MOLECULAR CHARACTERIZATION OF HIV DRUG RESISTANCE MUTATIONS IN PROTEASE AND REVERSE TRANSCRIPTASE GENES IN TREATMENT NAÏVE AND EXPERIENCED PATIENTS IN CARE IN ELDORET, KENYA

WINFRIDA CHERIRO

DOCTOR OF PHILOSOPHY

(Molecular Medicine)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2017

Molecular characterization of HIV drug resistance mutations in protease and reverse transcriptase genes in treatment naïve and experienced patients in care in Eldoret, Kenya

Winfrida Cheriro

A thesis submitted in fulfillment of the requirements for the award of the Degree of Doctor of Philosophy in Molecular Medicine in the Jomo Kenyatta University of Agriculture and Technology

2017

DECLARATION

This thesis is my original work and has not been presented for a degree in any other

University

Signature ……..…Date ……..

Winfrida Cheriro

The thesis has been submitted for examination with our approval as university supervisors.

Signature ……..…Date ……..

Prof. Gideon Kikuvi, PhD

JKUAT, Kenya

Signature ……..…Date ……..

Prof. Simeon K. Mining, PhD

Moi University, Kenya

Signature ……..…Date ……..

Prof. Michael Kiptoo, PhD

KEMRI, Kenya

Signature ……..…Date ……..

Prof. Elijah M. Songok, PhD

KEMRI, Kenya

DEDICATION

To my late parents Fredrick Cheriro and Afra Cheriro

To my son Sean Ruto, daughters Chantale Ruto and Charmaine Ruto

ACKNOWLEDGMENTS

I most sincerely want to thank Almighty God for seeing me through this study. My sincere gratitude goes to my supervisors Prof. Gideon Kikuvi, Professor Elijah Songok, Professor Simeon Mining and Professor Michael Kiptoo for working tirelessly in guiding me through conception of the study, project implementation, during field work, analysis and dissemination.

I am grateful to the Moi Teaching and Referral Hospital (MTRH) and to the Moi University School of Medicine (MUSOM) for their commitment to the mission of care, teaching and research. I would also like to express my gratitude to the institutional research and ethics committee (IREC) and the Academic Model Providing Access To Health Care Partnership Clinics (AMPATH) research office for permission to conduct this study. I wish to thank the directors and staff of AMPATH clinics and laboratories in Eldoret, Kenya Medical Research Institute (KEMRI) staff in Nairobi, National HIV and retro virology labs (NHRL) staff in Ottawa Canada, Ben Liang and his team at the University of Manitoba, Winnipeg, Canada for their technical support and cooperation. Special thanks go to the Canadian Commonwealth Scholarship Program (CCSP) of the Canada Ministry of Foreign Affairs and International Trade (DFAIT) for partly funding this study.

I highly acknowledge and appreciate the love, sacrifice and encouragement my husband, sisters and brother accorded me during the study period in order to make it a success. I want to thank all my friends, classmates and colleagues who encouraged me throughout this study. Most importantly, my sincere gratitude goes to the patients who participated in this study, whose outcome would help in improving the management of treatment of HIV/AIDS.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

ABC:	Abacavir
AIDS	Acquired immunodeficiency syndrome
AMPATH:	Academic Model Providing Access To Health care partnership clinics
APOBEC3G:	Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G
ARDR:	ARV Drug resistance
ART:	ARV treatment
ARV:	ARV
AZT:	Zidovudine
CXCR4:	Chemokine coreceptor 4
CCR5:	Chemokine coreceptor
CDNA:	Complementary Deoxyribonucleic acid
CD4:	Cluster of differentiation
CMV:	Cytomegalovirus
CTL:	Cytotoxic T-cells
ddI:	Didanosine
DNA:	Deoxyribonucleic acid
DR:	Drug resistance

DRMs: DRMs

ELISA: Enzyme-linked immunosorbent assay EIA

HAART: Highly active ARV therapy

HBV: Hepatitis B virus

HIV: Human immunodeficiency virus

HSR: Hypersensitivity reaction

IQR: Interquartile range

KIR: Killer cell immunoglobulin like receptors

LPV: Lopinavir

LPV/r: Lopinavir/ritonavir

LTNP: Long term Non-Progressors

MAC: Mycobacterium avium complex

MIP-1*α*: Macrophage Inhibitory Protein

MTRH: Moi Teaching and Referral Hospital

NASCOP: National AIDS control program

NGS: Next generation sequencing

NNRTI: Nucleoside reverse transcriptase

NRTI: Nucleotide reverse transcriptase

NVP: Nevirapine

OI: Opportunistic infection

PCR: Polymerase chain reaction

PI: Protease Inhibitors

PMTCT: Prevention of mother to child transmission

PR: Protease

Rantes: Regulated upon activation T cell expressed and secreted

RS: Resistance

RT: Reverse transcriptase

RRE: Rev Response elements

SD: Single dose

SDF-1: Stromal cell-derived factor 1

SIV: Simian immunodeficiency virus

SQ: Sequence

SQV: Saquinavir

SS: Sanger Sequencing

STI: Sexually transmitted infection

TAMS: Thymidine analogue mutations

TAR: Transactivation-response elements

TB: Tuberculosis

3TC: Lamivudine

TCR: T-cell receptors

TDF: Tenofovir

TDR: Transmitted DRMs

TLR: Toll-like receptors

TRIM5α: Tripartite motif containing protein 5α

UDPS: Ultra deep pyrosequencing

ABSTRACT

The Government of Kenya started offering ARV Therapy in public sector since 2003. Kenya is one of the six human immunodeficiency virus (HIV) 'high burden' countries in Africa. Kenya HIV Estimates Report in 2014 indicated that, nationally over 1.6 million patients were living with HIV and over 600,000 patients were receiving antiretroviral (ARV) Therapy (ART) with the national HIV prevalence at 7.6% in women and 5.6% in men. The use of ARV has resulted in reduction in acquired immunodeficiency syndrome (AIDS) related morbidity and mortality but led to emergence and spread of ARV drug resistance (DR) (ARVDR) which threatens to negatively impact on treatment regimens and compromise efforts to control the epidemic. Sanger sequencing of non-clonal Polymerase Chain Reaction (PCR) amplicons of plasma viral cDNA is widely used to detect drug resistance mutations (DRMs) in the molecular targets of HIV-1 namely reverse transcriptase (RT) and protease (PR) genes. A major limitation however, is its inability to detect low abundance of DR viruses (LADRVs) existing in a patient's plasma sample which have been shown by several studies to be clinically relevant often leading to failure of new ARTs. The use of next generation sequencing (NGS) has been shown to be more sensitive for LADRVS. The main objective of this study was to characterize inter subtype RT and PR gene mutations of viral isolates obtained from HIV infected ARV naive and ARV experienced patients failing therapy according to WHO guidelines. From October 2009 to October 2011, patients who met selection criteria were consecutively enrolled in this study through Moi Teaching and Referral Hospital (MTRH), Kenya. A total of 206 participants aged 6.6 years to 71.8 years were included in the study. Primers were specifically designed to amplify PR and RT. HIV-1 nucleic acid was extracted from plasma samples, reverse transcribed, amplified then sequenced to determine DR using Sanger sequencing and NGS, the new 454 pyrosequencing technologies. The mean age was 38.1 (SD: 12.2) years, 62.9% were female, 50.9% were married, engaged or cohabiting, 42.0% had secondary or tertiary level of education. Of the study participants 32.5% were ARV naïve and 52.5% in WHO clinical stage 3 or 4. There were 15 (7.6%) participants who reported opportunistic infections, 8 (4.0%) had tuberculosis, and 8 (4.4%) had sexually transmitted diseases. The median CD4 cell count per cubic milliliter was 210.0 (IQR: 70.0, 391.2) and the average viral load (log 10) was 3.1 (SD: 1.5), range (1.6 – 5.7). Of the 206 participants, 114 (55.3%) had their samples successfully amplified samples went through both sequencing techniques. A total of 83 participants were successfully sequenced using Sanger method. Up to 59.0% were ARV naïve. The average age was 36.9 (SD: 12.2) years with a range of 6.6 – 66.2 years. Sixty percent were female, 54.3% were married, and 42.0% had a secondary or a tertiary level of education From successfully edited sequences, 49 were from ARV naïve while 34 were from ARV experienced, 39.8 %(33/83) were male, mean age was 36. Among ARV naïve, 3/49 (6.1%) who were female patients were identified with DRMs of which, one had PI, two NRTI and 1 had NNRTI. None of their male counterparts had mutations. From the ARV experienced, Mean age was 35.85years (SD=14.06). Male were 18(52.9%). Most, 67.2% received 3TC + d4T/AZT + EFV/NVP as first-line treatment with 32.7% having EFV and 60% having NVP in their drug regimen. Those who reported treatment interruption or switch were 32.7%; mainly replacement of d4T or AZT by TDF or ABC and only 7% had been switched to protease inhibitor (PI) regimens. Subtype distribution were as follows; A 22(65%), A/D 2(6%), A/K 1(3%), AE 1(3%), B/A 1 (3%), D 3(9%), D/A 3(9%) and G 1(3%). Using the

new 454 pyrosequencing approach, 60 samples from ARV naïve were successfully sequenced of which 25 were subtype A, 11 subtype D, 1 Subtype C and the remaining were recombinants whereby,46 (76.6%) had at least one DRM; with 25 (41.6%) indicated as major and the rest 21 (35%) indicated as minor. The most prevalent mutation was NRTI position K219Q/R (11/46, (24%)) followed by NRTI M184V (5/46, (11%)) and NNRTI K103N (4/46, (9%)). The use of NGS technology in this study revealed a high prevalence of LADRVs among drug naive populations in Eldoret Kenya. The information obtained in this study can serve as an indicator of ARV program efficiency. DR testing would be necessary before initiating and /or changing ART in order to achieve optimal clinical outcome. DRMS were identified in ARV naïve patients with a prevalence of 6.1%. All naïve patients identified with DRMs were female. The most prevalent mutations identified in ARV naïve patients were those affecting NRTI 2/3(67%). DRMS were identified in ARV experienced patients with a prevalence of 6.1%. The most prevalent NRTI mutation observed was at position M184IV while the most prevalent NNRTI mutation observed was at position K103N. Drug Resistant Mutations across gender was statistically significant. Overall, all Male subjects from ARV experienced had DMRS when compared to Females. In this study a high prevalence of 41.6% of LADRVs among drug naive populations was revealed. Drug Resistance testing would be necessary before initiating ARV therapy so as to guide in the choice of susceptible combination ARV. It is highly recommended to use a feasible next generation sequencing technologies for surveillance of HIV drug resistance at population level to reliably detect and monitor emerging drug resistance patterns that may impact ARV treatment. There will be a need for a continued follow-up of persons with DRMS and LADRVS to determine clinical impact and help guide therapies for drug naïve populations

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Thirty years after the discovery of HIV-1, the early transmission, dissemination, and establishment of the virus in human populations remain unclear. The epidemic histories of HIV-1 group M underwent an epidemiological transition and outpaced regional population growth (Faria et al., 2014). The clinical management of HIV infection has greatly improved through the use of highly active ARV therapy (HAART). HAART is comprised of nucleoside or nucleotide reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), and fusion inhibitors. (HAART),(Palella et al., 1998)

The clinical effectiveness of these therapies is mediated by treatment-induced reduction of HIV viral replication as demonstrated by measurements of the amount of HIV RNA in the blood (the plasma viral load). Resistance of HIV to ARV agents was first reported within 2 years of the approval of the NRTI zidovudine (ZDV) for the treatment of persons with late-stage HIV infection. Subsequently, transmission of a ZDV-resistant isolate was first reported in 1992. The development of ARV resistance has since been reported with all other commercially available ARV agents within all classes (V. A. Johnson et al., 2011).

Previous studies have suggested that failing NNRTI-based regimens may have greater potential than other ART to induce the development of resistance mutations, which may limit options for second-line therapy (Mtambo et al., 2012). In resource-limited areas ART uptake involves many challenges which include inadequate supply of drugs. The durability of financial commitments from international donors compiled with the limited financial resources of in-country health ministries is a challenge. Adverse effects like

drug-related toxicities are some of the challenges. There is a need to monitor treatment failure, including the consequences of antiviral drug resistance is required (Ramadhani et al., 2007).Tremendous progress has been made with the scale-up of ART in Africa, with an estimated seven million people now receiving ART in the region.

A survey performed in Kampala between 2009 and 2010 showed a prevalence of transmitted drug resistance at 8.6% (Ndembi et al., 2011).Whilst public health surveillance forms the mainstay of the World Health Organization approach to ARV drug resistance, there is likely to be increasing demand for access to drug resistance testing as programs mature and as HIV clinical management becomes more complex. African-owned research initiatives have helped to develop affordable resistance testing appropriate for use in the region, and have developed delivery models for resistance testing at different levels of the public health system. The long-term population health impact and cost-effectiveness of resistance testing in the region will also require further investigation(Lessells, Avalos, & de Oliveira, 2013). The scale-up of resistance testing will require substantial expansion of clinical and laboratory capacity in the region, but the expertise and resources that exist in Africa to support this is limited. The availability of ARV therapy (ART) is increasing in low and middle-income countries including Kenya. As ART use continue to be scaled up, there is mounting evidence suggesting that drug resistance (DR) will develop and increase over time (Steegen et al., 2009). However, DR surveillance remains highly expensive and mostly nonexistent in many limited resource settings (Dudley et al., 2012). A cross-sectional study in Nairobi in 2005 found 4/53 (7.5%) new clients had TDR (Lihana, Khamadi, Lubano, et al., 2009). The multisite cross-sectional study from the PASER group, conducted between 2007 and 2009, reports TDR frequencies of 9/200 (4.5%) in Mombasa and 10/204 (4.9%) in Nairobi (Hamers et al., 2012). One of the biggest issues with the management of HIV disease in Kenya is the high rate of sub-optimal adherence due to stigma and cultural backgrounds which may affect ART compliance, resulting in an accelerated appearance

of drug-resistant mutants, which are a potential source of drug resistance (Lihana, Khamadi, Lubano, et al., 2009).

It is recommended by the World Health Organization (WHO) that surveillance of drug resistance occur in conjunction with scale-up efforts to ensure appropriate first-line therapy is offered relative to the resistance that exists (Bennett et al., 2009). The current standard genotypic resistance testing methods used in surveillance programs rely on Sanger sequencing (SS) a method that have detection limits of 20% of the virus quasispecies (Bennett et al., 2009).

Increased rates of virological failure to ART regimens, especially NNRTI have been noticed despite no evidence of DR mutations by SS at baseline. In fact, many studies have shown low abundance DR variants (LADRVs) at frequencies less than 20%, in both ART-naïve and heavily ART-treatment subjects (Paredes et al., 2010). Furthermore it has been noted that these LADRVs can increase and outcompete wild type strains under drug selection pressure leading to treatment failure (Dudley et al., 2012). Due to limitations of Sanger sequencing and the need for low cost genome sequencing, there has been a revolution in the large-scale genomics field. To date, three major next-generation sequencing (NGS) technologies Roche 454 Life Science FLX (454), Illumina (Solexa), and Ion PGM (Life technologies), have been commercialized (Bennett et al., 2009). These technologies share the paradigm of massive, parallel, clonal analysis of DNA templates with high data throughput. One application of these technologies is Ultra-deep pyrosequencing (UDPS), which allows identifying LADRV not detectable by standard Sanger Sequencing genotypic technique. Various studies from North America and Europe have shown that UDPS could identify LADRV at frequencies as low as 0.05% of the entire viral population and enabling detailed coverage of rare HIV DR variants (Ji et al., 2010). Baum and Wolf (2011) reported that LADRV studies done in Europe and North America are predominantly infected with HIV subtype B viruses. Less information is available from Sub-Sahara Africa, a region where non-B subtypes are

prevalent and reportedly with the highest projected rate of emerging transmitted HIV drug resistance(Bennett et al., 2009).

1.2 Statement of the Problem

Most of the data concerning non-B subtypes of HIV remain controversial. Given that the epidemics driven by subtypes other than B are occurring in countries with limited resources, there are currently limited ways to assess any of the problems that the increasing genetic diversity of HIV-1 brings into clinical practice. The emergence and spread of ARV drug resistance ARVDR threatens to negatively impact on treatment regimens and compromise efforts to control the epidemic. On the other hand, current standard resistance testing methods used in Sub-Saharan Africa are inadequate and rely on techniques that miss out on LADRVS, which have been documented to contribute to treatment failure.

1.3 Justification of the study

Whilst public health surveillance forms the mainstay of the World Health Organization approach to ARV drug resistance, there is increasing demand for access to drug resistance testing as programs mature and as HIV clinical management becomes more complex. The scale-up of resistance testing require substantial expansion of clinical and laboratory capacity, expertise and resources. It is believed that surveillance will maximize the utility of first-line therapy and help minimize the cost of providing ART thereby sustaining current ARV drug programs. Because DRMs often decrease the activity of many ARV agents within an individual class, the emergence of a single major resistance mutation can have important effects on a patient's response to multiple ARV agents. Additionally, the use of NGS has been shown to be more sensitive for LADRVS therefore 454 pyrosequencing will be vital in surveillance.

1.4 Hypothesis

1.4.1 Null hypothesis

Characterization of protease and reverse transcriptase gene mutations of viral isolates from HIV infected ARV naïve is not required for determining initial treatment strategies. Characterization of protease and reverse transcriptase gene mutations of viral isolates from HIV infected ARV experienced patients in care are not useful in changing therapy for those who fail ARV. Next-generation sequencing (NGS) technologies - Roche 454 Life Science FLX (454) pyrosequencing does not determine low abundance DR variants (LADRVs

1.5 Objectives

1.5.1 General objective

To characterize the inter subtype RT, and PR gene mutations of viral isolates from HIV infected ARV naïve and experienced patients in care.

1.5.2 Specific objectives

1. To determine the proportion, clinical and immunological characteristics of HIV infected ARV naïve patients with DRMs
2. To determine the proportion, clinical and virological characteristics of HIV infected ARV experienced patients failing therapy with DRMs
3. To determine low abundance DR variants (LADRVs) using next-generation sequencing (NGS) technologies - Roche 454 Life Science FLX (454) pyrosequencing

1.6 Significance of the study

The long-term success of ART programs depends on appropriate strategies to deal with potential threats, one of which is the emergence and spread of ARVDR. This information will be vital in the development of an in-country guideline for identification of HIV ARV factors to guide treatment options for HIV/AIDS patients.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of HIV drug resistance

Worldwide, the number of HIV-1 infected persons exceeds 40 million (Passaro, Pandhare, Qian, & Dash, 2015). Despite all the therapeutic advantages achieved during the last decade, including the development of HAART, once an individual has become infected, eradication of the virus has been impossible. In addition, new problems relating to the short- and long-term toxicity of drug treatments and the occurrence of DRMs in both circulating and transmitted viruses are emerging (Lennox et al., 2014).

2.2 ARV therapy

New drugs that offer new mechanisms of action, improvements in potency and activity even against multidrug-resistant viruses, dosing convenience, and tolerability have been approved. There has been a lot of improvement in the field of ART which has dramatically reduced HIV-associated morbidity and mortality and has transformed HIV disease into a chronic, manageable condition. In addition, effective treatment of HIV-infected individuals with ART is highly effective at preventing transmission to sexual partners (Aberg et al., 2014a).

2.2.1 History of ARV therapy

Great development has been done in the field of ARV therapy medicine. Few other areas have been subject to such fast- and short-lived trends. They have experienced the rapid developments of the last few years through many ups and downs. Following the hope of the early years, from 1987-1990, and the modest successes with monotherapy (Volberding, 1990).

Zidovudine (AZT), was introduced in March 1987. The nucleoside analogs ddC, didanosine (ddI), and stavudine (d4T), introduced between 1991 and 1994. The lack of treatment options led to a debate that lasted for several years about which nucleoside analogs should be used, when, and at what dose. One such question was if the alarm clock should be set to go off during the night for the fifth dose of AZT. Many patients, who were infected up until the mid-80s, began to die. Hospices were established, as well as more and more support groups and ambulatory nursing services. One became accustomed to AIDS and its resulting death toll. There was a definite progress in the field of opportunistic infections. Cotrimoxazole, pentamidine, gancyclovir, foscarnet, and fluconazole saved many patients lives, at least in the short-term. Between 1989 and 1994, the mortality rates hardly changed. In September 1995, the results of the American ACTG 175 revealed that two nucleoside analogs were more effective than monotherapy. The differences made on the clinical endpoints AIDS and death were highly significant. Both studies demonstrated that it was potentially of great importance to start treatment immediately with two nucleoside analogs, as opposed to using the drugs successively (Hammer et al., 1996).

The first studies with protease inhibitors (PIs) a completely new drug class, had been running for months. PIs had been designed using the knowledge of the molecular structure of HIV and protease. With the knowledge of the high turnover of the virus and the relentless daily destruction of CD4 cells, there was no consideration of a latent phase and no life without ART (Ho, 1995).

2.2.2 Key characteristics and uses of available ARV agents

Currently, there are five classes of drugs active against HIV (Table 2.1).

1. Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) also referred to as nucleoside/nucleotide analogues. NRTIs work by prematurely terminating DNA chain formation as the enzyme reverse transcriptase copies viral RNA into DNA.

2. Non-nucleoside reverse transcriptase inhibitors (NNRTIs) inhibit formation of viral DNA from viral RNA by tightly binding to the reverse transcriptase enzyme.
3. Protease inhibitors (PIs) bind to the viral protease enzyme and block the formation of viral proteins.
4. Entry inhibitors prevent entry of the virus into the host cell i.e. CD4
5. Integrase strand transfer inhibitors (INSTI) block the integrase enzyme which incorporates/integrates pro-viral DNA into the host cell DNA.

Table 2.1: Current ARV drugs by class

Nucleoside and Nucleotide reverse Transcriptase Inhibitors (NRTIs and NtRTI)	Non-nucleoside Reverse Transcriptase Inhibitors (NNRTIs)	Protease inhibitors (PIs)	Entry inhibitors	Integrase strand transfer inhibitors (INSTI)3
NRTIs Abacavir (ABC)* Didanosine (ddI) Emtricitabine (FTC)* Lamivudine (3TC)* Stavudine (d4T)* Zidovudine (AZT)*	Efavirenz* Nevirapine* Etravirine Rilpivirine	Indinavir (IDV) Nelfinavir (NFV) Ritonavir* Saquinavir (SQV) Lopinavir (LPV)* Atazanavir (ATV) Fosamprenavir Tipranavir Darunavir (DRV)	Fusion inhibitor enfuvirtide	Raltegravir Elvitegravir
NtRTI Tenofovir (TDF)*			CCR% antagonist Maraviroc	

* Agents recommended for use as first or second-line in Kenya

Adapted from Guidelines for ARV therapy in Kenya, 4th edition

2.2.3.: Nucleoside analogs (NRTIs) Mechanism of action

Nucleoside analogs (nukes) are also referred to as NRTIs. Their target is the HIV enzyme reverse transcriptase. Acting as alternative substrates or false building bricks, they compete with physiological nucleosides, differing from them only by a minor modification in the ribose molecule. The incorporation of nucleoside analogs induces the abortion of DNA synthesis, as phosphodiester bridges can no longer be built to stabilize the double strand. Nucleoside analogs are pro-drugs, which means that they are absorbed unchanged and only activated when three phosphates are attached by intracellular phosphorylation in a stepwise process. It is the triphosphate derivative that is efficacious. AZT and d4T are thymidine analogs, while FTC and 3TC are cytidine analogs. Combinations containing AZT + d4T or FTC + 3TC are therefore pointless, since both drugs compete for the same bases (Havlir, Vella, & Hammer, 2002).

DDI is an inosine analog, which is converted to dideoxyadenosine; abacavir is a guanosine analog. There is a high degree of cross-resistance between nucleoside analogs. Nucleoside analogs were the first drugs to be used in HIV treatment, and therefore, most of the experience is based on them. They are easy to take, and once-daily dosing is sufficient for most. Overall initial tolerability is fairly good. Frequent complaints during the first weeks are fatigue, headache and gastrointestinal problems, which range from mild abdominal discomfort to nausea, vomiting and diarrhea. The gastrointestinal complaints are easily treated symptomatically. Nucleoside analogs can cause a wide variety of long-term side effects, including myelotoxicity, lactate acidosis, polyneuropathy and pancreatitis. Although lipodystrophy was initially linked exclusively to treatment with PIs, many metabolic disorders, and especially lipoatrophy, are also attributed to nucleoside analogs (Galli et al., 2002).

Mitochondrial function requires nucleosides. The metabolism of these important organelles is disrupted by the incorporation of false nucleosides, leading to mitochondrial degeneration. There are probably considerable differences between the

individual drugs with regard to mitochondrial toxicity. Nucleoside analogs are eliminated mainly by renal excretion and do not interact with drugs that are metabolized by hepatic enzymes. There is therefore little potential for interaction. Ribavirin can also reduce intracellular phosphorylation of AZT or d4T. In contrast to the PIs and NNRTIs, the doses have to be adjusted for patients with renal insufficiency (Piscitelli & Gallicano., 2001).

2.2.3.1 ABC (Abacavir) (Ziagen.)

Abacavir is phosphorylated intracellularly to carbovir triphosphate, which has a long half-life (Harris, 2002). In October 2004, following larger studies, abacavir was licensed for once-daily therapy (Moyle et al., 2005). It is also a component of Trizivir and Kivexa. In combination with AZT+3TC (Trizivir, Triple Nuke), ABC was less effective than efavirenz (Gulick et al., 2004). The randomized, double blind CNA3005 Study also showed lower efficacy in comparison to indinavir, particularly with higher viral load (Staszewski et al., 2001). In contrast, efficacy was comparable to that of nelfinavir (Matheron et al., 2003). When combined with 3TC, the efficacy is similar to that of 3TC plus either AZT (DeJesus et al., 2004).

