

# Genetic Diversity and Acquired Drug Resistance Mutations Detected by Deep Sequencing in Virologic Failures among Antiretroviral Treatment Experienced Human Immunodeficiency Virus-1 Patients in a Pastoralist Region of Ethiopia

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**Purpose:** This study was conducted to investigate the drug resistance mutations and genetic diversity of HIV-1 in ART experienced patients in South Omo, Ethiopia.

**Patients and Methods:** A cross-sectional study conducted on 253 adult patients attending ART clinics for  $\geq 6$  months in South Omo. Samples with VL  $\geq 1000$  copies/mL were considered as virological failures (VF) and their reverse transcriptase gene codons 90–234 were sequenced using Illumina MiSeq. MinVar was used for the identification of the subtypes and drug resistance mutations. Phylogenetic tree was constructed by neighbor-joining method using the maximum likelihood model.

**Results:** The median duration of ART was 51 months and 18.6% (47/253) of the patients exhibited VF. Of 47 viraemic patients, the genome of 41 were sequenced and subtype C was dominant (87.8%) followed by recombinant subtype BC (4.9%), M-09-CPX (4.9) and BF1 (2.4%). Of 41 genotyped subjects, 85.4% (35/41) had at least one ADR mutation. Eighty-one percent (33/41) of viraemic patients harbored NRTI resistance mutations, and 48.8% (20/41) were positive for NNRTI resistance mutations, with 43.9% dual resistance mutations. Among NRTI resistance mutations, M184V (73.2%), K219Q (63.4%) and T215 (56.1%) complex were the most mutated positions, while the most common NNRTI resistance mutations were K103N (24.4%), K101E, P225H and V108I 7.5% each. Active tuberculosis (aOR=13, 95% CI= 3.46–29.69), immunological failure (aOR=3.61, 95% CI=1.26–10.39), opportunistic infections (aOR=8.39, 95% CI= 1.75–40.19), and poor adherence were significantly associated with virological failure, while rural residence (aOR 2.37; 95% CI: 1.62–9.10, P= 0.05), immunological failures (aOR 2.37; 95% CI: 1.62–9.10, P= 0.05) and high viral load (aOR 16; 95% CI: 5.35–51.59, P <0.001) were predictors of ADR mutation among the ART experienced and viraemic study subjects.

**Conclusion:** The study revealed considerable prevalence of VF and ADR mutation with the associated risk indicators. Regular virological monitoring and drug resistance genotyping methods should be implemented for better ART treatment outcomes of the nation.

**Keywords:** HIV-1, genetic diversity, acquired drug resistance, ART experienced, South Omo, Ethiopia

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## Introduction

At the end of 2018, 19.5 million people living with HIV were accessing ART globally.<sup>1</sup> However, virological failure and development of drug resistance are becoming a bottleneck for the success of ART program. A global study involving 36 countries and 1926 patients with treatment failure from 1998–2015 reported that 36.3% patients developed tenofovir resistance, where the highest (57%) was in sub-Saharan Africa. Of 700 individuals with tenofovir resistance, 578 (83%) had M184V/I mutation, 543 (78%) had major NNRTI resistance, and 457 (65%) had both.<sup>2</sup>

Since the first evidence of HIV infection in Ethiopia in 1984, AIDS has taken the lives of millions and left behind an estimated 744,100 orphans.<sup>3</sup> At the end of 2016, the national HIV prevalence stabilized at 1.12%, and 715,500 people living with HIV/AIDS, with 27,288 AIDS related deaths, and 19,743 new infections in the year.<sup>4</sup> Since free ART initiated in 2005 in Ethiopia, a total of 386,123 estimated adults living with HIV have been receiving ART with 73% coverage.<sup>5</sup>

Studies conducted in Ethiopia indicated that the prevalence of VF range from 5.3% to 18% among HIV/AIDS patients who were on ART for a median time of 6–24 months, while the dominant reported drug resistance mutations on RT gene were M184V, K103I, and no mutations reported on protease inhibitors associated gene.<sup>6–8</sup> Another study conducted in Addis Ababa, reported 31% VF, and 70% ADR mutations among the viraemic patients, out of which 62% against NRTIs, 68% against NNRTIs, 61% against any NRTI and NNRTI double class mutations.<sup>9</sup>

Data on virological failures and drug resistance mutations among patients on ART in Ethiopia is scarce due to lack of routine laboratory monitoring of HIV VL and genotype tests.<sup>10</sup> As the current study site borders to Kenya and South Sudan, and known world tourist destiny site, there is a concern that new HIV-1 variants may be introduced and intermixed from the neighboring and other countries.<sup>11,12</sup>

Furthermore, the available HIV drug resistance studies conducted in Ethiopia so far were based on the Standard Genotypic Resistance Testing (SGRT), that could not reliably detect clinically important low frequency (< 20%) drug resistance variant mutations of the virus quasispecies in a clinical sample.<sup>13–16</sup> Currently, the emerging NGS platforms like Illumina MiSeq allow the detection of

minority resistance mutation variants with 0.5–1% lower limit of detection while maintaining good accuracy.<sup>17–22</sup>

Therefore, this study was conducted to determine HIV-1 genetic diversity, rate of HIV-1 virological failure and ADR mutations using Illumina MiSeq in HIV-1 infected adults on ART for  $\geq 6$  months in South Omo, Ethiopia.

