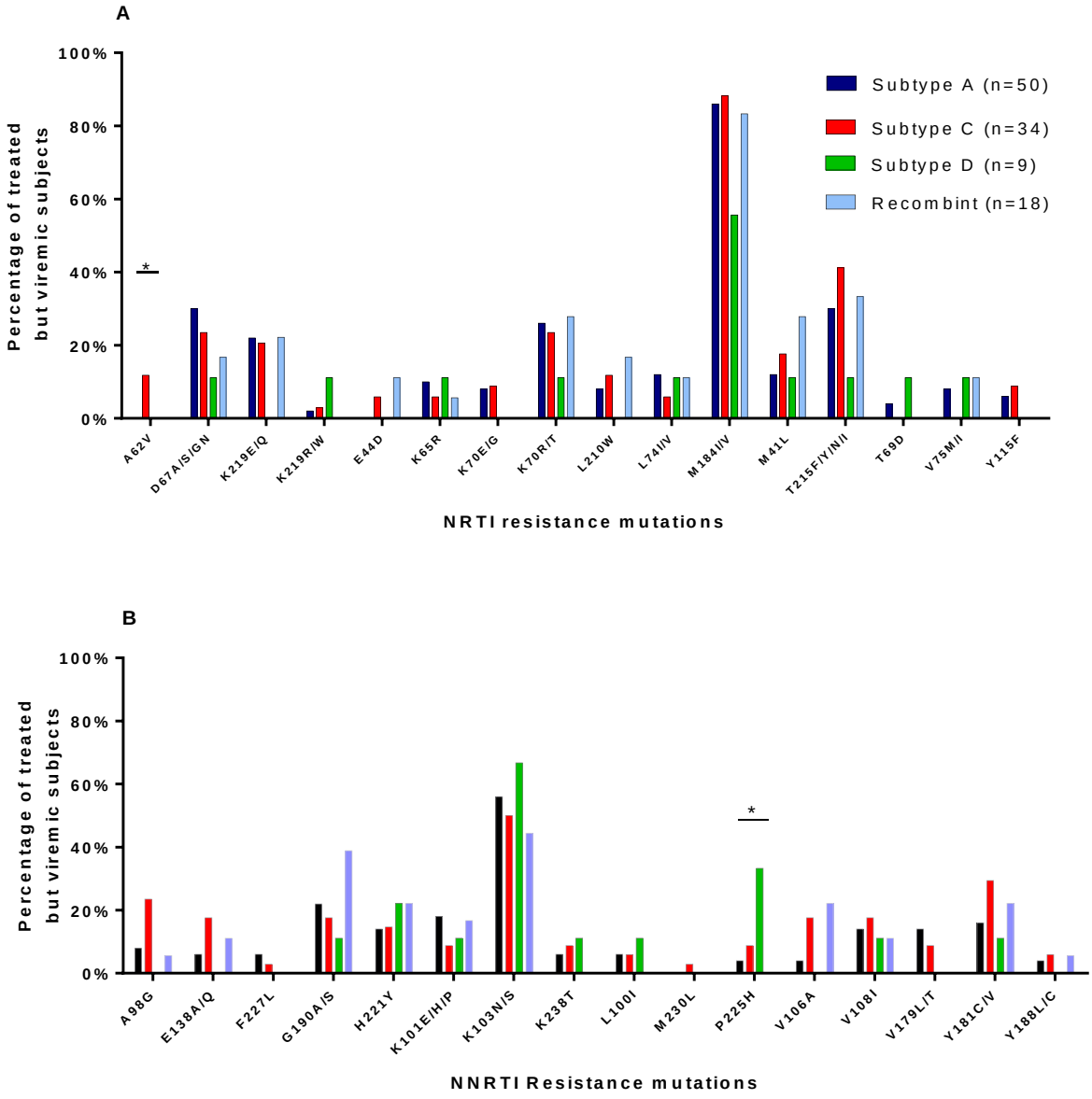

Figure 5. ***Distribution of Drug resistance mutations according to HIV subtype***