A regimen that is failing virologically can be successfully intensified with ABC if it is added early enough and if the viral load is not too high (Rozenbaum et al., 2001). ABC is also used to simplify HAART. Numerous randomized studies have demonstrated that patients on a successful PI- or NNRTI regimen can switch relatively safely to ABC plus two NRTIs (Clumeck et al., 2001) and (Bonjoch et al., 2005). There is a certain degree of risk associated with this, and particularly in extensively pretreated patients, virological failure is possible (Opravil et al., 2002).

With respect to mitochondrial toxicity, ABC is more favorable than several other substances. One drawback to the use of ABC is the risk of a hypersensitivity reaction (HSR), an allergic reaction that is associated with fever and lethargy. This occurs in 4-6

% of patients, almost always within the first six weeks of treatment. In acutely infected patients, the risk seems to be higher (up to 18 %), and ABC should be avoided (Stekler et al., 2006). The combination of strongly worded warnings contained in the package insert and the unspecific symptoms of HSR poses a constant challenge to the physician. A genetic predisposition exists, so that patients with HLA type B5701 are at a higher risk than others with HSR occurring in up to 80 % of them (Mallal et al., 2002).

2.9.3.2 AZT (Zidovudine, Retrovir.)

In contrast, two other early, very large studies, ACTG 016 and 019 demonstrated no significant survival benefit in asymptomatic patients, although the risk for progression was significantly reduced in both. The Long-term treatment almost always increases MCV (mean corpuscular volume of erythrocytes), which is useful as a means of assessing adherence. Gastrointestinal complaints, especially initially, may present a further problem. In contrast, AZT-related myopathy or even cardiomyopathy is quite rare. A logical disadvantage of AZT is that it has to be taken twice daily, disqualifying it as a substance for once-daily combinations. AZT finally came under pressure when in one study, it scored significantly worse than TDF, mainly due to poorer tolerability. Severe anemia was significantly increased in the AZT-arm in comparison to TDF, causing 5.5 % of cases to drop out (Gallant et al., 2006). Lack of neurotoxicity and good CNS penetration are some of the advantages of this drug. AZT still remains a component of many regimens and transmission prophylaxes. AZT is also a component of both Combivir and Trizivir at a slightly higher dose (300 instead of 250 mg), which may occasionally lead to higher myelotoxicity (Volberding, 1990).

2.9.3.4 ddI – (Didanosine, Videx.)

In 1991, it was the second nucleoside analog to be licensed. The introduction of acid-resistant tablets, which, in 2000, replaced the chewable tablets, improved tolerability and patient acceptance significantly. Early studies showed a survival advantage for

treatment-naïve patients with AZT+ddI compared to AZT monotherapy. This effect of ddI was less marked in AZT pretreated patients. The addition of ddI in another study led to significant survival benefit, although this was not the case in CPCRA007 (Saravolatz et al., 1996).

2.9.3.5 D4T (Stavudine, Zerit.)

It was the second thymidine analog to be introduced after AZT. Subjectively, d4T is often initially tolerated better than AZT (less gastrointestinal side effects and limited myelotoxicity), is certainly just as effective (Spruance et al., 1997) and used to be one of the most frequently prescribed HIV drugs. Based on current data, d4T should be avoided wherever possible and replaced, ideally with ABC or TDF if the resistance profile permits. In the developing countries, the situation is different, and it remains an important combination partner, particularly due to the lack of myelotoxicity (Moyle, Brown, Lysakova, & Barton, 2006).

2.9.3.6 3TC (Lamivudine, Epivir.)

It was in August 1996, the fifth NRTI to be licensed in Europe. It is a well-tolerated cytidine analog, whose substantial disadvantage is rapid development of resistance. A single point mutation (M184V) is sufficient to cause loss of efficacy. On monotherapy, this mutation is likely to lead to resistance after only a few weeks (Eron, 2008). The full effect of 3TC only emerges in combination with other NRTIs. As a component of Combivir Kivexa and Trizivir 3TC is actually one of the most frequently used ARV agents of all. In some studies 3TC significantly improved disease progression and survival when added to NRTI therapy (Staszewski et al., 2001).

2.9.3.7 FTC (Emtricitabine, Emtriva)

It is a cytidine analog, which is biochemically, very similar to 3TC, but has a longer half-life. FTC seems to have a low affinity for the mitochondrial polymerase, so the risk

of mitochondrial toxicity is likely to be relatively low. In monotherapy studies as well as in combination with AZT, FTC was at least as effective as 3TC (Benson et al., 2004), as Efficacy with 3TC is however limited by the M184V point mutation. Subsequent to data from the FTC-301 Study, the drug was licensed in 2003. Previously, a randomized, double blind trial showed that FTC was clearly more effective and tolerable than d4T, although this was probably not due to differences between FTC and 3TC (Saag, Bowers, Leitz, Levine, & Community, 2004). Another study demonstrated the good long-term tolerability and efficacy of a once-daily combination of FTC+ddI+efavirenz (Molina, Journot, et al., 2005).

2.9.3.8 TDF (TDF, Viread.)

TDF acts as a false building block similar to nucleoside analogs targeting the enzyme RT. In addition to the pentose and nucleic base, it is monophosphorylated and referred to as a nucleotide analog. The accurate description of the substance is TDF (disoproxil fumarate = TDF), referring to the phosphonate form from which the phosphonate component is only removed by a serum esterase, and which is activated intracellularly in two phosphorylation steps. In a previous study, TDF+FTC was significantly better than AZT+3TC (Gallant et al., 2006), particularly due to the improved tolerability. TDF can also help to improve d4T-induced lipoatrophy and dyslipidemias (Moyle et al., 2006).Reduced efficacies occur with particular triple nuke combinations. In the case of virological treatment failure on TDF, the K65R mutation, a problematic nucleoside analog resistance, is frequently found. The potential risk of nephrotoxicity is a serious problem for TDF that is associated with a mild to moderate disturbance of renal function. Severe disturbances are rare. Patients with renal disease should either not be treated with TDF, or at least receive a lower dose. Elderly and lighter patients are particularly at risk although it is not possible to predict patients at risk. In the Swiss cohort, 46 out of 2,592 patients (1.6 %) had to stop TDF because of renal toxicity, after on average 442 days (Fux et al., 2007).

2.9.3.9 TDF+3TC/FTC

Since the introduction of FTC and the combined tablet Truvada in August 2004, TDF has been administered more frequently together with FTC than with 3TC. TDF+FTC is currently the most commonly used backbone in Phase III/IV studies. In another study, using 509 treatment-naïve patients, TDF+FTC was compared to AZT+3TC (both with efavirenz). At 48 weeks, more patients on TDF+FTC reached a viral load of less than 50 copies per ml. The significant differences were primarily related to the poorer tolerability of AZT+3TC, which often resulted in the discontinuation of therapy (9 versus 4 %). Virological failure and resistance mutations were approximately equal in both arms and were infrequent. At 96 weeks, no further significant differences were observed, although lipoatrophy side effects were rare with TDF+FTC than with AZT+3TC. In the future, TDF therapy will play an important role providing no undesirable surprises arise with regard to nephrotoxicity (Gallant et al., 2006).

2.9.3.10 ABC+3TC

In a previous study, ABC+3TC had the same efficacy as d4T+3TC, but were also less toxic. So far, there are no comparable studies on TDF+FTC. It is important to note that ABC+3TC have a significantly shorter half-life. In comparison to TDF+FTC there could be an advantage in that L74V, usually occurring alongside the M184V mutation is associated with less cross-resistance than the TDF-associated K65R mutation. A significant disadvantage of the combination with NNRTIs is however the higher risk of occurrence of allergies under both ABC and NNRTIs, making it difficult to distinguish between a NNRTI rash and the ABC HSR (Berenguer et al., 2006).

2.9.3.11 AZT+3TC

In many guidelines, AZT+3TC is still regarded as the standard backbone for first line therapy. There is more experience with this combination than with any other. The

resistance profile is favorable: the M184V mutation that frequently develops during 3TC treatment probably increases sensitivity to AZT. AZT+3TC is usually given as Combivir. Furthermore, AZT+3TC were shown by the Gilead 934 Study to be less effective (tolerated less) than TDF+FTC (Gallant et al., 2006).

2.2.4: Entry inhibitors

Entry inhibitors differ from NRTIs, NNRTIs and PIs, which block the replication of HIV in the infected cell. They prevent HIV from entering its target cells. The first step in cell entry occurs when the HIV envelope glycoprotein, gp120, binds to the CD4 receptor of the target cell, leading to conformational changes in gp120 and therefore enabling binding of the V3 loop of gp120 to the chemokine co-receptors, CCR5 or CXCR4. Interactions between the two heptad repeat regions HR1 and HR2 within the transmembrane glycoprotein subunit gp41 induce a conformational change in gp41, leading to insertion of the gp41 fusion peptide into the target cell membrane, thereby enabling entry of the viral core into the target cell. CCR5 co-receptor antagonists function by binding specifically to the CCR5 molecule, which then is unable to bind to the viral gp120 subunit. The conformational changes leading to insertion of the gp41 fusion peptide are prevented and viral entry is stopped. Fusion inhibitors prevent fusion of viral and cell membranes. T-20 (enfuvirtide), a synthetic peptide consisting of 36 amino acids, mimics the C-terminal HR2 domain of gp41 and competitively binds to HR1. Interactions between HR1 and HR2 are blocked and the conformational change of gp41 that is necessary for fusion of virions to host cells is inhibited. A single amino acid substitution in gp41 can reduce the efficacy of T-20.

2.2.5 Poor and non-recommended backbones

Avoiding the previously popular d4T+ddI combination is highly recommended. Studies have shown that mitochondrial toxicity is too high, and it is inferior to AZT+3TC (Robbins et al., 2003). In cases where there is treatment failure, thymidine analog

mutations (TAMs) are usually present, which has been shown to limit future options. In NRTIs available today, ddI+d4T is no longer justified at least for first-line therapy. d4T+3TC is another combination recommended only in certain situations for first line therapy. Although it is subjectively very well tolerated initially, d4T leads to problems with long-term toxicity. Studies such have shown that d4T+3TC cause more lipoatrophy than ABC+3TC or TDF+3TC. d4T+3TC would only be used today when neither AZT nor TDF could be used due to co-morbidity. If therapy with d4T+3TC has been started, it should be rapidly replaced. Because ddI has to be taken on an empty stomach (whilst AZT is tolerated better when taken with a meal), and in particular due to the greater risk of gastrointestinal side effects, AZT+ ddI is contraindicated. TDF+ ddI are relatively toxic and, recently, many studies have shown lower efficacy TDF+ABC is likely to be problematic due to rapid development of resistance. AZT+d4T and FTC+3TC are antagonistic. Most data are derived from patients with subtype B viruses. Resistance pathways and patterns may differ in the various subtypes (Berenguer et al., 2006).

2.10 Laboratory Testing

2.10.1 Laboratory Testing for Initial Assessment and Monitoring of HIV-Infected Patients on ARV Therapy

A number of laboratory tests are important for initial evaluation of HIV-infected patients upon entry into care; during follow-up if ART is not initiated; and before and after initiation or modification of therapy to assess the virologic and immunologic efficacy of ART and to monitor for laboratory abnormalities that may be associated with ARV drugs. Two surrogate markers are used routinely to assess immune function and level of HIV viremia: CD4 T-cell count (CD4 count) and plasma HIV RNA (viral load), respectively. Resistance testing should be used to guide selection of an ARV regimen. A viral tropism assay should be performed before initiation of a CCR5 antagonist or at the time of virologic failure that occurs while a patient is receiving a CCR5 antagonist. HLAB*5701 testing should be performed before initiation of ABC (Aberg et al., 2014b).

2.10.2 Plasma HIV-1 RNA (Viral Load) Monitoring

HIV RNA (viral load) and CD4 T lymphocyte (CD4) cell count are the two surrogate markers of ARV treatment (ART) responses and HIV disease progression that have been used for decades to manage and monitor HIV infection. Viral load is a marker of response to ART. A patient's pre-ART viral load level and the magnitude of viral load decline after initiation of ART provide prognostic information about the probability of disease progression (Murray, Elashoff, Iacono-Connors, Cvetkovich, & Struble, 1999). The key goal of ART is to achieve and maintain durable viral suppression. Therefore, the most important use of the viral load is to monitor the effectiveness of therapy after initiation of ART. Measurement of CD4 count is particularly useful before initiation of ART. The CD4 cell count provides information on the overall immune function of an HIV-infected patient. The measurement is critical in establishing thresholds for the initiation and discontinuation of opportunistic infection (OI) prophylaxis and in assessing the urgency to initiate ART. The management of HIV-infected patients has changed substantially with the availability of newer, more potent, and less toxic ARV agents. In the United States, ART is now recommended for all HIV-infected patients regardless of their viral load or CD4 count. In the past, clinical practice, which was supported by treatment guidelines, was generally to monitor both CD4 cell count and viral load concurrently. Most HIV-infected patients in care now receive ART; the rationale for frequent CD4 monitoring is weaker. Several systematic reviews of data from clinical trials involving thousands of participants have established that decreases in viral load following initiation of ART are associated with reduced risk of progression to AIDS or death (Thiebaut et al., 2000). Viral load testing is an established surrogate marker for treatment response. The minimal change in viral load considered to be statistically significant (2 standard deviations) is a three-fold change (equivalent to a 0.5 log10 copies/mL change). Optimal viral suppression is defined generally as a viral load persistently below the level of detection (HIV RNA<20 to 75 copies/mL depending on the assay used (Damond et al., 2007).

2.10.3 CD4 Count Monitoring

The CD4 count is the most important laboratory indicator of immune function in HIV-infected patients. It is also the strongest predictor of subsequent disease progression and survival according to findings from clinical trials and cohort studies (Mellors et al., 1997). For most patients on therapy, an adequate response is defined as an increase in CD4 count in the range of 50 to 150 cells/mm (Thiebaut et al., 2000) during the first year of ART, generally with an accelerated response in the first 3 months of treatment. Subsequent increases average approximately 50 to 100 cells/mm3 per year until a steady state level is reached (Kaufmann et al., 2003). ART is now recommended for all HIV-infected patients. In patients who remain untreated for whatever reason, CD4 counts should be monitored every 3 to 6 months to assess the urgency of ART initiation and the need for OI prophylaxis. The CD4 count should be monitored more frequently, as clinically indicated, when there are changes in a patient's clinical status that may decrease CD4 count and prompt OI prophylaxis (Berglund, 1991).

2.10.4 Assays for resistance testing

There are two established assays for measuring resistance or sensitivity of HIV to specific ARV drugs, the genotypic and the phenotypic resistance tests. Genotypic and phenotypic resistance assays are used to assess viral strains and inform selection of treatment strategies. Standard assays provide information on resistance to NRTIs NNRTIs and PIs. Testing for integrase and fusion inhibitor resistance can also be ordered separately from several commercial laboratories. Co-receptor tropism assays should be performed whenever the use of a CCR5 antagonist is being considered. Phenotypic co-receptor tropism assays have been used in clinical practice. A genotypic assay to predict co-receptor use is now commercially available. Both assays are commercially available. Examples of commercially available genotypic resistance tests are: HIV-1 TrueGene., Bayer Healthcare Diagnostics/ Siemens Medical Solutions

Diagnostics; or ViroSeq, Celera Diagnostics/ Abbott Laboratories; both assays are approved by the FDA.

Other genotypic resistance assays such as virco.TYPE HIV-1, Virco, GenoSure (Plus), LabCorp, or GeneSeq, Monogram Biosciences (formerly Virologic) are established in the laboratories of the respective manufacturers and are used in clinical trials. Phenotypic resistance tests include: Antivirogram., Virco; PhenoSense., Monogram Biosciences (formerly ViroLogic); and Phenoscript., Viralliance. Genotypic assays detect drug-resistance mutations present in relevant viral genes. Most genotypic assays involve sequencing of the RT and PR genes to detect mutations that are known to confer drug resistance. Genotypic assays that assess mutations in the integrase and gp41 (envelope) genes are also commercially available. Genotypic assays can be performed rapidly and results are available within 1 to 2 weeks of sample collection. Interpretation of test results requires knowledge of the mutations selected by different ARV (ARV) drugs and of the potential for cross-resistance to other drugs conferred by certain mutations. The International AIDS Society-USA (IAS-USA) maintains an updated list of significant resistance-associated mutations in the RT, PR, integrase, and envelope genes.

Disadvantages of phenotypic testing include the lengthy procedure and high expense of the assay. The cost of genotyping ranges from 350 to 500 Euro per sample, depending on the assay and laboratory used. It is approximately twice as much for phenotyping. The drawback with both methods is that a minimum amount of virus is necessary in order to perform the test. A viral load below 1,000 copies/ml often does not allow any detection of resistance. Within the nucleotide sequences of the HIV genome, a group of three nucleotides, called a codon, defines a particular amino acid in the protein sequence. Resistance mutations are described using a number, which shows the position of the relevant codon, and two letters: the letter preceding the number corresponds to the amino acid specified by the codon at this position in the wild-type virus; the letter after

the number describes the amino acid that is produced from the mutated codon. M184V indicates a mutation in codon 184 of the reverse transcriptase gene leading to a valine for methionine substitution in the RT enzyme.

2.11 Mechanisms of resistance

Nucleoside and nucleotide reverse transcriptase inhibitors (NRTIs) are pro-drugs that only become effective after being converted to triphosphates. Nucleotide analogs require only two instead of three phosphorylation steps. Phosphorylated NRTIs compete with naturally occurring dNTPs (deoxynucleotide triphosphates). The incorporation of a phosphorylated NRTI into the proviral DNA blocks further elongation of the proviral DNA and leads to interruption of the chain. There are two main biochemical mechanisms that lead to NRTI resistance (de Mendoza et al., 2002).

Sterical inhibition is caused by mutations enabling the reverse transcriptase to recognize structural differences between NRTIs and dNTPs. Incorporation of NRTIs is then prevented in favor of dNTPs (e.g. in the presence of the mutations M184V, Q151M, L74V, or K65R (Naeger, Margot, & Miller, 2001), Phosphorylysis via ATP (adenosine triphosphate) or pyrophosphate leads to the excision of the NRTIs already incorporated in the growing DNA chain. This is the case with the following mutations: M41L, D67N, K70R, L210W, T215Y and K219Q. Phosphorylysis leads to cross-resistance between NRTIs, the degree of which may differ between substances (AZT, d4T > ABC > ddI > 3TC). Contrary to the excision mutations, K65R leads to a decreased excision of all NRTIs when compared to the wild-type, resulting in a greater stability once incorporated. For K65R, the combined effect of its opposing mechanisms on the one hand decreased incorporation and on the other, decreased excision results in a decreased susceptibility to most NRTIs but an increased susceptibility to AZT (White et al., 2005).

NNRTIs also inhibit the viral RT. NNRTIs are small molecules that bind to the hydrophobic pocket close to the catalytic domain of the RT. Mutations at the NNRTI

binding site reduce the affinity of the NNRTI to the RT and lead to loss of antiviral activity of NNRTI and treatment failure. PIs hinder the cleavage of viral precursor gag-polpolyprotein by the enzyme protease, thereby producing immature, non-infectious viral particles. PI resistance usually develops slowly, as several mutations must first accumulate. This is also referred to as the genetic barrier. For PIs, a distinction is made between major (or primary) and minor (or secondary) mutations. Major mutations are responsible for phenotypic resistance. They are selected for early on in the process of resistance to one drug, and are located within the active site of the target enzyme, the HIV protease. They reduce the ability of the protease inhibitor to bind to the enzyme. Major or primary mutations may also lead to a reduced activity of the protease. Minor mutations are located outside the active site and usually occur after major mutations. Minor mutations can be particularly found at polymorphic sites of non-B subtypes. Minor mutations can compensate for the reduction in viral fitness caused by major mutations (A. A. Johnson et al., 2006).

2.12 Transmission of resistant HIV strains

The prevalence of mutations already present in treatment-naïve patients differs among demographic regions. High prevalence of more than 20 % were observed in big US cities with large populations of homosexual men and a long period of access to ARV treatment. High rates of resistance transmission were observed in Madrid in the late nineties (Truong et al., 2006). In chronically infected patients the proportion with primary resistance was 11 % between 2001 and 2004 (Oette, Kaiser, et al., 2006). The proportion of NRTI mutations decreased over time, the frequency of NNRTI resistance mutations increased. The frequency of PI resistance remained relatively stable.

Primary resistance was mainly observed in subtype B infections. An increase over time was also observed in non-B subtypes. Follow-up data from the years 2002 and 2003 are derived from the SPREAD study whereby 9.1 % of the 1,050 newly diagnosed HIV patients were infected with a virus carrying resistance mutation (Wensing et al., 2006).

Transmitted resistance mutations can limit further treatment options and reduce treatment response. On careful consideration of any pre-existing resistance, primary treatment success is often possible (Oette, Kroidl, et al., 2006). The prevalence of TDR in sub-Saharan Africa has previously been reported to be <5 % (Kamoto, Aberle-Grasse, & Malawi, 2008; Pillay et al., 2008). Recent data suggest an increase in the prevalence of TDR in some settings. In Kampala, Uganda, the prevalence of TDR increased from 0% (2006–2007) to 8.6% (2009–2010)(Ndembi et al., 2011). The IAVI Early infection cohort conducted among the most at risk populations in East and Central Africa reported an increase in the prevalence of TDR in Zambia, from 0% (2005) to 16% (2009)(Wallis, Mellors, Venter, Sanne, & Stevens, 2010). A multisite, cross-sectional study conducted between 2007 and 2009 at 11 sites in sub-Sahara Africa reported a 38% increase in risk of TDR with each additional year from the start of the local ARV roll-out (Wallis, Mellors, Venter, Sanne, & Stevens, 2011). In Yaounde, Cameroon, a steady increase in the prevalence of TDR was observed, from 0% (1996–1999) to 12.3% (2007). The same study reported a TDR prevalence of 4.8% (2006–2007) in rural areas of Cameroon (Aghokeng et al., 2011).

In Kenya, a handful of studies have been done to assess the prevalence of TDR. A cross-sectional study in Nairobi in 2005 found 4/53 (7.5%) new clients had TDR (Lihana, Khamadi, Lwembe, et al., 2009). The IAVI early infection cohort reported an overall TDR prevalence of 3.1% from three sites in Kenya (Price et al., 2011) The multisite cross-sectional study from the PASER group, conducted from 2007 to 2009, reports TDR frequencies of 9/200 (4.5%) in Mombasa and 10/204 (4.9%) in Nairobi (Pillay et al., 2008). A cross-sectional survey among newly diagnosed ARV-naive adults attending four VCT centers from Mombasa in 2009–2010 reported an overall TDR prevalence of 13.2% (Sigaloff et al., 2012).

2.13 Interpretation of genotypic resistance profiles

2.13.1 NRTIs

A summary of mutations on the RT gene leading to NRTI resistance is shown on Table 2.2 (Castagna et al., 2006)

Table 2.2: Mutations on the reverse transcriptase gene leading to NRTI resistance

Zidovudine	**T215 Y/F (esp. with other TAMs)** **≥ 3 of the following mutations: M41L, D67N, K70R, L210W, K219Q/E** **Q151M (esp. with A62V/F77L/F116Y)** **T69SSX (insertion)***
Stavudine	V75M/S/A/T T215Y/F (usually in combination with other TAMs) ≥ 3 TAMs* Q151M (esp. with A62V/F77L/F116Y) T69SSX (insertion)*
ABC	≥ (4-) 5 of the following mutations M41L, D67N, L74V, M184V, L210W T215Y/F K65R+L74V+115F+ M184V Q151M (esp. with A62V/F77L/F116Y) T69SSX (insertion)* K65R (resistance possible)
Lamivudine	M184V/I T69SSX (insertion)* K65R (resistance possible)
Emtricitabine	M184V/I T69SSX (insertion)* K65R (resistance possible)
Didanosine	L74V, esp. with T69D/N or TAMs Q151M (esp. with A62V/F77L/F116Y) T69SSX (insertion)* K65R (partial resistance, esp. with T69D/N) T215Y/F and ≥ 2 of the following mutations: M41L, D67N, K70R, L210W, K219Q/E
TDF	T69SSX (insertion)* ≥ 3 TAMs with M41L or L210W (in some cases only partial resistance) (≥ 3 -) 6 of the following mutations: M41L, E44D, D67N, T69D/N/S, L74V, L210W, T215Y/F K65R (partial resistance)

TAMs = thymidine analog mutations

* T69SSX in combination with T215Y/F and other TAMs leads to a high degree of resistance to all NRTIs and TDF(Adapted from the rules of the DRMs Group of the International AIDS Society-USA (V. A. Johnson et al., 2011)

2.13.2 NNRTIs

Table 2.3: Summary of NNRTI mutations

NNRTIs	Resistance mutations
Efavirenz	L100l
	K101E
	K103N(H/S/T)
	V106M
	V108I (with other NNRTI mutations)
	Y181C(I)
	Y188L(C)
	G190S/A (C/E/Q/T/V)
	P225H (with other NNRTI mutations)
	M230L
Nevirapine	A98G
	L100l
	K101E
	K103N (H/S/T)
	V106A/M
	V108I
	Y181C/I
	Y188C/L/H
	G190A/S (C/E/Q/T/V)
	M230L
TMC125 (Etravirine	≥3 of the following mutations: V90I, A98G, L100I, K101E/P, V106I, V179D/F, Y181C/I/V, G190A/S.
	L100I+K103N
	F227C

(Adapted from the rules of the DRMs Group of the International AIDS Society-USA (V. A. Johnson et al., 2011) . Mutations associated with a high degree of resistance in bold font.