## Patients and Methods

### Study Area and Setting

This study was carried out in South Omo Zone of Southern Ethiopia bordering with Kenya in south and with South Sudan in southwest. The Zone is one of the least developed areas of the country with poor infrastructure, and inhabited by ethnically diversified population; possibly as many as 24 ethnic groups with unique cultural practices in the world, dominated by the ancient Nilotic pastoralist traditions.<sup>23</sup> The Zone inhabited by 573,435, of which 43,203 were urban inhabitants, and 25,518 were pastoralists.<sup>24</sup>

### Study Design and Setting

A cross-sectional study was conducted from January 2016 to December 2018 in patients attending ART clinic in four health centers and Jinka Zonal Hospital. Adult patients (age  $\geq 18$  years) who gave informed written consent and who had been on ART for 6 or more months and were still on ART at the time of enrolment were considered for the study. Sample size was determined using single population proportion formula assuming an HIV-1 VF prevalence of 21% after a median follow-up period of 12 months on ART in sub-Saharan setting,<sup>25</sup> and 5% of margin of error.

### Specimen Collection Procedure

A standardized drug resistance survey data collection questionnaire was used to collect socio-demographic and clinical data. Upon written consent, 10 mL EDTA blood samples were collected for CD4+T-cell count, viral load assays and genotyping. Plasma was temporarily stored in multiple aliquots at  $-30^{\circ}\text{C}$  at the study site and transported to Addis Ababa University and stored at  $-80^{\circ}\text{C}$ , until shipped to Kampala, Uganda on dry ice for VL measurement and genotyping at Makerere University. CD4+ T cell counts were monitored at the time of ART initiation and every 6 months as part of the ART program. All tests were done anonymously linked with unique code.

## CD4<sup>+</sup> T Cell Counts, RNA Extraction and Viral Load Measurement

CD4<sup>+</sup> T cell count was performed within 4–6 hours of blood collection using a FACS Caliber flow cytometer (FACScount Becton & Dickinson Immunocytometry, Oxford, UK) at the study site. HIV-1 genomic RNA was extracted from 140 µL of cryopreserved plasma using the QIAmp viral extraction kit (QIAGEN, Hilden, Germany) as per the manufacturer's protocol. Viral load was estimated using QuantiTect Probe RT-PCR kit (QIAGEN, Hilden, Germany) following the manufacturer's protocol<sup>26</sup> using the following primers and probes that targets the conserved portion of GAG and LTR genome of HIV-1 virus: Gag 183UF:CTA GCA GTGG CGCCGACAG, Gag187LR:CCATCTCTCTCCTTCTAGC CTCCGCTAGTCA, Probe Gag 187 PFAM-5'TCTCTC GACGCA G GACTC GCTTGCTG'3 –BHQ.

## Amplicons Generation for Illumina MiSeq Sequencing

Two rounds of PCR were used to generate HIV DNA fragments spanning the HIV reverse transcriptase (RT) codons 90–234, as described previously following the manufacturer's protocol.<sup>14</sup> Briefly, first strand DNA was generated using KAPA HiFi HotStart ReadyMix PCR kit (Boston, USA) following an *in vitro* reverse transcriptase using TaKaRa PrimeScript<sup>TM</sup> RT-PCR kit (Clontech Laboratories, Takara Bio Company). Using cDNA as a template, first round PCR was conducted using the following primers and cycling conditions: HIVRT3.1F: GAAGGGC ACACAGCCAGAAATTGCAG and HIVRT3.1R: GCTCCTACTATGGGTTCTTTCTCTAAC TGG. Using the first round PCR product as a template, second round PCR was done using internal sequence specific primer fused with Illumina Adaptors that adds the sequence to amplicon complementary to those on Illumina MiSeq flow cell as described elsewhere.<sup>14</sup> ILRT2796F: TCGTCCG CAGCGTCAGATGTGTATAAGAGACAGAGAACTC-AAGACTTCTGGA; ILRT3271R: GTCTCGTG GCTCGGAGATGTGTATAAGAGACAGACTGTCCAT-TTATCAGGATC.

Cycling conditions for both PCRs were the same: 1) Initial denaturation at 95°C, 2min., 2) denaturation at 98°C, 30 sec., 3) annealing at 60°C, 30 sec, 4) extension at 72°C, 1 min (Step 2–4 for 35 cycles) and 5) final extension at 72°C, 10 min. Following amplification, 3µL of each 2nd round PCR product was analyzed on the QIAxcel Advanced, using the Fast analysis Cartridge.

The expected amplicon is approximately 500bp, a short region spanning RT codon 90–234 was amplified in a nested second PCR with primers incorporating Illumina indexing adaptors (Lapointe et al 2015).

## MiSeq Library Preparation and Sequencing

### Multiplexing and Purification

Library preparation for MiSeq sequencing was done using a NextEra<sup>®</sup> XT DNA Sample Preparation and Index kit (Illumina, San Diego, CA, USA) as described elsewhere.<sup>14</sup> Briefly, eight base pair-long NextEra XT multiplexing indices were added using low cycle PCR to both the 5' and 3' ends of each amplicon used as a unique identifier in pooling up to 96 samples for subsequent sequencing simultaneously. A total of 9 i7 and 30 i5 indices were used to prepare up to 114 samples in two plates following Illumina NextEra XT indexing procedure.<sup>27</sup> Multiplexed MiSeq amplicons were purified using Agencourt AMPure<sup>®</sup> XP PCR purification systems (Beckman Coulter Company, Germany).

