Addis Ababa University, College of Health Sciences, School of Medicine,

Department of Microbiology, Immunology, and Parasitology

HIV-1 Genetic Diversity and Pre-treatment Drug Resistance Mutations among recently diagnosed HIV-1 Infected Antiretroviral-Naive individuals in Addis Ababa, Ethiopia

By:

Mulugeta Kiros

November 2019

Addis Ababa, Ethiopia

HIV-1 Genetic Diversity and Pre-treatment Drug Resistance Mutations among recently diagnosed HIV-1 Infected Antiretroviral-Naive individuals in Addis Ababa, Ethiopia

A thesis research submitted to the school of graduate studies of Addis Ababa University in partial fulfillment of the requirements for the degree of Master of Science in Medical Microbiology

By:

Mulugeta Kiros

Advisors:

Woldargay Erku Abegaz (Ph.D.), Associate Professor, AAU

Adane Mihret (Ph.D.), Associate Professor, AAU

Dawit Hailu (MSc), Assistant Researcher, AHRI

Andargachew Mulu (Ph.D.), Senior Researcher, AHRI

Collaborator:

Eleni Geberekidan (MSc), Associate Researcher, EPHI

November 2019

Addis Ababa, Ethiopia

Acknowledgments

First, I would like to thank and glorify the Almighty God who has been and will be the source of strength throughout my life. Secondly, I would like to thank Addis Ababa University College of health sciences, School of medicine, department of microbiology, parasitology and immunology for giving me this excellent opportunity to do this research. The same special thanks go to the Ethiopian Public Health Institute for the laboratory support and Armauer Hansen Research Institute for the budget allowance. I would like also to thank Debre Tabor University for the opportunity that they gave me.

My sincere gratefulness also goes to Woldeargay Erku, Ph.D. from Addis Ababa University School of medicine, college of health sciences, department of microbiology, parasitology and immunology, Andargachew Mulu, Ph.D., Adane Mihret, DVM, Ph.D., and Dawit Hilu, MSc from Armauer Hansen research institute for their unreserved help. In addition to this, I would like to thank the data collectors and study participants for their participation.

Lastly but not least I would like to thank all AHRI staff, my friends, colleagues and my beloved families for their unreserved help, encouragement and moral support throughout this research work.

Table of content

List of Abbreviations/Acronyms

AHRI	Armauer Hansen Research Institute
AIDS	Acquired Immuno Deficiency Syndrome
ALERT	All Africa Leprosy Rehabilitation and Training Center
ART	Antiretroviral Treatment
ARV	Antiretroviral Drugs
CCR5	C-C-chemokine Receptor type 5
CD	Cluster of Differentiation
CDC	Centers for Disease Control and Prevention
EDTA	Ethylene Diamine Tetra Acetic acid
EPHI	Ethiopian Public Health Institute
FIs	Fusion Inhibitors
FHAPCO	Federal HIV/AIDS Prevention and Control Office
GP	Glycoproteins
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
Stanford HIVdb	Stanford University HIV drug resistance database
INSTI	Integrase Strand Transfer Inhibitor
mL	milliliter
NRTI	Nucleoside reverse-transcriptase inhibitor
NNRTI	Nonnucleoside reverse-transcriptase inhibitor
PCR	Polymerase Chain Reaction
PDR	Pretreatment drug resistance
PDRM	Pretreatment drug resistance Mutation
PI	Protease inhibitor
PR	Protease
RT	Reverse transcriptase
RNA	Ribonucleic Acid
SIV	Simian Immunodeficiency virus
UNAIDS	Joint United Nations Program on HIV/AIDS
VCT	Voluntary Counseling Test

WHO	World Health Organization

List of tables

Contents Page

List of figures

Contents Page

Abstract

***Background**: Africa is a region hardest hit by the Human Immunodeficiency Virus (HIV) among other continents on the globe with the highest rise of the infection in the eastern and southern parts. Besides, the development of Pretreatment drug resistance (PDR) is becoming an obstacle to the success of antiretroviral therapy (ART). Despite ART scale-up, there is only limited information with regard to HIV-1 PDR in Ethiopia. Moreover, with increasing movement of people, HIV-1 variants other than the predominant subtype C may be introduced and intermixed from the neighboring countries. Therefore, this study was aimed to assess HIV-1 Genetic Diversity and Pre-treatment Drug Resistance Mutations among recently diagnosed HIV-1 Infected Antiretroviral-Naive individuals in Addis Ababa, Ethiopia.*

***Method**: Institutional based cross-sectional study was conducted from June 2018 up to December 2018 in Addis Ababa, Ethiopia. Plasma samples (n=72) from ART-naive study participants were collected for sequencing of partial HIV-1 pol region covering the complete protease (PR) and partial reverse transcriptase (RT) regions (nucleotides 2253 to 3539 of reference strain HXB2) using in-house assay. Both Stanford University HIV drug resistance database (Stanford HIVdb) and the International Antiviral Society-USA (IAS-USA) 2019 mutation list Algorithms were used to assess the presence of PDR mutations.*

***Result**: From 72 eligible plasma samples, 75% (54/72) of them were successfully amplified. Out of this, 51/54 (94.4%) were successfully sequenced and analyzed. According to the Stanford HIVdb and IAS-USA mutation list, 9.8% (5/51) of analyzed samples had at least one PDR Mutation. PDR mutations to Non-Nucleoside Reverse Transcriptase Inhibitor (NNRTIs) was the most frequently detected mutation (7.8% and 9.8%, according to Stanford HIVdb and IAS-USA Algorithm respectively) followed by Nucleoside Reverse Transcriptase Inhibitors (NRTIs) (1/51, 2% by both Algorithms) and Protease Inhibitors (PIs) (1/51, 2%, According to the Stanford HIVdb only). One individual had PDR mutations that confer resistance to NNRTI and NRTI simultaneously. In addition, a high rate of polymorphism was observed both in the PR and RT regions. With regard to HIV-1 genetic diversity, phylogenetic analysis showed that all 51/51 (100%) of the study participants were infected with subtype C virus.*

***Conclusion and Recommendations**: This study presents additional evidence for increased level of PDR and persistence HIV-1C clade homogeneity after 15 years of the rollout of ART and 3 decades of HIV-1C circulation in Ethiopia, respectively. Therefore, routine genotypic drug resistance testing is warranted for successful ART programs and the overall prevention of HIV transmission in the country to support the global efforts in achieving the third 90 of the UN target.*

***Keywords**: HIV, Genetic diversity, HIV drug resistance, HIV Subtype*

1. Introduction

1.1. Background

Human immunodeficiency virus (HIV), the etiologic agent of Acquired Immuno Deficiency Syndrome (AIDS), is a retrovirus that belongs to the family of *Retroviridae* genus *Lentivirus* (1). It is a rapidly evolving single-stranded positive-sense RNA Virus (1, 2) Based on genetic characteristics and differences in the viral antigens, HIV is classified into two genetically different types: types 1 and 2 (HIV-1, HIV-2) (3) which were both resulted due to cross-species transmissions of the simian immunodeficiency viruses (SIVs) (4). Phylogenetic analysis revealed that HIV -1 was evolved from chimpanzees (*Pan troglodytes troglodytes*) and HIV-2 from sooty mangabey monkey (*Cercocebus atys*) (5). HIV-1 is the most prevalent and pathogenic type than HIV-2 and is responsible for the vast majority of the global pandemic (6). It can be further classified into four groups; group M (Major), O (Outlier), N (non-M, non-O) and the most recent group P (4). In Ethiopia, HIV-1 was isolated for the first time in 1986, three years after the description of the virus in the US (7).

HIV Affects CD4 T cells (6) and causes HIV infection which is characterized by the following stages; primary viremia, chronic or asymptomatic phase, and AIDS (8). AIDS, the most advanced stage of HIV infection, was first described in United States (US) in 1981 (9) while in Ethiopia it was first documented in a young man and woman in 1984 in Addis Ababa (10). Since then, AIDS has become a worldwide epidemic, expanding in scope and magnitude affecting different populations and geographic regions (11). To date, HIV-1 is the major cause of AIDS in the world (12). HIV-1 shows greater genetic variability and rapid evolution which is resulted due to the high mutation and recombination rates of the reverse transcriptase enzyme, which lacks a proof-reading mechanism, along with high rates of viral replication (13). This poses a challenge to HIV prevention and vaccine development (13). Although there is no vaccine and cure for HIV, there are antiretroviral drugs for stopping viral replication to its undetectable state (14). In Ethiopia, antiretroviral therapy (ART) provision was started in 2005 (15).

1.2. Statement of the problem and justification for the study

HIV is a major global public health issue with millions of people infected worldwide (16). Many people have died since the start of the epidemic and it is still affecting so many people globally (17). Africa is the most affected region among other continents in the globe with the highest rise of the illness in the eastern and southern parts (18). Similarly, HIV/AIDS is among the top ten diseases in Ethiopia with high morbidity and mortality rate (19). Although the number of deaths due to the disease is decreasing due to ART scale-up, looking at the number of people newly infected with HIV, it is surging again since 2010 (20).

HIV/ AIDS leads to stigma, which negatively influences HIV care engagement (21-23). In children, HIV/ AIDS leads to School absenteeism due to their sick relatives, and they will be left emotionally and physically vulnerable by the illness due to the death of one or both parents and finally, they will become orphans. These effects lead to the decrement of human capital investment, further causing a decline in economic growth (24, 25). Similarly, due to the costs of illness, HIV declines the socioeconomic status of the victim (26). HIV/AIDS mortality causes a loss of productive labor time and a drop in household income. In addition, the morbidity associated with HIV/AIDS leads workers to be less attendant in work, which further leads them to be less productive (26, 27).

Although ART is contributing a lot in prolonging the life of HIV infected individuals (28), scaling up of this treatment option along with poor adherence and lack of drug resistance testing in resource-limited settings is paralleled by an increased prevalence of pretreatment drug resistance (PDR) (29, 30). This is becoming a significant obstacle in maintaining HIV replication suppression and leads to a higher probability of early virological failure (31) that further hinders the widespread use of ART (32, 33). Likewise, it is becoming a potential threat to the long-term success of ART and is emerging as a threat to the elimination of AIDS as a public health problem (34). Although it has been 15 years since the introduction of ART in the country only a few studies are done with regard to PDR with the majority of them being from the Northern part of Ethiopia (15, 35-37). However, there are only a few studies (38, 39) available with regard to PDR in the capital city of the country. Moreover, with increasing movement of people, HIV-1 variants other than the predominant subtype C may be introduced and intermixed from the neighboring countries.

Therefore, this study was aimed at generating useful information about genetic diversity and PDR magnitude in Addis Ababa that may be utilized in alleviating the problem treatment failure and consequently reducing the burden of the disease.

1.3. Significance of the Study

The finding of this study will provide information on the current magnitude of HIV-1 PDR, which will guide policymakers to prepare and/or update antiretroviral therapy guidelines, and develop effective, integrated testing for regional HIV-1 prevention and treatment programs. Likewise, the updated knowledge of PDR also helps to guide public health resource allocation and policy decisions with regard to treatment strategies and/or options that are currently available.

Similarly, updated data on HIV-1 genetic diversity helps control any new viral subtype introductions in the country. Besides, to the above-proposed implications, the result of this study can be used as recent information for those who are interested in the area. Moreover, understanding the genetic diversity and the magnitude of HIV-1 PDR in the population is crucial for setting national HIV control and prevention strategies to prevent the transmission of the virus.

.

2. Literature Review

2.1. HIV Epidemiology Globally

Since the start of the epidemic, 74.9 million [58.3 million–98.1 million] people have become infected with HIV while 32.0 million [23.6 million–43.8 million] people have died from AIDS-related illnesses (16). In 2018, there were 37.9 million [32.7 million–44.0 million] people living with HIV globally. Among this 36.2 million [31.3 million–42.0 million] of them are adults while 1.7 million [1.3 million–2.2 million] of them are children (<15 years). In the same year, 1.7 million [1.4 million–2.3 million] people became newly infected with HIV and 770,000 [570,000–1.1 million] people had died from AIDS-related illnesses (16).

As the World Health Organization has reported (40), the African region is the most affected region, with 25.7 million people living with HIV. This accounts for almost two-thirds of the global total of HIV infected people. Among these people living with HIV in Africa, 20.6 million of them are reported from eastern and southern Africa.

In Eastern and Southern Africa, which is the home of 54% of the world's people living with HIV (40), there were an estimated 800,000 [620,000 – 1,000 000] new HIV infections which account around 47% of the total global new infection and 310,000 [230,000– 400,000] people died from AIDS-related illness in the same year (40).

2.2. HIV Epidemiology in Ethiopia

HIV/AIDS is among the top five leading causes of age-standardized premature mortality and death rates in Ethiopia (18). In 2018, there were an estimated 23,000 new infections and 11,000 AIDS-related deaths (40) which showed a dramatic increment from the previous year which was 15,000 new infections and 16,000 AIDS-related deaths (20). There are an estimated 690, 000 [530 000–900 000] people living with HIV in the country (40).

According to data from the Federal HIV/AIDS Prevention and Control Office (FHAPCO), the current HIV prevalence in the country is 0.9%. Looking at HIV prevalence by region, Gambella ranks first (4.8 %), followed by Addis Ababa (3.4%), Dire Dawa (2.5%), and Harari (2.4%) (41). The prevalence is seven times higher in urban areas than in rural areas (41).