2.13.3 PIs

The spectrum of PI mutations is very large. Although there is a moderate to high degree of cross-resistance between PIs, the primary mutations are relatively specific for the individual drugs. If treatment is changed early on to another PI combination, i.e. before the accumulation of several mutations, the subsequent regimen may still be successful.

2.13.4 First generation PIs:

Most data on primary mutations selected for first in the presence of a PI, are derived from studies using unboosted PIs (Table 2.4).

Table 2.4: Mutations associated with PI resistance

PIs	Relevant resistance mutations and patterns	Further mutations associated with PI resistance
Indinavir	M46l/L V82A/F/S/T l84A/V when boosted with ritonavir, several mutations are required for a relevant loss of sensitivity	I54V/L/M/T, A71V/T, G73S/A, V77I and L90M ≥ 2 PRAMs*
Saquinavir/ Ritonavir (1000/100 mg BID)	≥ 4 of the following mutations: L10I/ R/V, G48V, I54V/L, A71V/T, V77I, V82A, I84V and L90M or ≥ 3-4 of: L10F/I/M/R/V, I15A/V, K20I/M/R/T, L24I, I62V, G73ST, 82A/F/S/T, I84V and L90M	≥ 2 PRAMs*
Nelfinavir	D30N l84A/V N88S/D L90M	V82A/F/S/T and at least 2 of the following mutations: L10I, M36I, M46l/L, I54V/L/M/T, A71V/T, V77I ≥ 2 PRAMs*
Fosamprenavir	I50V (esp. with M46I/L) V32I plus I47V I54L/M I84V	
Fosamprenavir/ Ritonavir (700/100 mg BID) or Amprenavir/ Ritonavir (600/100 mg BID)	≥ 6 of the following mutations: L10F/I/V, K20M/R, E35D, R41K, I54V/L/M, L63P, V82A/F/T/S, I84V V32I plus I47V or ≥ 3 mutations of: L10I/F/R/V, L33F, M36I, M46I/L, I54L/M/T/V, I62V, L63P, A71I/L/V/T, G73A/C/F/T, V82A/F/S/T, I84V and L90M	G73S
Lopinavir/ Ritonavir	≥ 8 of the following mutations: L10F/I/R/V, K20M/R, L24l, V32I, L33F, M46l/L, I47V/A, I50V, F53L, l54L/T/V, L63P, A71l/L/V/T, G73S, V82A/F/T, l84V, L90M L76V together with further P mutations I47V	5-7 of the following mutations: L10F/I/R/V, K20M/R, L24l, V32I, L33F, M46l/L, I47V/A, I50V, F53L, l54L/T/V, L63P, A71l/L/V/T, G73S, V82A/F/T, l84V, L90M ≥ 2 PRAMs*

(Adapted from the rules of the DRMs Group of the International AIDS Society-USA (V. A. Johnson et al., 2006)

2.13.4.1 Saquinavir:

Saquinavir is used together with other medications to treat or prevent HIV/AIDS. It is used with ritonavir or lopinavir/ritonavir. A previos retrospective analysis re-evaluated the genotypic interpretation of saquinavir resistance of 138 PI-experienced patients. In this retrospective study the mutations 10F/I/M/R/V, 15A/V, 20I/M/R/T, 24I, 62V, 73ST, 82A/F/S/T, 84V, and 90M were identified as those most strongly associated with virological response. The presence of 3 to 4 mutations was associated with a reduced response to boosted saquinavir in the 138 PI-experienced patients, (Molina, Marcelin, et al., 2005).

2.13.4.2 Nelfinavir:

The typical nelfinavir-specific resistance profile, with the D30N primary mutation and further secondary mutations, results in only a low degree of cross-resistance to other PIs. In subtype B viruses, treatment with nelfinavir generally leads to the emergence of D30N or M46I plus N88S. In subtype C, G and AE viruses, the mutations L90M and I84V occur more frequently. One reason for these different resistance pathways is the prevalence of natural polymorphisms: whereas the polymorphism M36I is present in only 30 % of subtype B viruses, M36I is present in 70 -100 % of non-B subtypes. For subtypes C or G primary resistance pathways are 82I/V + 63P + 36I/V or 82I + 63P + 36I + 20I, for subtype F resistance pathways are 88S or 82A + 54V (Babic et al., 2006).

A comparison between the replicative capacities of a virus with a single protease mutation (D30N or L90M) and that of the wild-type virus, demonstrated a significant loss of viral fitness in the presence of the D30N mutation selected by nelfinavir. In contrast, the L90M mutation only leads to a moderate reduction in the replicative capacity, which can be compensated for by the frequently occurring L63P polymorphism. Conversely, the L63P mutation hardly influences the reduced replicative capacity of D30N mutants, Unboosted indinavir and/or ritonavir mainly selected for the

major mutation V82A (T/F/S), which in combination with other mutations led to cross-resistance to other PIs.). Mutants that frequently developed under indinavir, harboring M46I, L63P, V82T, I84V or L10R, M46I, L63P, V82T, I84V, were just as fit as the wild-type virus.(Shafer et al 2002c)

2.13.4.3 Fos-/Amprenavir:

In the course of failing treatment with unboosted amprenavir or fosamprenavir, the following mutations have been selected: I54L/M, I50V or V32I plus I47V often together with the mutation M46I. A German team reported that even with 5-10 PI-mutations, which normally confer broad PI cross-resistance, re-sensitization is possible. The mutation L76V, which is primarily selected for by lopinavir and rarely by amprenavir, is associated with resistance to lopinavir,(fos-)amprenavir and darunavir, but can lead to resensitization to atazanavir, saquinavir and tipranavir (Gulick et al., 2006). The resistance profile of atazanavir, an aza-peptidomimetic PI, partly differs to that of other PIs. In patients, in whom first-line treatment with atazanavir failed, the mutation I50L often combined with A71V, K45R, and/or G73S were primarily observed. On the one hand, I50L leads to a loss of sensitivity to atazanavir; A study of 63 patients, activity of boosted atazanavir was reduced markedly in the presence of three or more mutations (Llibre & Clotet, 2012).

2.13.5 Second generation PIs

Tipranavir, the first non-peptidic protease inhibitor, shows good efficacy against viruses with multiple PI mutations. In a previous study, even in case of reduced susceptibility to darunavir, about half of 586 virus samples remained susceptible to tipranavir (Gulick et al., 2004). In pooled data analyses of phase II and III studies, a tipranavir mutation score was developed including 21 mutations at 16 positions (I10V, I13V, K20M/R/V, L33F, E35G, M36I, N43T, I47V, I54A/M/V, Q58E, H69K, T74P, V82L/T, N83D and I84V). Regression analyses showed that per increase of one in the mutation score, virological

response was decreased by 0.16 log. The presence of 4 to 7 mutations led to a moderately reduced tipranavir response.

The accumulation of 8 or more mutations was predictive for tipranavir failure (Karim, Baxter, Frohlich, & Karim, 2014). Darunavir, a non-peptidic PI, shows good activity, both in vitro and in vivo, against a broad spectrum of PI-resistant viruses. Pooled data analyses of the clinical studies Power 1, 2 and 3 showed that the presence of specific baseline mutations was associated with reduced virological response (i.e. V11I, V32I, L33F, I47V, I50V, I54L/M, G73S, L76V, I84V, and L89V).

The mutations V32I, L33F, I47V, I54L or L89V developed in ≥ 10 % of subjects into virological experienced failing therapy. Eleven baseline mutations at 10 positions were associated with reduced response to darunavir in PI-experienced patients: V11I, V32I, L33F, I47V, I50V/L, I54L/M, G73S, L76V, I84V und L89V. With at least three or four mutations response to darunavir was poor. The single mutations of this darunavir resistance score seemed to have different effects on darunavir susceptibility with a relative order of I50V, followed by I54M, L76V and I84V, and then by V32I, L33F and I47V. V11I, I54L, G73S and L89V had the smallest impact. This preliminary weighting of mutations must still be validated. New mutations emerging on failing darunavir were V32I, L33F, I47V, I54L and L89V. The corresponding median fold change in IC50 for darunavir was 8.14. Tipranavir did not show an increase in IC50, the respective median fold change was 0.82. About 50 % of virus isolates were still sensitive to tipranavir. Vice versa, in more than 50 % of isolates with reduced tipranavir susceptibility, sensitivity to darunavir was observed (M. O. Johnson, Gamarel, & Dawson Rose, 2006)

2.13.6 Fusion inhibitors

The focus here is on enfuvirtide (T-20) resistance. The gp41 genome has positions of high variability and highly conserved regions. There seems to be no differences between B and non-B subtypes. Polymorphic sites are observed in all regions of gp41 (Table 2.5).

Table 2.5: Mutations on the env (gp41) gene leading to t-20 resistance

Fusion inhibitors	Resistance mutations
T-20	G36A/D/E/S/V
	38A/M/E/K/V
	Q40H/K/P/R/T
	N42T/D/S
	N43D/K/H/S
	N42T+N43S
	N42T+N43K
	G36S+L44M
	L44M
	L45M/L/Q

(Adapted from the rules of the DRMs Group of the International AIDS Society-USA (V. A. Johnson et al., 2006)

2.13.7 New drugs

Etravirine (TMC125), a second generation NNRTI, is effective against viruses with NNRTI mutations such as L100I, K103N, Y188L and/or G190A/S. Etravirine has a higher genetic barrier than other NNRTIs due to its flexible binding to the reverse transcriptase. In a placebo-controlled study with etravirine, virological outcome was adjusted for other NNRTI mutations and the use of T-20 comparable with or without K103N. The mutation Y181C was related to reduce virological response. In patients with documented NNRTI resistance and at least three primary PI mutations, virological response to etravirine plus optimized backbone decreased with the number of nnrti

mutations. in patients without nnrti mutations at baseline, the mean viral load reduction at week 48 was 1.67 log in the 800 mg study arm. with one, two or three mutations viral load reductions were 1.38, 0.90 and 0.54 logs. in the duet trials, 13 tmc125 resistance associated mutations (rams) were identified: V90I, A98G, L100I, K101E/P, V106I, V179D/F, Y181C/I/V, and G190A/S. In the presence of 0-2 TMC125 RAMs virological response was not compromised, but with three or more RAMs, virological response was markedly reduced (Mills & Nachega, 2007).

2.13.8 Integrase inhibitors

Genotypic analysis of patients with failing first-line therapy with raltegravir, TDF and lamivudine in a study indicated two cases with the signature mutation N155H, in one of two along with additional integrase resistance mutations. Some patients failed while harbouring only a 3TC mutation (Markowitz et al., 2007). In other studies, in treatment experienced patients raltegravir failure was generally associated with one of two genetic pathways: N155H or Q148K/R/H. Secondary mutations commonly observed with N155H included V151I, T97A, G163R, L74M and E92Q. Viruses that evolved resistance via the Q148H/R/K pathway tended to select E138K and G140S/A. Another pathway involved in raltegravir resistance is Y143R/C together with L74A/I, E92Q, T97A, I203M, and S230R (Cooper, 2007).

2.14 Fusion inhibitors Resistance mutations

The reduction in susceptibility is generally higher for double mutations than for single mutations. In countries with access to ARV treatment primary resistance mutations are observed in ≥10% of treatment naïve patients. With the aid of HIV resistance tests prior to initiation of ARV treatment, virological response rates can be improved. Virological rebound occurs primarily due to the emergence of resistant HIV Variants. Several years, national and international HIV treatment guidelines have recommended the use of resistance testing. With some delay, the costs for resistance testing prior to ART

initiation and in case of virological failure are covered by public health insurances in several countries. Currently, both genotypic and phenotypic tests show good intra- and inter-assay reliability. The interpretation of genotypic resistance profiles has become very complex and requires constant updating of the guidelines. New ARVs such as CCR5 antagonists or integrase inhibitors must be implemented in resistance evaluation. The determination of the thresholds associated with clinically relevant phenotypic drug resistance is crucial for the effective use of (virtual) phenotypic testing.

2.15 Subtype-specific mutations

A previous study carried out evaluated treatment success and development of ART drug resistance after short-term treatment among patients attending the Comprehensive HIV Care Centre (CCC) of Coast Province General Hospital, Mombasa, Kenya. One hundred and fifty HIV-infected individuals receiving ART were consecutively recruited to participate in the study. After determination of plasma viral load, patients with detectable viral load levels were subjected to genotypic drug resistance testing. At the time of sampling, 132 of the 150 participants were on ART for more than 6 months (median 21 months, IQR = 12–26). An efficient viral load reduction to below 50 copies/ml was observed in 113 (85.6%) of them. Eleven (11) of these 16 patients were infected with a subtype A1 virus. Major PR mutations were absent, but mutations associated with drug resistance in RT were detected in 14 of the 16 patients (87.5%). High-level resistance against at least 2 drugs of the ART regimen was observed in 9/14 (64.3%). The 3TC mutation M184V and the NNRTI mutation K103N were most frequent but also the multi-drug resistance Q151M and the broad NRTI cross-resistance K65R were observed. The results of this study revealed a high rate of treatment success after short term ART in patients treated at a public provincial hospital in a resource limited area (Steegen et al., 2009).

In Uganda, an analysis of 66 patients on HAART treatment reported that 52% had genotypic mutations for ARV resistance. The mutations were more prevalent in patients

infected with HIV subtype D than in subtype A (Eshleman et al., 2006). In a preliminary study on ART resistance carried out in Nairobi, it was found that of the 55 children who were on ARV treatment at Nyumbani children's home, 22% developed treatment failure. Three of the children had genotypic mutations which had been reported earlier as "secondary' in subtype B strains. This emerging disparity between traditional subtype B mutation signatures and non-B subtypes is calling for larger studies to define viral genotypic correlates for ARV resistance in Africa where non-B subtypes predominate (Lwembe et al., 2007). There is a solid body of evidence to indicate that drug resistance pathways vary between different subtypes. One obvious consequence of the genetic diversity of HIV-1 is the potential impact on the efficacy of a future vaccine. Less obvious and largely controversial is the impact of genetic diversity on disease progression, vertical transmission, ARV therapy, and drug-resistance pathways (Angelis et al., 2014).

2.16 ARV resistance in ARV naive patients

2.16.1 ART guidelines for Kenya

The optimum time for is clear that initiation of treatment should be done before irreversible damage has occurred to the immune system. Early initiation of treatment is important as it is easier to control viral replication, there is lower risk of resistance with complete viral suppression being achieved in many cases and this also decreases the risk of HIV transmission. Kenya has adopted WHO recommendations for early initiation of ART. While the decision to start therapy is based on medical criteria, other factors may impact on the patient's capacity to adhere to treatment such as social circumstances and support systems (Table 2.6).

Table 2.6: When to start ARV in ART-naïve in Kenya

Clinical event	CD4 not available	CD4 > 350/mm3	CD4 < 350/mm3
WHO stage 1 & 2	Defer ART	Defer ART	Start ART
WHO stage 3 & 4	Start ART	Start ART	Start ART
Active TB with HIV	Start ART	Start ART	Start ART

Adapted from Guidelines for ART in Kenya 2015

2.17 ARV resistance in ARV experienced patients

2.17.1 Laboratory monitoring for treatment failure

2.17.1.1 Immunological monitoring

Laboratory monitoring is important for HIV infected patients, especially in determining when to commence ART. CD4 and Viral load testing is on the rise in Kenya. The coverage of CD4 testing services in Kenya is about 85%. The placement of CD4 equipment in Kenya is based on geographical location and distance from each placement.

2.17.1.2 Biological monitoring:

Virological monitoring in assessment for treatment failure is more accurate than the clinical or immunological criteria for determining treatment failure. Due to cost limitations, viral load testing is not offered as a routine test but to a targeted group of patients that includes patients suspected to be failing 1st and 2nd line of treatment. Where there is a clear indication to switch regimen based on clinical and immunological criteria, a viral load test is not essential to change the regimen. Prior to requesting for a viral load test, additional evaluation is done and includes evaluation of adherence, presence of opportunistic infections/recent vaccination and presence of drug interactions. The Viral load cut off figure of 1,000 copies per ml is used as an optimal viral load threshold for defining virological failure. For patients with VL

>1,000copies/ml, there is need to reinforce adherence and repeat a viral load test after 3 months in adults. For children suspected to be failing treatment, adherence should be enforced and more frequent clinical reviews for such children before a repeat VL after 3 months.

2.18 Significance of HIV DRMs

During recent years significant progress has been made in the treatment of HIV-1, at least in part due to the availability of potent ARV drugs. Increase in ART in resource-limited settings (RLS) will successfully reduce HIV-related morbidity and mortality (Steegen et al., 2009). A previous study carried out to evaluate treatment success and development of ART drug resistance after short-term treatment among patients attending the Comprehensive HIV Care Centre (CCC) of Coast Province General Hospital, Mombasa, Kenya, revealed a high rate of treatment success after short term ART in patients treated at a public provincial hospital in a resource limited area (Steegen et al., 2009). A survey performed in Kampala between 2009 and 2010 showed a prevalence of transmitted drug resistance at 8.6% (Bennett et al., 2009). The ever-expanding rollout of ART in RLS without routine virological monitoring has been accompanied with development of drug resistance that has resulted in limited treatment success. Another previous cross-sectional study to determine treatment failure and DRMs among adults receiving first-line (3TC_d4T/AZT_NVP/EFV) and second-line (3TC/AZT/LPV/r) in Nairobi, Kenya, showed that HIV-1 drug resistance was significantly high in the study population. They concluded that the detected accumulated resistance strains due to emergence of HIV drug resistance will continue to be a big challenge (Koigi, Ngayo, Khamadi, Ngugi, & Nyamache, 2014).

The public health approach to providing ART has been implemented in many Lower-Income Countries (ART-LINC) Collaboration sites. Most patients start ART at advanced disease, with CD4 cell counts well below the recommended thresholds (AIDS et al., 2008). A study investigated the efficacy and safety of ARV regimens with once daily

compared to twice daily dosing in diverse areas of the world. In their conclusion EFV+FTC-TDF had similar high efficacy compared to EFV+3TC-ZDV in this trial population, recruited in diverse multinational settings. They concluded that superior safety, especially in HIV-1-infected women, and once daily dosing of EFV+FTC-TDF were advantageous for use of this regimen for initial treatment of HIV-1 infection in resource-limited countries and that ATV+DDI+FTC had inferior efficacy and is not recommended as an initial ARV regimen (Campbell et al., 2012). As part of the AIDS Relief program, a retrospective review of patient medical chart information along with a cross-sectional viral load, and adherence measurement was conducted between 2004 and 2009. An on-treatment analysis excluded patients who died, transferred out of care, or were lost to follow-up. A switch of ARVs for any reason was considered a failure in the intent-to-treat analysis. Patients with only clinically relevant reasons for switching such as toxicity, adverse effects, viral failure or clinical/immunological failure, lost to follow-up, and death were considered experienced failing therapy as part of the modified-intent-to-treat analysis. In the on-treatment analysis, older age ($P < 0.004$) and baseline CD4 <100 cells per cubic millimeter ($P < 0.021$) were the most significant variables impacting viral load. (Amoroso et al., 2012).

Viral load monitoring has been proposed as a tool to reinforce adherence, but outcomes have never been systematically assessed. A meta-analysis was conducted to systematically analyze the research on viral load monitoring as a tool to reinforce adherence. Viremic re-suppression was defined as a decrease in viral load beneath a particular threshold following viral load levels that had been elevated despite ARV treatment. Six databases were searched for studies published up to November 2012, which reported the use of viral load monitoring as a tool to identify patients in need of adherence support.

2.19 Significance of minor sequence variants

ARV drugs have been remarkably successful in suppressing HIV-1 infection; but transmitted drug resistance can reduce the efficacy of first-line regimens. A considerable proportion of transmitted HIV-1 drug resistance is undetected by conventional genotyping and that minority mutations can have clinical consequences (V. A. Johnson et al., 2011). NRTIs are essential components of ARV (ARV) therapy. NNRTIs have a low genetic barrier to resistance: a single mutation is often sufficient to cause resistance to the currently recommended first-generation NNRTIs, nevirapine and efavirenz. The RT mutation K103N is the most commonly occurring NNRTI-resistant mutation in patients with acquired and transmitted NNRTI resistance. K103N reduces susceptibility to efavirenz and nevirapine by; 20-fold and; 50-fold, respectively, but has no effect on susceptibility to the most recently approved NNRTI, etravirine. In another study, Roger Paredes from Harvard Medical School and colleagues looked at the effect of pre-existing minority NNRTI-resistant variants on the risk of virological failure in people starting ART for the first time. The study authors concluded that in adherent patients, pre-existing minority Y181C mutants more. They noted that 70% of participants with minority Y181C HIV variants still achieved long-term viral suppression (Paredes & Clotet, 2010).

A cross-sectional analysis of transmitted HIV-1 DR and a case control study of the impact of minority drug resistance on treatment response suggested that a considerable proportion of transmitted HIV-1 drug resistance was undetected by conventional genotyping and that minority mutations can have clinical consequences. With no treatment history to help guide therapies for drug-naive persons, the findings suggested an important role for sensitive baseline drug resistance testing (J. A. Johnson et al., 2008). A recent pooled analysis found that low-frequency NNRTI resistance mutations confer a greater than 2-fold risk of virologic failure in treatment-naive individuals

initiating a first-line NNRTI-containing cART regimen. A dose-dependent association of drug-resistant minority variants with increased risk of virologic failure was observed.

Absolute numbers of drug-resistant minority variants over 1 $\log_{10}$ copies/mL plasma appeared to be associated with a statistically significant higher risk of virological failure (Li, Xu, Zhao, Wang, & Cao, 2014).

2.20 Comparison of Pyrosequencing with Sanger sequencing

Ultra-deep pyrosequencing using the GS20 Sequencer can reliably detect minor HIV-1 variants present within a 1000-bp region encompassing the >50 HIV-1 drug-resistance mutations (Wang, Mitsuya, Gharizadeh, Ronaghi, & Shafer, 2007). Dideoxynucleotide (Sanger) sequencing (direct PCR sequencing) of plasma viral cDNA is widely used to detect more than 50 drug-resistance mutations in the molecular targets of HIV-1 therapy—reverse transcriptase (RT) and protease—in clinical settings. As a result of the low genetic barrier to NNRTI resistance, multiple NNRTI-resistant lineages often emerge in plasma samples from patients experiencing ongoing viral replication although receiving an NNRTI-containing regimen.

Standard genotypic resistance testing (SGRT) performed by direct PCR sequencing typically detects HIV-1 variants comprising 20% of the viruses within a clinical sample but may miss less prevalent drug-resistant variants. A previous study performed an ultradeep pyrosequencing (UDPS; 454 Life Sciences a Roche Company, Branford, CT) of plasma virus samples from 13 treatment-naive and NNRTI-experienced patients in whom standard genotypic resistance testing revealed K103N but no other major NNRTI-resistance mutations treatment-naive patients, UDPS did not detect additional major NNRTI-resistant mutations suggesting that etravirine may be effective in patients with transmitted K103N concluded that in NNRTI-experienced patients, UDPS often detected additional major NNRTI-resistant mutations suggesting that etravirine may not be fully active in patients with acquired K103N (Varghese et al., 2009).

HIV-1 DRMs are conventionally detected by bulk sequence analysis of the virus sample. Bulk genotyping may detect certain mutations in clinical samples at frequencies as low as 10%. This detection limitation is however a concern because most newly diagnosed persons have been infected for several months to years, providing time for DR viruses to decay to levels that conventional testing is not able to detect. DRMs at frequencies detectable by conventional genotyping are known to reduce the efficacy of ART. There is increasing interest in the clinical consequences of these minority DRMs not detected by conventional genotyping which have shown by several studies to be clinically relevant in that they are often responsible for the virological failure of a new ARV treatment regimen (Palmer et al., 2006). Over time, revertants out compete the drug-resistant viruses to become the predominant viruses in circulation. The rates of mutant virus decay can vary substantially because of differences in fitness cost. (Little et al., 2012).

In comparison, SS has demanding instrumental, technical requirements and subjective data evaluation and limited sensitivity (20-30%). On the other hand SS has limitation of having multistep processing, prohibitive expense, labour intensity, unideal reproducibility and long turnaround time as shown in Figure 2.1

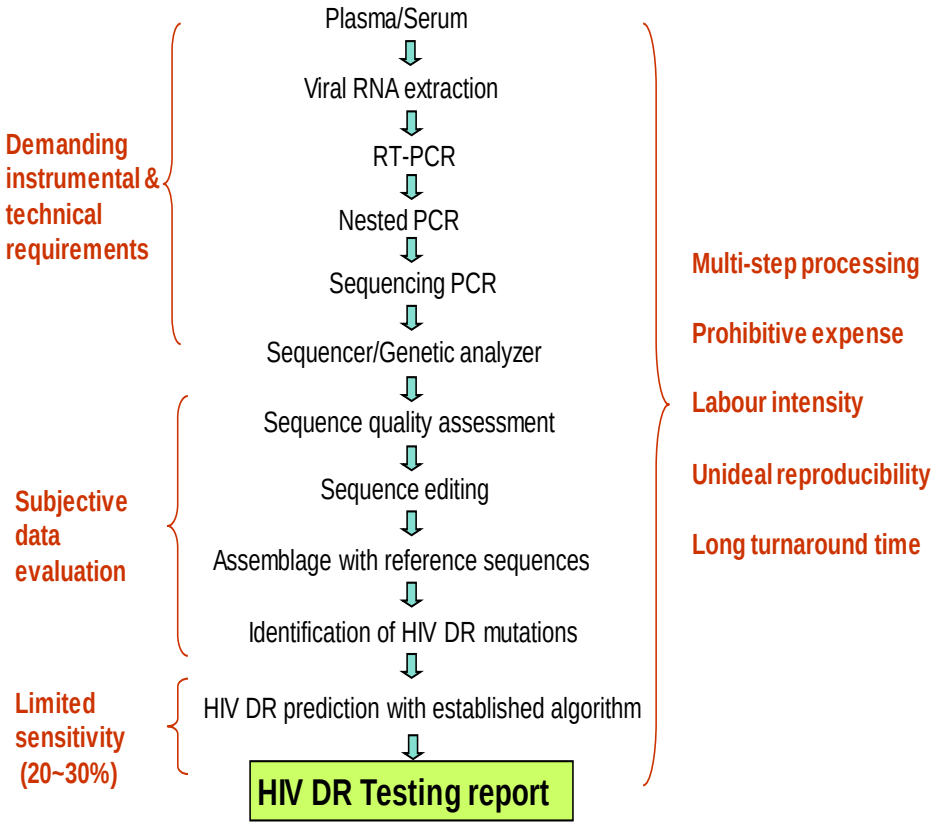


Figure 2.1: Conventional Genotypic HIV DR genotyping

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was a prospective cohort design.