### Library Quantification, Normalization, and Pooling

Library quantification was determined using Qubit<sup>®</sup> dsDNA HS (High Sensitivity) Assay kits using the Qubit<sup>®</sup> 2.0 Fluorometer. A final library with equimolar concentration of 4nM was prepared using Re-suspension Buffer, and 5 µL of diluted DNA was aliquoted from each library and then the two libraries were pooled with unique indices prior to MiSeq sequencing with a 2×300 cycles v3 reagent kit.

### Denature and Dilute PhiX Control

In preparation for cluster generation and sequencing, pooled libraries were denatured using freshly diluted 0.2N NaOH with hybridization buffer, and then heat denatured before MiSeq sequencing. A 5% PhiX spike-in was added to serve as an internal control for these low diversity libraries according to Illumina recommendation.

### MiSeq Data Processing

Sequences were de-multiplexed automatically on the MiSeq and two paired fastq files were generated for each sample representing the two paired-end reads. MiSeq short-read data in fastq format were imported and processed by freely available pipeline, MinVar, rapid and versatile tool for HIV-1 drug resistance genotyping by deep sequencing (Huber et al 2017). MiSeq consensus sequences spanning RT codons 90 to 234 were produced

from the empirical raw nucleotide frequency distributions in the aligned and merged read data. The main output was a table with subtype and amino acid mutations with respect to HIV-1 consensus B, annotated according to the class of resistance defined in the Stanford HIVdb.<sup>28</sup> Drug resistance associated mutations were grouped as NRTI, NNRTI and others.

## Phylogenetic Analysis

All sequences were automatically aligned with reference sequences of all known HIV-1 group M (sub-) subtypes (A1, A2, B, C, D, F1, F2, G, H, J, and K) and circulating recombinant forms (CRFs) retrieved from the Los Alamos database using MUSCLE in MEGA 6 software.<sup>29</sup> HIV-1 subtyping was determined using REGA version 3 of Stanford HIVdb.<sup>30</sup> Phylogenetic tree was constructed by the neighbor-joining method using the Maximum Likelihood Model. The reliability of the tree was assessed by bootstrapping of 1000 replicates. Clustering of sequences with a bootstrap value of more than 70% was considered significant for subtyping. DNA polymorphism was analyzed using DnaSP software.

## Variable Definitions

The major outcomes of interest in this study were virological Failure (VF), HIV-1 genetic diversity and ADR mutations. Detectable VL was defined as HIV-1 RNA levels  $\geq 126$  copies/mL and VF as HIV-1 RNA  $\geq 1000$  copies/mL. The prevalence of HIV-1 VF was determined as percentage of plasma samples with detectable viral load  $\geq 1000$  copies/mL. The prevalence of ADR was determined as percentage of samples with detectable resistance associated mutations as determined by the Stanford HIV drug resistance database divided by the total number of samples with VF that were successfully sequenced.

Based on the Stanford HIV drug resistance database, mutation levels were classified as high resistant (H), intermediate (I), low (L), potential low (PL), and susceptible (S). In this study, a read frequency of 5% and 20% was considered as a minor variant, and a read frequency  $>20\%$  was classified as a major variant. All Food and Drug Administration (FDA) approved first line ARVs were considered for analysis that were covered in the sequenced region (codon 90–234) of RT gene.<sup>31</sup>

## Data Analysis

Descriptive statistics were employed to describe socio-demographic, clinical characteristics, HIV-1 VF and

different types of drug resistant mutations among the study subjects. Continuous data were presented using mean and medians, and categorical data were presented as frequencies and percentages. To determine the independent predictor variables of ADR mutations among the study subjects, bivariate and multivariate logistic regression analysis were conducted. Crude and adjusted odd ratios (OR), 95% confidence intervals (CI) and p-values  $<0.05$  were considered significantly associated. Data analysis was performed using SPSS for Windows, Version 23.0. Chicago, SPSS Inc.

## Ethics Approval and Consent to Participate

Institutional permission to conduct the study was obtained from Aklilu Lemma Institute of Pathobiology Institution Review Board, Addis Ababa University. In addition, written consent was received from each study participant. We confirm that the revised manuscript of this study was conducted in accordance with the Declaration of Helsinki.

## Results

### Baseline Characteristics of the Study Participants

A total of 253 adults on first line ART for a median duration of 51 (IQR: 49–61) months were recruited from January 2016–December 2018. Majority of them were females (60.5%), mean age was 33.5 years (95% CI: 32.5–34.5/]) and 70% the study subjects were taking tenofovir (TDF) based ART, while 30% were on Zidovudine (AZT) based regimen during the study period. Majority (71.9%) of them started ART in WHO III/IV stage, with the median base line CD4 count of 239 (95% CI: 204–284.5) and IQR of 228 (95% CI: 208–268). About 40% (102/253) of the participants reported poor ART adherence. Majority (94%) of study subjects had base line CD4 cell counts  $\leq 350$  cells/ $\mu$ L at the start of the study (Table 1). Based on the fall of follow up CD4 count to baseline, severe immunological failure was observed in 11.5% (27/235) of the subjects and it was significantly associated with older age and malnutrition in the bivariate analysis (OR= 5.9, 95% CI: 1.7–20.1, P= 0.005) and (OR= 3.1, 95% CI: 1.22–8.10, P=0.02) respectively (data not shown).