2.3. HIV-1 Structure and Genetic Diversity

An HIV virion has an icosahedral structure and measures a diameter of 120 nm (42, 43). It is composed of two identical single strands of positive-sense RNA which measures ~ 9.8 kb in size (1, 44) (Figure 1). In the direction, 5' to 3' the reading frame, the HIV genome contains nine genes that encode fifteen viral proteins (44) (Figure 2). Three major genes, gag, pol and env, code for structural proteins, enzymes and envelope proteins respectively (3, 45). The structural proteins include; outer core membrane (MA, p17), the capsid protein (CA, p24), the nucleocapsid (NC, p7) and a smaller, nucleic acid-stabilizing protein (3). Enzymes like protease (PR, p12), reverse transcriptase (RT, p51) and RNase H (p15) or RT plus RNase H (together p66) and integrase (IN, p32) encoded by the pol reading frame (3). Adjacent to the pol gene follows the env reading frame from which the two envelope glycoproteins gp120 (surface protein, SU) and gp41 (transmembrane protein, TM) are derived (3). The remaining genes code for regulatory (Tat, Rev) and accessory proteins (Vif, Vpr, Vpu, Nef) (44).

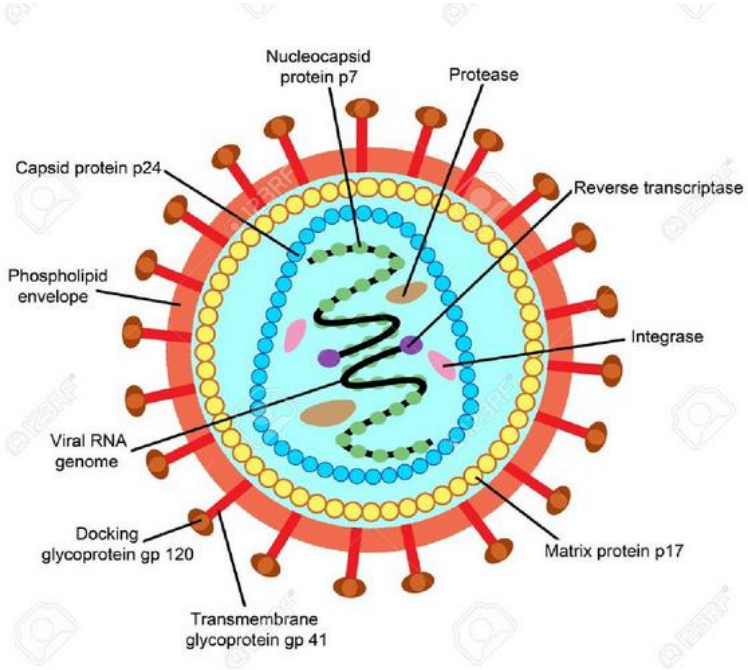

Figure 1: Structure of HIV-1

(Source: https://www.123rf.com/photo_18649988_structure-of-human-immunodeficiency-virus-hiv-illustration-for-basic-medical-education-for-clinics-a.html?fromid=Y083blZiN3R3S2EwMTNiUWNIeVl5QT09)

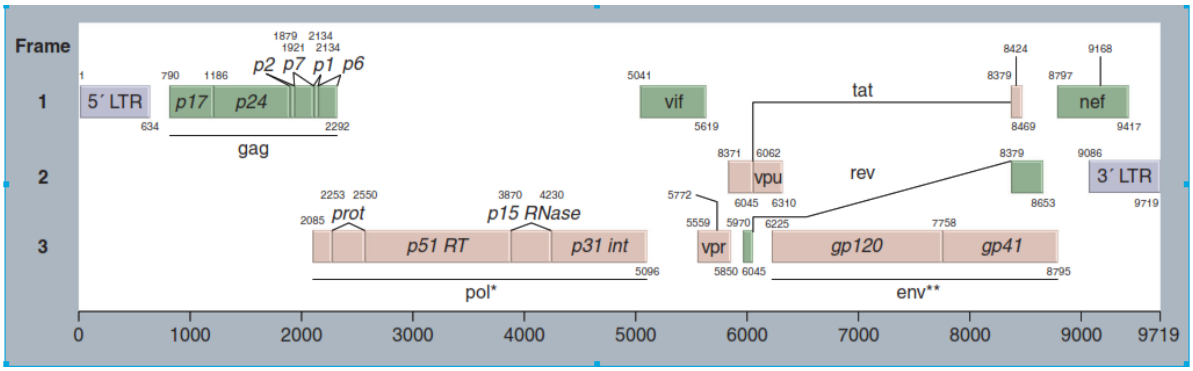

Figure 2. HIV-1 Genome organization.

Source: https://www.hiv.lanl.gov/content/sequence/HIV/MAP/landmark.html.

Based on *env* gene sequences, HIV-1 is classified into four distinct virus groups (M, N, O, and P); among these groups, group M is the predominant one and it is responsible for HIV pandemic making up more than 97 % of HIV infections worldwide (46). It contains at least 9 subtypes or "clades" A-D, F-H and J-K (A–J) (47, 48). Additionally, different subtypes can combine genetic material to form a hybrid virus, known as a 'circulating recombinant form' (CRFs). Around 101 of these are known to exist (49). These subtypes have varied geographical distribution (Figure 3). Type B is the main clade in the USA, type C in Southern Africa, while in East Africa, A and D are the most common viral subtypes (50). In contrary to neighboring countries, HIV-1 subtype C is the most common viral subtype in Ethiopia (51, 52). Within the Ethiopian HIV-1C, two distinct subtype C strains and/or sub-clusters (designated main C and C') are co-circulating in the country (53).

Each of these subtypes discussed above shows extensive variability between them (47). Intermixing of these viral subtypes leads to the expansion of novel recombinant mosaics to different regions (54).

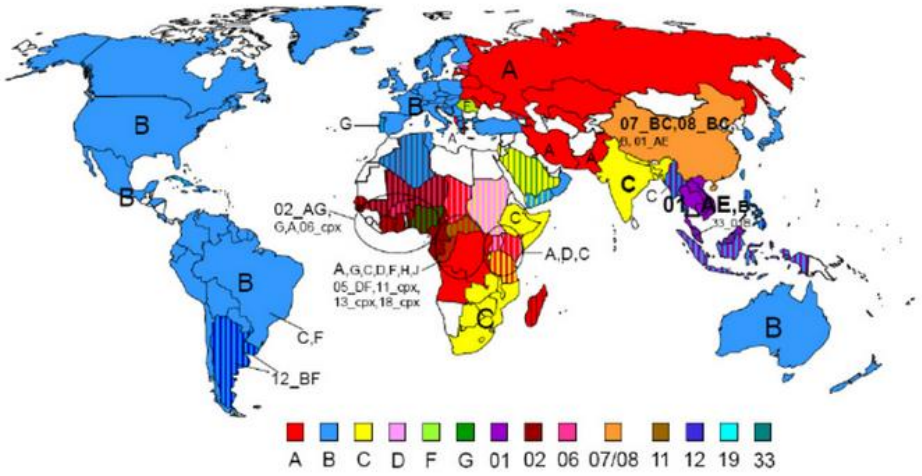

Figure 3: Global geographic distribution of HIV-1 subtypes and recombinant forms (55)

2.4. HIV-1 Life Cycle, pathogenesis, and transmission

HIV replication comprises the following sequential steps: the first step is attachment of HIV-1 envelope glycoprotein gp120 to the host cells expressing CD4 (cluster of differentiation 4) marker and/or co-receptors such as C-C chemokine receptor type 5 (CCR5; R5) and C-X-C chemokine receptor type 4 (CXCR4; X4) (56). This virus-cell receptor binding leads to exposure of hydrophobic regions of viral glycoprotein gp41 and insertion of the fusion peptide into the host cell membrane that again leads to translocation of the viral capsid into the cytoplasm (6). HIV-1 capsid entry is another crucial step in viral replication, which further leads to reverse transcription of viral RNA into double-stranded DNA via reverse transcriptase enzyme. This DNA is transported via nucleopores into the cell nucleus and is incorporated into the human host cell genome by integrase and become proviral DNA (57). This is followed by transcription, from integrated provirus of large mRNA that translates into a large protein inside the cytoplasm; and a genomic positive-sense RNA. The large protein is cleaved into the component proteins with the help of the enzyme protease that comes into the host cell along with the virion. Assembly of the newly produced viral proteins and genomic RNA occur in the cytoplasm, and the new virus particle is released by budding from the cell surface (58).

This process will be repeated so many times and leads to the elimination of infected T lymphocytes within days from the blood of an HIV-infected person via either cytotoxic HIV components and/or lysis of virus-producing cells or by cytotoxic T lymphocytes (3). This continuous loss of T helper lymphocytes results in immunodeficiency and makes it harder and harder for the body to fight off infections and some other diseases. Opportunistic infections or cancers take advantage of a very weak immune system and signal that the person has AIDS (3, 59).

HIV can be transmitted via the exchange of a variety of body fluids from infected individuals, such as blood, breast milk, semen, and vaginal secretions. Individuals cannot become infected through ordinary day-to-day contact such as kissing, hugging, shaking hands, or sharing personal objects, food or water (59, 60).

2.5. HIV-1 Antiretroviral drugs, drug resistance, and drug resistance assay

2.5.1. HIV-1 Antiretroviral drugs

Although HIV has no cure (2), by using antiretroviral drugs it is possible to control the replication of the virus and prolong the life of people living with HIV (11). Antiretroviral drugs inhibit the replication of the virus and lead to alteration of key viral replication steps (61). To date, there are 25 Food and Drug Administration (FDA) approved drugs are available for the treatment of HIV-1 infections (62). These drugs are classified into six classes; nucleoside analogue reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), entry/ fusion inhibitors (FIs), C-C-chemokine receptor type 5 (CCR5) antagonists, and integrase strand transfer inhibitors (INSTIs). The following figure (Figure 4) illustrates the mechanisms of how the above listed antiretroviral drug classes work.

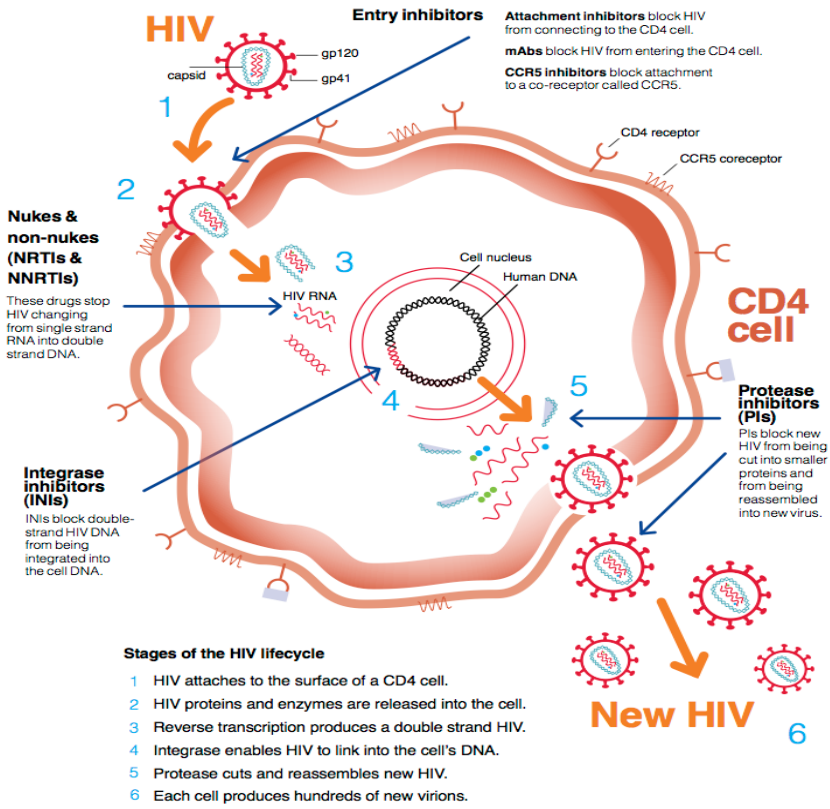

Figure 4: HIV-1 life cycle and the mechanism of action of antiretroviral drugs

(Source: http://i-base.info/htb/wp-content/uploads/2017/07/Viral-lifecyle-and-drug-targets-slide-2017-1024x729.png)

Currently, HIV-1 is treated using combination therapy of three or more active substances in a regimen designated as highly active antiretroviral therapy (HAART) (47, 61). World Health Organization (WHO) recommends INSTIs combined with two nucleoside reverse-transcriptase inhibitors (NRTIs) as a first-line drug. For those individuals with a first-line regimen failure, a boosted PI-containing regimen should be used as a second-line treatment (63). In Ethiopia, the publicly funded ART program was started in 2005 and the number of people using ART has increased since then (15, 64). In 2016, there were 420,000 people receiving ART (20). Below the table (Table 1) summarizes the current treatment guidelines in Ethiopia.

Table 1: Currently preferred first-, second- and third-line ART regimens for Adolescents and adults in Ethiopia

Population	1st line regimens	2nd line regimens	3rd line regimens
Adults , adolescents , Pregnant/breastfeeding women	*AZT+3TC + EFV/NVP*	*TDF+3TC + ATV/r or LPV/r*	*DRV/r [o] + DTG (or RAL) ± ABC*
	TDF+3TC+EFV/NVP	*AZT+3TC + ATV/r or LPV/r*	*DRV/r [o] + DTG (or RAL) ± ABC*
	ABC+3TC+EFV/NVP	*AZT+3TC + ATV/r or LPV/r*	*DRV/r [o] + DTG (or RAL) ± TDF*

(Federal Ministry of Health, 2017)

2.5.2. HIV-1 Drug resistance

Although the global scale-up of ART has led to dramatic reductions in HIV-1 mortality and incidence, the development of HIV-1 drug resistance poses a potential threat to the long-term success of ART (65). Due to the high error rate and lack of proofreading ability of HIV-1 reverse transcriptase, HIV mutates easily, and this enables the virus to acquire mutations that allow resistance to anti-HIV drugs (66). HIV-1 drug resistance can be acquired (developing in a person receiving the antiretroviral treatment) or transmitted (occurring because a virus with drug-resistance mutations was transmitted to a drug-naive person (67). Although both acquired and transmitted HIV-1 drug resistance are public health concerns, PDR has the potential to be more rapidly reverse the effectiveness of first-line antiretroviral therapy at the population level (67).