3.2 Study site

The study was carried out at Moi Teaching and Referral Hospital- AMPATH (Academic Model Providing Access to Health care partnership clinics), Eldoret (Einterz et al., 2007).
Moi Teaching and Referral Hospital (MTRH) provides both routine and referral health services for Western Kenya region. The region includes the expansive Rift Valley,

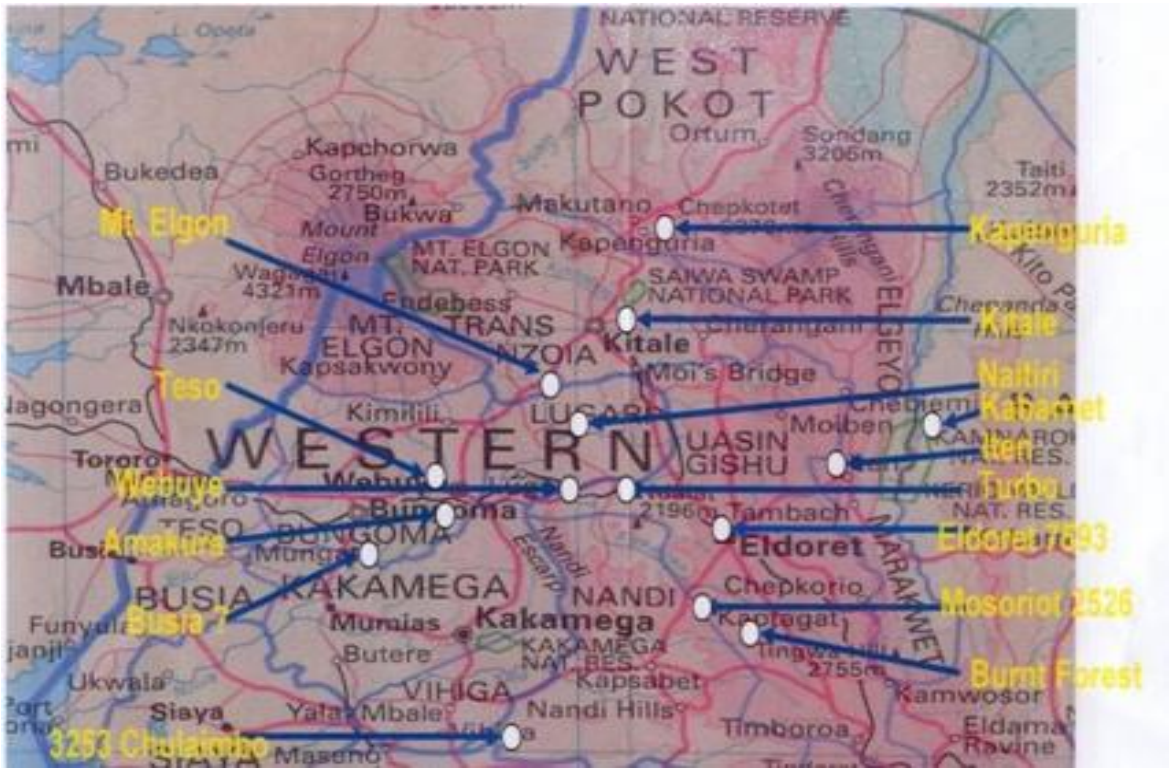


Figure 3.1: Map of study centres

Western and Nyanza provinces, with a cumulative population of about 15 million. The hospital is located in Eldoret Town in Uasin-Gishu District, which forms part of the Uasin-Gishu Plateau West of the Great Rift Valley, at an altitude of 2118m above the sea level, latitude 00°30’52”N and longitude 035°17’52”E.The hospital is also used for teaching Medical, Nursing, and Environmental Health students of Moi University. AMPATH is a partnership between Moi University School of Medicine, Moi Teaching and Referral Hospital in Eldoret, Kenya in collaboration with U.S Medical Schools headed by Indiana School of Medicine. AMPATH has 25 comprehensive HIV care clinics in urban and rural centers in western Kenya region (Figure 3.1).

3.3Study population

Patients included in this study were treatment-naïve and those who were on ARV and presented with treatment failure to the study clinic between October 2009 and October 2011. Study site provides free HIV-related counseling, testing, prevention advice and AIDS care for the population of western Kenya. After measurement of plasma CD4+ T-cell (CD4) concentrations, patients with < 200 cells/ml or an AIDS-related illness as defined by WHO and diagnosed as having AIDS were given ART in accordance with WHO guidelines. Treatment adherence was encouraged through counseling, home visits, pill counts and social support. All patients were seen by a social worker for three adherence counseling sessions before starting therapy and again every month after treatment had commenced. If during therapy a patient came late for a visit or reported poor adherence, additional counseling was provided. Social workers attempted to identify barriers to adherence and to work with each patient in developing an individualized plan to improve it.

3.4 Selection criteria

3.4.1 Inclusion criteria

ARV naïve were included from patients who were HIV positive and had no history of exposure to ARV drugs and ARV drug naïve status according to a medical chart review and personal interview. ARV experienced were included from patients who were HIV positive who were

recipient of ARV drug therapy uninterrupted for at least twelve (12) months and had failed ARV drugs according to WHO guidelines

3.4.2 Exclusion criteria

ARV naïve patients who were recipient of ARV drug therapy were excluded. ARV experienced patients who had been exposed to ARV drugs but were off therapy for a period longer than two weeks before time of enrollment were excluded

3.5 Sampling Procedure

3.5.1 Patients and treatment

The study was conducted on isolates from patients who were known HIV positive attending the study site and met the selection criteria. The study clinics provided ART according to the national guidelines for ART scale-up as recommended by WHO surveillance and monitoring surveys. During September 2009 and October 2011, patients receiving ARV therapy for at least 12 months and were suspected to be failing clinically, immunologically and virologically according to WHO guidelines were consecutively enrolled. Clinical failure was defined as new or recurrent WHO stage 4 condition. Certain WHO clinical stage 3 conditions (e.g. pulmonary TB, severe bacterial infections), may be an indication of treatment failure. Immunological failure was defined as fall of CD4 count to baseline (or below), or 30% fall from on-treatment peak value or persistent CD4 levels below 100 cells/mm3 without concomitant infection to cause transient CD4 cell decrease. Virological failure was defined as plasma viral load above 1000 copies/ml. The optimal viral load threshold for defining virological failure had not been determined. Values of >1000 copies/ml were associated with clinical progression and/or a decline in the CD4 cell count. After informed consent was obtained, a standardized questionnaire was administered to assess demographic, epidemiologic, clinical, and treatment information. ART-naive patients were also enrolled during the same period at the same study clinics. Samples from patients who had no history of exposure to ARV drugs and ARV drug naïve status according to a medical chart review and personal interview were collected consecutively (APPENDIX II, III).

3.5.2 Variables

Patient variables collected were, age, sex, place of residence, initial encounter date, CD4+ cell count, viral load, drug regimen and adherence history. The specimen had information on specimen code, and date of collection.

3.5.3 Ethical considerations

3.5.3.1 Ethical Approval

The study was approved by the Institutional Research and Ethics Committee of the Moi University School of Medicine (MUSOM) and MTRH Review Board (IREC):(IREC/2010/06) and AMPATH (RES/STUD/17/2010)

3.5.3.2 Informed consent

Informed consent was sought and obtained from patients who met selection criteria and was signed by those who accepted to participate in the study (Appendix I and II).

3.5.3.3 Data protection

All information obtained about the patients and the results of the research were treated confidentially. The information was coded and kept under a password protected database. The study files were kept electronically at the Centre. The results of the research do not appear in medical record nor were they shared with other medical personnel with identifying information.

3.5.3.4 Reliability

All procedures followed were well established methods.

3.6 Limitations and assumptions of the study

The information observed in this study provides useful information for clinicians managing patients and can serve as an indicator of ARV program efficiency in patients still on treatment.

However the study estimates of drug resistance in treated patients are likely minimal estimates. One of our limitations was the fact that in ARV experienced, samples analyzed were from patients whom the criteria identified as possible experienced failing therapy and viral load determined according to WHO guidelines. Another limitation of our study is that out of clinics in other sites in western Kenya and public hospitals that are involved in ART care; we studied only patients who attended the Moi Teaching and Referral Hospital ART care clinics, which was the main specialized site in HIV/AIDS.

3.7 Sample size

The sample size was determined based on a Study carried out to evaluate treatment success and development of ART drug RS after short-term treatment among patients attending the Comprehensive HIV Care Centre (CCC) of Coast Province General Hospital, Mombasa, Kenya. Of the 19 patients with a detectable viral load, sequencing of the protease (PR) and reverse transcriptase (RT) gene was successful in 16. Major PR mutations were absent, but mutations associated with drug RS in RT were detected in 14 of the 16 patients (87.5%).(Lihana, Khamadi, Lubano, et al., 2009; Steegen et al., 2009).

The aim of the study was to characterize acquired and transmitted antiretroviral drug resistant mutations among HIV positive patients failing therapy. Based on the above study, it was anticipated that the proportion of patients failing therapy due to antiretroviral drug resistant associated mutations would be approximately 90%.

In order to be able to estimate this proportion within plus or minus 5%, a sufficient sample size was determined using the following formula (Cochran, 1963).

$$n = \left(\frac{Z_{1-\alpha/2}}{d}\right)^2 \times P \times (1-P)$$

$$= \left(\frac{1.96}{0.05}\right)^2 \times 0.90 \times (1-0.9)$$

$$= 139$$

Where Z_c is the quantile of the standard normal distribution corresponding to c x 100% percentile, c = (1-a/2), where "a" is the type I error equal to 5%, d is the margin of error, equal to 5%, and P is the proportion of patients with ART associated resistance mutations, assumed to be 90.0%.

Given that all the extracted samples undergoing sequencing would not be fully processed, we corrected for the possibility of losing some samples. We hypothesized that there would be 30% lose of samples during processing. Correcting the sample size for this likely lose gave

$\frac{n}{1-r} = \frac{139}{1-0.3} \approx 200$ as the number of participants whose samples would be extracted for drug resistance mutation evaluation. ARV naïve and experienced participants were recruited in the ratio of 2:1 based on the AMPATH data of September 2009, which indicated that out of a total number of 46,773 adults, 31,718 (67.8%), were on treatment.

3.8 Laboratory Procedures

3.8.1 DNA extraction

HIV-1 nucleic acid was extracted from 400 µl of plasma using the Nuclisens EasyMag system (Biomerieux, Canada) following manufacturer's instructions (APPENDIX IV, V and VI). Negative Human plasma (NHP) was included with the isolation of nucleic acids. The same sample was processed in parallel with test specimens up to the point of sequencing. Previously isolated Negative Human plasma (NHP) nucleic acid was included during the amplification of nucleic acids to monitor processes downstream of nucleic acid isolation whereby test passed if both negative controls showed no signal on electrophoresis of PCR products at any stage of the algorithm. Positive control used was Accurun (BBI Diagnostics, cat# 5524-500) diluted to 10000 copies/ml in negative human plasma, was processed from the isolation of nucleic acids through the entire algorithm including sequencing of generated PCR product. Previously isolated Accurun nucleic acid was included during the amplification of nucleic acids to monitor processes downstream of nucleic acid isolation whereby test passed if both positive controls produced correct pedigreed sequence. All biological materials were handled as potentially

infectious, work with plasma was performed in a Biological safety cabinet (BSC) wearing a gown and double gloves. Extraction was performed in Sample prep room, serum/plasma samples were thawed at room temperature. EasyMAG robot and computer were turned on as per manufacturers instructions

3.8.2 RT-PCR

40µl master mix per 0.2ml thin-walled PCR tube was dispensed using sterile individually wrapped Gilson DistriTip cap tubes. PCR tubes were transported and RNA samples retrieved (if stored in -80°C freezer, thawed at room temperature and placed on ice). Quick spin of samples @10,000 x g for 30 sec was done to pellet any residual silica 10µl RNA template was added to corresponding PCR tube and mixed by pipetting several times (avoiding producing bubbles), only opening one tube at a time. A negative control (water) and an Accurun positive control for each batch were added.

Table 3.1: RT-PCR master mix

	Volume per reaction (µl)	final concentration
RNase-free water	13.75	-
5x RT-PCR buffer (contains12.5mM $MgCl_2$)	10	1x
dNTP mix (10mM each)	2	0.4 mM each
forward primer (5µM)	6	0.6 µM
reverse primer (5µM)	6	0.6 µM
RNasin Ribonuclease Inhibitor (40U/µl)	0.25	10 U
RT-PCR enzyme mix	2	-
TOTAL	40µl	

+ 10µl template = 50µl total volume

3.8.2.1 RT-PCR thermocycling conditions (for Algortithm# 1-4)

PCR tubes were placed into thermocycler once it reached the RT incubation temperature of 50°C, and the lid closed not tightly. PCR products were stored at 4°C (short term). In the second round PCR, all master mix solutions were prepared in a BSC Clean room. (Table 3.2)

Table 3.2: RT-PCR thermocycling conditions (for Algortithm# 1-4)

1X	50°C	40 min	reverse transcriptase step
1X	95°C	15 min	inactivates RT enzyme and activates Taq mixture
35X	94°C 53°C 72°C	30 sec 30 sec 2 min 30 sec	cycling
1X	72°C	10 min	final extension
	4°C	HOLD	

45µl master mix was dispensed into 0.2ml thin-walled PCR tubes using sterile Gilson

DistriTip. 5µl of first-round RT-PCR product was added into corresponding nested PCR tube, mixed by pipetting. Cycles on thermocycler (GeneAmp PCR System 9700, ABI) was verified.(Table 3.3). PCR tubes were placed into thermocycler, run thermocycler, conditions below. PCR products were stored at 4°C (short term) or -20°C (long term).(Table 3.4)

Table 3.3: Nested PCR master mix:

	Volume per rxn (µl)	final concentration
RNase-free water	22.5	-
10X Gold buffer	5	1X
25 mM MgCl2	4	2 mM
dNTP mix (10mM each)	1	0.4 mM each
forward primer (5µM)	6	0.6 µM
reverse primer (5µM)	6	0.6 µM
AmpliTaq Gold (5U/µl)	0.5	2.5 U
TOTAL	45 µl	

+ 5 µl template = 50 µl total

Table 3.4: Second/nested PCR thermocycling conditions (for algorithm #1-4):

1 X	95°C	10 min
35 X	94°C 53°C 72°C	20 sec 30 sec 2 min 30 sec
1 X	72°C	10 min
	4°C	HOLD

3.8.2.1 PCR Product Evaluation and Quantification

3.8.2.1 Gel Electrophoresis Protocol (optional) or QIAxcel

Second round PCR products went through gel electrophoresis and/or QIAxcel to identify amplified products. PCR amplicons were then purified and diluted to 15ng/ml for DNA. 1.5% agarose gel was prepared containing EtBr (0.35µg/ml final concentration). 1X TAE buffer #1 was used, which did NOT contain EtBr. It was poured into the electrophoresis mould with the desired comb(s).Once the gel solidified, it was placed in electrophoresis unit and submerged with 1X TAE running buffer #2 (contains EtBr). 5µl of each nested PCR product + 2µl loading dye per lane was loaded on the gel including a lane for low DNA mass ladder (4µl + 2µl loading dye) and a lane with 100bp or 1kb plus ladder (6µl already in loading dye) 0.5µg/6µl loaded. The gel was run at 100V for 1hour and a photo of gel taken. Gel was transferred to UV transilluminator, camera settings adjusted and photo of gel using UVP BioDoc-It system (Mitsubishi) taken. Agarose gels containing EtBr were considered hazardous chemical waste. Place gel in container in fume hood to dehydrate. Once dehydrated, dispose of gel in designated waste containers for later incineration. Interpretation of gel results: Correct PCR product generated was verified, size was dependent on primers used.

3.8.2.2 Automated Extraction Protocol

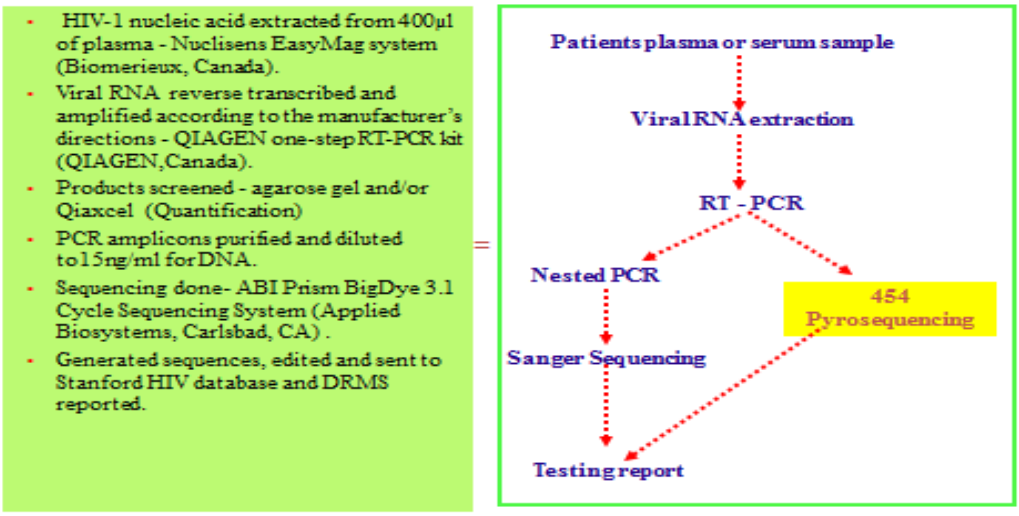
EasyMAG robot and computer was used as per manufacturer's instructions (NucliSens easyMAG Application Training Manual, Doc. Code: GCS TM0336, version 2 Revision 2005/08/01). launch easyMAG software. Briefly, 400µl of plasma sample was added to appropriate predefined well in sample vessel containing lysis buffer and 200µl of controls to appropriate predefined wells. Samples were allowed to incubate for 10 minutes at room temperature for complete lysis to occur. Silica thoroughly vortexed, using the electronic multi-channel pipette (EMP) provided set to program 1 and a single BioHit tip, 550µl dH2O added to silica tube, vortexed using the EMP set to program 2 and a single BioHit tip, aliquots of premix silicadispensed to the premix strips. Using the EMP set to program 3 and 8 BioHit tips, silica was transfered to sample vessel and sample vessels containing; sample, lysis and silica transported to easyMAG instrument. EasyMAG run was labelled one 1.5ml RNase-free tube per sample with the specimen ID and date. Once run was complete, within 30min extracted nucleic acid was transfered to labeled tube and silica discarded, proceeded directly to RT-PCR and eluate stored at -80°C.

Sequencing was done using ABI Prism BigDye 3.1 Cycle Sequencing System (Applied Biosystems, Carlsbad, CA) following manufacturer's instructions. The sequencing PCR primers included both nested PCR primers and two additional primers PS1 59-CTGGTGTYTCATTRTTKRTACTAGGT- 39 and PS2 59-TTYTGGGARGTYCARY TAGGRATACC-39.

3.8.3 Bulk Sanger Sequencing-Based HIV-1 DR Genotypic Test

Genotypic drug resistance testing was performed on plasma samples using a previously described in-house assay (Ji et al., 2010). HIV-1 PR and RT were bidirectional sequenced with an in-house protocol. Briefly, viral RNA was reverse transcribed and amplified according to the manufacturer's directions using the QIAGEN one-step RT-

PCR kit (QIAGEN, Canada). The PCR products from the first round went through a second round nested PCR. Second round PCR products went through gel electrophoresis and/or QIAxcel to identify amplified products (APPENDIX VII). PCR amplicons were then purified and diluted to 15ng/ml (Appendix VIII). The sequencing PCR primers included both nested PCR primers and two additional (APPENDIX X). Sequencing was done using ABI Prism BigDye 3.1 Cycle Sequencing System (Applied Biosystems, Carlsbad, CA) following manufacturer's instructions (APPENDIX X, XI, and XII) (Figure 3.1). Generated sequences were edited using BioEdit v7.0.5B. Conventional sequences were assembled and edited in Seqscape v2.5 (Applied Biosystems, USA). Aligned fasta files were uploaded to Stanford database. Phylogenetic trees were constructed and visualized (APPENDIX XIII) All Mutations were identified using a surveillance drug resistance mutation (SDRM) list with additional clinical protease mutations identified according to the IAS-USA HIV DR mutation list (APPENDIX XIV) DRMs and subtype data collected from the Stanford HIV database sequence analysis program were manually input into appropriate excel spreadsheet file, verified and corrections made as needed(Figure 3.2)



.

Figure 3.2: The workflow of conventional sequencing based HIV DR genotyping

3.8.4 Pyrosequencing

Briefly, viral RNA was reverse transcribed and amplified according to the manufacturer's directions using the QIAGEN one-step RT-PCR kit (QIAGEN, Canada) (Figure 3.2). Nested PCR was conducted using fusion primers with forward primers tagged with multiplex identifiers (MID) (Figure 3.3) (APPENDIX XV). The sequences of the MID tagged primers are shown in Table 3.1. All MID tagged forward fusion primers consisted of a forward primer adaptor sequence and a reverse adaptor(Figure 3.4). All nested PCR procedures were performed using common reaction conditions at annealing temperature of 58.9 °C. Resulting PCR amplicons were purified, quantified and pyrosequenced using 1/16 the capacity of a full GS FLX Titanium PicoTiter Plate. Reads that passed the quality control software, was of sufficient read length to cover the amplicon and could be successfully mapped to the HXB-2 reference sequence underwent further analysis

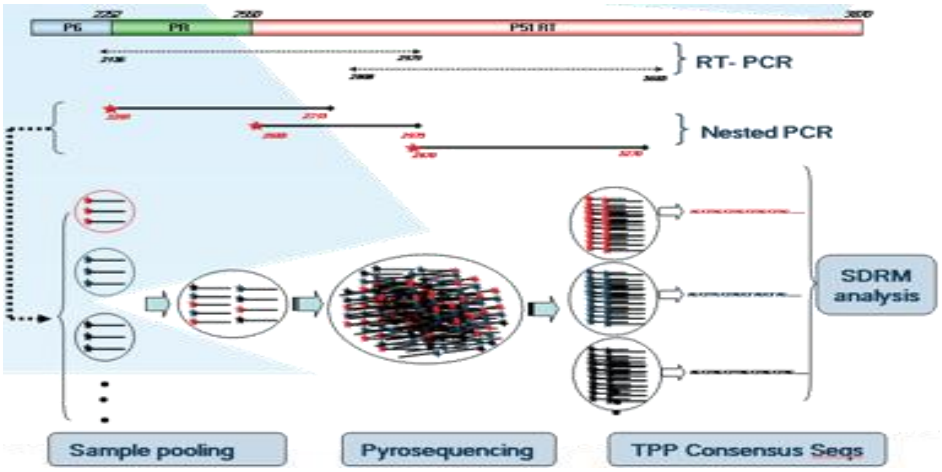


Figure 3.3: TPP Experimental workflow

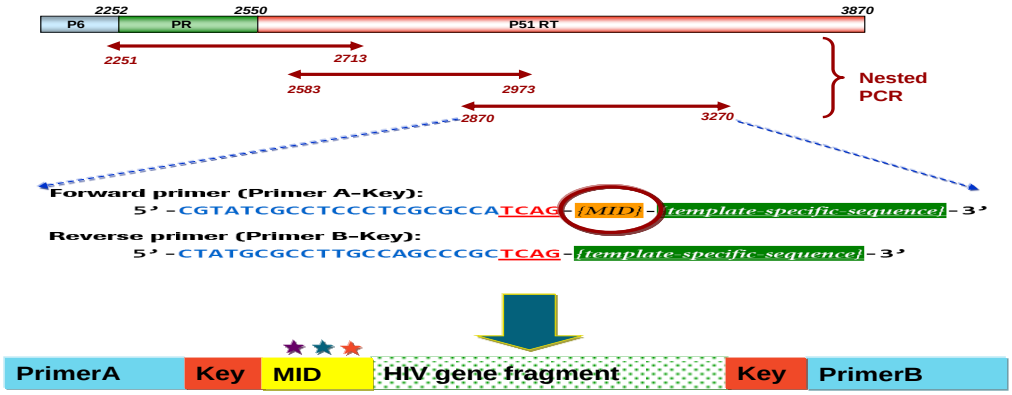


Figure 3.4: DNA bar coding or tagging

Generated sequences were edited using Bio Edit v 7.0.5B. Aligned fasta files were uploaded to Stanford HIV Drug resistance http://hivdb6.stanford .edu/asi/deployed/hiv _central.pl?program=hivdb&action=showSequenceForm Phylogenetic relationships of

newly derived viral sequences for comparisons with those of previously reported HIV group M from the Los Alamos database by CLUSTAL W profile alignment. To improve the accuracy of HIV-1 sub typing, the genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) was used and the REGA sub typing tool, (http://dbpartners.stanford.edu/RegaSubtyping/) was utilized as needed. Drug resistance mutation and subtype data collected from the Stanford HIV database sequence analysis program were manually input into appropriate excel spreadsheet file, verified and corrections made as needed (APPENDIX XIII, XIV).