### Phylogenetic Analysis of HIV-1 Sequences of RT Gene from ART Experienced Patients

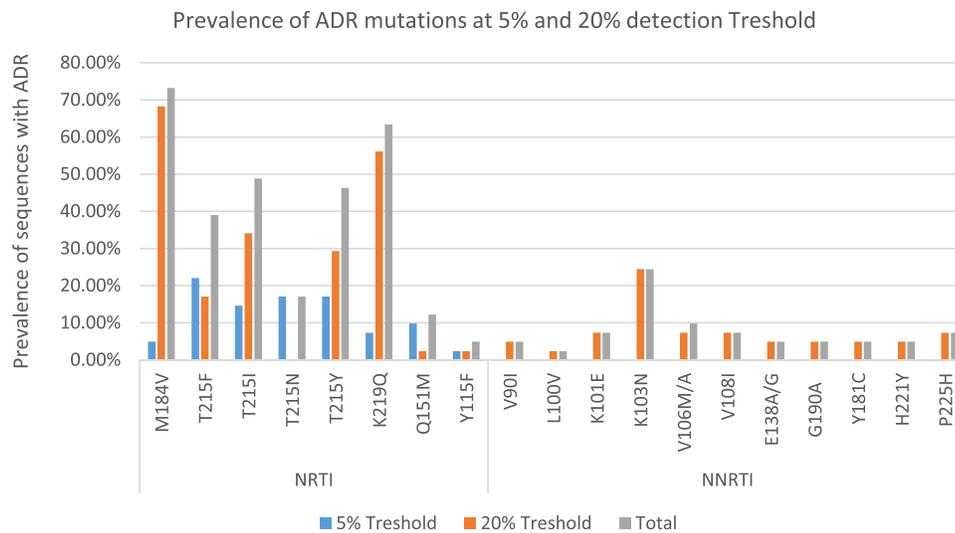
From a total of 253 ART experienced study subjects, 47 (18.6%) showed VF ( $\geq 1000$  copies/mL), of which 41

**Table I** Socio-Demographic and Clinical Characteristics of the Study Subjects Among First Line ART Experienced Patients at South Omo, Ethiopia (n=253)

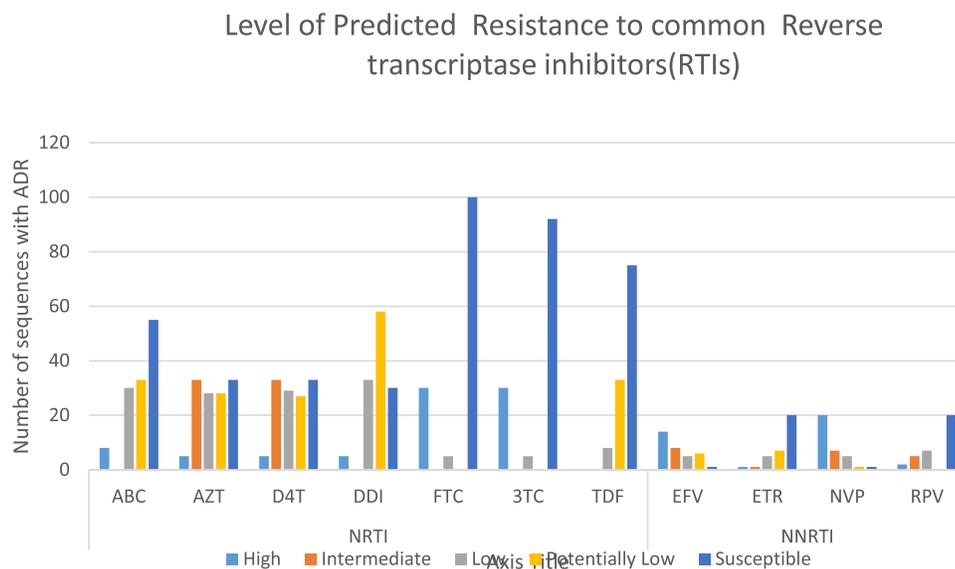
Characteristics	Categories	Frequency (%)
Sex	Male Female	100(39.5%) 153(60.5%)
Age (years)	Mean	33.5(95% CI:32.5–34.5), Sd=8.4
Age Group(years)	≤ 30 >30	103(40.7%) 150(59.3%)
Marital status	Married Divorced Single Widowed	131(51.8%) 59(23.3%) 25(10.3%) 37(14.6%)
Educational status	Illiterate Read & write Primary Secondary Tertiary	126(49.8%) 19(7.5%) 61(24.1%) 36(14.2%) 11(4.3%)
Occupation	Agro pastoralist Housewife Government Employee Daily Laborer	30(15%) 76(30%) 46(28.2%) 93(36.8%)
First line ART regimen Categorized	Zidovudine based Tenofovir based	76(30%) 177(70%)
Base Line WHO staging	I/II III/IV	71(28.1%) 182(71.9%)
Baseline CD4 count(cells/μL)	Median IQR	239(95% CI: 204–284.5) 228 (95% CI: 208–268)
Baseline CD4 count(cells/μL)	≤350 >350	238(94.1%) 15(5.9%)
Current CD4 Count(cells/μL)	Median IQR	501(95% CI:462–540) 337(95% CI:288–399)
Duration on ART (Months)	Median IQR	51(95% CI:42–59) 57(95% CI:49–60)
Follow up WHO staging	I/II III/IV Missing	232(91.7%) 11(4.3%) 10(4.0%)
Duration of ART group	≤ 12 months > 12 months	27(10.7%) 226(89.3%)
Active tuberculosis	Yes No	38(15%) 215(85%)
Any OIs diagnosed	Yes No	38(15%) 215(85%)
Any STIs diagnosed	Yes No	35(13.8%) 215(85%)

(Continued)





**Figure 2** ADR mutation frequency at 5% and 20% detection sensitivity thresholds, as determined by MiSeq sequencing.



**Figure 3** Predicted ARV drug responses of 35 patients with ADR mutations. Drug responses were based on the Stanford HIVdb. The y-axis indicates the number of sequences with ADR while the x-axis indicates the different ARV drug classes that were affected by the ADR mutations.