Each bar represents frequency of DRM on individual codons for HIV subtype in 100 subjects with at least one resistance mutation. a) NRTI resistance mutation b) NNRTI resistance mutation. HIV subtypes was determine using REGA HIV subtyping tool v.2.0. on protease–reverse transcriptase sequences.“”Mutation with significant differences in frequencies across HIV subtypes. Abbreviations; NRTI, nucleos(t)ide reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor.*

7.4 Selection of Drug resistance mutations according to HIV subtype

Influence of HIV subtype variation in favouring selection of specific DRM has been described before.[484950] However, in these studies, comparison is based on circulation HIV strains from different geographical location and thus effects of genetic variation due to geographical regions or host factors that may influence the findings cannot be ruled out. We thus analysed for differential selection of DRM across HIV subtypes exploiting the unique opportunity of co-circulation of multiple HIV subtype in Tanzania to better illuminate the influence of HIV subtype in DRM selection. In NRTI resistance mutation only A62V showed to be preferentially selected by HIV subtype A where five subtype A had this DRM and none form other subtypes.[50](Figure 5a) Interestingly, tenofovir resistance mutation K65R did not show preferential selection across subtype different from previous reports that HIV subtype C preferentially select this mutation.[51] NNRTI Substitution P225H was significantly more common in HIV subtype D while frequency of selection of other NNRTI DRM did not show statistical significant deference's across HIV subtypes circulating in Tanzania. (Figure 5b)

7.5 DRM in treated but viremic subjects on tenofovir containing regimen at failure

Since the introduction of tenofovir in Tanzania, a single drug substitution between a thymidine analogue and tenofovir occurs frequently without confirmation of viral suppression, thus risking acquisition of DRM against both drugs. To assess the impact of this, treated viremic subjects harbouring at least one DRM were stratified to tenofovir (36/100; 36%) and non-tenofovir-containing regimen at failure (64/100; 64%), and their DRM patterns were analysed (Figure 6). As expected, we observed significantly higher prevalence of tenofovir-associated DRM, K65R and K70E/G in tenofovir-containing regimen group (13/36; 36%) compared to non-

tenofovir containing regimen group (3/64; 4.9%) (P <0.001) (Figure 2). On the other hand, prevalence of subjects harbouring DRM against NNRTI and lamivudine/emtricitabine was comparable in both groups (both P >0.24). Interestingly, we observed high burden of TAM (18/36; 50%) in tenofovir containing regimen group despite not being on thymidine analogues at failure (Figure 6). This rate of TAM was comparable to that observed in subjects on non-tenofovir containing regimen at failure (32/64 50%) (*i.e.*, on zidovudine or abacavir containing regimen). Indeed, three or more TAM including M41L and L210W (henceforth denoted as $\geq 3TAM_{M41L/L210W}$), that are known to confer reduced susceptibility to tenofovir in combination,[52] were present in (5/36; 13.9%) among tenofovir containing regimen group. In contrast, such patterns of $\geq 3TAM_{M41L/L210W}$ were not observed in a multi-centre cohort study (n=712) spanning seven SSA countries involving treated but viremic subjects after first-line regimen consisting only of tenofovir + lamivudine/emtricitabine + NNRTI with no previously known exposure to additional NRTI.[43] Overall, in this study, prevalence of tenofovir-associated DRM (K65R, K70E/G and $\geq 3TAM_{M41L/L210W}$) reached to 18/36 (50%) among subjects on tenofovir containing regimen at failure (Figure 6), suggesting the epidemiological importance of TAM in tenofovir resistance in the setting where tenofovir has been used to replace thymidine analogues.