3.9 Data Management

All the data generated in this study was saved in Microsoft Excel worksheets with a detailed database established to capture all the necessary information.

3.10 Statistical Methods

Categorical variables such as gender, education level, WHO clinical stage, drug resistance mutations among others were summarized using frequencies and the corresponding percentages. Continuous variables such age, CD4 cell count, and viral load were summarized using mean and the corresponding standard deviation (SD) if the Gaussian assumptions were satisfied. Whenever the Gaussian assumptions were violated we summarized the continuous variables using median and the corresponding inter quartile range (IQR). Gaussian assumptions were assessed using Shapiro-Wilk test, and graphically using histograms. The viral load variable was log transformed using logarithm to base 10. This variable was log transformed to achieve a normal distribution.

Fisher's exact test was used to assess the association between the presence of drug resistance mutations (DRMs) and independent categorical variables such as gender, WHO clinical stage among others. Independent samples t-test was used to compared the average viral load (log 10) level per milliliter among those who had DRMs to those who

did not have DRMs while two-sample Wilcoxon rank-sum test was used to compare age, and CD4 cell count between those who had DRMs and those who had no DRMs. We reported the corresponding p-values. There was evidence of difference from the data if the p-value was less than the nominal value of 0.05, type I error.

Analysis was stratified by the ARV status, ARV experienced experienced failing therapy and ARV naïve. Data analysis was done using R: A language and environment for statistical Computing (R Core Team, 2015)

Results were presented using graphs and tables.

CHAPTER FOUR

RESULTS

4.1 Baseline characteristics of the recruited patients

A total of 206 participants aged 6.6 years to 71.8 years were included in the study. Baseline characteristics were as shown in Table 4.1 (APPENDIX XVI).

Table 4.1: Baseline characteristics of all the recruited patients

Variable	N	N = 206 n (%) or Mean (SD) or Median (IQR)
Socio-demographic characteristics		
Age at sample collection (Years)	195	38.1 (12.2)
Range (Min. – Max.)		6.6 – 71.8
Female	205	129 (62.9%)
Marital status		
Single		7 (13.2%)
Married/Engaged/cohabiting	53	27 (50.9%)
Divorced/separated/Widowed		19 (35.8%)
Education level		
No formal education		3 (7.0%)
Primary		22 (51.2%)
Secondary	43	15 (34.9%)
Tertiary		3 (7.0%)
Clinical characteristics		
ARV status		
Experienced failing therapy	206	139 (67.5%)
Naïve		67 (32.5%)
WHO clinical stage		
Stage 1		54 (30.5%)
Stage 2	177	30 (16.9%)
Stage 3		70 (39.5%)
Stage 4		23 (13.0%)
Have opportunistic infections	198	15 (7.6%)
Tuberculosis	198	8 (4.0%)
Sexually transmitted diseases	182	8 (4.4%)
CD4 count (Cells per cubic mm)	192	210.0 (70.0, 391.2)
Viral load (Log base 10)	92	3.1 (1.5)
Range (Min. – Max.)		1.6 – 5.7

4.2 Characteristics from all the Amplified samples

Of the 206 participants, 114 (55.3%) had their samples successfully amplified and processed. Their characteristics stratified by ARV status were as shown in Table 4. 2.

Table 4.2: ARV Naïve and experienced failing therapy whose samples were amplified

Variable	N	Total N = 114	ARV Experienced failing therapy N= 49	ARV Naïve N = 65
		n (%) or Mean (SD) or Median (IQR)		
Socio-demographic characteristics				
Age at sample collection (Years)	104	36.2 (11.6)	36.7 (12.4)	35.7 (11.0)
Range (Min. – Max.)		6.6 – 66.2	6.6 – 63.6	7.5 – 66.2
Female	114	75 (65.8%)	28 (57.1%)	47 (72.3%)
Marital status				
Single		5 (11.9%)	0 (0.0%)	5 (14.3%)
Married/Engaged/cohabiting	42	21 (50.0%)	4 (57.1%)	17 (48.6%)
Divorced/separated/Widowed		16 (38.1%)	3 (42.9%)	13 (37.1%)
Education level				
No formal education		2 (5.7%)	1 (16.7%)	1 (3.4%)
Primary	35	20 (57.1%)	3 (50.0%)	17 (58.6%)
Secondary		12 (34.3%)	2 (33.3%)	10 (34.5%)
Tertiary		1 (2.9%)	0 (0.0%)	1 (3.4%)
Clinical characteristics				
WHO clinical stage				
Stage 1		33 (36.7%)	4 (8.7%)	29 (65.9%)
Stage 2	90	15 (16.7%)	8 (17.4%)	7 (15.9%)
Stage 3		32 (35.6%)	25 (54.3%)	7 (15.9%)
Stage 4		10 (11.1%)	9 (19.6%)	1 (2.3%)
Have opportunistic infections	108	10 (9.3%)	6 (12.2%)	4 (6.8%)
Tuberculosis	108	7 (6.5%)	2 (4.1%)	5 (8.5%)
Sexually transmitted diseases	93	4 (4.3%)	2 (4.8%)	2 (3.9%)
CD4 count (Cells per cubic mm)	101	266.0 (80.0, 442.0)	163.0 (62.0, 294.5)	386.0 (138.0, 528.5)
Range (Min. – Max.)		0.0 – 1986.0	2.0 – 849.0	0.0 – 1986.0
Viral load (Log base 10)	39	4.1 (1.1)	41. (1.1)	4.2 (0.6)
Range (Min. – Max.)		1.6 – 5.6	1.6 – 5.6	3.5 – 4.6

4.2.1 Characteristics from all the Amplified samples from ARV Naïve

Of the 206 participants, 114 (55.3%) had their samples processed. Up to 65 (57.0%) of the study participants were ARV naïve. The socio-demographic and clinical characteristics were as shown in Table 4.3.

Table 4.3: Characteristics from all the Amplified samples from ARV Naïve

Variable	N	ARV Naïve N = 65 n (%) or Mean (SD) or Median (IQR)
Socio-demographic characteristics		
Age at sample collection (Years)	65	35.7 (11.0)
Range (Min. – Max.)		7.5 – 66.2
Female	65	47 (72.3%)
Marital status		
Single		5 (14.3%)
Married/Engaged/cohabiting	35	17 (48.6%)
Divorced/separated/Widowed		13 (37.1%)
Education level		
No formal education		1 (3.4%)
Primary	29	17 (58.6%)
Secondary		10 (34.5%)
Tertiary		1 (3.4%)
Clinical characteristics		
WHO clinical stage		
Stage 1		29 (65.9%)
Stage 2	44	7 (15.9%)
Stage 3		7 (15.9%)
Stage 4		1 (2.3%)
Have opportunistic infections	59	4 (6.8%)
Tuberculosis	59	5 (8.5%)
Sexually transmitted diseases	52	2 (3.9%)
CD4 count (Cells per cubic mm)	51	386.0 (138.0, 528.5)
Range (Min. – Max.)		0.0 – 1986.0
Viral load (Log base 10)	34	4.2 (0.6)
Range (Min. – Max.)		3.5 – 4.6

4.2.2 Characteristics from all the Amplified samples from ARV experienced failing therapy

The mean age of the ARV experienced participants was 36.7 (SD: 12.4) years with a minimum and a maximum of 6.6 and 63.6 years respectively. The proportion of female participants was 57.1%, and the proportion married was 57.1%. Up to 33.3% had a secondary level of education. None had tertiary level of education. Up to 26.1% were in WHO clinical stage 1 or 2, and the proportion reporting opportunistic infections was 19.6%. Up to 12.2% and 4.1% had history of TB, and sexually transmitted diseases respectively.The median CD4 cell count per cubic milliliter among the ARV experienced group was 163.0 (IQR: 62.0, 294.5), and the average viral load (log 10) was 4.1 (SD: 1.1) copies per ml.(Table 4.4)

Table 4.4: Characteristics from all the Amplified samples from ARV experienced failing therapy

Variable	N	ARV Failures N= 49 n (%) or Mean (SD) or Median (IQR)
Socio-demographic characteristics		
Age at sample collection (Years)	49	36.7 (12.4)
Range (Min. – Max.)		6.6 – 63.6
Female	49	28 (57.1%)
Marital status		
Single		0 (0.0%)
Married/Engaged/cohabiting	7	4 (57.1%)
Divorced/separated/Widowed		3 (42.9%)
Education level		
No formal education		1 (16.7%)
Primary	5	3 (50.0%)
Secondary		2 (33.3%)
Tertiary		0 (0.0%)
Clinical characteristics		
WHO clinical stage		
Stage 1		4 (8.7%)
Stage 2	46	8 (17.4%)
Stage 3		25 (54.3%)
Stage 4		9 (19.6%)
Have opportunistic infections	49	6 (12.2%)
Tuberculosis	49	2 (4.1%)
Sexually transmitted diseases	42	2 (4.8%)
CD4 count (Cells per cubic mm)	38	163.0 (62.0, 294.5)
Range (Min. – Max.)		2.0 – 849.0
Viral load (Log base 10)	31	4.1 (1.1)
Range (Min. – Max.)		1.6 – 5.6

4.3 Sanger Sequenced data

A total of 83 participants who were successfully sequenced using Sanger method. Up to 59.0% were ARV naïve (Figure 4.1). The average age was 36.9 (SD: 12.2) years with a range of 6.6 – 66.2 years. Sixty percent were female, 54.3% were married, and 42.0% had a secondary or a tertiary level of education (Table 4.4).

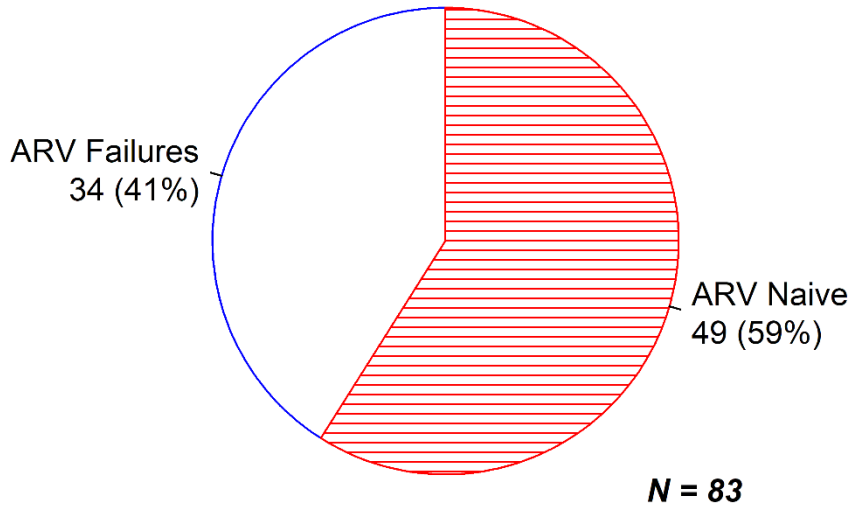


Figure 4.1: Distribution Sanger Sequenced by the ARV status

Of this number, 9.6% were in WHO clinical stage 3 or 4, and 2.4% had reported some opportunistic infections. HIV subtype A was the most common strain of HIV 43 (51.8%). The other was HIV subtype D representing 20.5%. The median CD4 cell count was 281.0 (IQR: 121.2, 430.0), and the average viral load (log 10) was 3.8 (SD: 1.1) with a minimum and maximum of 1.6 and respectively 5.4 (Table 4.5).

Table 4.5: Overall descriptive characteristics among the participants whose samples were Sanger sequenced

Variable	N	n (%) or Median (IQR) or Mean (SD)
Socio-demographic characteristics		
Age at sample collection (Years)	83	36.9 (12.2)
Range (Min. – Max.)		6.6 - 66.2
Female	83	50 (60.2%)
Marital status		
Single	35	4 (11.4%)
Married/engaged/cohabiting		19 (54.3%)
Separated/Divorced/Widowed		12 (34.3%)
Education level		
No formal education		2 (6.5%)
Primary	31	16 (51.6%)
Secondary		12 (38.7%)
Tertiary		1 (3.2%)
Clinical characteristics		
WHO clinical stage		
Stage 1		50 (60.2%)
Stage 2		9 (10.8%)
Stage 3	83	18 (21.7)
Stage 4		6 (7.2%)
Opportunistic infection	83	2 (2.4%)
HIV subtypes		
A		43 (51.8%)
A/D		5 (6.0%)
A/K		1 (1.2%)
AE		3 (3.6%)
AE/C		1 (1.2%)
B		1 (1.2%)
B/A	83	2 (2.4%)
B/AE		1 (1.2%)
B/C		1 (1.2%)
C		3 (3.6%)
C/EC		1 (1.2%)
D		17 (20.5%)
D/A		3 (3.6%)
G		1 (1.2%)
CD4 count (Cells per cubic mm)	82	281.0 (121.2, 430.0)
Range (Min. – Max.)		1.0 – 1260.0
Viral load copies per ml (Log Base 10)	34	3.8 (1.1)
Range (Min. – Max.)		1.6 – 5.4

4.3.1 Sanger sequenced with DRMS

Participants who had DRMs were compared to those who did not have DRMS (Figure 4.2)

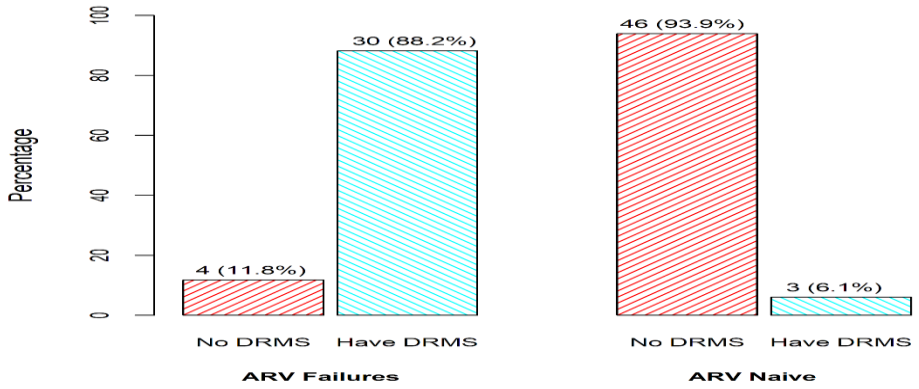


Figure 4.2: Sanger Sequenced Drug resistance by ARV status

4.3.2 Comparison between Sanger sequenced with DRMs and without from ARV Naïve

Up to 59.0% were ARV naïve. Of this number 3 (6.1%) had drug resistance mutations. There were 2 (4.1%) ARV naïve participants with NRTI based resistance gene mutations, 1 (2.0%) with NNRTI based resistance gene mutation, and 1 (1.2%) with PI drug resistance mutation. The demographic and clinical characteristics of the ARV naive participants who had DRMs to those who had no DRMs was compared (Table 4.6).

Table 4.6: Comparison of the characteristics of participants whose samples were Sanger Sequenced from ARV naïve with DRMs and without DRMS

Variable	N	DRMS		P-value
		No (n = 46)	Yes (n = 3)	
		n (%) or Median (IQR)		
Age at sample collection (Years)	49	36.1 (30.2, 42.3)	36.0 (35.9, 51.1)	0.428[w]
Range (Min. – Max.)		7.5 – 63.1	35.9 – 66.2	
Female	49	31 (67.4%)	3 (100.0%)	0.543[f]
Marital status				
Single		4 (16.0%)	0 (0.0%)	>0.999[f]
Married/engaged/cohabiting	28	14 (56.0%)	2 (66.7%)	
Separated/Divorced/Widowed		7 (28.0%)	1 (33.3%)	
Education level				
No formal education		1 (4.8%)	0 (0.0%)	
Primary	47	10 (47.6%)	2 (66.7%)	>0.999[f]
Secondary		9 (42.9%)	1 (33.3%)	
Tertiary		1 (4.8%)	0 (0.0%)	
WHO clinical stage				
Stage 1		40 (87.0%)	3 (100.0%)	
Stage 2	49	2 (4.3%)	0 (0.0%)	>0.999[f]
Stage 3		3 (6.5%)	0 (0.0%)	
Stage 4		1 (2.2%)	0 (0.0%)	
HIV subtypes				
A		18 (39.1%)	3 (100.0%)	
A/D		3 (6.5%)	0 (0.0%)	
AE		2 (4.3%)	0 (0.0%)	
AE/C		1 (2.2%)	0 (0.0%)	0.737[f]
B	49	1 (2.2%)	0 (0.0%)	
B/A		1 (2.2%)	0 (0.0%)	
B/AE		1 (2.2%)	0 (0.0%)	
B/C		1 (2.2%)	0 (0.0%)	
C		3 (6.5%)	0 (0.0%)	
C/EC		1 (2.2%)	0 (0.0%)	
D		14 (30.4%)	0 (0.0%)	
CD4 count (Cells per cubic mm)	48	326.0 (190.0, 511.0)	341.0 (305.5, 368.0)	0.915[w]
Range (Min. – Max.)		7.0 – 1260.0	270.0 – 395.0	

[f]Fisher's Exact test; [w]Two sample Wilcoxon rank-sum test

4.3.3 Characteristics of Sanger sequenced ARV experienced failing therapy with and without DRMS

Up to 59.0% were ARV naïve. Of this number 3 (6.1%) had drug resistance mutations.There were 2 (4.1%) ARV naïve participants with NRTI based resistance gene mutations, 1 (2.0%) with NNRTI based resistance gene mutation, and 1 (1.2%) with PI drug resistance mutation (Table 4.7).

Table 4.7: Characteristics of Sanger sequenced ARV experienced failing therapy with and without DRMS

Variable	N	DRMS		P
		No (n = 4)	Yes (n = 30)	
		n (%) or Mean (SD) or Median (IQR)		
Age at sample collection (Years)	34	32.9 (30.6, 38.1)	36.2 (28.2, 46.3)	0.728[w]
Range (Min. – Max.)		26.4 – 51.1	6.6 – 63.6	
Female	34	4 (100.0%)	12 (40.0%)	0.039[f]
Marital status				
Married/engaged/cohabiting	7	0 (0.0%)	3 (50.0%)	>0.999[f]
Separated/Divorced/Widowed		1 (100.0%)	3 (50.0%)	
Education level				
No formal education		0 (0.0%)	1 (16.7%)	
Primary	31	1 (100.0%)	3 (50.0%)	>0.999[f]
Secondary		0 (0.0%)	2 (3.3%)	
WHO clinical stage				
Stage 1		3 (75.0%)	4 (13.3%)	
Stage 2	34	1 (25.0%)	6 (20.0%)	0.035[f]
Stage 3		0 (0.0%)	15 (50.0%)	
Stage 4		0 (0.0%)	5 (16.7%)	
Have opportunistic infections	34	1 (25.0%)	1 (3.3%)	0.225[f]
HIV subtypes				
A		2 (50.0%)	20 (66.7%)	
A/D		0 (0.0%)	2 (6.7%)	
A/K		0 (0.0%)	1 (3.3%)	0.339[f]
AE		1 (25.0%)	0 (0.0%)	
B/A		0 (0.0%)	1 (3.3%)	
D	34	0 (0.0%)	3 (10.0%)	
D/A		1 (25.0%)	2 (6.7%)	
G		0 (0.0%)	1 (3.3%)	
CD4 count (Cells per cubic mm)	34	150.0 (95.2, 196.0)	192.0 (118.2, 308.5)	0.273[w]
Range (Min. – Max.)		39.0 – 226.0	1.0 – 713.0	
Viral load copies per ml (Log Base 10)	34	4.1 (0.9)	3.8 (1.1)	0.769[t]
Range (Min. – Max.)		3.1 – 5.0	1.6 – 5.4	

[f]Fisher's Exact test; [t]Independent samples t-test; [w]Two sample Wilcoxon rank-sum test

The distribution of the NRTI based resistance gene mutations were as shown in Figure 4.3.

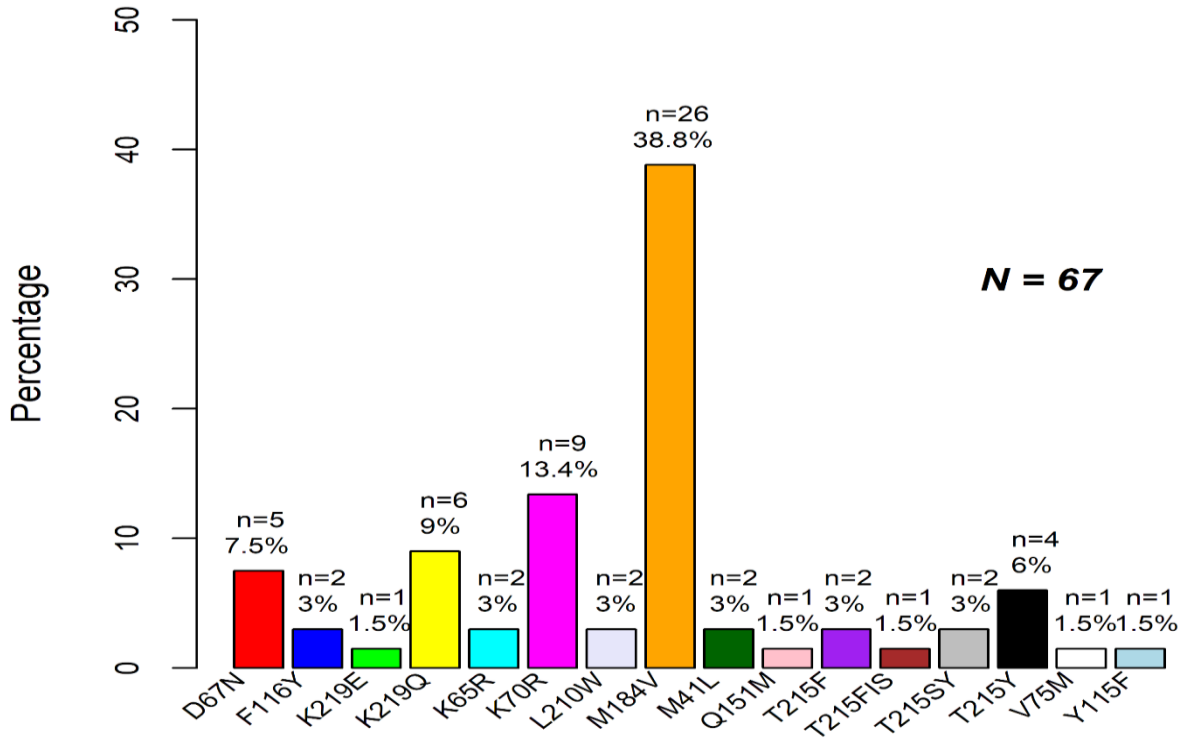


Figure 4.3: NRTI Drug resistant mutations among the ARV failures

There were 30 (88.2%) participants who had NNRTI based resistant gene mutations among the ARV experienced participants. (Figure 4.4)

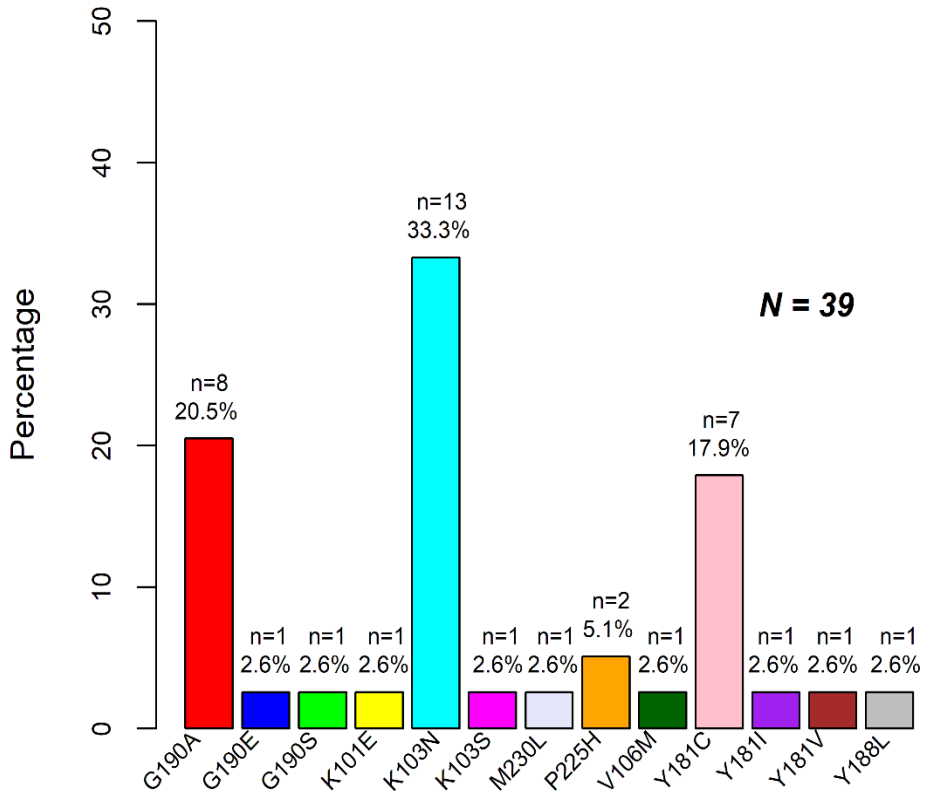


Figure 4.4: NNRTI DRMs among the participants who were ARV experienced failing therapy

4.4 Drug resistant mutations by ARV regimens

The relationship between ARV regimens and specific types of drug resistant gene mutations was as is shown in Table 4.8. Drug resistant mutants M184V, and K70R were common across the six classes of ARV combinations.