**Abbreviations:** ADR, acquired drug resistance; NRTIs, nucleoside reverse transcriptase; NNRTI, nonnucleoside reverse transcriptase inhibitors; ABC, abacavir; AZT, zidovudine; DDI, didanosine; 3TC, lamivudine; D4T, stavudine; TDF, tenofovir; NVP, nevirapine; EFV, efavirenz; ETR, etravirine; RPV, rilpivirine.

(4.9%), and BF1 (2.4%) were found. DNA polymorphism analyses indicated that HIV-1 isolates exhibited high haplotype ( $h > 0.99$ ) and nucleotide ( $\pi > 0.08$ ) diversity.

## Prevalence of Acquired Drug Resistance Mutations (ADR)

Out of 41 genotyped isolates, 85.4% (35/41) had at least one ADR mutations on RT gene, while 14.6% (6/41) viraemic samples did not have any detectable resistance mutations, or had very low frequency (2–4%) drug resistance mutants.

Among study subjects who harbored at least one DRM, 60% (14/35) were taking tenofovir (TDF) based regimen, while 40% (11/35) were on zidovudine (AZT). With regard to NNRTI class, 21 (60%) were taking efavirenz-containing ART regimen, while 14 (40%) were on nevirapine combined regimen. However, there was no significant associations between regimen type and development of drug resistance mutations among both drug class combinations.

Out of the 41 viraemic subjects 33 (80.5%) harbored NRTI resistance mutations, and 20 (48.8%) were found to

have NNRTI resistance mutations, while 13 (31.7%) harbored other resistance mutations like V90A, K101Q and K103R. Both NRTI and NNRTI resistance mutations were simultaneously detected among 18 (43.9%) viraemic patients (Table 2).

Among NRTI resistance mutations, M184V (73.2%), and K219Q, (63.4%) were the most common resistance mutations detected from viraemic subjects, respectively. Among the drug resistance harboring subjects, 23 (56.1%) were found to have T215 complex which is

Thymidine Analog-associated Mutations (TAMs) that are known to affect all NRTIs currently approved by the US FDA other than emtricitabine and lamivudine (Figure 2). In addition, multi-NRTI resistance mutation, Q151M was detected among 5 (12.2%) of the study subjects, which affects all NRTIs currently approved by the US FDA except tenofovir<sup>33</sup> (Table 2).

Of the 41 viraemic specimens genotyped, the major NNRTI resistance-associated mutations detected were: K103N (24.4%), P225H (7.3%), K101E (7.3%), V108I

**Table 2** HIV-1 Drug Resistance Conferring Mutations in the RT Gene Among First Line ART Experienced Adults with ADR Mutations, South Omo, Ethiopia, 2017 (N=41)

Drug Class	AA Mutations	Frequency N (%)			Confer Resistance to
		5–20%	>20%	Total	
NRTI					
	M184V	2(4.9)	28(68.3)	30(73.2)	<b>3TC, FTC</b> , ABC, DDI
	T215F	9(22.0)	7(17.1)	16(39.0)	ABC, DDI, TDF, <b>D4T, ZDV</b>
	T215I	6(14.6)	14(34.1)	20(48.8)	
	T215N	7(17.1)	0(0%)	7(17.1)	
	T215Y	7(17.1)	12(29.3)	16(46.3)	ABC, DDI, TDF, <b>D4T, ZDV</b>
	K219Q	3(7.3)	23(56.1)	26 (63.4)	ABC, DDI, TDF, <b>D4T, ZDV</b>
	Q151M	4(9.8)	1(2.4)	5(12.2)	<b>AZT, D4T, DDI, ABC</b> , 3TC, FTC, TDF
	Y115F	1(2.4)	1(2.4)	2(4.9)	
	<b>Any NRTI</b>	<b>20(48.8)</b>	<b>32(75)</b>	<b>33(80.5)</b>	
NNRTI	V90I	0	2(4.9)	2(4.9)	EFV, ETR, RPV
	L100V	0	1(2.4)	1(2.4)	NVP, <b>EFV, ETR, RPV</b>
	K101E	0	3(7.3)	3(7.3)	<b>NVP</b> , EFV, ETR, RPV
	K103N	0	10(24.4)	10(24.4)	NVP, EFV
	V106M/A	0	3(7.3)	4(9.8)	NVP
	V108I	0	3(7.3)	3(7.3)	NVP
	E138A/G	0	2(4.9)	2(4.9)	ETR, RPV
	G190A	0	2(4.9)	2(4.9)	<b>EFV</b> , ETR, RPV
	Y181C	0	2(4.9)	2(4.9)	NVP
	H221Y	0	2(4.9)	2(4.9)	
	P225H	0	3(7.3)	3(7.3)	EFV
		<b>Any NNRTI</b>	<b>0</b>	<b>20(48.7)</b>	<b>20(48.8)</b>
NRTI and/or NNRTI	<b>NRTI or NNRTI</b>	<b>20</b>	<b>35</b>	<b>35(85.4)</b>	
	<b>NRTI &amp; NNRTI</b>	<b>0</b>	<b>18</b>	<b>18(43.9)</b>	

Notes: (hivdb.stanford.edu). \*Red bold: Highest level resistance. Bold: high level, Plain text: low level.