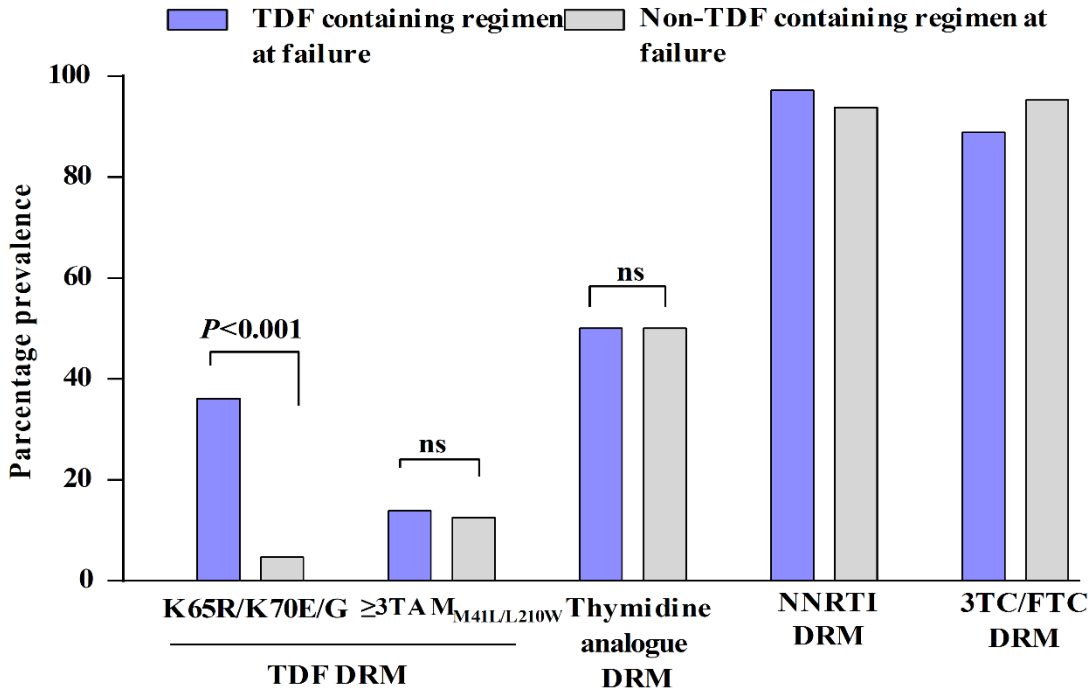

Figure 6. ***Distribution of selected DRM in subjects on tenofovir and non-tenofovir containing regimen at failure***

Pattern of DRM from 100 sequences (36 and 64 on tenofovir and non-tenofovir containing regimen at failure, respectively) in association with tenofovir and thymidine analogs is shown. Thymidine analogue mutation (TAM) is defined as M41L, D67N, K70R, L210W, T215F/Y and K219Q/E. Combinations of greater than 3 TAMs including M41L and L210W are known to confer cross-resistance to tenofovir and denoted as ≥3TAM$_{M41L/L210W}$. P value was obtained by Fisher's exact test. ns, not significant

7.6 Prediction of antiviral activity of first and second-line antiretroviral drugs available in Tanzania in treated but viremic subjects

We then sought to predict potential impact of accumulated DRM to the antiviral activity of the available antiretroviral drugs. Subjects' RT and protease sequences were interpreted using the REGA algorithm where each drug was classified as resistant, intermediate resistant or susceptible (refer Materials and Methods section). We found that 80/89 (90%) of viremic subjects on first-line regimen exhibited resistance to efavirenz and niverapine (GSS=0); and that 78/89 (87.6%) and 15/22 (68.2%) of subjects on first and second-line regimens exhibited resistance to emtricitabine and lamivudine (GSS=0), respectively (Figure 7). In contrast, all subjects on first-line regimen at failure were susceptible to PI (GSS=1.5) (Figure 7). Protease sequence also revealed that the majority of subjects on second-line regimen at failure (18/22; 82%) remained susceptible to PI (GSS =1.5), suggesting that they remained viremic due to reasons other than drug resistance. When we fixed our analysis to subjects on second-line regimen with triple class DRM (8/22, 36.4%), half of them were predicted to be sensitive to PI (GSS=1.5) while the other half were predicted to be resistant/intermediate resistant to all PI, NNRTI, and NRTI available in Tanzania, making it impossible to tailor a viable salvage regimen (GSS>2) for them. (Figure 8)