Table 4.8: Drug resistant mutations by ARV regimens

	ARV REGIMENS						
DRMS	2nd Line	A	C	F	G	H	**Total**
D67N	0 (0.0%)	3 (15.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (18.2%)	**5 (7.5%)**
F116Y	1 (16.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (9.1%)	**2 (3.0%)**
K219E	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (9.1%)	**1 (1.5%)**
K219Q	1 (16.7%)	2 (10.0%)	1 (25.0%)	1 (5.3%)	0 (0.0%)	1 (9.1%)	**6 (9.0%)**
K65R	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (18.2%)	**2 (3.0%)**
K70R	1 (16.7%)	2 (10.0%)	1 (25.0%)	2 (10.5%)	2 (28.6%)	1 (9.1%)	**9 (13.4%)**
K210W	0 (0.0%)	1 (5.0%)	0 (0.0%)	1 (5.3%)	0 (0.0%)	0 (0.0%)	**2 (3.0%)**
M184V	2 (33.3%)	9 (45.0%)	2 (50.0%)	9 (47.4%)	2 (28.6%)	2 (18.2%)	**26 (38.8%)**
M41L	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (5.3%)	1 (14.3%)	0 (0.0%)	**2 (3.0%)**
Q151M	1 (16.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	**1 (1.5%)**
T215F	0 (0.0%)	0 (0.0%)	0 (0.0%)	2 (10.5%)	0 (0.0%)	0 (0.0%)	**2 (3.0%)**
T215FIS	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (14.3%)	0 (0.0%)	**1 (1.5%)**
T215SY	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (5.3%)	1 (14.3%)	0 (0.0%)	**2 (3.0%)**
T215Y	0 (0.0%)	3 (15.0%)	0 (0.0%)	1 (5.3%)	0 (0.0%)	0 (0.0%)	**4 (6.0%)**
V75M	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (5.3%)	0 (0.0%)	0 (0.0%)	**1 (1.5%)**
Y115F	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (9.1%)	**1 (1.5%)**
Total	6	20	4	19	7	11	**67**

A - d4T(30gm)-3TC-NVP; C - d4T(30gm)-3TC-EFV; F - AZT-3TC-NVP; G - TDF-3TC-EFV; H - TDF-3TC-NVP

4.5 The new 454 pyrosequencing

A total of 101 participants had their samples pyro sequenced. The average age was 35.8 (SD: 12.4) years with a minimum and a maximum of 0.0 and 66.2 years respectively. Two thirds were female participants, 47.2% were married, and 64.3% were in primary school (Table 9). Of this number, 19.6% were in WHO clinical stage 3 or 4, and 9.5% reported opportunistic infections. There were 7 (7.4%) with tuberculosis, and 2 (2.4%)

who had sexually transmitted diseases. HIV subtype A was the most common subtype (50.0%) followed by subtype D (18.2%). The median CD4 cell count was 266.0 (IQR: 80.0, 441.0) while the mean viral load (log 10) was 4.2 (SD: 1.1), Table 4.9.

Table 4.9: Overall descriptive characteristics among the participants whose samples were pyro sequenced

Variable	N	n (%) or Median (IQR) or Mean (SD)
Socio-demographic characteristics		N = 101
Age at sample collection (Years)	101	35.8 (12.4)
Range (Min. – Max.)		0.0 – 66.2
Female	101	68 (67.3%)
Marital status		
Single		4 (11.1%)
Married/engaged/cohabiting	36	17 (47.2%)
Separated/Divorced/Widowed		15 (41.7%)
Education level		
No formal education		0 (0.0%)
Primary	28	18 (64.3%)
Secondary		10 (35.7%)
Tertiary		0 (0.0%)
Clinical characteristics		
ARV status		
Experienced failing therapy	101	41 (40.6%)
Naïve		60 (59.4%)
WHO clinical stage		
Stage 1		29 (26.7%)
Stage 2	79	15 (19.0%)
Stage 3		27 (34.2%)
Stage 4		8 (10.1%)
Opportunistic infection	95	9 (9.5%)
Tuberculosis	95	7 (7.4%)
Sexually transmitted diseases	82	2 (2.4%)
HIV subtypes		
A		54 (53.5%)
A/AE		3 (3.0%)
A/B		1 (1.0%)
A/D		5 (5.0%)
A/G		1 (1.0%)
A/K		1 (1.0%)
AE		3 (3.0%)
AE/C	101	1 (1.0%)
B		1 (1.0%)
B/A		3 (3.0%)
B/AE		2 (2.0%)
B/C		1 (1.0%)
C		2 (2.0%)
C/EC		1 (1.0%)
D		18 (17.8%)
D/A		3 (3.0%)
K/A		1 (1.0%)
CD4 count (Cells per cubic mm)	85	266.0 (80.0, 441.0)
Range (Min. – Max.)		0.0 – 1986
Viral load copies per ml (Log Base 10)	31	4.2 (1.1)
Range (Min. – Max.)		1.7 – 5.6

4.5.1 454 pyrosequenced samples among the ARV naïve participants with DRMS

Up to 60 (59.4%) were ARV naïve. Of this number 46 (76.7%) had drug resistance mutations. A total of 127 drug resistant gene strains were observed among the ARV naïve participants. The most common was K219Q (11.0%). It was followed by K101E and N88D each representing 6.3%. The drug resistance gene mutations were as shown in Figure 4.5.

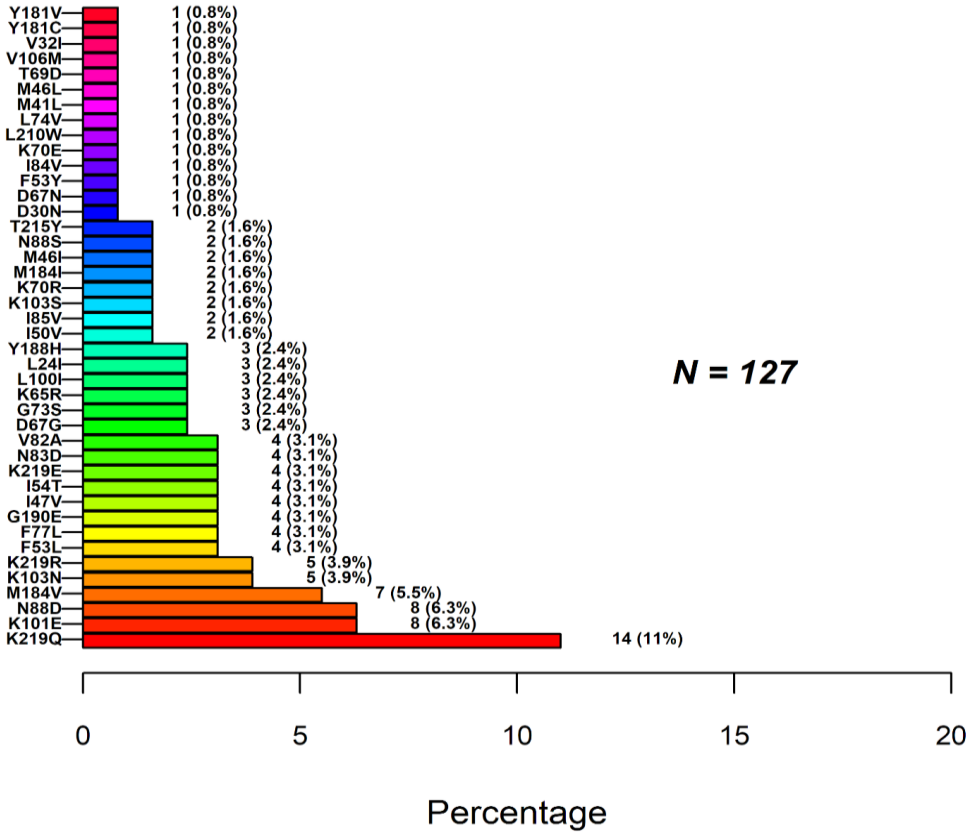


Figure 4.5: 454 pyrosequenced samples among the ARV naïve participants With DRMS

The demographic and clinical characteristic of the ARV naïve participants was compared by the presence or absence of drug resistant gene mutations (Table 4.10).

Table 4.10: Comparison of characteristics from ARV naïve participants of DRMs to non DRMs

Variable	N	DRMS		P-value
		No (n = 14)	Yes (n = 46)	
		n (%) or Median (IQR)		
Age at sample collection (Years)	53	28.6 (25.3, 41.5)	34.0 (28.8, 40.3)	0.280[w]
Range (Min. – Max.)		11.3 – 51.1	0.0 – 66.2	
Female	60	11 (78.6%)	33 (71.7%)	0.740[f]
Marital status				
Single		0 (0.0%)	4 (17.4%)	
Married/Engaged/cohabiting	32	4 (44.4%)	11 (47.8%)	0.425[f]
Divorced/separated/Widowed		5 (55.6%)	8 (34.8%)	
Education level				
Primary		0 (0.0%)	0 (0.0%)	
Secondary	26	4 (50.0%)	13 (72.2%)	0.382[f]
Tertiary		4 (50.0%)	5 (27.8%)	
WHO clinical stage				
Stage 1		7 (77.8%)	18 (56.2%)	
Stage 2	41	2 (22.2%)	6 (18.8%)	0.506[f]
Stage 3		0 (0.0%)	7 (21.9%)	
Stage 4		0 (0.0%)	1 (3.1%)	
Have opportunistic infections	54	0 (0.0%)	4 (9.8%)	0.562[f]
Tuberculosis	54	0 (0.0%)	6 (14.6%)	0.317[f]
HIV subtypes				
A		8 (57.1%)	25 (54.3%)	
A/AE		1 (7.1%)	1 (2.2%)	
A/B		0 (0.0%)	1 (2.2%)	
A/D		0 (0.0%)	2 (4.3%)	
A/G		1 (7.1%)	0 (0.0%)	
A/K	60	0 (0.0%)	1 (2.2%)	0.510[f]
AE		0 (0.0%)	2 (4.3%)	
AE/C		1 (7.1%)	0 (0.0%)	
B/A		0 (0.0%)	1 (2.2%)	
B/AE		1 (7.1%)	1 (2.2%)	
C		0 (0.0%)	1 (2.2%)	
D		2 (14.3%)	10 (21.7%)	
D/A		0 (0.0%)	1 (2.2%)	
CD4 count (Cells per cubic mm)	51	403.0 (48.0, 537.0)	368.0 (148.0,484.8)	0.888[w]
Range (Min. – Max.)		0.0 – 849.0	0.0 – 1986.0	

[f]Fisher's Exact test; [w]Two sample Wilcoxon rank-sum test

4.5.2 454 pyrosequenced samples with DRMS from the ARV experienced failing therapy participants

There were 41 (40.6%) ARV experienced participants who samples were pyro-sequenced. Of this number were 36 (87.8%) participants who had drug resistance gene mutations. The distribution of the drug resistance gene mutation strains were as shown in Figure 4.6. There were a total 149 drug resistant gene mutations that were observed. Of this, the most common one was M184V representing 13.4%. It was followed by K103N (10.7%). Mutants D67N and G190A each represented 5.4% (Figure 4.6).

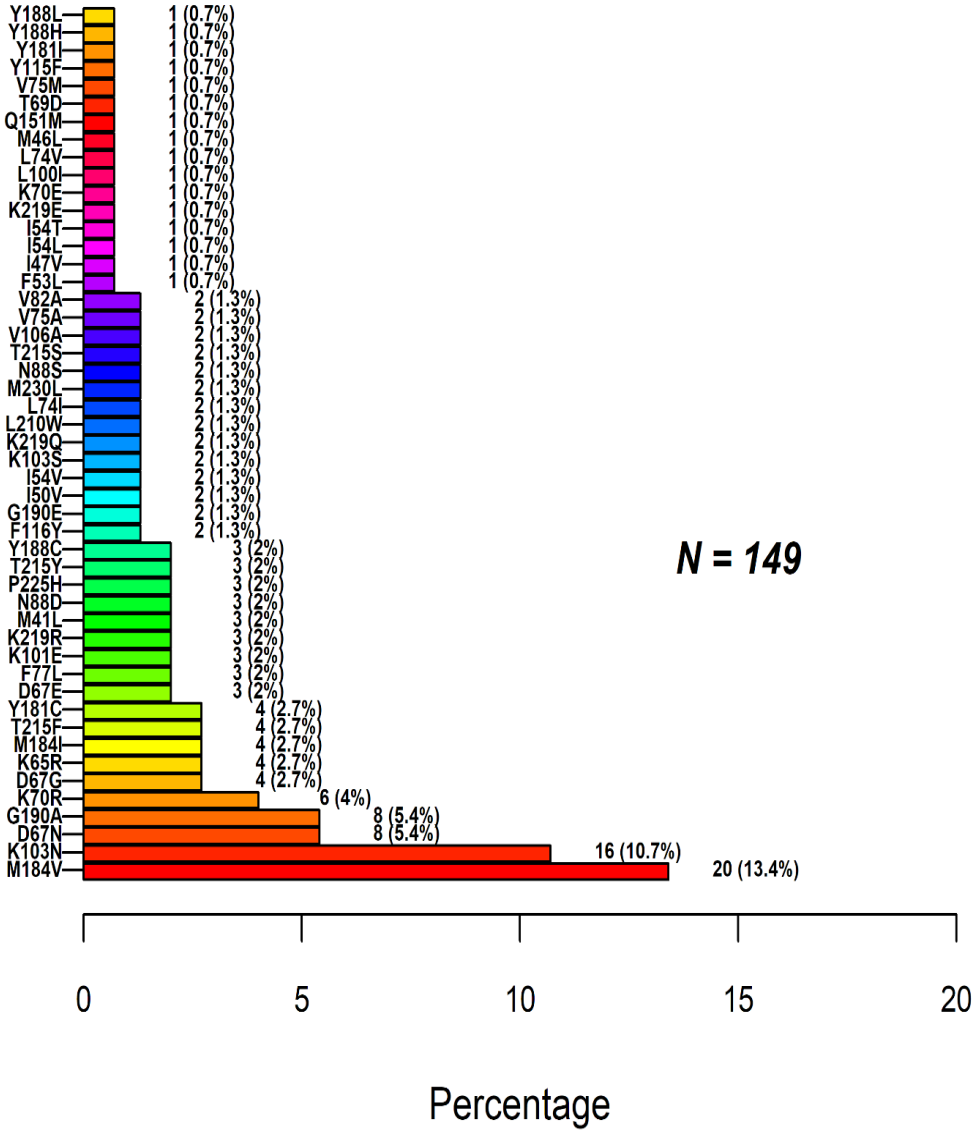


Figure 4.6: Drug resistant gene mutations among the ARV experienced.

ARV experienced failing therapy who had drug resistant mutation genes was compared to those who did not have. The results were as shown in Table 4.11.

Table 4.11: 454 pyrosequenced samples with and without DRMS from the ARV experienced failing therapy participants

Variable	N	DRMS		P-value
		No (n = 5)	Yes (n = 36)	
		n (%) or Mean (SD) or Median (IQR)		
Age at sample collection (Years)	40	33.8 (32.0, 39.1)	36.7 (31.0 – 42.9)	0.935[w]
Range (Min. – Max.)		27.4 – 56.0	6.6 – 63.1	
Female	41	4 (80.0%)	20 (55.6%)	0.382[f]
WHO clinical stage				
Stage 1		1 (20.0%)	3 (9.1%)	
Stage 2	38	1 (20.0%)	6 (18.2%)	0.212[f]
Stage 3		1 (20.0%)	19 (57.6%)	
Stage 4		4 (40.0%)	5 (15.2%)	
Have opportunistic infections	41	1 (20.0%)	4 (11.1%)	0.497[f]
Tuberculosis	41	0 (0.0%)	1 (2.8%)	>0.999[f]
Sexually transmitted infections	33	0 (0.0%)	2 (6.7%)	>0.999[f]
HIV subtypes				
A		3 (60.0%)	18 (50.0%)	
A/AE		0 (0.0%)	1 (2.8%)	
A/D		1 (20.0%)	2 (5.6%)	
AE		0 (0.0%)	1 (2.8%)	
B		1 (20.0%)	0 (0.0%)	0.547[f]
B/A	41	0 (0.0%)	2 (5.6%)	
B/C		0 (0.0%)	1 (2.8%)	
C		0 (0.0%)	1 (2.8%)	
C/EC		0 (0.0%)	1 (2.8%)	
D		0 (0.0%)	6 (16.7%)	
D/A		0 (0.0%)	2 (5.6%)	
K/A		0 (0.0%)	1 (2.8%)	
CD4 count (Cells per cubic mm)	34	214.0	139.0	0.331[w]
		(188.5, 350.0)	(68.5, 294.5)	
Range (Min. – Max.)		163.0 – 486.0	2.0 – 713.0	
Viral load (Log base 10)	28	4.1 (1.6)	4.3 (1.1)	0.859[t]
Range (Min. – Max.)		1.7 – 5.2	1.7 – 5.6	

[f]Fisher's Exact test; [t]Independent samples t-test; [w]Two sample Wilcoxon rank-sum test

The participants who had drug resistant gene mutations were older than those who did not have drug resistant gene mutation, 36.7 (IQR: 31.0, 42.9) vs. 33.8 (IQR: 32.0, 39.1) years. However, the difference was not statistically significant, p = 0.935. The proportion of female participants in both groups were similar, 80.0% vs. 55.6%, p =

0.382. There was no evidence from the data to link WHO clinical stage with the presence of drug resistant gene mutations, p = 0.212. A higher proportion of the participants with HIV subtype A was seen among those who had drug resistant gene mutations (60.0%) compared to 50.0% among those who had no drug resistant gene mutations. There difference was however, not statistically significant, p = 0.547. The median CD4 cell count, and the mean viral load levels were similar in both groups, p 0.331, and 0.859 respectively.

4.5.4 Susceptibility to change of the ARV regimens

The probability that the participant was susceptible to the new regimen after it was changed was 85.7%, and the probability that the participant remained susceptible without change of the regimen was 25.0%. Susceptibility to change of the ARV regimens was assessed the findings were as shown in Table 4.12.

Table 4.12: Susceptibility to change of ARV regimen

		Susceptible to new regimen		
		No	Yes	Total
Changed regimen 16 months later	No	15 (75.0%)	5 (25.0%)	20
	Yes	2 (14.3%)	12 (85.7%)	14
	Total	17	17	34

CHAPTER FIVE

DISCUSSION

5.1 Discussion

Baseline characteristics

A total of 206 participants aged 6.6 years to 71.8 years were included in the study. The mean age was 38.1 (SD: 12.2) years, 62.9% were female, 50.9% were married, engaged or cohabiting, 42.0% had secondary or tertiary level of education. Of the study participants 32.5% were ARV naïve and 52.5% in WHO clinical stage 3 or 4. There were 15 (7.6%) participants who reported opportunistic infections, 8 (4.0%) had tuberculosis, and 8 (4.4%) had sexually transmitted diseases. The median CD4 cell count per cubic milliliter was 210.0 (IQR: 70.0, 391.2) and the average viral load (log 10) was 3.1 (SD: 1.5), range (1.6 – 5.7).

Characteristics of Sanger sequenced

The ARV naïve and experienced failing therapy were comparable in their age, 36.7 (SD: 12.4) vs.35.7 (SD: 11.0) years. The proportion of female in the ARV naïve group was high compared to the experienced group, 72.3% vs. 57.1%. The proportion married did not differ significantly in the groups, 48.6% vs. 57.1%. The level of education was also similar among the ARV naïve and ARV experienced group. The participants in WHO clinical stage 3 or 4 was high among the experienced participants, 31.8% vs. 8.1%. Also, the proportion reporting opportunistic infections was high among the ARV experienced group.

The median CD4 cell count per cubic milliliter was high among the ARV naïve group, 386.0 (IQR: 138.0, 528.5) compared to the ARV experienced participants, 163.0 (IQR:

62.0, 294.5). The average viral load (log 10) was similar for the two groups of participants From the ARV naive group the prevalence of DRMs was similar to findings from a cross-sectional study carried out in Nairobi in 2005 which found 4/53 (7.5%) (Lihana, Khamadi, Lubano, et al., 2009) and higher than multisite cross-sectional study from the PASER group, conducted between 2007 and 2009 which reported 9/200 (4.5%) in Mombasa and 10/204 (4.9%) in Nairobi (Hamers et al., 2012) . It was also higher compared to the prevalence of TDR in sub-Saharan Africa which has previously been reported to be <5 % (Kamoto et al., 2008; Pillay et al., 2008). Recent data suggest an increase in the prevalence of TDR in some settings. In Kampala, Uganda, the prevalence of TDR increased from 0% (2006–2007) to 8.6% (2009–2010)(Ndembi et al., 2011).

The mean age of the ARV naïve participants was 35.7 (SD: 11.0) years with a minimum and a maximum of 7.5 and 66.2 years respectively. The proportion of female participants was 72.3%, and the proportion married was 48.6%. Up to 37.9% had a secondary or tertiary level of education. Up to 81.8% were in WHO clinical stage 1 or 2, and the proportion reporting opportunistic infections was 6.8%. Up to 8.5% and 3.9% had history of TB, and sexually transmitted diseases respectively. The median CD4 cell count per cubic milliliter among the ARV naïve group was 386.0 (IQR: 138.0, 528.5), and the average viral load (log 10) was 4.2 (SD: 0.6) copies per ml.

Characteristics of Sanger sequenced ARV naïve with DRMS

The most prevalent mutations among ARV naïve group were those affecting NRTI. Mutations affecting NNRTI were also observed in this study. Overall there were 39 NNRTI based drug resistant gene mutations. The most common one was K103N representing 33.3%, followed G190A, and Y181C representing 20.5% and 17.9% respectively. Among the ARV naïve participants, there was one participant with K103N NNRTI based drugs resistant gene mutation. Studies have shown that while NNRTIs have a low genetic barrier to resistance, a single mutation is often sufficient to cause resistance to the currently recommended first-generation NNRTIs, nevirapine and

efavirenz (Paredes & Clotet, 2010). The RT mutation K103N observed which has been seen to be the most commonly occurring NNRTI-resistant mutation in patients with acquired and transmitted NNRTI resistance. K103N reduces susceptibility to efavirenz and nevirapine by; 20-fold and; 50-fold, respectively, but has no effect on susceptibility to the most recently approved NNRTI, etravirine (Paredes & Clotet, 2010).

Characteristics of Sanger sequenced ARV experienced failing therapy with DRMS

The second group was ARV experienced failing therapy according to WHO guidelines. Among patients who harbored NRTIs, most harbored a mutation at position M184IV 26 (76.47%). The M184Vmutation has been seen to cause high-level in vitro resistance to 3TC and FTC and low-level in vitro RS to ddI and ABC. It is known to increase susceptibility to AZT, TDF, and d4T. Drug RS generally increase the risk of treatment failure and disease progression, but the presence of the M184V mutation appears to decrease viral fitness by reducing its ability to replicate and increasing susceptibility to other NRTIs (Johnson et al., 2011).

Up to 34 (41.0%) of the participants who were sanger sequenced were experienced. Of this number 30 (88.2%) had drug resistance gene mutations. Among the ARV experienced participants, 28 (82.4%) had gene mutations that were resistant to NRTI based regimens and 30 (88.2%) participants had NNRTI based resistance gene mutations. Majority of patients who reported with drug RS mutations, were resistant to AZT and/or d4T because they harbored either mutation in the RT gene associated with RS to RT inhibitors. A recent study found that, even with the M184V mutation in individuals using 3TC in an NNRTI- based regimen containing a boosted protease inhibitor and an NRTI backbone of 3TC or FTC with another NRTI was as effective in lowering viral load as were changes to either 3TC-sparing regimens or those with more intensive multi-drug combinations. When associated with TAMs, M184V have been reported to increase ABC RS (Johnson et al., 2011).

The broad NRTI cross-RS K65R was observed in one patient (PID: KE12-060). The above patient was already resistant to ABC and EFV, which were two out of the three drugs in the combination therapy. K65R, has been shown to be selected frequently (4%−11%) in patients with non-subtype-B clades for whom Stavudine-containing regimens are failing in the absence of TDF. According to Stanford report, K65R causes intermediate RS to ddI, ABC, 3TC, FTC, and TDF, and low-level RS to d4T. The K65R causes AZT hypersusceptibility. Importantly, presence of the K65R mutation compromises also the use of second-line regimens (Wallis et al., 2010).

The participants who had DRMs were comparable in age to those who did not have ARV gene mutations, median age: 32.9 (IQR: 30.6, 38.1) vs. 36.2 (IQR: 28.2, 46.3) years, p = 0.728. A significantly higher proportion of the female participants had no gene mutations, 100.0% vs. 40.0%, p = 0.039. There was no sufficient evidence from the data to link marital status, and level of education to DRMs (p > 0.05). A significantly higher proportion of participants without DRMs were in WHO clinical stage 1 compared to those who had DRMs, p = 0.035. A higher proportion of participants with HIV subtype A had drug resistant gene mutations (66.7%) compared to those who did not have drug resistant gene mutations (50.0%). The distribution of CD4 cell count were comparable for the two groups, median CD4: 150.0 (IQR: 95.2, 196.0) vs. 192.0 (IQR: 118.2, 308.5), p =0.273. Similarly, the average viral load (log 10) were comparable, 4.1 (SD: 0.9) vs. 3.8 (SD: 1.1), p = 0.769Some nucleoside (or nucleotide) analogue reverse transcriptase inhibitor (NRTI) mutations, like T215Y, may lead to viral hypersusceptibility to the non-nucleoside analogue reverse transcriptase inhibitors (NNRTIs), including etravirine, in NRTI treated individuals.