PDR is an emerging phenomenon with important clinical and public health implications (68). In the US, the first data on PDR was documented in 1999 and the prevalence of any PDR mutation was 16.3% and the individuals were predominantly Men Sex with Men (68). In Africa, PDR prevalence ranges from low (4%) in Uganda (69) to high (15.9%) in Nigeria (70). Until recently, the prevalence of PDR was lower in Ethiopia (0 – 4.9 %) (38, 52, 71). However, increments have been observed in recent reports from the northern part of the country (36, 72, 73) while there is limited data in central Ethiopia.

2.5.2.1. Mechanisms of HIV-1 Drug Resistance

2.5.2.1.1. Mechanism of resistance to NNRTI

NNRTI drugs like Nevirapine, Efavirenz, Etravirine, Rilpivirine, etc. work by binding to an allosteric site, a pocket near the active site of the viral RT enzyme and induce conformational changes that subsequently result in inhibition of DNA synthesis. However, in the presence of mutations like L100I, K101E/P, K103N/S, V106A/M, E138K, Y181I/V, Y188L/C/H, G190A/S/E, M230L, etc., this will not happen because these mutations cause conformational change of the pocket so that the NNRTIs will not bind to the target allosteric site anymore (74).

2.5.2.1.2. Mechanism of resistance to NRTI

These drugs are artificial analogues of naturally occurring deoxynucleotides and due to lack of a 3'-hydroxyl group or an altered sugar moiety, once incorporated into the growing chain of viral DNA they result in premature chain termination (74). However, in the presence of mutations like M41L, D67N, K70R, L210W, T215F/Y, and K219E/Q in the RT gene results in the removal of chain terminators or nucleotide excision by pyrophosphate-dependent hydrolysis (pyrophosphorolysis) (75). Similarly, other mutations like K65R, L74V, Q151M, Y115F and M184V/I in this gene confers the RT enzyme to an ability to discriminate between naturally occurring deoxynucleotides and artificial analogues (74).

2.5.2.1.3. Mechanism of resistance to PIs

In contrast to NNRTIs, PIs bind to the active site of the protease enzyme and inhibit the function of the enzyme by competitively occupying and preventing the natural substrate protein from accessing the active site. However, in the presence of the mutations like G48M, I54M, V82A/F, L90M, etc., this competitive binding does not happen because the mutation in the PI gene causes conformational change in the active site of this enzyme and this subsequently results in widening of the enzyme pocket so that the PIs will not bind anymore. Unfortunately, this widening of the active site does not have any effect on the enzyme activity (74).

2.5.2.2. HIV-1 Drug resistance assay

HIV-1 drug resistance testing can be performed phenotypically or genotypically (76). Phenotypic resistance testing refers to in vitro susceptibility assays that measure ARV susceptibility in the cell culture while genotypic resistance testing involves the sequencing of enzyme coding regions to identify established clinically significant drug-resistant mutations (76). The phenotypic assay is a preferred assay for the detection of new mutations that arise in a particular HIV variant since genotypic testing can only identify documented HIV mutations and are not able to identify new mutations (77). In addition, this assay does have a comparable ability with old genotypic assays like population-based Sanger sequencing in detecting mutations of minority variants (78). However, this assay is time-consuming, expensive and not as sensitive as those recently developed advanced genotypic assays like next-generation sequencing that can detect mutations in viral populations that are present in less than 1 % (78-80). This makes it hard to be chosen as the preferred test by different laboratories. On the contrary, genotypic assays are highly reproducible and highly sensitive. In addition, genotypic resistance test results provide molecular epidemiological data (76).

In this study, we used population-based Sanger sequencing genotypic assays to detect the drug resistance mutations.

3. Objectives

3.1. General Objective

- To assess HIV-1 Genetic Diversity and Pre-treatment Drug Resistance Mutations among recently diagnosed HIV-1 Infected Antiretroviral-Naive individuals in Addis Ababa, Ethiopia

3.2. Specific Objectives

- To determine the magnitude of pre-treatment non-nucleoside reverse-transcriptase inhibitor-associated drug resistance mutations
- To determine the magnitude of pre-treatment nucleoside reverse-transcriptase inhibitor-associated drug resistance mutations
- To determine the magnitude of pre-treatment protease inhibitor-associated drug resistance mutations
- To genetically characterize circulating HIV-1 strains

4. Methods and Materials

4.1. Study Area and design

Institutional based cross-sectional study was conducted in Addis Ababa, which is the capital city of Ethiopia. Based on the 2007 census conducted by the central statistical agency of Ethiopia, Addis Ababa has a total population of 2,739,551, of whom 1,305,387 are male; and all of the populations are urban inhabitants (81).

The study was conducted by collecting serum specimens from participants visiting Voluntary Counseling Test (VCT) centers of the following hospitals: Alert Hospital (Kolfe), woreda 11 heath center (Nifas-Silk), Akaki health center (Akaki Kality), and Addis Ketema Health center (Addis Ketema). These sites were selected based on previous data (HIV positive reports from VCT) from the Addis Ababa health bureau, which showed that HIV positive results from these sites were higher.

4.2. Study Period

The study was conducted from June 2018 up to December 2018.

4.3. Population

4.3.1. Source Population

The source population for this study were all HIV infected individuals in Addis Ababa.

4.3.2. Study Population

The study population was all ART naïve HIV infected individuals visiting VCT centers who reported their ART status as ART-naive or who were identified by caregivers as ART-naive during the time of sample collection.

4.4. Sample size calculation and sampling methods

For a single population proportion using the following assumptions.

Where n = sample size

Z (α / 2) (statistic for a level of confidence) =1.96, which is the upper percentile of the standard normal distribution

P (proportion) = 4.9 %, which is the prevalence of PDR in Addis Ababa (Ph.D. dissertation)

d (precision) = 5 %, difference from the actual figures of source population or margin of error.

The sample size was calculated using the following formula:

$$n = \left(Z\frac{\alpha}{2}\right)^2 \frac{p\,(1-p)}{d^2} \qquad n = (1.96)^2 \frac{0.049\ (1-0.049)}{0.05^2} = 71.60$$, which is approximately **72**.

4.5. Eligibility criteria

4.5.1. Inclusion criteria

- Being ART naïve
- Age ≥ 18 years old

4.5.2. Exclusion criteria

- Unwilling to participate

4.6. Study variables

4.6.1. Dependent variables

- HIV-1 Genetic diversity
- HIV-1 PDR drug resistance

4.6.2. Independent variables

- Socio-demographic characteristics like Age, Sex, occupation, etc.
- Viral load

4.7. Operational definitions

Pretreatment drug resistance (PDR): Resistance to one or more antiretroviral drugs found in individuals with no history of drug exposure.

Viral load: The amount or concentration of HIV-1 RNA in a sample of blood (copies/ml).

4.8. Data Collection Tools and Techniques

4.8.1. Demographic data collection

A structured questionnaire was used to collect Socio-demographic data like Age, Sex, occupation, etc. of study participants.

4.8.2. Blood Sample Collection and Transport

Trained medical personnel collected a blood sample (10 mL) from study participants aseptically. Vacutainer test tubes containing ethylene diamine tetra-acetic acid (EDTA) were used to collect the blood samples from the vein of each study participant. The sample was labeled with appropriate patient information. Plasma was then separated from blood cells in the 10 mL tube within two hours in the laboratory by centrifugation at the speed of 1200g (3000 rpm) for 10 to 15 minutes in accordance with the recommendation of WHO (2009). The plasma was aliquotted into Nunc tubes of 1.5-ml capacity, and transported on the same day to the AHRI laboratory, where it was stored at -80^0C until required for molecular analysis.

4.9. Laboratory investigations

4.9.1. Viral load and RNA extraction

HIV-1 RNA extraction and viral quantification were done by Abbott Real-time HIV-1 M2000rt extraction machine (Abbott Laboratories, Abbott Park, USA) at Ethiopian Public Health Institute (EPHI), WHO accredited National HIV laboratory in Addis Ababa. From each participant's plasma, 200 µL was automatically pipetted for RNA extraction. This yielded a total of 60-µL RNA extract. From this, 50 µL was used for viral quantification procedure while the remaining 10 µL of the elute was transported to AHRI and used for cDNA synthesis.

4.9.2. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA for the partial polymerase (pol) gene including the PR/RT region was performed in a 20 microliter reaction mixture using superscript IV Reverse Transcriptase enzyme and HIVrt primer (5'-TGTTTTACATCATTAGTGTG-3', HXB2 location: 3630-3649). The thermal cycling for cDNA synthesis was; 50 °C for 1 hour (36). After cDNA was synthesized, we used Platinum Taq High Fidelity (Invitrogen, Carlsbad, CA, USA) polymerase enzyme and two in-house outer primers (HIVpcrFor1: 5'-TGATGACAGCATGTCAGGGAGTGG-3', HXB2 location 1826–1849) and HIVpcrRev1: 5'-GGCTCTTGATAAATTTGATATGTCCATTG-3', HXB2 location 3555–3583) for this first-round PCR. The reaction was performed in a 50 microliter reaction mixture and the cycling conditions used were initial denaturation at 94 °C for 2 min, 35 cycles of 30 sec at 94 °C, 1 min at 54 °C, 1 min at 72 °C followed by a final extension for 5 min at 72 °C (36). This yields 1757 bp amplicon.

4.9.3. Nested PCR and DNA purification

Second-round PCR was performed to re-amplify an amplicon from first-round PCR. This was carried out using High-Fidelity Taq polymerase (Life Technologies, USA) and two inner in-house primers: HIVpcrFor2: 5'-AGCCAACAGCCCCACCAG-3', HXB2 location 2150–2167) and HIVpcrRev2: 5'-CTGTATTTCTGCTATTAAGTCTTTTG-3', HXB2 location 3514–3539). The reaction was performed in a 50 microliter reaction mixture like the first-round PCR and the thermal cycling consisted of initial denaturation at 94 °C for 2 min, 35 cycles of 30 sec at 94 °C, 1 min at 54 °C, 1 min at 72 °C followed by a final extension for 5 min at 72 °C (36). Agarose gel electrophoresis (using 1.5% agarose gel) was then performed to confirm the final amplified PCR product (1389 bp; Figure 5) (36). This was followed by DNA purification using GeneJET Gel Extraction and DNA Cleanup Micro Kit (Thermo Fisher Scientific, Inc., United States), following the manufacturer's instruction. The Quality and concentration of purified DNA were checked using both NanoDrop and gel electrophoresis.

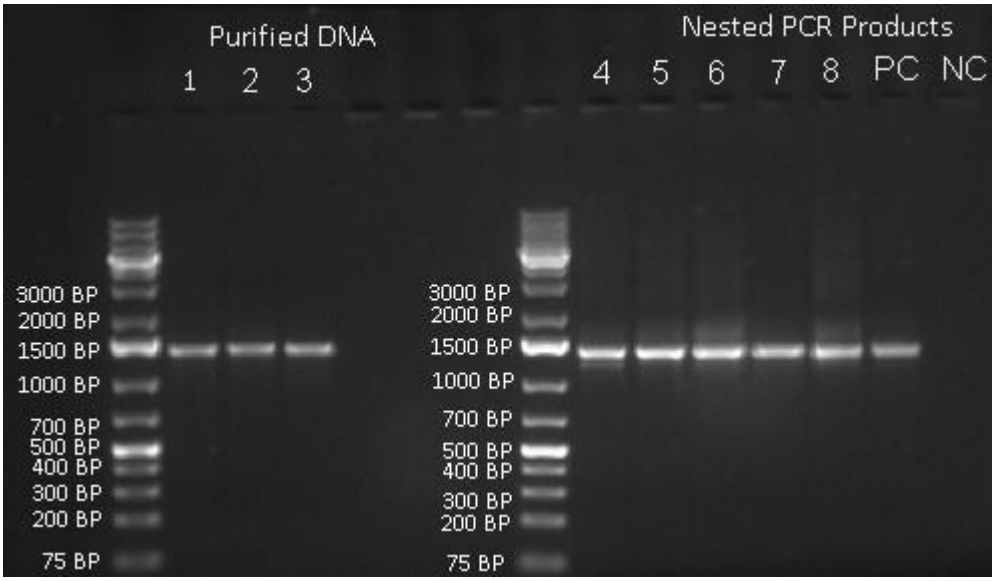

Figure 5: Agarose gel electrophoresis of PCR products from eight representative samples

(Lane 1 – 3 represents purified DNA samples while Lane 4 – 8 represents Nested PCR products). Lane 9 and 10 are controls (i.e. Lane 9 positive control and Lane 10 Negative control)

4.9.4. DNA sequencing

Cycle sequencing reaction (by Sanger sequencing method) for each purified DNA sample was performed using Big Dye Terminator Cycle Sequencing Ready Reaction mix v.3.1 (Applied Biosystems, USA) and four in-house inner primers (HIVpcrFor2: 5'-AGCCAACAGCCCCACCAG-3', HXB2 location 2150–2167, HIVSeq1: 5'-GTTAAACAATGGCCATTGACAGA-3', HXB2 location 2610–2632, HIVSeq4: 5'-CCATCCCTGTGGAAGCACATT -3', HXB2 location 2988–3008, HIVpcrRev2: 5'-CTGTATTTCTGCTATTAAGTCTTTTG -3', HXB2 location, 3514–3539). The thermal cycling consisted of initial denaturation at 96 °C for 2 min, 39 cycles of 30 sec at 96 °C, 15 sec at 56 °C, 5 min at 60 °C and a final extension for 5 min at 60 °C (36). Following Cycle sequencing, the excess dye terminators were removed using DyeEx 2.0 Spin Kit (Qiagen, Germany) following the manufacturer's instruction, further dried using vacuum centrifuge, and stored at 20°C until sequenced. These dried DNA samples were transported to the EPHI, for further processing. Upon arrival, the DNA samples were treated with 20 µl of formamide and subsequently processed with an automated ABI 3500 xL Genetic Analyzer (Applied Biosystems).