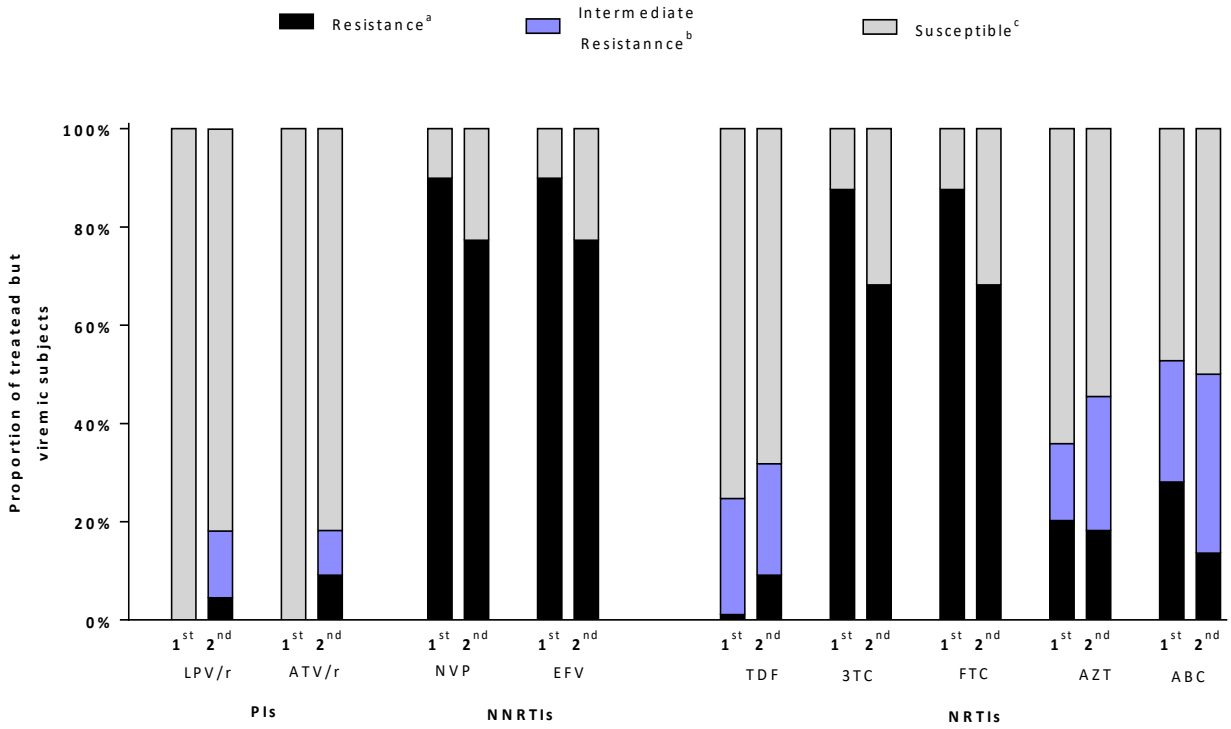

Figure 7 ***Prediction of susceptibility to antiretroviral drugs that are available in Tanzania in treated viremic subjects failing first and second-line regimen***

All 111 sequences (89 and 22 failing first and second-line regimen, respectively) were interpreted by REGA algorithm and scored based on the genotypic susceptibility score (GSS) as resistant (GSS=0), intermediate resistant (GSS=0.5/0.75) or susceptible (GSS=1/1.5) to each of the available drug in Tanzania. Each drug susceptibility pattern is represented by a stacked bar and separated for subject on first or second-line regimen. ATV/r, atazanavir boosted with ritonavir; LPV/r, lopinavir boosted with ritonavir; EFV, efavirenz; NVP, nevirapine; TDF, tenofovir; 3TC-lamivudine; FTC, emtricitabine; AZT, zidovudine; ABC, abacavir; NRTI, nucleos(t)ide reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor

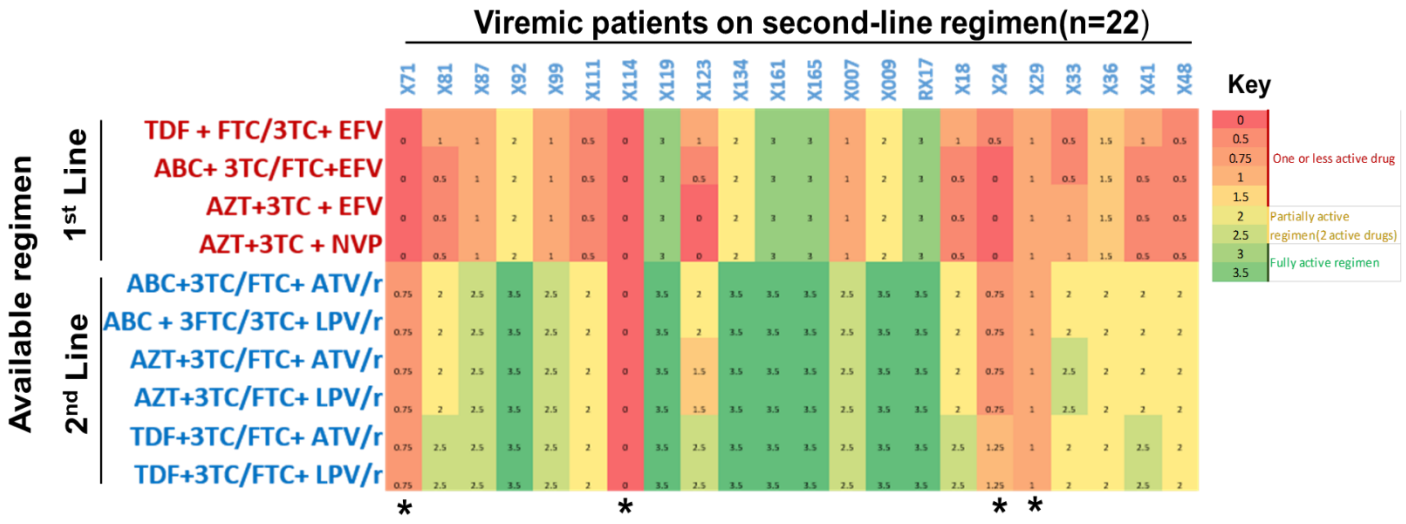

Figure 8. ***Prediction of susceptibility to first and second line regimen that are available in Tanzania in treated viremic subjects failing second-line regimen***

All 22 sequences of patient failing second line regimen were interpreted by REGA algorithm and scored based on the genotypic susceptibility score (GSS) as resistant (GSS=0), intermediate resistant (GSS=0.5/0.75) or susceptible (GSS=1/1.5) and the summation of individual drug score was used to predict antiviral activity of each regimen available in Tanzania. ' denote patients with low antiviral activity to all regimen available in Tanzania (GSS <2). ATV/r, atazanavir boosted with ritonavir; LPV/r, lopinavir boosted with ritonavir; EFV, efavirenz; NVP, nevirapine; TDF, tenofovir; 3TC- lamivudine; FTC, emtricitabine; AZT, zidovudine; ABC, abacavir; NRTI, nucleos(t)ide reverse transcriptase inhibitor; NNRTI, nonnucleoside reverse transcriptase inhibitor; PI, protease inhibitor*

7.7 Prevalence of intergrade inhibitor resistance associated mutations

Some natural occurring polymorphism has been associated with HIV drug resistance.[53] However, frequency of occurrence of these polymorphisms associated with integrase strand transfer inhibitors (INSTI) resistance in Tanzania has yet to be described before. To obtain this data, all patients IN sequences were analysed for IN associated drug resistance mutation using the Stanford HIV drug resistance mutation interpretation algorithm (https://hivdb.stanford.edu/hivdb). We found that none of the patient's sequences harbored any major drug resistance mutations in the Integrase region. However, we observed accessory drug resistance mutations in 8/158 (5%) of patients with or without exposure to other ART drugs. (Table4)