Despite agreement regarding the significance of most individual mutations; experts continue to show some disagreement in the interpretation of genotypes (Steegen et al., 2009). In this study, it was observed that among patients who harbored NNRTIs, the mutation K103N 13(38.24%) was most frequent but also G190A 8(23.52%) and Y181C

7(20.59%). Implications on treatment as per 2011 update of the DRMs in HIV-1, K103N has been shown to cause high-level RS to NVP, and EFV. By itself it has been shown to have no effect on ETR susceptibility but have a synergistic effect with L100I and possibly K101P on ETR susceptibility. On the other hand, Y181C has been shown to cause high-level RS to NVP, ~2-fold decreased susceptibility to EFV, and ~5-fold decreased susceptibility to ETR and RPV.

Y181C has also been shown to form the foundation for high-level ETR and RPV RS as the addition of some single mutations and many double mutations cause high-level RS to these drugs. Although Y181C causes 2-fold decreased EFV susceptibility, salvage therapy has generally not been successful in NVP-treated patients who harbor Y181C. Y181C increases susceptibility to AZT and TDF. The presence of mutations (A98G (2), K101E (1), Y181C/1(9), G190S/A (10), M230L (1) had already compromised the use of next generation NNRTI etravirine (Johnson et al., 2011)

Multi NRTI 69 insertion complex b observed in this study, affects all NRTIs and includes T215YF(n=5), K219QE(n=4).The 69 insertion complex is associated with resistance to all NRTIs currently approved by the US FDA when present with 1 or more thymidine analogue associated mutations (TAMs) at codons 41, 210, or 215). Multi NRTI resistance 151 complex C was also observed which affects all NRTIs currently approved except TDF and include and not limited to Q151M (n=1). By itself, Q151M causes intermediate to-high level RS to AZT, ddI, d4T, and ABC; and low-level RS to TDF. With changes at the associated positions 75, 77, and 116, Q151M confers high-level RS to AZT, ddI, d4T, and ABC, intermediate RS to TDF, and low-level RS to 3TC and FTC. The above patient was on second line therapy but failing clinically. From our results we observed that the patient had both NRTI and NNRTI multiple mutations and susceptible to second line drugs with a high viral load of 211,082 copies/ml. Multi NRTI resistance Thymidine Analogue Associated Mutations d,e Mutations (TAMs) observed in this study were (M41L, D67N, K70R, L210W, T215Y/F, and K219Q/E).

This study results are similar to the one carried out to evaluate treatment success and development of ART drug RS after short-term treatment among patients attending the Comprehensive HIV Care Centre (CCC) of Coast Province General Hospital, Mombasa, Kenya. Of the 19 patients with a detectable viral load, sequencing of the protease (PR) and reverse transcriptase (RT) gene was successful in 16. Eleven (11) of these 16 patients were infected with a subtype A1 virus. Major PR mutations were absent, but mutations associated with drug RS in RT were detected in 14 of the 16 patients (87.5%). High-level RS against at least 2 drugs of the ART regimen was observed in 9/14 (64.3%). The 3TC mutation M184V and the NNRTI mutation K103N were most frequent but also the multi-drug RS Q151M and the broad NRTI cross-RS K65R were observed ((Lihana, Khamadi, Lubano, et al., 2009; Steegen et al., 2009).

454 pyrosequenced samples with DRMS

Results show that the participants who had drug resistant gene mutations were older than those who did not have drug resistant gene mutations, median age: 34.0 (IQR: 28.8, 40.3) vs. 28.6 (IQR: 25.3, 41.5) years. The proportions of female participants in the two groups were similar, 78.6% vs. 71.7%, $p = 0.740$.

Marital status, education level, and WHO clinical stage were not associated with the presence of drug resistant gene mutations among the ARV naïve participants, $p = 0.425$, 0.382, and 0.506 respectively.

HIV subtype A was the highly presented among those who had no drug resistant gene mutations compared to those who had, 57.1% vs. 54.3%. HIV subtype D was also highly presented among the group who had drug resistant gene mutations (21.7%) compared to those who did not have drug resistant gene mutations (14.3%). There was however no evidence from the data to link HIV subtypes to drug resistant gene mutations, $p = 0.510$.

The median CD4 cell count were similar for the two groups of participants, 403.0 (IQR: 48.0, 537.0) vs. 368.0 (IQR: 148.0, 484.8), p = 0.888.

454 pyrosequenced samples with DRMS among the ARV naïve participants

Up to 60 (59.4%) were ARV naïve. Of this number 46 (76.7%) had drug resistance mutations. A total of 127 drug resistant gene strains were observed among the ARV naïve participants. The most common was K219Q (11.0%). It was followed by K101E and N88D each representing 6.3%.

Described in this study is a method to sequence together patient samples to simultaneously test for HIV drug resistance using Roche/454 pyrosequencing in a RLS. Successful DR surveillance programs typically acquire sequencing results from large numbers of antiretroviral naive and experienced subjects and produce an estimate of the percentage of drug resistance based upon the aggregate results. The percentage resistance to protease and reverse transcriptase inhibitors is not described in the context of the individual but instead is attributed to the population under study. Thus far, pyrosequencing of HIV has been used to explore HIV DR in a population of viruses within an individual. However, in this proof of concept study, we use pyrsosequencing on pooled specimens in order to survey for protease (PR) and reverse transcriptase (RT) DRM contained in viruses within a population. The findings presented here are strongly supportive of analyzing pooled specimens for the determination of the prevalence of HIV DR in HIV PR and RT. From this study, use of NGS technology revealed a high prevalence of low abundance drug resistant variants among the drug naive populations in North Rift Kenya. This is the first report whereby NGS technology has been utilized to inform on the status of HIV drug resistance in HIV infected populations in the country. One of earlier studies on HIV DR on drug naïve antenatal clinic attendees in the region using direct Sanger sequencing had revealed a paltry 3.2 % prevalence of transmitted DRMs (Kiptoo et al., 2008).

454 pyrosequenced samples with DRMS among the ARV naïve participants

This high outcome with the use of next generation sequencing is in concordance with results observed elsewhere in Africa with a prevalence of 80% among a drug naïve populations in Zambia infected by HIV subtype C (Gonzalez-Serna et al., 2014) confirming the high sensitivity of this technique for the detection and quantification of DRMs. The most prevalent variants observed in our study were the mutants carrying the thymidine analogue mutations, TAM, K219Q/R, M184V/I and none TAM- K103N. Viruses with K219Q mutations have been noted to evolve rapidly to zidovudine (AZT) resistance and shows high replicative fitness in presence of AZT. The M184V on the other hand confer high level resistance to 3TC, a key backbone to first line ARV treatment regimens in Kenya. The M184I mutation has been noted to be the first to appear but is quickly replaced by the M184V since this mutation has greater ability to induce higher replicative capacity (Gonzalez-Serna et al., 2014).

In the WHO drug resistance reports an increase of transmitted drug resistant variants have been observed in sub-Saharan Africa over time with the most commonly observed DRMs being M184V and K103N (WHO Drug resistance report 2012). The K103N mutation in particular, was noted in more than half of HIV infected patients presenting with NNRTI resistance. Despite such evidence of increasing rates of transmitted and acquired NNRTI resistance, efavirenz or nevirapine are still key components in first line ART in Africa. Due to competitive costs and the existing scaling, the pooled pyrosequencing approach may be useful in global TDR surveillance through its implementation at specialized HIV DR laboratories (Hezhao Ji et al 2010).This finding emphasizes the importance of surveying for HIV drug resistance and highlights the significance of developing tools such as those provided here to perform these surveys faster and cheaper to choose optimal first-line/second-line or salvage therapies.

5.2 Conclusions

1. Drug Resistant Mutations were identified in ARV naïve patients with a prevalence of 6.1%. All naïve patients identified with DRMs were female. The most prevalent mutations identified in ARV naïve patients were those affecting NRTI
2. Drug Resistant Mutations were identified in ARV experienced patients failing therapy with a prevalence of 91%. The most prevalent NRTI mutation observed was at position M184IV while the most prevalent NNRTI mutation observed was at position K103N. Drug Resistant Mutations across gender was statistically significant. Overall, all Male subjects from ARV experienced had DMRS when compared to Females
3. In this study a high prevalence of 76.6% of LADRVs among drug naive populations was revealed.

5.3 Recommendations

1. Drug Resistance testing would be necessary before initiating ARV therapy so as to guide in the choice of susceptible combination ARV. The new 454 pyrosequencing is highly recommended
2. It is highly recommended to use a feasible next generation sequencing technologies for surveillance of HIV drug resistance at population level to reliably detect and monitor emerging drug resistance patterns that may impact ARV treatment.
3. Further research is necessary for a continued follow-up of persons with DRMS and LADRVS to determine clinical impact and help guide therapies for drug naïve populations.

REFERENCES

Aberg, J. A., Gallant, J. E., Ghanem, K. G., Emmanuel, P., Zingman, B. S., Horberg, M. A., & Infectious Diseases Society of, A. (2014a). Primary care guidelines for the management of persons infected with HIV: 2013 update by the HIV Medicine Association of the Infectious Diseases Society of America. *Clin Infect Dis, 58*(1), 1-10. doi: 10.1093/cid/cit757

Aberg, J. A., Gallant, J. E., Ghanem, K. G., Emmanuel, P., Zingman, B. S., Horberg, M. A., & Infectious Diseases Society of, A. (2014b). Primary care guidelines for the management of persons infected with HIV: 2013 update by the HIV medicine association of the Infectious Diseases Society of America. *Clin Infect Dis, 58*(1), e1-34. doi: 10.1093/cid/cit665

Aghokeng, A. F., Kouanfack, C., Laurent, C., Ebong, E., Atem-Tambe, A., Butel, C., . . . Peeters, M. (2011). Scale-up of antiretroviral treatment in sub-Saharan Africa is accompanied by increasing HIV-1 drug resistance mutations in drug-naive patients. *AIDS, 25*(17), 2183-2188. doi: 10.1097/QAD.0b013e32834bbbe9

AIDS, A.-L. C. o. I. D. t. E., Keiser, O., Anastos, K., Schechter, M., Balestre, E., Myer, L., . . . Egger, M. (2008). Antiretroviral therapy in resource-limited settings 1996 to 2006: patient characteristics, treatment regimens and monitoring in sub-Saharan Africa, Asia and Latin America. *Trop Med Int Health, 13*(7), 870-879. doi: 10.1111/j.1365-3156.2008.02078.x

Amoroso, A., Etienne-Mesubi, M., Edozien, A., Ojoo, S., Sheneberger, R., Obiefune, M., . . . Redfield, R. R. (2012). Treatment outcomes of recommended first-line antiretroviral regimens in resource-limited clinics. *J Acquir Immune Defic Syndr, 60*(3), 314-320. doi: 10.1097/QAI.0b013e31824e5256

Angelis, K., Albert, J., Mamais, I., Magiorkinis, G., Hatzakis, A., Hamouda, O., . . . Paraskevis, D. (2014). Global Dispersal Pattern of HIV Type 1 Subtype CRF01_AE: A Genetic Trace of Human Mobility Related to Heterosexual Sexual Activities Centralized in Southeast Asia. *J Infect Dis*. doi: 10.1093/infdis/jiu666

Babic, D. Z., Zelnikar, M., Seme, K., Vandamme, A. M., Snoeck, J., Tomazic, J., . . . Poljak, M. (2006). Prevalence of antiretroviral drug resistance mutations and HIV-1 non-B subtypes in newly diagnosed drug-naive patients in Slovenia, 2000-2004. *Virus Res, 118*(1-2), 156-163. doi: 10.1016/j.virusres.2005.12.006

Bennett, D. E., Camacho, R. J., Otelea, D., Kuritzkes, D. R., Fleury, H., Kiuchi, M., . . . Shafer, R. W. (2009). Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. *PLoS One, 4*(3), e4724. doi: 10.1371/journal.pone.0004724

Benson, C. A., van der Horst, C., Lamarca, A., Haas, D. W., McDonald, C. K., Steinhart, C. R., . . . Group, F. T. C. W. (2004). A randomized study of emtricitabine and lamivudine in stably suppressed patients with HIV. *AIDS, 18*(17), 2269-2276.

Berenguer, J., Perez-Elias, M. J., Bellon, J. M., Knobel, H., Rivas-Gonzalez, P., Gatell, J. M., . . . Zidovudine Cohort Study, G. (2006). Effectiveness and safety of abacavir, lamivudine, and zidovudine in antiretroviral therapy-naive HIV-infected patients: results from a large multicenter observational cohort. *J Acquir Immune Defic Syndr, 41*(2), 154-159.

Berglund, O. (1991). [Communicable disease control regulations have no place in the prevention of HIV]. *Lakartidningen, 88*(12), 1048-1049.

Bonjoch, A., Paredes, R., Galvez, J., Miralles, C., Videla, S., Martinez, E., . . . Simplifi, H. S. G. (2005). Antiretroviral treatment simplification with 3 NRTIs or 2 NRTIs plus nevirapine in HIV-1-infected patients treated with successful first-line HAART. *J Acquir Immune Defic Syndr, 39*(3), 313-316.

Campbell, T. B., Smeaton, L. M., Kumarasamy, N., Flanigan, T., Klingman, K. L., Firnhaber, C., . . . ACTG, P. s. t. o. t. (2012). Efficacy and safety of three antiretroviral regimens for initial treatment of HIV-1: a randomized clinical trial in diverse multinational settings. *PLoS Med, 9*(8), e1001290. doi: 10.1371/journal.pmed.1001290

Castagna, A., Danise, A., Menzo, S., Galli, L., Gianotti, N., Carini, E., . . . Lazzarin, A. (2006). Lamivudine monotherapy in HIV-1-infected patients harbouring a lamivudine-resistant virus: a randomized pilot study (E-184V study). *AIDS, 20*(6), 795-803. doi: 10.1097/01.aids.0000218542.08845.b2

Clumeck, N., Goebel, F., Rozenbaum, W., Gerstoft, J., Staszewski, S., Montaner, J., . . . Team, C. N. A. S. (2001). Simplification with abacavir-based triple nucleoside therapy versus continued protease inhibitor-based highly active antiretroviral therapy in HIV-1-infected patients with undetectable plasma HIV-1 RNA. *AIDS, 15*(12), 1517-1526.

Cooper, C. L. (2007). HIV antiretroviral medications and hepatotoxicity. *Curr Opin HIV AIDS, 2*(6), 466-473. doi: 10.1097/COH.0b013e3282f0dd0b

Damond, F., Roquebert, B., Benard, A., Collin, G., Miceli, M., Yeni, P., . . . Descamps, D. (2007). Human immunodeficiency virus type 1 (HIV-1) plasma load discrepancies between the Roche COBAS AMPLICOR HIV-1 MONITOR Version 1.5 and the Roche COBAS AmpliPrep/COBAS TaqMan HIV-1 assays. *J Clin Microbiol, 45*(10), 3436-3438. doi: 10.1128/JCM.00973-07

de Mendoza, C., Ramos, J. T., Ciria, L., Fortuny, C., Garcia, F. J., de Jose, M. I., . . . Soriano, V. (2002). Efficacy and safety of stavudine plus didanosine in asymptomatic HIV-infected children with plasma HIV RNA below 50,000 copies per milliliter. *HIV Clin Trials, 3*(1), 9-16. doi: 10.1310/FAJF-7A8G-QAR4-Q0X5

DeJesus, E., Herrera, G., Teofilo, E., Gerstoft, J., Buendia, C. B., Brand, J. D., . . . Team, C. N. A. S. (2004). Abacavir versus zidovudine combined with lamivudine and efavirenz, for the treatment of antiretroviral-naive HIV-infected adults. *Clin Infect Dis, 39*(7), 1038-1046. doi: 10.1086/424009

Dudley, D. M., Chin, E. N., Bimber, B. N., Sanabani, S. S., Tarosso, L. F., Costa, P. R., . . . O'Connor, D. H. (2012). Low-cost ultra-wide genotyping using Roche/454 pyrosequencing for surveillance of HIV drug resistance. *PLoS One, 7*(5), e36494. doi: 10.1371/journal.pone.0036494

Einterz, R. M., Kimaiyo, S., Mengech, H. N., Khwa-Otsyula, B. O., Esamai, F., Quigley, F., & Mamlin, J. J. (2007). Responding to the HIV pandemic: the power of an academic medical partnership. *Acad Med, 82*(8), 812-818. doi: 10.1097/ACM.0b013e3180cc29f1

Eron, J. J. (2008). Managing antiretroviral therapy: changing regimens, resistance testing, and the risks from structured treatment interruptions. *J Infect Dis, 197 Suppl 3*, S261-271. doi: 10.1086/533418

Eshleman, S. H., Lie, Y., Hoover, D. R., Chen, S., Hudelson, S. E., Fiscus, S. A., . . . Taha, T. E. (2006). Association between the replication capacity and mother-to-child transmission of HIV-1, in antiretroviral drug-naive Malawian women. *J Infect Dis, 193*(11), 1512-1515. doi: 10.1086/503810

Faria, N. R., Rambaut, A., Suchard, M. A., Baele, G., Bedford, T., Ward, M. J., . . . Lemey, P. (2014). HIV epidemiology. The early spread and epidemic ignition of HIV-1 in human populations. *Science, 346*(6205), 56-61. doi: 10.1126/science.1256739

Fux, C. A., Simcock, M., Wolbers, M., Bucher, H. C., Hirschel, B., Opravil, M., . . . Swiss, H. I. V. C. S. (2007). Tenofovir use is associated with a reduction in calculated glomerular filtration rates in the Swiss HIV Cohort Study. *Antivir Ther, 12*(8), 1165-1173.

Gallant, J. E., DeJesus, E., Arribas, J. R., Pozniak, A. L., Gazzard, B., Campo, R. E., . . . Study, G. (2006). Tenofovir DF, emtricitabine, and efavirenz vs. zidovudine, lamivudine, and efavirenz for HIV. *N Engl J Med, 354*(3), 251-260. doi: 10.1056/NEJMoa051871

Galli, M., Ridolfo, A. L., Adorni, F., Gervasoni, C., Ravasio, L., Corsico, L., . . . Moroni, M. (2002). Body habitus changes and metabolic alterations in protease inhibitor-naive HIV-1-infected patients treated with two nucleoside reverse transcriptase inhibitors. *J Acquir Immune Defic Syndr, 29*(1), 21-31.

Gonzalez-Serna, A., Min, J. E., Woods, C., Chan, D., Lima, V. D., Montaner, J. S., . . . Swenson, L. C. (2014). Performance of HIV-1 drug resistance testing at low-level viremia and its ability to predict future virologic outcomes and viral evolution in treatment-naive individuals. *Clin Infect Dis, 58*(8), 1165-1173. doi: 10.1093/cid/ciu019

Gulick, R. M., Ribaudo, H. J., Shikuma, C. M., Lalama, C., Schackman, B. R., Meyer, W. A., 3rd, . . . Team, A. C. T. G. A. S. (2006). Three- vs four-drug antiretroviral regimens for the initial treatment of HIV-1 infection: a randomized controlled trial. *JAMA, 296*(7), 769-781. doi: 10.1001/jama.296.7.769

Gulick, R. M., Ribaudo, H. J., Shikuma, C. M., Lustgarten, S., Squires, K. E., Meyer, W. A., 3rd, . . . Team, A. C. T. G. S. A. (2004). Triple-nucleoside regimens versus efavirenz-containing regimens for the initial treatment of HIV-1 infection. *N Engl J Med, 350*(18), 1850-1861. doi: 10.1056/NEJMoa031772

Hamers, R. L., Schuurman, R., Sigaloff, K. C., Wallis, C. L., Kityo, C., Siwale, M., . . . PharmAccess African Studies to Evaluate Resistance, I. (2012). Effect of pretreatment HIV-1 drug resistance on immunological, virological, and drug-resistance outcomes of first-line antiretroviral treatment in sub-Saharan Africa: a multicentre cohort study. *Lancet Infect Dis, 12*(4), 307-317. doi: 10.1016/S1473-3099(11)70255-9

Hammer, S. M., Katzenstein, D. A., Hughes, M. D., Gundacker, H., Schooley, R. T., Haubrich, R. H., . . . Merigan, T. C. (1996). A trial comparing nucleoside monotherapy with combination therapy in HIV-infected adults with CD4 cell counts from 200 to 500 per cubic millimeter. AIDS Clinical Trials Group Study 175 Study Team. *N Engl J Med, 335*(15), 1081-1090. doi: 10.1056/NEJM199610103351501

Harris, N. (2002). HIV and AIDS: an update on impact in the United States and in the Department of Health and Human Services Region IV. *Ethn Dis, 12*(1), S2-48-50.

Havlir, D., Vella, S., & Hammer, S. (2002). The Global HIV Drug Resistance Surveillance Program: a partnership between WHO and IAS. International AIDS Society. *AIDS, 16*(10), 7-9.

Ho, D. D. (1995). Time to hit HIV, early and hard. *N Engl J Med, 333*(7), 450-451. doi: 10.1056/NEJM199508173330710

Ji, H., Masse, N., Tyler, S., Liang, B., Li, Y., Merks, H., . . . Brooks, J. (2010). HIV drug resistance surveillance using pooled pyrosequencing. *PLoS One, 5*(2), e9263. doi: 10.1371/journal.pone.0009263

Johnson, A. A., Sayer, J. M., Yagi, H., Patil, S. S., Debart, F., Maier, M. A., . . . Pommier, Y. (2006). Effect of DNA modifications on DNA processing by HIV-1 integrase and inhibitor binding: role of DNA backbone flexibility and an open catalytic site. *J Biol Chem, 281*(43), 32428-32438. doi: 10.1074/jbc.M605101200

Johnson, J. A., Li, J. F., Wei, X., Lipscomb, J., Irlbeck, D., Craig, C., . . . Heneine, W. (2008). Minority HIV-1 drug resistance mutations are present in antiretroviral treatment-naive populations and associate with reduced treatment efficacy. *PLoS Med, 5*(7), e158. doi: 10.1371/journal.pmed.0050158

Johnson, M. O., Gamarel, K. E., & Dawson Rose, C. (2006). Changing HIV treatment expectancies: a pilot study. *AIDS Care, 18*(6), 550-553. doi: 10.1080/09540120500241439

Johnson, V. A., Brun-Vezinet, F., Clotet, B., Kuritzkes, D. R., Pillay, D., Schapiro, J. M., & Richman, D. D. (2006). Update of the drug resistance mutations in HIV-1: Fall 2006. *Top HIV Med, 14*(3), 125-130.

Johnson, V. A., Calvez, V., Gunthard, H. F., Paredes, R., Pillay, D., Shafer, R., . . . Richman, D. D. (2011). 2011 update of the drug resistance mutations in HIV-1. *Top Antivir Med, 19*(4), 156-164.

Kamoto, K., Aberle-Grasse, J., & Malawi, H. I. V. D. R. T. F. (2008). Surveillance of transmitted HIV drug resistance with the World Health Organization threshold survey method in Lilongwe, Malawi. *Antivir Ther, 13 Suppl 2*, 83-87.

Karim, S. A., Baxter, C., Frohlich, J., & Karim, Q. A. (2014). The need for multipurpose prevention technologies in sub-Saharan Africa. *BJOG, 121 Suppl 5*, 27-34. doi: 10.1111/1471-0528.12842

Kaufmann, G. R., Perrin, L., Pantaleo, G., Opravil, M., Furrer, H., Telenti, A., . . . Swiss, H. I. V. C. S. G. (2003). CD4 T-lymphocyte recovery in individuals with advanced HIV-1 infection receiving potent antiretroviral therapy for 4 years: the Swiss HIV Cohort Study. *Arch Intern Med, 163*(18), 2187-2195. doi: 10.1001/archinte.163.18.2187

Kiptoo, M., Ichimura, H., Wembe, R. L., Ng'Ang'a, Z., Mueke, J., Kinyua, J., . . . Songok, E. M. (2008). Prevalence of nevirapine-associated resistance mutations after single dose prophylactic treatment among antenatal clinic attendees in north rift Kenya. *AIDS Res Hum Retroviruses, 24*(12), 1555-1559. doi: 10.1089/aid.2008.0018

Koigi, P., Ngayo, M. O., Khamadi, S., Ngugi, C., & Nyamache, A. K. (2014). HIV type 1 drug resistance patterns among patients failing first and second line antiretroviral therapy in Nairobi, Kenya. *BMC Res Notes, 7*, 890. doi: 10.1186/1756-0500-7-890

Lennox, J. L., Landovitz, R. J., Ribaudo, H. J., Ofotokun, I., Na, L. H., Godfrey, C., . . . Team, A. A. (2014). Efficacy and tolerability of 3 nonnucleoside reverse transcriptase inhibitor-sparing antiretroviral regimens for treatment-naive volunteers infected with HIV-1: a randomized, controlled equivalence trial. *Ann Intern Med, 161*(7), 461-471. doi: 10.7326/M14-1084

Lessells, R. J., Avalos, A., & de Oliveira, T. (2013). Implementing HIV-1 genotypic resistance testing in antiretroviral therapy programs in Africa: needs, opportunities, and challenges. *AIDS Rev, 15*(4), 221-229.