4.10. Data processing and analysis

4.10.1. Data Management and Quality Assurance

The questionnaire was checked for spelling errors and completeness of the response given by participants before data entry to ensure quality of the result. In addition, Visual inspection of the reagent bottles and expiration date checks were performed before each laboratory work. The lab procedures were conducted in separately designated laboratory spaces for quality control purposes. Both positive and negative controls were used for each laboratory work. Overall, standard operating procedures will be strictly followed to ensure the quality of the test result and to maintain high standard of accuracy of the test result. Quality of sequence data was checked using an online data management and quality assurance tool found in the Stanford University HIV drug resistance database (http://Stanford HIVdb.stanford.edu).

4.10.2. Statistical analyses

Demographic and clinical data recorded from each participant questionnaire response (age, gender, etc.) were checked for completeness and entered into Epi data v3.1 software and exported to SPSS version 25.0 (SPSS Inc. the United States) for analysis. Logistic regression was used to assess the associations between PDR and demographic or virological characteristics. A p-value < 0.05 was considered significant. Drug resistance mutations for PI, NRTI, and NNRTIs were characterized by their frequency and percentages.

4.10.3. Sequence Analysis

4.10.3.1. Sequence editing, alignment, and subtype determination

SeqA5.4 software, which was contained in the ABI PRISM® 3500 xL Genetic Analyzer (Applied Biosystems) collects, processes, and stores the data automatically after each run. Then sequences were exported to other computer and were first edited using chromas software v.2.6.6 (http://technelysium.com.au/wp/chromaspro/) (Figure. 6) and Geneious prime® v.2019.2.1 (https://www.geneious.com/academic/). Then the fragment sequences were aligned using Geneious prime® v.2019.2.1 software (https://www.geneious.com/academic/) (Figure. 7). Then HIV-1 subtype determination was done using the REGA HIV subtyping tool (Leuven University, Leuven, Belgium; https://www.genomedetective.com/app/typingtool/hiv).

4.10.3.2. Pretreatment drug resistance determination

PDR determination was performed using the Stanford Genotypic Resistance calibrated population resistance (CPR) tool version 6.0 contained in the Stanford HIVdb (http://Stanford HIVdb.stanford.edu) algorithm and the IAS-USA 2019 mutation list. Classification of PDR level (low: < 5%, moderate: 5–15%, or high: >15%) was made based on the WHO threshold survey protocol (82).

4.11. Ethical Approval

Ethical approval was obtained from Addis Ababa University, College of health sciences, department of Medical Microbiology, Immunology and Parasitology research and ethical review committee (DRERC) (Reference Number: DERC//17/18/ 02-H), and Armauer Hansen Research Institute (AHRI)/ All Africa Leprosy Rehabilitation and Training Center (ALERT)

ethical review committee (Protocol Number: PO16/18). Written informed consent was obtained from adult HIV infected individuals before including them to the study. Samples were coded in order to keep the confidentiality of the study participant.

5. Result

5.1. Sociodemographic characteristics of study participants

Out of the 72 antiretroviral-naive, individuals who had plasma samples available for RNA extraction, 54 were PR/RT amplified (75%); partial HIV-1 pol sequence covering the complete PR and partial RT regions (nucleotides 2253 to 3539 of reference strain HXB2) were amplified. Out of 54 amplified samples, 51 (94.4%) were successfully sequenced and analyzed (Figure 6 and 7).

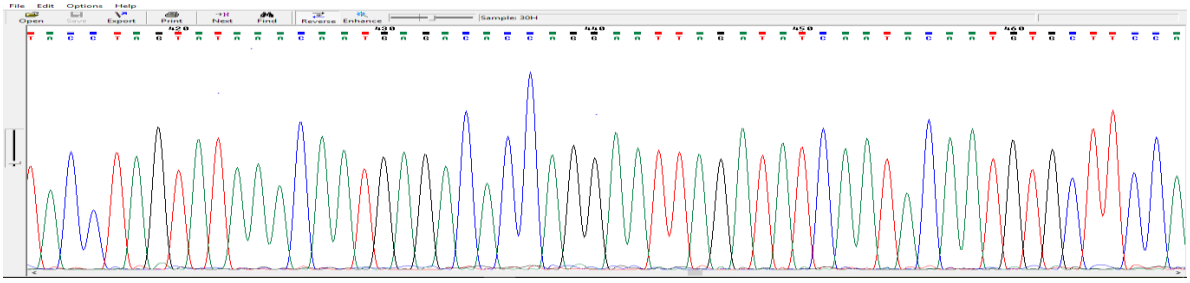

Figure 6: A representative Screenshot of chromas software v.2.6.6 edited sequence fragment

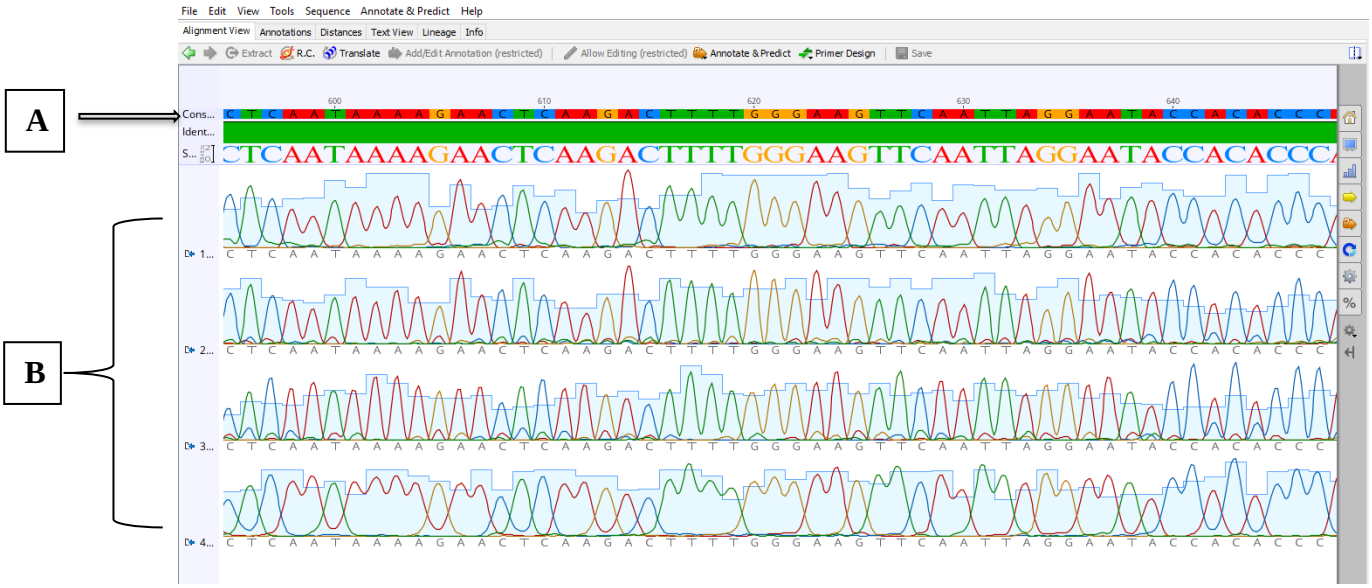

Figure 7: A representative Screenshot of Geneious prime® v.2019.2.1 software aligned sequence that was constructed from ABI PRISM® 3500 xL Genetic Analyzer produced overlapping fragments

'A' represents the final consensus sequence while 'B' represents each fragment

Of those 51 studied subjects, 54.9% were female. The average age of study participants is 37 years. Most (80.4%) of the study participants reported 12 years and/or less than 12 years of schooling as shown in the table (Table 2). With regard to viral load, 58.8 % of study participants had > 100, 0000 copies/ml (Table 2). No significant associations were observed between participant characteristics and Pretreatment drug resistance Mutations (PDRMs) (Table 2).

Table 2: Sociodemographic and virological characteristics and their bivariate analysis of included study participants

Characteristics	Frequency (N)	Percentage (%)	Individuals with PDRM (N)	Bivariate Analysis P-Value
Sex				
Male	23	45.1	2	R
Female	28	54.9	4	0.81
Age Category				
18-28	23	45.1	2	0.99
29-38	17	33.3	2	0.99
39-48	8	15.7	2	0.99
>49	3	5.9	0	R
Baseline Viral load (Copies/ml)				
2000-10000	2	3.9	0	R
10001-100000	19	37.3	3	0.99
≥100000	30	58.8	3	0.95
Occupation				
Unemployed	31	60.8	4	R
Employed	20	31.2	2	0.97
Marital status				
Married	19	37.3	1	R
Single	14	27.5	1	0.99
Divorced	14	27.5	4	0.96
Widowed/widower	4	7.8	0	0.99
Educational status				
No schooling	8	15.7	0	0.99
Primary	15	25.4	3	0.95
Secondary	18	35.3	2	0.82
College (diploma)	3	5.9	1	0.99
University degree	7	13.7	1	R

5.2. The magnitude of Pretreatment drug resistance

According to both the Stanford HIVdb and IAS-USA algorithms, 9.8% (5/51) of analyzed samples had at least one PDR Mutation. PDR mutations to NNRTIs were the most frequently detected mutations (7.8% and 9.8%, according to Stanford HIVdb and IAS-USA mutation list respectively) followed by NRTIs (1/51, 1.96% by both criteria's) and PIs (1/51, 1.96%, according to the Stanford HIVdb only). Simultaneous resistance to NNRTI and NRTI was observed in 1/51, 1.96% patient.

NNRTIs resistance-associated mutations K103N in one patient (1.96%), Y188L and H221Y in another patient (1.96%), K101E in one patient (1.96%), and V106A in another patient (1.96%) were presented by both criteria. L234I, which confer major resistance to NNRTIs was seen in one individual (1.96%) according to Stanford HIVdb only (Table 3). On the contrary, E138A mutation, which is associated with reduced RPV and ETR susceptibility was detected by the IAS-USA mutation list only in two individuals (3.92%) (Table 3).

Y115F and M184V PDR mutations that confer limited resistance against NRTI drugs (abacavir, emtricitabine, lamivudine) were observed in one patient (1.96%) by both criteria (Table 3). G75S PDR mutation that confers low-level resistance to PI drug class atazanavir/ritonavir was observed in another study participant which confer limited resistance against NRTI drugs (abacavir, emtricitabine, lamivudine) according to Stanford HIVdb only (Table 3).

Table 3: Pretreatment drug resistance mutations detected and their resistance pattern to common Drugs

Sample ID	Age/Sex	Viral Load (copies/ml)	Mutations type					
			NNRTIs	Resistant to	NRTIs	Resistant to	PIs	Resistant to
AL-078	32/F	134,263	None		None		G73S*	**ATV/r[a]**
NI-033A	37/F	124,246	**K103N**	EFV[c], NVP[c]	None		None	
AL-074	45/M	19,652	**Y188L, H221Y**, L234I*	ETR[a], DOR[c], EFV[c], NVP[c], RPV[c]	**Y115F, M184V**	ABC[c], FTC[c], 3TC[c]	None	
AL-082	40/M	443,919	**K101E**	ETR[a], EFV[a],NVP[b], RPV[b], DOR[a]	None		None	
AK-135	25/F	66,791	**V106A,** *E138A*	ETR[a], DOR[c], EFV[b], NVP[c], RPV[a]	None		None	
PA-146D	27/F	14,498	*E138A*	ETR[a], RPV[a]	None		None	

F = Female, M= Male, [a]low-level resistance, [b]Intermediate-level drug resistance, [c]High-level drug resistance, ATV/r = Atazanavir/ritonavir, ABC = Abacavir, FTC = Emtricitabine**,** 3TC = Lamivudine, DOR = Doravirine, EFV = Efavirenz**,** ETR = Etravirine**,** NVP = Nevirapine**,** RPV = Rilpivirine, NRTI = nucleoside reverse transcriptase inhibitor; NNRTI = non-NRTI; PI = protease inhibitor.

Mutations in bold, are PDRMs by both IAS-USA and Stanford HIVdb algorithm; Mutation in Italics are reported by IAS-USA only. While indicated by * only detected by Stanford HIVdb algorithm.

In addition to the above-mentioned major mutations, several additional minor drug-related mutations and/or polymorphisms were also observed on the RT and PR sequences based on both algorithms (Table. 5). In the current study, viruses with such minor drug-related mutations and/or polymorphisms were present in all participants. Single accessory resistance mutations and/or polymorphisms were present in over 15.6 % (n=8) of RT sequences with A98S (17.6%) and V179I (3.9%) being the most frequently observed mutations while all PR sequences harbored at least two minor PIs resistance mutations and/or polymorphisms (Table.

5). The most frequent mutations observed in the PR sequence were H69K (100%) and M36L (98%), followed by L89M (58.8%), I15V (25.5%), K20R (25.5%), T74S (17.6%) and L89I (5.88%) (Table 4).