(7.3%), V90I (4.9%), V106M/A (9.8%), H221Y (4.9%), E138G/A (4.9%), and Y181C (4.9%). Among the NNRTI associated mutations, Y188D was detected in all viraemic patients at low frequency (2–4%), which is not common in earlier reports. All NNRTI resistance associated mutations were found as major variants (>20% detection level; Figure 2 and Figure 3).

We examined risk factors associated with HIV-1 ADR mutations among first line ART experienced patients (Table 3). In bivariate analysis, resident type, occupation, immunological failure, follow up CD4+ T cell count, missed ART pills within the last week, and VL were found to be associated with ADR mutations. However, in multivariate model analysis only residence type, immunological status and VL at the time of sampling were found to be independent predictors of acquired drug resistance mutation among the ART experienced and viraemic study subjects. Accordingly, study subjects that resided in rural areas were three times more likely to develop ADR (aOR 2.37; 95% CI: 1.62–9.10,  $P = 0.05$ ) than urban dwellers. Similarly, study subjects that showed immunological failure during ART (aOR 2.37; 95% CI: 1.62–9.10,  $P = 0.05$ ) had a higher prevalence of ADR, compared to those with immunological recovery. Likewise, participants with higher VL ( $\geq 3.7$  log copies/mL) had a higher prevalence of ADR, in comparison to those with lower viral loads ( $< 3.7$  log copies/mL (aOR 16; 95% CI: 5.35–51.59,  $P < 0.001$ )) (Table 3).

## Discussion

In this study, we investigated HIV-1 genetic diversity and acquired drug resistance mutations among 253 adults on first-line antiretroviral treatment for  $\geq 6$  months in South Omo, Ethiopia.

Detection of a significant number of HIV-1 subtype C in the current study in Ethiopia is in agreement with the previous studies that unanimously reported the HIV-1 epidemic in Ethiopia is dominated by subtype C viruses with two co-circulating sub-clusters named C and C' that were identified since 2000.<sup>34–37</sup> It is well established fact that the dominant circulating HIV-1 subtype in East Africa is subtype C with different geographical clusters identified as East African HIV-1 subtype C, and Ethiopian unique subtype C' lineage.<sup>35,37</sup> However, 8.5% HIV-1 isolates from in Djibouti patients were identified as subtype B,<sup>38</sup> and two isolates (1%) from Addis Ababa were detected as subtype B.<sup>9</sup>

The identification recombinant HIV-1 subtype BC, BF1 and M-09-cpx in the current study might indicate the introduction of new subtypes as a result of high tourist flow and population movement between the bordering countries in the study area. However, this finding should be interpreted with precaution since we sequenced a very short RT region used for drug resistance mutation analysis that may not be suitable for HIV subtyping. As a result, future studies that involve long and conserved region of the virus will be required to proof that we had non-C subtypes in Ethiopia.

Eighty five percent (35/41) of the genotyped study subjects had at least one RTI-associated drug resistance mutations, is similar to the South African study where 84% viraemic samples had one or more ADR mutations,<sup>39</sup> and comparable to the pooled ADR mutation prevalence of 70.7% reported by WHO at the end of 201,<sup>1</sup> where resistance to NNRTI had the highest observed rate (61%), followed by NRTI (55%). Similarly 70% prevalence of ADR mutation in Addis Ababa was reported by Abegaz in 2011.<sup>9</sup>

In contrast, more than 80% of NRTI resistance mutation described in this study is much higher than 55.3% reported from West Africa, while 48.8% NNRTI associated resistance mutation prevalence is comparable to 57% NNRTI prevalence reported from other East African Countries.<sup>10,40</sup> The disparity may emanate from the genotyping methods used. Most previous studies used the conventional Sanger based genotyping tests to identify HIV drug resistance associated mutations that could miss the lower abundance variants, unlike the current study which applied NGS platforms that could detect low frequency mutations.

Similar to our study, 83% for reverse transcriptase inhibitors (RTIs) drug resistance mutations among viraemic patients was reported from Rwanda.<sup>41</sup> Moreover, another study from Spain, indicated 76.9% NRTI drug resistance mutations and 36.5% NNRTI associated mutations among ART experience patients.<sup>42</sup> Similar to our report, a decade review from India identified drug resistance mutation prevalence of 78.4% to any RTIs, 68.8% to NRTI, and 73.13% to NNRTI among first line ART experienced adults.<sup>43</sup> The most recent WHO report also indicated that NNRTI resistance ranged from 47% to 90% among people with viral failure on first-line NNRTI containing regimens, which are the backbone of the WHO first line ART regimen.<sup>1</sup>

**Table 3** Logistic Regression Analysis Describing Associations of HIV-1 Drug Resistance Mutations Among First Line ART Experienced Patients in South Omo, Ethiopia (n=253)