Table 4. Illustrates the distribution of INI accessory drug resistance mutations among n=8 patients

Patient ID	Treatment status (non INSTIs)	INI Major resistance mutations	INI Accessory resistance mutations
V01	Treatment naïve	-	T97TA
V08	Treatment naive	-	T97A
V10	Treatment naïve	-	E157Q
V60	Treatment naïve	-	T97A
X73	Virological failure	-	G163EK
X81	Virological failure	-	E157Q
X111	Virological failure	-	E157Q
X165	Virological failure	-	A128AT

8. DISCUSSION

Our study reports DRM in the entire spectrum of HIV treatment, from baseline resistance to resistance in first and second-line regimen, using recently (June-October 2017) collected samples that should reflect contemporary circulating DRM after ~5 years of major programmatic shifts in HIV treatment in Tanzania. Our results can be summarized into three key findings; (1) pre-treatment DRM were highly prevalent (29.9%), (2) at detection of virological failure, virtually all subjects harbour multiclass DRM, and (3) emergence of essentially untreatable HIV-1 variants that harbour DRM conferring cross-resistance to all currently available ART in Tanzania.

Higher prevalence of pre-treatment DRM (29.8%) are described in our study compared to previous studies in Tanzania, which report prevalence ranging from <5% to 22%, on samples collected between 2003 and 2013.[36,54–56] This is in line with upward trend of pre-treatment DRM due to wide spread availability of ART in this era of 'treat all' as reported in a recent meta-analysis study by Gupta *et al*.[44] Initiation/re-initiation to a partly active first-line regimen may lead to a poor virological outcome and increase chances for further accumulation of DRM and regimen switch.[57–59] WHO now recommends integrase inhibitor (dolutegravir) based first-line regimen in situations of pre-treatments resistance to NNRTI as identified by baseline drug resistance testing or alternatively, a national representative data of NNRTI resistance above the threshold of 10%.[31,60] However, individualized baseline drug resistance testing is yet unavailable in Tanzania and unlikely to be feasible in the near future. Our findings therefore, accentuates the urgent need of national representative data for circulating pre-treatment DRM to guide the decision of a viable first-line regimen in Tanzania.

Analysis of failing subjects in the present study revealed that majority had developed multiclass DRM that restrict treatment options to already limited alternatives. This is in line with multiple other reports elsewhere.[61–63] High viremia at detection of treatment failure in this study suggest that our subjects may have had long periods of undiagnosed viral replications. Sustaining high viremia could also lead to transmission of resistant HIV strain and further accumulation of DRM. It is therefore warranted that more frequent individualized virological monitoring than that stipulated in the current HIV treatment guideline (once annually)[31] could be implemented at least to some selected patients to ensure less periods of uncontrolled viremia. On the other hand, antiretroviral with high genetic barrier may be necessary to mitigate the burden of DRM in resource-limited settings.

A multicentre retrospective cohort study involving studies from 36 countries in SSA demonstrated that incidence of tenofovir resistance in subjects failing first-line treatment is very high and reaches 57% of subjects failing treatment.[45] We also found similar prevalence (50%) of resistance to tenofovir in our study. However, selection of K65R (31%) was not as high as described in other SSA countries which range from 53% to 69%.[64–67] Prevalence of subtype C, which is linked to rapid selection of this mutation,[67,68] may explain this difference. However, it seems unlikely because a significant proportion of our study subjects (33.5%) were infected with HIV-1 subtype C. Of note, we did not find any difference in K65R distribution between subtypes in our subjects. Instead, accumulation of multiple TAM in our subjects on tenofovir containing regimen may explain at least in part the lower prevalence of K65R because these mutations are rarely selected in combination.[69] Thus, the evidence demonstrated here highlights an important role of concomitant use of thymidine analogues and tenofovir in shaping the epidemiology of

tenofovir-resistant viral variants, and raises a concern in tenofovir-based public health approach both for treatment of HIV-1 and oral pre-exposure prophylaxis.[70]