Table 4: Minor drug-resistant mutations and/or polymorphisms

Drug Class	Minor drug-resistant mutations and/or polymorphisms	Frequency (N)	Percentage (%)
NNRTIs	A98S	9	17.64
	V179I	2	3.92
	K238R	1	1.96
	K103R	1	1.96
	K101R	1	1.96
NRTIs	T69S	1	1.96
	A62V	1	1.96
	E44D	1	1.96
PIs	M36L	51	100
	H69K/S	50/1	98.04/1.96
	L89M/I	30/3	58.82/5.88
	I15V	13	25.49
	K20R/T	13/1	25.49/1.96
	I64V	1	1.96
	T74S/A	9/1	17.64/1.96
	V82I	1	1.96
	A71T	2	3.9
	K20K/R	1	1.96
	V11I	1	1.96
	L10I/V	2/1	3.92/1.96
	G16E	1	1.96
	E35D	1	1.96
	L33V	1	1.96
	I62V	1	1.96
	K43R	1	1.96

5.3. HIV-1 genetic diversity

With regard to HIV-1 genetic diversity, phylogenetic analysis showed that all 51/51 (100%) of the study participants were infected with HIV -1 subtype C virus.

6. Discussion

Drug resistance poses a challenge for viral suppression, which in turn jeopardizes prevention plan against HIV infection (83). In this study, we sequenced the partial pol gene from supposedly ART naïve HIV infected individuals to show the current viral diversity and the detection rate of ARV resistance mutations in Addis Ababa, Ethiopia. The finding of this study indicates that all of the study participants were infected with the Subtype C virus. In agreement with previously published studies (35, 36), this study indicated the HIV-1 subtype C virus still dominates the HIV-1 epidemic in Ethiopia. Therefore, this is the latest evidence, which showed persistence HIV-1C clade homogeneity in the country.

WHO categorizes PDR in to three: low (<5%), moderate (5%–15%) or high (>15%) categories (82). Based on this PDR classification, this study revealed that the prevalence of PDR 15 years after the rollout of ART is moderate (9.8%) in the study area but with an increased level of resistance. In contrast to this finding, low levels of PDR among pregnant women (38) and among ART naïve adult individuals (71) were reported previously from the same study area. Similarly, a study done in Brazil among ART naïve pregnant women indicated a lower PDR detection rate (84). The absence of PDRM screening before starting ART and poor level of adherence might have contributed to this increment of PDR in the current study. In agreement with the finding of this study a moderate level (5.6 – 7.2 %) of PDR among HIV-1 ART-naive individuals was reported from Northern Ethiopia (15, 35, 36). This study, combined with all the above previously published reports in Ethiopia, showed a stable (moderate level) or slowly increasing level of PDR 15 years after the rollout of ART in the country.

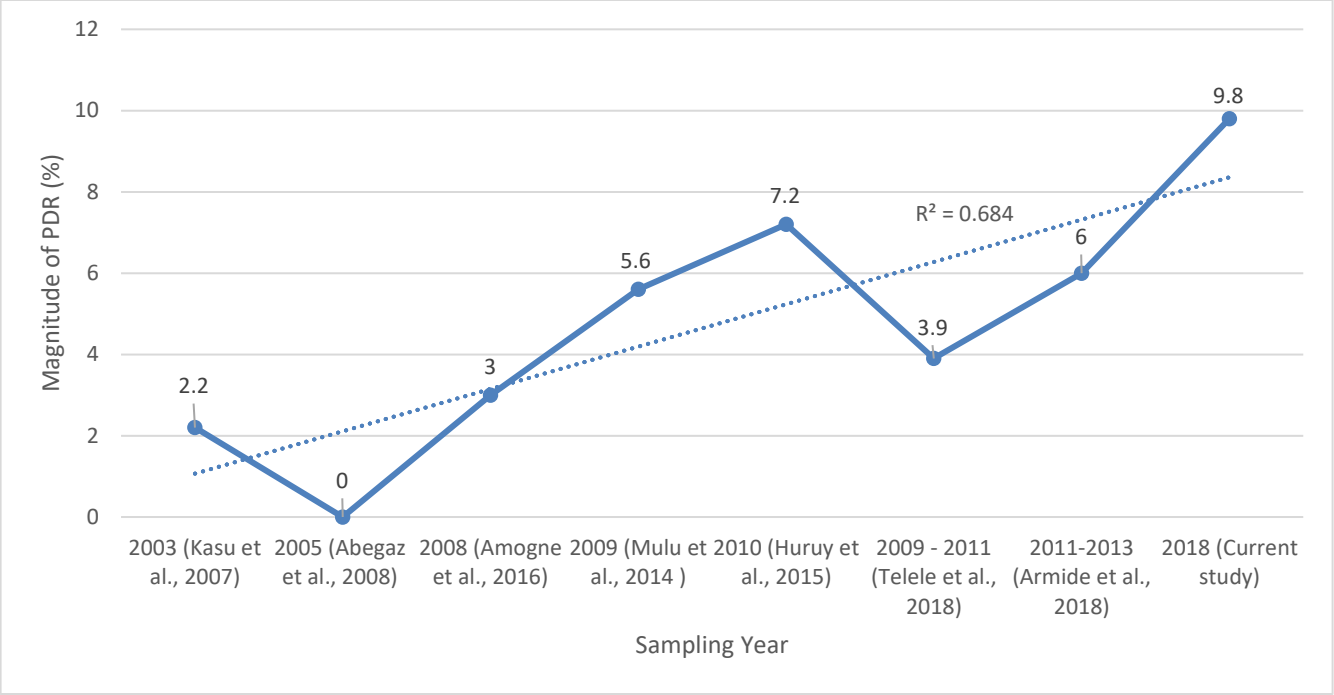

Figure 8: Trend of PDR Magnitude over years in Ethiopia

In addition to the above previously published data's from Ethiopia, this finding is in agreement with studies done in India (85), Brazil (86-88), China (89), and Iran (90) which reported a moderate magnitude of PDR among HIV-1 antiretroviral adults.

Similar to previously published studies in Ethiopia (35, 71), NNRTI-associated mutations were the most common mutations observed relative to others (NRTI and PI-associated) major mutations. Despite this, the frequency of these NNRTI-associated mutations observed in this study is higher compared to previous studies done in Ethiopia which reported lower (1.5 – 3.6 %) frequency of PDR (35, 71, 91). This might be due to the high rate transmission of viruses harboring this mutation conferring resistance. Meanwhile, moderate (6%) frequency of NNRTIs associated mutation were reported previously from Gonder (15) in agreement with the frequency observed in this study (7.8%).

The lower frequency of NRTI and PI associated mutations observed in this study are in agreement with previously conducted studies among ART naïve individuals in Ethiopia (35, 36, 71, 91, 92). Likewise, similar NRTI and PI associated PDRMs were reported in other countries like Brazil (93) and India (85).

Mutations in the RT sequence which confer resistance to NNRTIs; K101E, K103N, and E138A which were observed in this study had been reported previously in Ethiopia among ART naïve individuals in agreement to our study (36, 71, 91). In contrary, V106A (Mutation in the RTs sequence that confers resistance to NNRTIs), M114V and Y115F (Mutations in the RTs sequence that confers resistance to NRTIs) and G73S (Mutation in the PI sequence that confers resistance to PIs) were detected for the first time (Figure. 9) among ART naïve individuals in Ethiopia. This might be due to high risky sexual exercise of individuals failing ART (viruses with such drug resistance mutations are transmitting from individuals on ART to healthy individuals) and/or due to the higher evolutionary rate of HIV-1C in Ethiopia and those HIV-1 variants containing these mutations are circulating in the country. Although they are seen for the first time among ART naïve individuals in the country, these mutations were reported among ART naïve individuals from other countries (94).

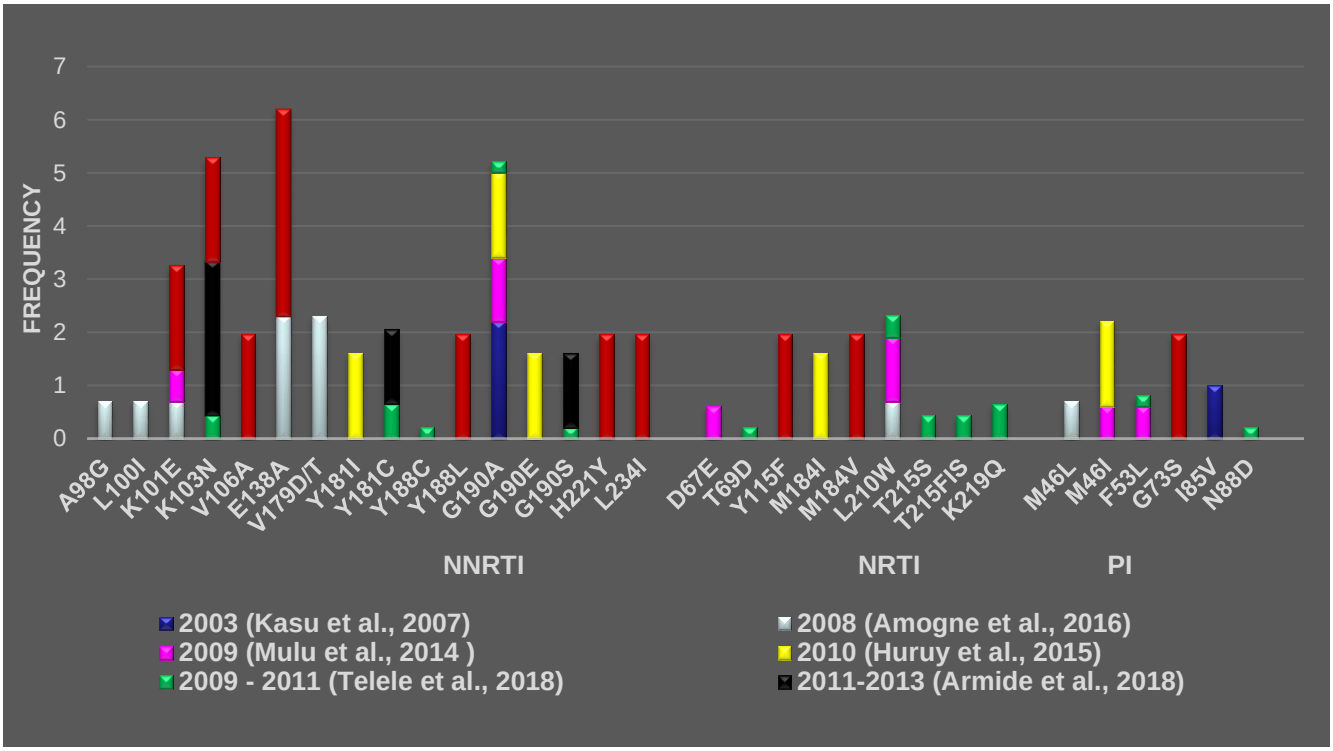

Figure 9: Comparison of major primary drug resistance mutations identified in treatment-naive Ethiopian individuals infected with HIV-1C.

E138A mutation which is associated with a decreased response to ETR and RPV was found in 3.9% (2/51) of the specimens according to the IAS-USA mutation list only were also reported in Ethiopia with similar frequency (36). In agreement with the previous report from

Gonder (73), one individual had PDR mutations associated with two combined drug classes (NRTIs plus NNRTIs).

With regard to minor mutations and/or polymorphisms, a high rate of polymorphic change was observed in the PR region of sequenced specimens most frequently than the RT region in this study. This, the so-called accessory mutations or resistance-related mutations usually may not reflect PDR but may represent natural HIV-1 genetic variability with possible clinical implications if present with other mutations. The most frequent minor mutations observed in the PR sequence were H69K (100%) and M36L (98%), followed by L89M (58.8%), I15V (25.5%), K20R (25.5%), and T74S (17.6%). These mutations with relatively the same frequencies were reported from Ethiopia (36) and India (95). The presence of these mutations indicates the genetic variability of the virus across the globe.

7. Limitation and strength of the study

Since we used the population-based Sanger sequencing method, which detects only sequences from the viral population represented by over 20% of the total population, minor variant PDR may be missed. Therefore, the reported magnitude of PDR might be underestimated. The strength of this study is the fact that it was able to detect ARV drug resistance mutations among drug-naïve patients after several years of previous work, which is very rarely done in the country without transporting it to laboratories abroad.

8. Conclusion and recommendation

In conclusion, this study presents additional evidence that the genetic diversity of HIV-1 in Ethiopia is still dominated by the HIV-1 subtype C virus. In addition to this, the study also an increased level of HIV-1 PDR 15 years after the rollout of ART in Ethiopia. PDRMs to NNRTIs were the most frequent mutations observed (K103N, Y188L, H221Y, L234I, K101E, V106A, and E138A) followed by PDRMs to NRTIs (Y115F and M184V) and PIs (G73S) with 7.8%, 1.96%, and 1.96% mutation frequency respectively. Therefore, high follow-up and counseling strategies warranted to those who are experiencing virological failure so as to prevent further dissemination such viruses. In general, this study showed increased levels of HIV-1 PDR in Ethiopia that potentially compromises the effectiveness of ARV drugs especially those based on NNRTI regimens. This highlights the need for routine HIV-1 drug resistance testing before ART and a broader public health action to prevent the emergence and transmission of drug-resistant variants. In addition, consistent follow-up and strengthening of adherence patterns, and robust monitoring of viral load to identify early treatment failure is warranted for successful ART programs and overall prevention of HIV transmission in the country and to support the global efforts in achieving the third 90 of the UNAIDS target.