Risk Factor	Categories	Drug Resistance Mutations		Bivariate Analysis			Multivariate Analysis		
		Yes, N (%)	No, N (%)	Crude OR	95% C.I	P-value	Adjusted OR	95% C.I	P-value
Residence type	Rural	25(30.9%)	56(69.1%)	7.32	3.27–15.99	<0.001	3.08	1.5–9.0	0.04*
	Urban	10(5.8%)	161(93.6%)	I			I		
Age Group	≤30 years	9(8.7%)	94(91.3%)	2.59	1.01–4.89	0.06	0.51	0.15–1.73	0.28
	> 30 years	26(17.3%)	124(82.7%)	I			I		
Occupation	Agro-pastoralist	11(28.9%)	27(71.1%)	3.04	1.18–7.79	0.02	1.42	0.35–5.74	0.62
	House wife	8(10.5%)	68(89.5%)	0.87	0.33–2.34	0.79	0.51	0.14–2.09	0.35
	Employed	5(10.9%)	11(11.8%)	0.91	0.30–2.79	0.87	0.63	0.15–2.71	0.53
	Daily laborer	41(89.1%)	82(88.2%)	I			I		
Immunological Failure	Yes	10(37.0%)	17(63.0%)	4.97	2.03–12.20	<0.001	2.37	1.62–9.10	0.05*
	No	22(10.6%)	186(89.4%)	I			I		
Active TB	Yes	5(29.4%)	12(70.6%)	2.86	0.94–8.69	0.06	0.59	0.10–3.49	0.56
	No	30(12.7%)	206(87.3%)	I			I		
Baseline WHO staging	I/II	5(7.2%)	64(92.8%)	I			I		
	III/IV	28(16.5%)	142(83.5%)	2.05	0.80–5.18	0.13	2.69	0.56–12.95	0.22
	Any OIs diagnosed	9(23.7%)	29(76.3%)	2.26	0.96–5.26	0.06	0.40	0.09–1.92	0.25
Follow up CD4 count	Yes	26(12.1%)	189(87.9%)	I			I		
	No	24(19.2%)	101(80.8%)	2.51	1.20–5.37	0.02	1.16	0.36 –3.76	0.80
Duration on ART in month	≤12	11(8.7%)	116(91.3%)	I			I		
	>12	1(3.7%)	26(96.3%)	I			I		
Missed Pills within last week	Yes	34(15%)	192(85.0%)	4.60	0.60–35.07	0.14	3.99	0.41–38.86	0.23
	No	20(19.6%)	15 (9.9%)	2.21	1.70–4.56	0.03	0.89	0.30–2.62	0.84
Missed Pills within last week	Yes	82(8.4%)	136(90.1%)	I			I		
	No								

Viral Load (RNA copies/mL)	≤3.7 log	11 (5.2%)	201 (94.8%)	1			
	>3.7 log	24 (58.8%)	17 (41.5%)	25	10.8–61.49	< 0.001	16
							5.35 51.59
							<0.001*

Note: \*Bold indicate statistically significant associations with Pvalue < 0.05.

Mathematical modeling on global trend on drug resistance pattern suggesting that NNRTI resistance mutations harboring individuals were found to be more likely to acquire new HIVDR mutations and experience death, this in turn may be a challenge to achieve the global targets to end AIDS as a public health threat by 2030.<sup>1</sup>

In this study, majority (60%) participants who had ADR mutations were on tenofovir (TDF), while the remaining 40% were on Zidovudine (AZT) based regimen. However, regimen type did not show any association with viral failure and drug resistance mutations. A recent global research conducted in an attempt to find a point of care genotypic resistance test to detect HIV-1 drug resistance mutations revealed that the most common major DRMs were M184V (91.5%) and the TAMs complex 34.5%,<sup>44</sup> which is comparable to the prevalence of major NRTIs in the present study. Moreover, this is in line with another study conducted in Northern India that reported 89.8% NRTI and NNRTI ADR mutations among virological failures with M184V, T215Y were the most frequent NRTI associated mutations and K103N, G190A, Y181C from NNRTI associated ones.<sup>45</sup>

In consistent with this study, quite a number of global and regional genetic analyses of HIV-1 have unanimously reported that the most globally prevalent NRTI resistance mutations is M184V followed by thymidine analog-associated mutations (TAM) including M41L, D67N, K70R, L210W, T215Y/F and K219Q/E, and, the most prevalent NNRTI was K103N.<sup>10,33,40,46–48</sup> Similarly, WHO 2017 drug resistance mutations revealed that the most commonly observed NRTI-associated resistance mutations were at codons 184, and the most commonly observed NNRTI-associated mutations were at positions 103, 181 and 190 globally.<sup>1</sup>

In this study, all NNRTI resistance mutations were found at a higher frequencies and no minor mutations were detected. The most frequent NNRTI resistance mutation detected were K103N (24.4%), followed by K101E, V108I and P225H found at 7.3% prevalence each. Interestingly, the finding identified all the four RT gene positions (K103N, Y181C, G190A and V106M) recommended for point of care genotypic test for DRMs in low and middle income countries.<sup>44</sup>