DRM alone could not explain viremia in 82% of subjects failing second-line regimen due to lack of PI resistance in this study. Being sensitive to PIs alone should be enough to control viremia regardless of the predicted antiviral activity of drugs forming the NRTI backbone of the second-line regimen.[71–73] Poor adherence may partly explain viremia in these subjects. In fact, a South African study demonstrated that when intensive adherence counselling was implemented to subjects failing second-line regimen without PI resistance mutation, a significant fraction (67%) of individuals were able to re-suppress viremia.[74] Our data therefore highlights the importance of DRM genotyping to viremic subjects failing second-line regimen in order to identify potential third-line regimen candidates because only small fraction of viremic subjects on second-line regimen developed PI DRM. Alternatively, mutations at other locus of the viral genome may affect sensitivity to PI. For example, mutations in *gag* gene has been linked to PI resistance with or without resistance mutations on *PR* gene [75,76] although clinical significance of the *gag* mutations to PI resistance is still the subject of research.

Perhaps the most concerning finding in our study is the detection of HIV variants resistant to virtually all currently available ART regimens in Tanzania. To our knowledge, this is the first report to describe such viral variants in Tanzania although similar observations were reported in multiple SSA countries including Uganda, Kenya, Botswana, Zimbabwe and South Africa.[77–80] Third-line regimens tailored from integrase inhibitors and newer generation of PI have shown potent virological outcome and tolerability when used to treat multidrug-resistant variants.[81–83] WHO now recommends a third-line regimen for adults which involves newer generation NNRTI, PI and integrase inhibitors.[31] However, none of the drug is currently available in most SSA

countries including Tanzania. This therefore underscore the urgent need for third-line regimen drugs in SSA region and clear eligibility criteria, which guarantees cost effectiveness.

Amid the introduction of dolutegravir in Tanzania, our study demonstrated that naturally occurring polymorphisms that are associated with integrase inhibitors resistance are rare in Tanzania which is consistent with reports from Ethiopia and Mozambique before INSTI introduction to these countries[8485]. E157Q which was detected in 3/158 (1.8%) patient in our study when alone, has been reported to have no impact on phenotypic susceptibility to dolutegravir.[86] However, it has been reported to increase dolutegravir resistance mediated by R263K that tend to be disproportionately selected by dolutegravir in patients with no previous exposure to INSTI.[24] Mutation T97A was also detected in 3/158(1.8%) in the present study. This mutation alone seems to have no effect of dolutegravir however, it has been reported that T97A in combination with other mutations, reduces susceptibility to dolutegravir.[87] Thus, importance of E157Q and T97A polymorphism in the subsequent development of resistance to dolutegravir in Tanzania settings should be investigated with strategic longitudinal studies.

Some limitations merit mention. Our analysis based on self-reported account of ART exposure prior to initiation of treatment. Given that undisclosed previous exposure of treatment happen relatively often (incidence of 10-30%) in SSA region,[88] we suspect a certain proportion of the observed pre-treatment DRM to be a result of undisclosed previous exposure of treatment. Also, this was a cross-sectional study in a single HIV treatment clinic in an urban tertiary hospital with greater resources available for HIV management including on site viral load monitoring, compared to majority of care and treatment clinics. Thus, what we observed in the current study could be different in rural clinics where resources for HIV treatment and monitoring is more

limited. In addition, our approach could not distinguish experimentally between transmitted and acquired DRM. For this, follow-up studies that includes tests for ART exposure is necessary.

9. CONCLUSION

In conclusion, our study documents drug resistance in Dar es Salaam-Tanzania where significant changes to its ART program has been adopted. The findings that significant proportion (14.9%) of individuals starting treatment harbours resistant viral strain to default regimen, and that one fifth of subjects failing second-line regimen were resistant to all available ART is a major concern to the efficacy of the ART program. Naturally occurring resistance to INSTI is rare in Tanzania. Thus, the current study underscores the need for newer generation of ART with higher genetic barrier to resistance and more robust treatment monitoring.

10 FUNDING INFORMATION

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