9. References

1. Carroll KC, Butel J, Morse S. Jawetz Melnick and Adelbergs Medical Microbiology 27 E: McGraw-Hill Education; 2015.
2. Andrews SM, Rowland-Jones S. Recent advances in understanding HIV evolution. [version 1; referees: 2 approved] *F1000Research*. 2017;6 (F1000 Faculty Rev):597.
3. German Advisory Committee Blood (Arbeitskreis Blut) Subgroup 'assessment of pathogens transmissible by Blood'. Human Immunodeficiency Virus (HIV). Transfusion medicine and hemotherapy : offizielles Organ der Deutschen Gesellschaft fur Transfusionsmedizin und Immunhamatologie. 2016;43(3):203-22.
4. Bbosa N, Kaleebu P, Ssemwanga D. HIV subtype diversity worldwide. Curr Opin HIV AIDS. 2019;14(3):153-60.
5. Sharp PM, Hahn BH. The evolution of HIV-1 and the origin of AIDS. Philosophical Transactions of the Royal Society B: Biological Sciences. 2010;365(1552):2487-94.
6. Deeks SG, Overbaugh J, Phillips A, Buchbinder S. HIV infection. Nature Reviews Disease Primers. 2015;1:15035.
7. Ayehunie S JB, Sonnerborg A, Salminen M, Zewdie DW, Fehniger T, Britton S, Strannegard O. New subtype of HIV-1 in Ethiopia. Lancet. 1990;336(8720):942.
8. Levin BR, Bull, J., & Stewart, F. M.,, . Epidemiology, Evolution, and Future of the HIV/AIDS Pandemic. Emerging Infectious Diseases. 2001;7(7):505-11.
9. Centers for Disease Control (CDC). Pneumocystis pneumonia--Los Angeles. MMWR Morbidity and mortality weekly report. 1981;30(21):250-2.
10. Abebe A, Kuiken CL, Goudsmit J, Valk M, Messele T, Sahlu T, et al. HIV type 1 subtype C in Addis Ababa, Ethiopia. AIDS research and human retroviruses. 1997;13(12):1071-5.
11. World Healh Organization(WHO). HIV/AIDS fact sheet 2017 [Available from: http://www.who.int/mediacentre/factsheets/fs360/en/.
12. Hoffmann C, Kamps BS. HIV Medicine 2003. Paris: Flying; 2003.
13. Hemelaar J. Implications of HIV diversity for the HIV-1 pandemic. The Journal of infection. 2013;66(5):391-400.
14. Cenderello G, De Maria A. Discordant responses to cART in HIV-1 patients in the era of high potency antiretroviral drugs: clinical evaluation, classification, management prospects. Expert review of anti-infective therapy. 2016;14(1):29-40.
15. Arimide DA, Abebe A, Kebede Y, Adugna F, Tilahun T, Kassa D, et al. HIV-genetic diversity and drug resistance transmission clusters in Gondar, Northern Ethiopia, 2003-2013. PloS one. 2018;13(10):e0205446.
16. The Joint United Nations Programme on HIV/ AIDS (UNAIDS). FACT SHEET – GLOBAL AIDS UPDATE 2019. 2019.
17. UNAIDS(Joint United Nations Program on HIV/AIDS). Fact sheet - Latest statistics on the status of the AIDS epidemic. 2017.
18. UNAIDS (Joint United Nations Program on HIV/AIDS). UNAIDS DATA 2017.
19. Misganaw A, Haregu TN, Deribe K, Tessema GA, Deribew A, Melaku YA, et al. National mortality burden due to communicable, non-communicable, and other diseases in Ethiopia, 1990–2015: findings from the Global Burden of Disease Study 2015. Population health metrics. 2017;15(1):29.
20. World Health Organization (WHO). Ethiopia HIV Country Profile 2017 2019 [Available from: http://cfs.hivci.org/country-factsheet.html#.

21. Velloza J, Watt MH, Choi KW, Abler L, Kalichman SC, Skinner D, et al. HIV/AIDS-related stigma in South African alcohol-serving venues and its potential impact on HIV disclosure, testing and treatment-seeking behaviours. Global public health. 2015;10(9):1092-106.
22. Emlet CA, Brennan DJ, Brennenstuhl S, Rueda S, Hart TA, Rourke SB. The impact of HIV-related stigma on older and younger adults living with HIV disease: does age matter? AIDS care. 2015;27(4):520-8.
23. Girma E, Gebretsadik LA, Kaufman MR, Rimal RN, Morankar SN, Limaye RJ. Stigma against people with HIV/AIDS in rural Ethiopia, 2005 to 2011: signs and predictors of improvement. AIDS and Behavior. 2014;18(6):1046-53.
24. Fortson JG. Mortality risk and human capital investment: The Impact of HIV/AIDS in Sub-Saharan Africa. The Review of Economics and Statistics. 2011;93(1):1-15.
25. Gachuhi D. The Impact of HIV/AIDS on Education Systems in Eastern and Southern Africa Region. draft, New York: UNICEF. 1999.
26. Tekola F, Reniers G, Haile Mariam D, Araya T, Davey G. The economic impact of HIV/AIDS morbidity and mortality on households in Addis Ababa, Ethiopia. AIDS care. 2008;20(8):995-1001.
27. Fox MP, Rosen S, MacLeod WB, Wasunna M, Bii M, Foglia G, et al. The impact of HIV/AIDS on labour productivity in Kenya. Tropical Medicine & International Health. 2004;9(3):318-24.
28. De Clercq E. Antiretroviral drugs. Current opinion in pharmacology. 2010;10(5):507-15.
29. Stecher M, Chaillon A, Eis-Hubinger AM, Lehmann C, Fatkenheuer G, Wasmuth JC, et al. Pretreatment human immunodeficiency virus type 1 (HIV-1) drug resistance in transmission clusters of the Cologne-Bonn region, Germany. Clinical microbiology and infection : the official publication of the European Society of Clinical Microbiology and Infectious Diseases. 2019;25(2):253.e1-.e4.
30. Zazzi M, Hu H, Prosperi M. The global burden of HIV-1 drug resistance in the past 20 years. PeerJ. 2018;6:e4848.
31. Panichsillapakit T, Smith DM, Wertheim JO, Richman DD, Little SJ, Mehta SR. Prevalence of Transmitted HIV Drug Resistance Among Recently Infected Persons in San Diego, CA 1996-2013. J Acquir Immune Defic Syndr. 2016;71(2):228-36.
32. Yendewa GA, Sahr F, Lakoh S, Ruiz M, Patino L, Tabernilla A, et al. Prevalence of drug resistance mutations among ART-naive and -experienced HIV-infected patients in Sierra Leone. The Journal of antimicrobial chemotherapy. 2019;74(7):2024-9.
33. World Health Organization (WHO). HIV DRUG RESISTANCE REPORT 2019. Geneva, Switzerland: World Health Organization; 2019 (WHO/CDS/HIV/19.21). Report No.: Licence: CC BY-NC-SA 3.0 IGO.
34. Do HT, Nguyen DT, Nguyen LAT, Do DH, Le HX, Trinh XMT, et al. An Alarmingly High Proportion of HIV-1 Isolates Carrying Mutations Corresponding to Resistance to Antiretroviral Drugs among HIV-Positive High-Risk Groups in Central Vietnam: a Substudy of the National Sentinel Survey. Japanese journal of infectious diseases. 2017;70(6):621-7.
35. Huruy K, Maier M, Mulu A, Liebert UG. Limited increase in primary HIV-1C drug resistance mutations in treatment naive individuals in Ethiopia. J Med Virol. 2015;87(6):978-84.

36. Mulu A, Lange T, Liebert UG, Maier M. Clade homogeneity and Pol gene polymorphisms in chronically HIV-1 infected antiretroviral treatment naive patients after the roll out of ART in Ethiopia. BMC infectious diseases. 2014;14(1):158.
37. Ethiopian Public Health Insitute. HIV Drug Resistance Early Warning Indicators Survey in Ethiopia. 2013/14.
38. Abegaz WE, Grossman Z, Wolday D, Ram D, Kaplan J, Sibide K, et al. Threshold survey evaluating transmitted HIV drug resistance among public antenatal clinic clients in Addis Ababa, Ethiopia. Antiviral therapy. 2008;13:89.
39. Ekici H, Amogne W, Aderaye G, Lindquist L, Sonnerborg A, Abdurahman S. Minority drug-resistant HIV-1 variants in treatment naive East-African and Caucasian patients detected by allele-specific real-time PCR. PLoS One. 2014;9(10):e111042.
40. The Joint United Nations Programme on HIV/ AIDS (UNAIDS). UNAIDS Data. 2019.
41. Federal HIV/AIDS Prevention and Control Office. HIV Prevention in Ethiopia National Road Map 2018 - 2020. 2018.
42. Kasper D, Fauci A, Hauser S, Longo D, Jameson J, Loscalzo J. Harrison's principles of internal medicine, 19e. 2015.
43. Rodger AJ, Mahungu TW, Johnson MA. HIV/AIDS: An atlas of investigation and management: Clinical Publishing; 2011.
44. Rojas VK, Park I-W. Role of the Ubiquitin Proteasome System (UPS) in the HIV-1 Life Cycle. International journal of molecular sciences. 2019;20(12):2984.
45. Li G, Piampongsant S, Faria NR, Voet A, Pineda-Peña A-C, Khouri R, et al. An integrated map of HIV genome-wide variation from a population perspective. Retrovirology. 2015;12(1):18.
46. Tebit DM, Patel H, Ratcliff A, Alessandri E, Liu J, Carpenter C, et al. HIV-1 Group O Genotypes and Phenotypes: Relationship to Fitness and Susceptibility to Antiretroviral Drugs. AIDS Res Hum Retroviruses. 2016;32(7):676-88.
47. Brooks G, Carroll K, Butel J, Morse S, Mietzner T. Jawetz, Melnick, Adelberg Medical Microbiology: Placebo doo; 2015.
48. Sayan M, Willke A, Ozgunes N, Sargın F. HIV-1 subtypes and primary antiretroviral resistance mutations in antiretroviral therapy naive HIV-1 infected individuals in Turkey. Japanese journal of infectious diseases. 2013;66(4):306-11.
49. HIV sequence database. HIV Circulating Recombinant Forms (CRFs) 2017 [Available from: https://www.hiv.lanl.gov/content/sequence/HIV/CRFs/CRFs.html.
50. Whiteside A. HIV/AIDS: A Very Short Introduction. New York: Oxford University Press 2008.
51. Kalu AW, Telele NF, Gebreselasie S, Fekade D, Abdurahman S, Marrone G, et al. Monophylogenetic HIV-1C epidemic in Ethiopia is dominated by CCR5-tropic viruses-an analysis of a prospective country-wide cohort. BMC Infect Dis. 2017;17(1):37.
52. Woldaregay E. Genomic characterization of hiv-1 isolates from Ethiopian patients: baseline studies on antiretroviral drug resistance and sub-type variations. 2011.
53. Abebe A. HIV-1 subtype C in Ethiopia: genotypic and phenotypic variation. 2000.
54. Brenner B, Wainberg MA, Roger M. Phylogenetic inferences on HIV-1 transmission: implications for the design of prevention and treatment interventions. AIDS (London, England). 2013;27(7):1045.
55. Kiptoo M. The Changing Trends of HIV Subtypes and Its Implication on Mother-to-Child Transmission. Recent Translational Research in HIV/AIDS. 2011:71.

56. Woollard SM, Kanmogne GD. Maraviroc: a review of its use in HIV infection and beyond. Drug design, development and therapy. 2015;9:5447-68.
57. Yi HA, Fochtman BC, Rizzo RC, Jacobs A. Inhibition of HIV Entry by Targeting the Envelope Transmembrane Subunit gp41. Current HIV research. 2016;14(3):283-94.
58. Li G, De Clercq E. HIV genome-wide protein associations: a review of 30 years of research. Microbiol Mol Biol Rev. 2016;80(3):679-731.
59. CDC (Centers for Disease Control and Prevention). HIV/AIDS 2017 [Available from: https://www.cdc.gov/hiv/basics/whatishiv.html.
60. Sobieski M, Przystupski D, Korzeniewska A, Kwiatkowski S, Górska A, Kotowski K, et al. A review of current literature on the diagnosis, prophylaxis and treatment of HIV/AIDS. World Scientific News. 2018;110:129-46.
61. Arts EJ, Hazuda DJ. HIV-1 antiretroviral drug therapy. Cold Spring Harbor Perspectives in Medicine. 2012;2(4):a007161.
62. AIDSinfo. FDA-Approved HIV Medicines Understanding HIV/AIDS United States: U.S. Department of Health and Human Services; 2019 [Available from: https://aidsinfo.nih.gov/understanding-hiv-aids/fact-sheets/21/58/fda-approved-hiv-medicines.
63. World Health Organization (WHO). Update of recommendations on first- and second-line antiretroviral regimens Policy brief. 2019.
64. Fekade D, Weldegebreal T, Teklu AM, Damen M, Abdella S, Baraki N, et al. Predictors of survival among adult Ethiopian patients in the national ART program at Seven University Teaching Hospitals: A prospective cohort study. Ethiopian journal of health sciences. 2017;27(1):63-71.
65. Zhao J, Chang L, Wang L. Nucleic acid testing and molecular characterization of HIV infections. European journal of clinical microbiology & infectious diseases : official publication of the European Society of Clinical Microbiology. 2019;38(5):829-42.
66. Lipscomb JT, Switzer WM, Li JF, Masciotra S, Owen SM, Johnson JA. HIV reverse-transcriptase drug resistance mutations during early infection reveal greater transmission diversity than in envelope sequences. J Infect Dis. 2014;210(11):1827-37.
67. Shafer RW, Rhee S-Y, Pillay D, Miller V, Sandstrom P, Schapiro JM, et al. HIV-1 protease and reverse transcriptase mutations for drug resistance surveillance. AIDS (London, England). 2007;21(2):215.
68. Taiwo B. Understanding transmitted HIV resistance through the experience in the USA. International journal of infectious diseases : IJID : official publication of the International Society for Infectious Diseases. 2009;13(5):552-9.
69. Reynolds SJ, Ssempijja V, Galiwango R, Ndyanabo A, Nakigozi G, Lyagoba F, et al. Low Rates of Transmitted Drug Resistance Among Newly Identified HIV-1 Seroconverters in Rural Rakai, Uganda. AIDS Res Hum Retroviruses. 2017;33(5):448-51.
70. Boerma RS, Boender TS, Sigaloff KC, Rinke de Wit TF, van Hensbroek MB, Ndembi N, et al. High levels of pre-treatment HIV drug resistance and treatment failure in Nigerian children. J Int AIDS Soc. 2016;19(1):21140.
71. Amogne W, Bontell I, Grossmann S, Aderaye G, Lindquist L, Sönnerborg A, et al. Phylogenetic analysis of Ethiopian HIV-1 subtype C near full-length genomes reveals high intrasubtype diversity and a strong geographical cluster. AIDS research and human retroviruses. 2016;32(5):471-4.