In the current study the most abundantly identified resistance mutations were M184V, K103N and TAMs which are known to be the key resistance mutation types among ART naïve and experienced subjects.<sup>49</sup> It is known that M184V causes high level resistance to 3TC and FTC

and low level resistance to ddI and ABC, however, it increases susceptibility to TDF, AZT and d4T susceptibility and decreases viral replication fitness, and it also appears to delay or prevent emergence of TAMs.<sup>50</sup> TAMs were first reported among patients receiving AZT monotherapy.<sup>51–53</sup> The presence of TAMs in this study may be a warning sign for clinicians in charge of the AIDS patients to closely monitor their clients, as TAMs are known to reduce all NRTIs susceptibility currently approved by the US FDA other than emtricitabine and lamivudine.<sup>33,54</sup> K103N is a non-polymorphic mutation selected in patients receiving NVP and EFV,<sup>55</sup> which is known to reduce NVP and EFV susceptibility by about 50 and 20-fold, respectively.<sup>56</sup>

Hence, the high prevalence of M184V and K103N in the present study may be challenging to control HIV viraemia among ART experienced patients, as these mutations almost affect all FDA approved first line regimen commonly used in Ethiopia.

In our study, 4 (9.8%) study subjects harbored Q151M, multi NRTI resistance mutation, which is known to cause high level resistance to AZT, D4T, DDI, ABC and low level resistance to 3TC, FTC, TDF.<sup>57,58</sup> The detection of Q151 complex in the present study might be a big challenge to manage and control HIV/AIDS patients among the ART users in the country, this in turn calls for vigilant monitoring of drug resistance in the area.

Contrary to most earlier reports,<sup>59</sup> Y188D was detected in low frequency (2–4%) among all the genotyped virae-mic samples in this study as NNRTI associated mutation. This might require further investigation to confirm its significance as NNRTI associated resistance mutation in the study area in particular, and in Ethiopia in general.

In our study, residence type, immunological status and viral load of the study participants were found to be independent predictors of acquired drug resistance mutation in multivariate logistic analysis. In this study, unlike several research findings, age was not significantly associated with drug resistance mutations in multivariate analysis.<sup>60</sup>

Higher viral load ( $> 3.7$  log copies/mL) showed strong correlation with acquired drug resistance mutations in this study. It is well established fact that high viral load had strong correlation with acquired drug resistance mutation.<sup>60–62</sup> Hence, World Health Organization recommendation of switching to second line ART after 2 consecutive viral loads of  $\geq 5000$  copies/mL, appeared to be the most appropriate strategy.<sup>63</sup> Although immunological

failure seems inferior to confirm development of drug resistance, it has been found to be associated with both virological failure and drug resistance mutations.<sup>63</sup>

In the current study, rural patients were significantly more likely to have higher rates of NRTI and NNRTI resistance mutations, compared to the urban dwellers (aOR 2.37; 95% CI: 1.62–9.10,  $P= 0.05$ ). This is similar to a recent study conducted in South Africa comparing urban and rural HIV treatment programs, reported that the rural group were more likely to have high rate of drug resistance mutations (88.8%, vs 64.1%) than the urban inhabitants.<sup>64</sup> According to the authors, the prevalence of acquired drug resistance mutations in adults with first-line therapy failure differed between the urban and rural sites. Several reasons might be suggested to this differential prevalence of drug resistance mutation between the urban and rural groups. The current study site is one of the poorest area in road access and other infrastructures, hence, rural dwellers faces transportation problem to access the health facilities, and higher stigma than the urban inhabitants.

## Conclusion and Recommendation

The degree of HIV-1 virological failure (18.6%) and acquired drug resistance mutations acquired drug resistance (85.4%) detected from the current study conducted in South Omo, Ethiopia, were similar with global reports found from other resource limited countries. High virological failure was observed among participants diagnosed with opportunistic infections, active tuberculosis, immunological failures and poor treatment adherence. Similarly, rural residents, having a high viral load and immunological failures were more prone to develop acquired drug resistance HIV-1 mutants. Hence, special attention should be given to those who developed opportunistic infections including active tuberculosis, poor treatment adherence, and immunologically failed individuals for better ARV treatment outcomes. In addition, ART service providers should give special attention to the rural residents and with viral load of  $\geq 5000$  copies per mL. We would also recommend that, at the very least, viral load measurements and genotypic drug resistance testing should be in place to inform the timely switch to second line treatment in case of virological failures. This will improve the quality of HIV/AIDS care and treatment program in Ethiopia. This study also highlights the utility of high-throughput sequencing in a relatively low resource limited settings for deep HIV genotyping and analysis of drug resistance mutation.

## Abbreviations

AIDS, Acquired immune deficiency syndrome; DRMs, drug-resistance mutations; ADR, Acquired Drug Resistance; ART, Antiretroviral Therapy; cDNA, complementary DNA; NRTI, Nucleoside Reverse Transcriptase Inhibitor; NNRTI, Non-Nucleoside Reverse Transcriptase Inhibitor; RT, Reverse Transcriptase; RT-PCR, Real Time Polymerase Chain Reaction; CRF, Circulating Recombinant Form.

## Data Sharing Statement

The nucleotide sequences discussed in this study were deposited into National Centre for Biotechnology Information (NCBI) GenBank under accession numbers MG570974-MG571084.

## Consent for Publication

Not applicable, as the manuscript does not contain data from any individual person.

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## Author Contributions

All authors made a significant contribution to the work reported, whether that is in the conception, study design, execution, acquisition of data, analysis and interpretation, or in all these areas; took part in drafting, revising or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

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## Disclosure

Rembert Pieper is an employee of Janssen Biopharma. The authors declare that they have no other potential conflicts of interest for this work.

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