72. Ethiopian Public Health Insitute. Surveillance of transmitted HIV drug resistance among voluntary counseling and testing centers in Gondar town Ethiopia. 2014.
73. Huruy K, Maier M, Mulu A, Liebert UG. Limited increase in primary HIV-1C drug resistance mutations in treatment naïve individuals in Ethiopia. Journal of medical virology. 2015;87(6):978-84.
74. Iyidogan P, Anderson KS. Current perspectives on HIV-1 antiretroviral drug resistance. Viruses. 2014;6(10):4095-139.
75. 75. Sluis-Cremer N, Arion D, Parniak M. Molecular mechanisms of HIV-1 resistance to nucleoside reverse transcriptase inhibitors (NRTIs). Cellular and Molecular Life Sciences CMLS. 2000;57(10):1408-22.
76. Clutter DS, Jordan MR, Bertagnolio S, Shafer RW. HIV-1 drug resistance and resistance testing. Infection, genetics and evolution : journal of molecular epidemiology and evolutionary genetics in infectious diseases. 2016;46:292-307.
77. Zdanowicz MM. The pharmacology of HIV drug resistance. American journal of pharmaceutical education. 2006;70(5):100.
78. Hanna GJ, D'Aquila RT. Clinical use of genotypic and phenotypic drug resistance testing to monitor antiretroviral chemotherapy. Clinical infectious diseases : an official publication of the Infectious Diseases Society of America. 2001;32(5):774-82.
79. Hassan AS, Bibby DF, Mwaringa SM, Agutu CA, Ndirangu KK, Sanders EJ, et al. Presence, persistence and effects of pre-treatment HIV-1 drug resistance variants detected using next generation sequencing: A Retrospective longitudinal study from rural coastal Kenya. PLoS One. 2019;14(2):e0210559.
80. Smit E. Antiviral resistance testing. Current opinion in infectious diseases. 2014;27(6):566-72.
81. Population Census Commission. Summary and statistical report of the 2007 population and housing census. Population size by age and sex. Ethiopia. 2008.
82. Bennett DE, Myatt M, Bertagnolio S, Sutherland D, Gilks CF. Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. Antiviral therapy. 2008;13:25.
83. Chauhan CK, Lakshmi PVM, Sagar V, Sharma A, Arora SK, Kumar R. Primary HIV Drug Resistance among Recently Infected Cases of HIV in North-West India. AIDS Res Treat. 2019;2019:1525646.
84. Pimenta ATM, Correa IA, Melli P, Abduch R, Duarte G, Couto-Fernandez JC, et al. HIV-1 genetic diversity and resistance to antiretroviral drugs among pregnant women in Ribeirao Preto (SP), Brazil. Cross-sectional study. Sao Paulo medical journal = Revista paulista de medicina. 2018;136(2):129-35.
85. Nesakumar M, Haribabu H, Cheedarla N, Karunaianantham R, Kailasam N, Sathyamoorthy P, et al. Transmitted HIV-1 drug resistance in a treatment naive cohort of recently infected individuals from Chennai, India. AIDS Res Hum Retroviruses. 2019.
86. Tanaka TSO, Leite TF, Freitas SZ, Cesar GA, de Rezende GR, Lindenberg ASC, et al. HIV-1 Molecular Epidemiology, Transmission Clusters and Transmitted Drug Resistance Mutations in Central Brazil. Front Microbiol. 2019;10:20.
87. Coelho LPO, Matsuda EM, Nogueira RS, de Moraes MJ, Jamal LF, Madruga JVR, et al. Prevalence of HIV-1 transmitted drug resistance and viral suppression among recently diagnosed adults in Sao Paulo, Brazil. Archives of virology. 2019;164(3):699-706.

88. Bahls LD, Canezin PH, Reiche EMV, Fernandez JCC, Dias JRC, Meneguetti VAF, et al. Moderate prevalence of HIV-1 transmitted drug resistance mutations in southern Brazil. AIDS research and therapy. 2019;16(1):4.
89. Song YX, Xin RL, Li ZC, Yu HW, Lun WH, Ye J, et al. Prevalence of transmitted drug resistance among HIV-1 treatment-naive patients in Beijing. Epidemiology and infection. 2018;146(3):339-44.
90. Vahabpour R, Bokharaei-Salim F, Kalantari S, Garshasbi S, Monavari SH, Esghaei M, et al. HIV-1 genetic diversity and transmitted drug resistance frequency among Iranian treatment-naive, sexually infected individuals. Archives of virology. 2017;162(6):1477-85.
91. Telele NF, Kalu AW, Gebre-Selassie S, Fekade D, Abdurahman S, Marrone G, et al. Pretreatment drug resistance in a large countrywide Ethiopian HIV-1C cohort: a comparison of Sanger and high-throughput sequencing. Scientific reports. 2018;8(1):7556.
92. Kassu A, Fujino M, Matsuda M, Nishizawa M, Ota F, Sugiura W. Molecular epidemiology of HIV type 1 in treatment-naive patients in north Ethiopia. AIDS Res Hum Retroviruses. 2007;23(4):564-8.
93. Tanaka TSO, Leite TCNF, Freitas SZ, Cesar GA, Rezende GR, Lindenberg ASC, et al. HIV-1 Molecular epidemiology, transmission clusters and transmitted drug resistance mutations in Central Brazil. Frontiers in microbiology. 2019;10:20.
94. World Health Organization (WHO). The HIV drug resistance report. Geneva: Switherland World Health Organization; 2017. Report No.: CC BY-NC-SA 3.0 IGO.
95. Azam M, Malik A, Rizvi M, Rai A. Zero prevalence of primary drug resistance-associated mutations to protease inhibitors in HIV-1 drug-naive patients in and around Aligarh, India. The Journal of Infection in Developing Countries. 2014;8(01):079-85.

Annexes

Annex I: Information sheet for study participants (English Version)

Project Title: HIV-1 Genetic Diversity and Pretreatment drug resistance Mutations among Recently diagnosed HIV-1 Infected Antiretroviral-Naive individuals in Addis Ababa, Ethiopia

Principal Investigator: Mulugeta Kiros

Greetings,

My name is _____. I am a data collector in this study. I would like to invite you to participate in this collaborative study between AAU and AHRI that will have a role in improving the health of the people.

Background

HIV is a virus that causes HIV infection, which leads to the decrement of immunity against various diseases. HIV spreads by having unprotected sex, by sharing needles, by getting an injection with a needle, which has not been properly cleaned, or by a mother with HIV passing the virus to her baby during pregnancy or breastfeeding. HIV infection is a serious health problem in Addis Ababa.

Objectives of the study

The purpose of this study is to assess HIV-1 Genetic Diversity and Pretreatment drug resistance Mutations among HIV-1 Infected Antiretroviral-Naive individuals in Addis Ababa, Ethiopia

Duration of the study: The study will be conducted from June up to December 2018

Procedures: If you agree to participate in the study, we will interview you and ask you questions about yourself such as age, education and marital status, occupation, etc. We will also ask you to provide a blood sample (approximately 10 ml blood or 2 teaspoons) taken from a vein in your arm. In addition, we will ask you to provide us information that helps us assess questions such as what might affect the transmission of HIV between people. The information you provide in the questionnaire and your HIV results will be kept strictly confidential unless you and your partner both agree to share this information with one another. We strongly encourage all participants who have a sexual partner to disclose their HIV results to their partner and to receive couples' counseling, but we will not disclose the information you provide or HIV test results to a partner, family member or the community, without direct permission from a participant. The interview and sample collection will take

approximately 45 minutes. You can agree to participate in the study; even if you do not, you will not lose your access to study site clinics or any other services.

Potential benefits and Risks from being in the study

Benefits: Currently, real-time phylodynamics of HIV-1 transmission networks in Addis Ababa is unknown. Therefore, although you will not personally benefit from the study, the result of this study will be used to halt HIV transmission in Addis Ababa.

Potential risks: There will not be any risks or discomfort in this survey, except for minor pain or bruise at the site of the needle stick, which is common with every blood draw.

Assurance of confidentiality: Research records of your participation in this study will be maintained by storing them in AHRI. Only authorized project personnel (approved by the project Principal Investigators) will have access to these files. All data and medical information collected from you will be kept confidential (no names will be included in the study).

Participation is voluntary: Your participation in this study is voluntary. You are free to withdraw at any time the study (interview, sample collection).

Cost of participation: The procedures used are safe. Therefore, the potential risk due to your participation is minimal. You will not be given any payment for participating in this study. However, if you are injured from participating in the study, treatment will be available. If you experience any symptoms, which you feel may be related to the study, be sure to report them immediately to the persons collecting the sample.

If there is any portion of this consent explanation sheet that you do not understand, feel free to ask by using the following address or to the person collecting the information.

Name: Mulugeta Kiros Phone number: 0919989310

AAERC Address: +251113481289

Annex II: ለጥናቱ ተሳታፊዎች የተዘጋጀ የመረጃ ቅጽ (አማርኛ ቅጂ)

የጥናቱ ርእስ: በአዲስ አበባ አዲስ በ ኤች አይቪ ቫይረስ ተጠቂ በሆኑ ስዎች ላይ ያለዉን መድሃኒት መቋቋም የሚችል ኤች አይቪ ቫይረስ መጠንና የ ቫይረስ አይነት ብዛት

<u>ዋና አጥኚ፡ ሙሉጌታ ኪሮስ</u>

ሰላም፤ ስሜ _____ ይባላል። እኔ ብዚህ ጥናት ውስጥ መረጃ ሰብሳቢ ነኝ። በዚህ የህብረተሰቡን ጠና ለማጎልበት በሚጠቅመው በአዲስ አበባ ዩኒቨርስቲ እና በአህሪ ትብብር በሚጠናው ይህ ጥናታችን ላይ እንዲሳተፉ ልጋብዞት እፈልጋለው።

<u>መግብያ</u>

ኤች አይቪ ማለት የኤች አይቪ በሽታ ማለትም ሁኖ የሰውነታችን በሽታ መከላከል አቅም የሚቀንስ በሽታ የሚያመጣ ቫይረስ ነው። ኤች አይቪ ጥንቃቄ በጎደለው የግብረ ስጋ ግኙኝነት፣ ስለታማ የሆኑ ነገሮችን በጋራ በመጠቀም፣ በተበከለ መርፌ በመወጋት ወይም ከእናት ወደ ልጅ (በእርግዝና ወቅት ወይም በጡት ማጥባት ጊዜ) ይተላለፋል። የኤች አይቪ በሽታ በአዲስ አበባ አደገኛ የሆነ የጤና እክል ነው።

<u>የጥናቱ አላማ</u>

የዚህ ጥናት አላማ በአዲስ አበባ አዲስ በ ኤች አይቪ ቫይረስ ተጠቂ በሆኑ ስዎች ላይ ያለዉን መድሃኒት መቋቋም የሚችል ኤች አይቪ ቫይረስ መጠንና የ ቫይረስ አይነት ብዛት ለማወቅ ነው።

<u>ጥናቱ የሚፈጀው ጊዜ:</u> ጥናቱ ከሰኔ እስከ ታህሳስ 2010 አ.ም ይካሄዳል።

<u>በቅደም ተከተል የሚተገበሩ ተግባሮች</u>

እርሶ በዚህ ጥናት ላይ ለመሳተፍ ፍቃደኛ ከሆኑ፤ ስለእርሶ እድሜ ፣ የትምህርት ደረጃ፣ የጋብቻ ሁኔታ፣ ስለስራዎ ወዘተ ጥያቄዎችን በመጠየቅ ቃለመጠይቅ እናደርግሎታለን። ከእጅዎት የደም ስር ላይ የደም ናሙና እንዲሰጡም እንጠይቆታለን። ይህ ናሙና (ወደ 10 ሚሊ ሊትር ወይም 2 የሻይ ማንክያ የሚጠጋ ደም) የኤች አይ ቪ ቫይረስ በእርሶ ደም ላይ አለ ወይስ የለም ለማረጋገጥ ይጠቅማል። በተጨማሪም፣ የእርሶን ያገቡት ወይም እውቅና ያለው የትዳር አጋር ለማወቅ የሚጠቅመንን መረጃ እንዲሰጡን እንጠይቆታለን። ይህ መረጃ በሰዎች ላይ ያለውን የኤች አይ ቪ ስርጭትን በምን ሊባባስ ይችላል የሚለውን ጥያቄ እንድንመልስ ይረዳናል። በመጠይቁ ላይ የሚሰጡን መረጃ እና የእርሶን የኤች አይ ቪ ውጤት እርሶ ካል ፈቀዱ ድረስ አስተማማኝ በሆነ ሁኔታ ይጠበቃል። ነገር ግን እርሶ እና የእርሶ የትዳር አጋር ሁለታችሁም ውጤቱን በፍላጎት አብራችሁ ለማየት ከፈለገቹ ይቻላል። ሁሉንም የፍቅር አጋር ያለው ተሳታፊ አጋሮች የሚሰጠውን ምክር ለማግኘት የኤች አይ ቪ ውጤቱን እንዲያሳውቅ አጥብቀን እናበረታታለን። ነገር ግን እርሶ በቀጥታ እስካልፈቀዱ ድረስ እርሶ የሚሰጡት መረጃ ወይም የእርሶ የኤች አይ ቪ ውጤት ለትዳር አጋር፣ ለቤተሰብ ወይም ለህብረተሰብ አናሳውቅም። ቃለመጠይቁ እና ናሙና ስብሰባው እስከ 45 ደቂቃ ይወስዳል። በዚህ ጥናት ላይ መሳተፍ ይችላሉ፤ ባይችሉ እንኳን ጥናቱ በሚካሄድበት ሃኪም ቤት ላይ የሚሰጠውን ማንኛውም አገልግሎት ማግኘት ይችላሉ።

<u>በዚህ ጥናት በመሳተፍዎ የሚያገኙት ጥቅም እና ልያጋጥማቸው የሚችሉ ጉዳቶች</u>

<u>የሚያገኙት ጥቅም:</u> ከዚህ በፊት በአዲስ አበባ ያለዉን መድሃኒት መቋቋም የሚችል ኤች አይቪ ቫይረስ መጠንና የ ቫይረስ አይነት ብዛት የሚግልጽ መረጃ ቢኖርም አንኳ ፤ በአሁኑ ጊዜ ለውጥ ይኑር አይኑር አይታወቅም። ስለዚህ እርሶ ከዚህ ጥናት በግል በቀጥታ ባይጠቀሙ እንካን የጥናቱ ውጤት በበአዲስ አበባ ላይ ያለውን የኤች አይቪ ስርጭት ለመግታት ይጠቅማል።

<u>ልያጋጥምዎ የሚችሉ ጉዳቶች:</u> የደም ናሙና በሚወሰድበት ግዜ በማንኛዉም ሰው ልሙድ የሆነዉነ ትንሽ የእጅ መበለዝ እና ደም የመፍሰስ ምልክት ልታይቦት ይችላል። ከዚህ ውጪ ጥናቱ ምንም አይነት ጉዳት አያስከትልም።

<u>ሚስጥር የመጠበቅ ሁኔታ:</u> ማንኛዉም የእርስዎ የጥናት ውጤት በ አርማዎር ሃንሰን የምርምር ማእከል የሚቀመጥ ይሆናል። ጥናቱን የሚያካሂደው ሰው ወይም ጥናቱን የሚያካሂድ ሰው ማረጋገጫ የተሰጣቸው ሰዎች ብቻ እነዚህን መረጃዎች የሚያዩ ይሆናል። ማንኛዉም ከእርሶ የተገኘ ህክምና ነክ የሆነ ወይም ያልሆነ መረጃ ሚስጥራዊ በሆን መልኩ ይጠበቃል (ምንም አይነት ስም በትናቱ ላይ አይካተትም)።

<u>ተሳትፎው በፍላጎት ነው:</u> የእርሶ በዚህ ጥናት ላይ ተሳትፎ ሙሉ ለሙሉ በፍላጎት ነው። በማንኛዉም ጊዜ (በቃለ መጠይቅ ፣ ናሙና ስብሰባ ጊዜ) መውጣት ይችላሉ።

<u>የተሳትፎ ክፍያ:</u> በዚህ ጥናት የሚተገበሩ ተግባራት ላስተማመኝ ናቸው። ስለዚህ፤ በእርሶ ላይ ሊፈጠሩ የሚችሉ ጉዳቶች በጣም አነስተኛ ናቸው። በዚህ ጥናት ላይ በመሳተፍዎ ምንም አይነት ክፍያ አያገኙም።

በዚህ መረጃ ቅጽ ማንኛዉም ያልገባዎት ክፍል ካለ የሚቀጥለዉን አድራሻ በመጠቀም ወይም ለናሙና ሰብሳቢው ነጻ ሁነው ይጠይቁ።

ስም፡ ሙሉጌታ ኪሮስ ስልክ ቁጥር፡ +251919989310

Annex III: Consent form for study participants (English Version)

Statement of participant consent

Code _____

Age _____

I have been asked to participate in this research study The principal investigator Mulugeta Kiros has explained the study to me, how long it will last, the testing I will undergo, and risks that I might take due to my participation in the study. The information above has been read to me. I have been given an opportunity to ask questions about this research project. All questions were answered in a way that I understand. If I have other questions about this research, I can ask Mulugeta Kiros who is the representative of this study.

I understand that my participation is voluntary and if I decline to participate in the study, I will not lose any benefits or access to study site clinic services. I am signing my name below to indicate my consent to participate in this project. I will be given a copy of the signed consent form.

Code: _____ Participants Address: _____

Signature of participant_____ Date: _____

(Thumb print if non-literate)

Signature of investigator eliciting consent: _____ Date: _____

Signature of principal investigator: _____ Date: _____

You will receive a copy of this consent form.

Thank you in advance for participating.

Annex IV: ለጥናቱ ተሳታፊዎች የተዘጋጀ የስምምነት መግለጫ ቅጽ (አማርኛ ቅጂ)

በአዲስ አበባ ያለዉን መድሃኒት መቋቋም የሚችል ኤች አይቪ ቫይረስ መጠንና የ ቫይረስ አይነት ብዛት

የተሳታፊው የስምምነት መግለጫ

ሚስጥራዊ መለያ _____

እድሜ, _____

እኔ በዚህ ጥናት ላይ እንድሳተፍ ተጠይቄአለው። ጥናቱ ስንት ጊዜ እንደሚፈጅ፣ ምን ምን ምርመራ እንደሚያካሂድ፣ እና በመሳተፌ ልያጋጥሙኝ የሚችሉ ተጓዳኝ ጉዳቶችን በጤና ባለሞያው ግልጽ ተደርገውልኛል ። ከላይ የተጠቀሰውን መረጃ ለኔ ተነባቢ ነበር።. ስለ ጥናቱ ጥያቄ እንድጠየቅ እድል ተሰጥቶኛል። ሁሉም ጥያቄዎች እንደሚገባኝ ተመልሰውልኛል። ስለዚህ ጥናት ሌላ ጥያቄ ካለኝ፤ ለጤና ባለሞያው መጠየቅ እንደምችል ተነግሮኛል።

ጥናቱ በፍላጎት እንደሆነ እና እንዲሁም በጥናቱ ላይ ኣልሳተፍም ብል እንኳ ከክሊንኩ ጥቅምም ሆነ አገልግሎት እንደማላጣ ገብቶኛል። በዚህ ጥናት ለመሳተፍ መስማማቴን ለማሳየት ፊርማየን ከታች አስቀምጣለው። የዚህ የስምምነቴን ፊረማ ቅጽ ግልባጭ ይሰጠኛል።

ሚስጥራዊ መለያ፤ _____ የተሳታፊው አድራሻ፤ _____

የተሳታፊው ፊርማ: _____ ቀን፤_____

(ያልተማረ ከሆነ የእጅ አሻራ)

ስምምነቱን ያካሄደዉ መርማሪ ፊርማ: ፤_____ ቀን፤_____

የዋና አጥኚዉ ፊርማ፤_____
ቀን፤_____

የዚህኛውን መረጃ ቅጽ ግልባጭ ይቀበላሉ።

ስለተሳተፎዎ እናመሰግናለን ።

Annex V. Questionnaire for study participants (English Version)

Human Immunology Virus-1 Genetic Diversity and Pre-treatment Drug Resistance Mutations among recently diagnosed HIV-1 Infected Antiretroviral-Naive individuals in Addis Ababa, Ethiopia

Health facility: _____

Name of data collector: _____

Date (DD/MM/YY E.C): _____

Instruction: Encircle a number that describes you best.

Part I: Socio-demographic characteristics				
S.No.	**Questions**	**Response**	Skip	Code
1.	Code	_____		
2.	Sex	_____		
3.	Age (years)	_____		
4.	Education status	1. No schooling 2. Primary or incomplete secondary 3. Secondary 4. Tertiary 5. Unknown		
5.	Marital status	1. Married 2. Single 3. Divorced 4. widowed/widower 5. other, specify_____		
6.	Religion	1. Muslim 2. Orthodox 3. Protestant 4. Catholic 5. Others, specify_____		

6.	Occupation	1. Unemployed 2. Self employed 3. Government employee 4. Employee at private enterprise 5. Student 6. Farmer 7. Others, specify_____		

Part II: Laboratory Data				
S.No.	**Questions**	**Response**	Skip	Code
1.	Baseline Viral load (Copies/ml)	_____		

Thank you in advance for participating.

Name and signature of the data collector

Name _____ Signature _____ Date _____

Name and signature of the principal investigator

Name _____ Signature _____ Date _____

Annex VI: Laboratory Procedure

A. cDNA Synthesis

1. Take samples out of -80°C and put it in ice.
2. Label small PCR tubes
3. Let thaw, Mix, and spin down briefly all the required reagents (no enzyme), and put on ice.
3. Label a new Eppendorf tube and prepare enough for 1-2 samples more than you need, plus a blank.
4. Calculate all other math and Combine the below-mentioned components in a reaction tube (Eppendorf tube).

Component	20-µL rxn (1X)
DEPC-treated water	6
5× Superscript IV RT Buffer	4.0 µL
10 mM dNTP mix (10 mM each)	1.0 µL
100 mM DTT	1.0 µL
RNaseOUT™ RNase Inhibitor (40 U/µL)	1.0 µL
50 µM HIVrt primer	1.0 µL
Superscript IV RT	1 µL

5. Add 15 µL of Master Mix to each small PCR tubes
6. Add template 5 µL RNA and put it into thermocycler and
   Note: Be sure the tubes are closed completely!
7. Store the product at -20°C for a short period of time or -80°C for a longer period.

B. **PCR 01**

1. Take samples out of -80 and put it in ice.
2. Label small PCR tubes
3. Let thaw, Mix, and spin down briefly all the required reagents (no enzyme), and put on ice.
3. Label a new Eppendorf tube and prepare enough for 1-2 samples more than you need, plus a blank.
4. Calculate all other math and Combine the below-mentioned components in a reaction tube (Eppendorf tube).

Component	50-µL reaction **(1X)**
Molecular Grade water	to 50 µL
10X High Fidelity PCR Buffer	5 µL
50 mM $MgSO_4$	2 µL
10 mM dNTP Mix	1 µL
10 µM PCRfor1 primer	1 µL
10 µM PCRrev1 primer	1 µL
Platinum® *Taq* DNA Polymerase High Fidelity (5 U/µL)	0.2 µL

5. Add 45 µL of Master Mix to each small PCR tubes
8. Add template 5 µL template DNA form the thawed reaction 1 product (cDNA synthesis) and put it into thermocycler and adjust it using the following cycling condition: initial denaturation at 94 °C for 2 min, 35 cycles of 30 sec at 94 °C, 1 min at 54 °C, 1 min at 72 °C followed by a final extension for 5 min at 72 °C.
   Note: Be sure the tubes are closed completely!
9. Store the product at -20°C for a short period of time or -80°C for a longer period.

C. PCR 02

1. Take samples out of -80 and put it in ice.
2. Label small PCR tubes
3. Let thaw, Mix, and spin down briefly all the required reagents (no enzyme), and put on ice.

3. Label a new Eppendorf tube and prepare enough for 1-2 samples more than you need, plus a blank.
4. Calculate all other math and Combine the below-mentioned components in a reaction tube (Eppendorf tube).

Component	50-µL reaction **(1X)**
Molecular Grade water	to 50 µL
10X High Fidelity PCR Buffer	5 µL
50 mM $MgSO_4$	2 µL
10 mM dNTP Mix	1 µL
10 µM PCRfor2 primer	1 µL
10 µM PCRrev2 primer	1 µL
Platinum® *Taq* DNA Polymerase High Fidelity (5 U/µL)	0.2 µL

5. Add 49 µL of Master Mix to each small PCR tubes
10. Add template 1 µL template DNA from thawed PCR01 product and put it into thermocycler and adjust it using the following cycling condition: initial denaturation at 94 °C for 2 min, 35 cycles of 30 sec at 94 °C, 1 min at 54 °C, 1 min at 72 °C followed by a final extension for 5 min at 72 °C.
    Note: Be sure the tubes are closed completely!
11. Store the product at -20°C for a short period of time or -80°C for a longer period.

Annex VII: Declaration

I the undersigned declare that this MSc thesis is my original work and has not been presented for a degree in any other university and all sources of material used for the thesis have been duly acknowledged.

Name of the principal investigator: Mulugeta Kiros (BSc, MSc candidate)
Signature: _____Date of submission: _____

This thesis has been submitted with our approval as advisors.

Name of Advisors	Signature	Date
Woldargay Erku (PhD), Associate Professor, DMIP, CHS, AAU	_____	_____
Adane Mihret (DVM, PhD), Associate Professor, AAU	_____	_____
Dawit Hailu (MSc), Assistant Researcher, AHRI	_____	_____
Andargachew Mulu (Ph.D.) Associate Professor, AHRI	_____	_____