Li, B., Xu, X. H., Zhao, Z. C., Wang, M. H., & Cao, Y. P. (2014). High prevalence of metallo-beta-lactamase among carbapenem-resistant Klebsiella pneumoniae in a teaching hospital in China. *Can J Microbiol, 60*(10), 691-695. doi: 10.1139/cjm-2014-0291

Lihana, R. W., Khamadi, S. A., Lubano, K., Lwembe, R., Kiptoo, M. K., Lagat, N., . . . Ichimura, H. (2009). HIV type 1 subtype diversity and drug resistance among HIV type 1-infected Kenyan patients initiating antiretroviral therapy. *AIDS Res Hum Retroviruses, 25*(12), 1211-1217. doi: 10.1089/aid.2009.0007

Lihana, R. W., Khamadi, S. A., Lwembe, R. M., Kinyua, J. G., Muriuki, J. K., Lagat, N. J., . . . Songok, E. M. (2009). HIV-1 subtype and viral tropism determination for evaluating antiretroviral therapy options: an analysis of archived Kenyan blood samples. *BMC Infect Dis, 9*, 215. doi: 10.1186/1471-2334-9-215

Little, K. M., Kilmarx, P. H., Taylor, A. W., Rose, C. E., Rivadeneira, E. D., & Nesheim, S. R. (2012). A review of evidence for transmission of HIV from children to breastfeeding women and implications for prevention. *Pediatr Infect Dis J, 31*(9), 938-942. doi: 10.1097/INF.0b013e318261130f

Llibre, J. M., & Clotet, B. (2012). Once-daily single-tablet regimens: a long and winding road to excellence in antiretroviral treatment. *AIDS Rev, 14*(3), 168-178.

Lwembe, R., Ochieng, W., Panikulam, A., Mongoina, C. O., Palakudy, T., Koizumi, Y., . . . Ichimura, H. (2007). Anti-retroviral drug resistance-associated mutations among non-subtype B HIV-1-infected Kenyan children with treatment failure. *J Med Virol, 79*(7), 865-872. doi: 10.1002/jmv.20912

Mallal, S., Nolan, D., Witt, C., Masel, G., Martin, A. M., Moore, C., . . . Christiansen, F. T. (2002). Association between presence of HLA-B*5701, HLA-DR7, and HLA-DQ3 and hypersensitivity to HIV-1 reverse-transcriptase inhibitor abacavir. *Lancet, 359*(9308), 727-732.

Markowitz, M., Slater, L. N., Schwartz, R., Kazanjian, P. H., Hathaway, B., Wheeler, D., . . . McCallister, S. (2007). Long-term efficacy and safety of tipranavir boosted with ritonavir in HIV-1-infected patients failing multiple protease inhibitor regimens: 80-week data from a phase 2 study. *J Acquir Immune Defic Syndr, 45*(4), 401-410. doi: 10.1097/QAI.0b013e318074eff5

Matheron, S., Descamps, D., Boue, F., Livrozet, J. M., Lafeuillade, A., Aquilina, C., . . . Group, C. N. A. S. (2003). Triple nucleoside combination zidovudine/lamivudine/abacavir versus zidovudine/lamivudine/nelfinavir as first-line therapy in HIV-1-infected adults: a randomized trial. *Antivir Ther, 8*(2), 163-171.

Mellors, J. W., Munoz, A., Giorgi, J. V., Margolick, J. B., Tassoni, C. J., Gupta, P., . . . Rinaldo, C. R., Jr. (1997). Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. *Ann Intern Med, 126*(12), 946-954.

Mills, E. J., & Nachega, J. B. (2007). A wake-up call for global access to salvage HIV drug regimens. *Lancet, 370*(9603), 1885-1887. doi: 10.1016/S0140-6736(07)61790-5

Molina, J. M., Journot, V., Morand-Joubert, L., Yeni, P., Rozenbaum, W., Rancinan, C., . . . Team, A. S. (2005). Simplification therapy with once-daily emtricitabine, didanosine, and efavirenz in HIV-1-infected adults with viral suppression receiving a protease inhibitor-based regimen: a randomized trial. *J Infect Dis, 191*(6), 830-839. doi: 10.1086/428091

M**olina, J. M., Marcelin, A. G., Pavie, J., Heripret, L., De Boever, C. M., Troccaz, M., . . . Team, A. J. S.** (2005). Didanosine in HIV-1-infected patients experiencing failure of antiretroviral therapy: a randomized placebo-controlled trial. *J Infect Dis, 191*(6), 840-847. doi: 10.1086/428094

Moyle, G. J., Brown, S., Lysakova, L., & Barton, S. E. (2006). Long-term safety and efficacy of poly-L-lactic acid in the treatment of HIV-related facial lipoatrophy. *HIV Med, 7*(3), 181-185. doi: 10.1111/j.1468-1293.2006.00342.x

Moyle, G. J., DeJesus, E., Cahn, P., Castillo, S. A., Zhao, H., Gordon, D. N., . . . Ziagen Once-Daily in Antiretroviral Combination Therapy Study, T. (2005). Abacavir once or twice daily combined with once-daily lamivudine and efavirenz for the treatment of antiretroviral-naive HIV-infected adults: results of the Ziagen Once Daily in Antiretroviral Combination Study. *J Acquir Immune Defic Syndr, 38*(4), 417-425.

Mtambo, A., Chan, K., Shen, A., Lima, V., Hogg, R., Montaner, J., & Moore, D. (2012). Treatment limitations imposed by antiretroviral drug resistance mutations: implication for choices of first line regimens in resource-limited settings. *HIV Med, 13*(3), 141-147. doi: 10.1111/j.1468-1293.2011.00950.x

Murray, J. S., Elashoff, M. R., Iacono-Connors, L. C., Cvetkovich, T. A., & Struble, K. A. (1999). The use of plasma HIV RNA as a study endpoint in efficacy trials of antiretroviral drugs. *AIDS, 13*(7), 797-804.

Naeger, L. K., Margot, N. A., & Miller, M. D. (2001). Increased drug susceptibility of HIV-1 reverse transcriptase mutants containing M184V and zidovudine-associated mutations: analysis of enzyme processivity, chain-terminator removal and viral replication. *Antivir Ther, 6*(2), 115-126.

Ndembi, N., Hamers, R. L., Sigaloff, K. C., Lyagoba, F., Magambo, B., Nanteza, B., . . . Rinke de Wit, T. F. (2011). Transmitted antiretroviral drug resistance among newly HIV-1 diagnosed young individuals in Kampala. *AIDS, 25*(7), 905-910. doi: 10.1097/QAD.0b013e328346260f

Oette, M., Kaiser, R., Daumer, M., Petch, R., Fatkenheuer, G., Carls, H., . . . Haussinger, D. (2006). Primary HIV drug resistance and efficacy of first-line antiretroviral therapy guided by resistance testing. *J Acquir Immune Defic Syndr, 41*(5), 573-581. doi: 10.1097/01.qai.0000214805.52723.c1

Oette, M., Kroidl, A., Gobels, K., Stabbert, A., Menge, M., Sagir, A., . . . Haussinger, D. (2006). Predictors of short-term success of antiretroviral therapy in HIV infection. *J Antimicrob Chemother, 58*(1), 147-153. doi: 10.1093/jac/dkl189

Opravil, M., Ledergerber, B., Furrer, H., Hirschel, B., Imhof, A., Gallant, S., . . . Swiss, H. I. V. C. S. (2002). Clinical efficacy of early initiation of HAART in patients with asymptomatic HIV infection and CD4 cell count > 350 x 10(6) /l. *AIDS, 16*(10), 1371-1381.

Palella, F. J., Jr., Delaney, K. M., Moorman, A. C., Loveless, M. O., Fuhrer, J., Satten, G. A., . . . Holmberg, S. D. (1998). Declining morbidity and mortality among patients with advanced human immunodeficiency virus infection. HIV Outpatient Study Investigators. *N Engl J Med, 338*(13), 853-860. doi: 10.1056/NEJM199803263381301

Palmer, S., Boltz, V., Martinson, N., Maldarelli, F., Gray, G., McIntyre, J., . . . Coffin, J. (2006). Persistence of nevirapine-resistant HIV-1 in women after single-dose nevirapine therapy for prevention of maternal-to-fetal HIV-1 transmission. *Proc Natl Acad Sci U S A, 103*(18), 7094-7099. doi: 10.1073/pnas.0602033103

Paredes, R., & Clotet, B. (2010). Clinical management of HIV-1 resistance. *Antiviral Res, 85*(1), 245-265. doi: 10.1016/j.antiviral.2009.09.015

P**aredes, R., Lalama, C. M., Ribaudo, H. J., Schackman, B. R., Shikuma, C., Giguel, F., . . . Team, A. C. T. G. A. S. (**2010). Pre-existing minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure. *J Infect Dis, 201*(5), 662-671. doi: 10.1086/650543

Passaro, R. C., Pandhare, J., Qian, H. Z., & Dash, C. (2015). The Complex Interaction Between Methamphetamine Abuse and HIV-1 Pathogenesis. *J Neuroimmune Pharmacol*. doi: 10.1007/s11481-015-9604-2

Pillay, V., Ledwaba, J., Hunt, G., Rakgotho, M., Singh, B., Makubalo, L., . . . Morris, L. (2008). Antiretroviral drug resistance surveillance among drug-naive HIV-1-infected individuals in Gauteng Province, South Africa in 2002 and 2004. *Antivir Ther, 13 Suppl 2*, 101-107.

Price, M. A., Wallis, C. L., Lakhi, S., Karita, E., Kamali, A., Anzala, O., . . . Group, I. E. I. C. S. (2011). Transmitted HIV type 1 drug resistance among individuals with recent HIV infection in East and Southern Africa. *AIDS Res Hum Retroviruses, 27*(1), 5-12. doi: 10.1089/aid.2010.0030

Ramadhani, H. O., Thielman, N. M., Landman, K. Z., Ndosi, E. M., Gao, F., Kirchherr, J. L., . . . Crump, J. A. (2007). Predictors of incomplete adherence, virologic failure, and antiviral drug resistance among HIV-infected adults receiving antiretroviral therapy in Tanzania. *Clin Infect Dis, 45*(11), 1492-1498. doi: 10.1086/522991

Robbins, G. K., De Gruttola, V., Shafer, R. W., Smeaton, L. M., Snyder, S. W., Pettinelli, C., . . . Team, A. C. T. G. (2003). Comparison of sequential three-drug regimens as initial therapy for HIV-1 infection. *N Engl J Med, 349*(24), 2293-2303. doi: 10.1056/NEJMoa030264

Rozenbaum, W., Katlama, C., Massip, P., Bentata, M., Zucman, D., Delfraissy, J. F., . . . Mamet, J. P. (2001). Treatment intensification with abacavir in HIV-infected patients with at least 12 weeks previous lamivudine/zidovudine treatment. *Antivir Ther, 6*(2), 135-142.

Saag, M. S., Bowers, P., Leitz, G. J., Levine, A. M., & Community, H. I. V. A. M. P. S. S. G. (2004). Once-weekly epoetin alfa improves quality of life and increases hemoglobin in anemic HIV+ patients. *AIDS Res Hum Retroviruses, 20*(10), 1037-1045.

Saravolatz, L. D., Winslow, D. L., Collins, G., Hodges, J. S., Pettinelli, C., Stein, D. S., . . . Abrams, D. (1996). Zidovudine alone or in combination with didanosine or zalcitabine in HIV-infected patients with the acquired immunodeficiency syndrome or fewer than 200 CD4 cells per cubic millimeter. Investigators for the Terry Beirn Community Programs for Clinical Research on AIDS. *N Engl J Med, 335*(15), 1099-1106. doi: 10.1056/NEJM199610103351503

Sigaloff, K. C., Mandaliya, K., Hamers, R. L., Otieno, F., Jao, I. M., Lyagoba, F., . . . Rinke de Wit, T. F. (2012). Short communication: High prevalence of transmitted antiretroviral drug resistance among newly HIV type 1 diagnosed adults in Mombasa, Kenya. *AIDS Res Hum Retroviruses, 28*(9), 1033-1037. doi: 10.1089/AID.2011.0348

Spruance, S. L., Pavia, A. T., Mellors, J. W., Murphy, R., Gathe, J., Jr., Stool, E., . . . Dunkle, L. (1997). Clinical efficacy of monotherapy with stavudine compared with zidovudine in HIV-infected, zidovudine-experienced patients. A randomized, double-blind, controlled trial. Bristol-Myers Squibb Stavudine/019 Study Group. *Ann Intern Med, 126*(5), 355-363.

Staszewski, S., Keiser, P., Montaner, J., Raffi, F., Gathe, J., Brotas, V., . . . Team, C. I. S. (2001). Abacavir-lamivudine-zidovudine vs indinavir-lamivudine-zidovudine in antiretroviral-naive HIV-infected adults: A randomized equivalence trial. *JAMA, 285*(9), 1155-1163.

Steegen, K., Luchters, S., Dauwe, K., Reynaerts, J., Mandaliya, K., Jaoko, W., . . . Verhofstede, C. (2009). Effectiveness of antiretroviral therapy and development of drug resistance in HIV-1 infected patients in Mombasa, Kenya. *AIDS Res Ther, 6*, 12. doi: 10.1186/1742-6405-6-12

Stekler, J., Maenza, J., Stevens, C., Holte, S., Malhotra, U., McElrath, M. J., . . . Collier, A. C. (2006). Abacavir hypersensitivity reaction in primary HIV infection. *AIDS, 20*(9), 1269-1274. doi: 10.1097/01.aids.0000232234.19006.a2

Thiebaut, R., Morlat, P., Jacqmin-Gadda, H., Neau, D., Mercie, P., Dabis, F., & Chene, G. (2000). Clinical progression of HIV-1 infection according to the viral response during the first year of antiretroviral treatment. Groupe d'Epidemiologie du SIDA en Aquitaine (GECSA). *AIDS, 14*(8), 971-978.

Truong, H. M., Grant, R. M., McFarland, W., Kellogg, T., Kent, C., Louie, B., . . . Klausner, J. D. (2006). Routine surveillance for the detection of acute and recent HIV infections and transmission of antiretroviral resistance. *AIDS, 20*(17), 2193-2197. doi: 10.1097/01.aids.0000252059.85236.af

Varghese, V., Shahriar, R., Rhee, S. Y., Liu, T., Simen, B. B., Egholm, M., . . . Shafer, R. W. (2009). Minority variants associated with transmitted and acquired HIV-1 nonnucleoside reverse transcriptase inhibitor resistance: implications for the use of second-generation nonnucleoside reverse transcriptase inhibitors. *J Acquir Immune Defic Syndr, 52*(3), 309-315. doi: 10.1097/QAI.0b013e3181bca669

Volberding, P. A. (1990). Rationale for variations in clinical trial design in different HIV disease stages. *J Acquir Immune Defic Syndr, 3 Suppl 2*, S40-44.

Wallis, C. L., Mellors, J. W., Venter, W. D., Sanne, I., & Stevens, W. (2010). Varied patterns of HIV-1 drug resistance on failing first-line antiretroviral therapy in South Africa. *J Acquir Immune Defic Syndr, 53*(4), 480-484. doi: 10.1097/QAI.0b013e3181bc478b

Wallis, C. L., Mellors, J. W., Venter, W. D., Sanne, I., & Stevens, W. (2011). Protease Inhibitor Resistance Is Uncommon in HIV-1 Subtype C Infected Patients on Failing Second-Line Lopinavir/r-Containing Antiretroviral Therapy in South Africa. *AIDS Res Treat, 2011*, 769627. doi: 10.1155/2011/769627

Wang, C., Mitsuya, Y., Gharizadeh, B., Ronaghi, M., & Shafer, R. W. (2007). Characterization of mutation spectra with ultra-deep pyrosequencing: application to HIV-1 drug resistance. *Genome Res, 17*(8), 1195-1201. doi: 10.1101/gr.6468307

Wensing, A. M., Boucher, C. A., van Kasteren, M., van Dijken, P. J., Geelen, S. P., & Juttmann, J. R. (2006). Prevention of mother-to-child transmission of multi-drug resistant HIV-1 using maternal therapy with both enfuvirtide and tipranavir. *AIDS, 20*(10), 1465-1467. doi: 10.1097/01.aids.0000233587.94961.4f

White, K. L., Margot, N. A., Ly, J. K., Chen, J. M., Ray, A. S., Pavelko, M., . . . Miller, M. D. (2005). A combination of decreased NRTI incorporation and decreased excision determines the resistance profile of HIV-1 K65R RT. *AIDS, 19*(16), 1751-1760.

APPENDICES

Appendix i: Study Questionnaire

MOLECULAR CHARACTERIZATION OF PROTEASE AND REVERSE TRANSCRIPTASE HIV DRMS IN TREATMENT NAÏVE AND EXPERIENCED PATIENTS IN CARE IN ELDORET, KENYA

CCC No. _____ Study No. _____

Interviewer Date: ____/____/____ Hospital: _____

Part A: Socio-demographic information

1. Name:_____ Sex_____
   Date of Birth: ____/____/____
2. District of residence: _____
3. District of origin:_____
4. Ethnicity:_____
5. Religion: (a).Protestants (b) Catholic (c) Islam (d) Traditional (e) other(s), specify_____
6. Location:_____
7. Sublocation:_____
8. Contact information: Cell phone /landline or other person if client has no telephone contact no:_____
9. Marital status:(1) Single (2) Married/Engaged/Cohabit (3) Divorced/Separated/Widowed (4) Inherited
10. If married, type of marriage: (1) Monogamous (2) Polygamous
11. Occupation:_____
12. Family monthly income (Kshs.): (1) 0-2,000 (2) 3,000-5,000 (3)6,000-8,000 (4) 9,000-10,000(5) 11,000-20,000 (6) 21,000-30,000 (7) ≥31,000

Part B: Clinical information

1. Date of first HIV positive test: ____/____/____
2. Does your spouse/partner know your HIV status? (1) Yes (2) No
3. If no, please give reason(s)_____
4. WHO staging 1.I 2.II 3. III 4. IV
5. History of STIs? (1) Yes (2) No
6. If yes (a)Specify STIs in last one month _____

   (b) Specify STIs in last 6 months _____

   (c) Specify STIs in the last one year _____

7. History of OIs? (1) Yes (2) No
8. If yes

   (a) Specify OIs in last one month _____

   (b) Specify OIs in last 6 months _____

   (c) Specify OIs in the last one year _____

9. History of TB? (1) Yes (2) No
10. If Yes

    (a) Specify TB in last one month_____

    (b) Specify TB in last 6 months _____

    (c) Specify TB in the last one year _____

11. Have you used any antiretroviral drugs? (1) Yes (2) No
12. If yes, which drugs?

a. d4T (30gm)-3TC-NVP b. d4T (40gm)-3TC-NVP c. d4T (30gm)-3TC-EFV d. d4T (40gm)-3TC-EFV e. AZT-3TC-EFV f. AZT-3TC-NVP g. TDF-3TC-EFVh. TDF-3TC-NVP i. ABC-AZT-EVF

Other _____

13. Date of initiation of ART: ____/____/____
14. Have you switched to 2nd line regimen? (1) Yes (2) No
15. If yes, give reason(s)_____
16. If yes, which drugs? _____
17. Date of switch to 2nd line regimen: ____/____/____
18. Which method do you use to remember taking your drugs?

a) Using my phone
b) My spouse/Relative
c) Myself

19. During the last 7 days, how many antiretroviral pills did the patient MISS ☐
20. During the last 30 days, how many antiretroviral pills did the patient MISS ☐
21. If the patient missed any doses, please specify the reason _____

22. Do you use any method of protection when having sexual intercourse?

a) Male condom
b) Female condom
c) Both
d) None

Part C : Examination

1. Weight _____ Temperature_____ Height _____

Part D: Laboratory information

1. CD4 Count: Visit 1_____Date:____/____/____

Visit 2_____Date:____/____/____

Visit 3_____Date:____/____/____

Visit 4_____Date:____/____/____

2. Viral Load: _____Date:____/____/____

3. LFTs: Visit 1_____ Date:____/____/____

Visit 2_____ Date:____/____/____

Visit 3_____ Date:____/____/____

Visit 4_____ Date:____/____/____

4. Hb: Visit 1_____ Date:____/____/____

Visit 2_____ Date:____/____/____

Visit 3_____ Date:____/____/____

Visit 4_____ Date:____/____/____

Part E: Problems:

1. What major problems are you experiencing to access ARVs?

(1) Distance to clinic (2) Long clinic waiting time (3) High cost of laboratory tests (4) Lack of adequate information on ARV use (5) Supply of drugs (6) Side effects (7) Forgetting my clinic dates (8) Confidentiality (9) Other(s), Specify_____

_____

2. If 3 above is ticked/circled then specify the test and cost:_____

_____

Appendix ii: **Consent Information**

MOLECULAR CHARACTERIZATION OF PROTEASE AND REVERSE TRANSCRIPTASE HIV DRMS IN TREATMENT NAÏVE AND EXPERIENCED PATIENTS IN CARE IN ELDORET, KENYA

a) Description/Purpose of the study

The use of ARV drugs has greatly prolonged lives of people infected by Human Immunodeficiency virus. In Kenya more than 120,000 people are on treatment with ARV drugs. Though these drugs are effective, they may fail when the virus develops resistance towards them. The reasons why resistance develops in other people and not in others is unknown. We are intending to carry out an investigative study to determine the extent of HIV drug resistance among patients attending AMPATH center and possible reasons for the resistance. This study is very important, for it will help doctors treating HIV/AIDS patients to know the alternative type of drugs to give when patients fail treatment. It will also help us to test newer drugs that may be more effective. As you are about to begin ARV treatment or has been on treatment, we are asking for your participation in this study.

If you agree, we will use the blood sample that you have given out for CD4+ analysis and other blood tests also for this study. We will use the blood sample to culture the virus, classify the virus and compare it with other viruses that have been known to have defeated ARV drugs. We will also use the same sample to determine if there are other genetic factors within your body that may help HIV viruses to easily evade some types of ARV drugs.

Use of DNA Material

The DNA fragment obtained from your sample shall be sequenced to determine if you are at risk of developing ARV treatment failure. The DNA material will not be modified or engineered in any way.

Laboratory Sites

Sequencing will be done at the University of Nairobi Institute of Tropical Medicine laboratories. This is a WHO accredited lab for viral sequencing. The Public Health Agency of Canada laboratories (PHAC) in Winnipeg act as a site for external quality assurance for the UoN labs. As a quality assurance procedure, representative sequenced samples will be picked randomly and sent to PHAC for confirmation. PHAC lab has been involved in genetic studies for the past 10 years. The high number of publications in peer review journals on genetic studies from PHAC is proof of the facility to handle such studies.

Research team

You are being asked to participate in a research study called MOLECULAR CHARACTERIZATION OF PROTEASE AND REVERSE TRANSCRIPTASE HIV DRMS IN TREATMENT NAÏVE AND EXPERIENCED PATIENTS IN CARE IN ELDORET, KENYA

Winfrida Cheriro is a senior laboratory scientist at the Moi Teaching and referral hospital Eldoret and a PhD student in molecular medicine at Jomo Kenyatta University Institute of Tropical Medicine, Nairobi. Prof Elijah songok, an Assistant Professor, Department of Medical Microbiology, Faculty of Medicine, University of Manitoba, Canada and Principal Research Officer, Kenya Medical Research Institute, Nairobi Kenya is the main supervisor assisted by Prof Simeon Mining, a senior lecturer and head department of immunology, school of medicine, Moi university, Dr Gideon Kikuvi lecturer, Institute of Tropical Medicine, Jomo Kenyatta University of Agriculture and Technology, Prof Michael Kiptoo, Principal research officer, Centre for Virus Research, Kenya Medical Research Institute Nairobi and postgraduate coordinator ITROMID-KEMRI. Dr Wilfred Emonyi is the manager, AMPATH reference lab.

b) Benefits of Participation in the Study

You will receive no personal benefit from your participation in this study. The information generated from this study will however be provided to the Ministry of Health to help them make decisions on the type of ARV drugs to use for people who fail first line of treatment. To you in particular, it will help you directly when you develop resistance to the type of drugs that you will using. Based on our observations, we will provide information to your doctor to use to decide on the possible effective drug regimen to change to as an alternative.

c) Archiving of specimens

Your sample will be stored at AMPATH reference lab, Kenya Medical Research Institute (CVR and CGHR), University of Nairobi (UNITID) as a dried blood spot on a filter paper. The AIDS virus is in constant evolution. The type that is in circulation now will likely be different from the one that will be prevalent in 5, 10, 15 or 25 years time. We will wish to compare the current virus and the type that will be in circulation then. In addition newer and better technologies for analyzing the AIDS virus keep emerging every year; we will need to test your sample with these new methods. In this regard we will store your sample (about 5-10ug) for a period of 25 years after the end of the study. In the event of our need to do future comparative studies using your sample, we will apply again to the Directors, AMPATH through the Ethical Review Board for approval. No information which may reveal your identity will be attached to the sample. We will protect the confidentiality of the samples by assigning them a specific code. Your DNA sample will not be specifically identified but a code will link you to the sample. Similarly, as we have to compare our study methods with those of others abroad, or these newer technologies may appear earlier in developed countries, your sample may be transported to Canada (Public Health Agency of Canada) or/ and USA (Brown University) for training, quality control and confirmation purposes. At end of the storage and study period, the DNA material shall be incinerated and disposed of as per the prevailing regulations of disposal of genetic materials of Public Health Agency of Canada.

Sharing of samples

Your samples will not be shared with any member outside the investigating team and their students. However, any member of the team may use the sample for other genetic studies. The use of the material for other studies will however require a re-approval from ethical committee.

Risks of participation

Since this research is being performed with samples that have already been taken for other purposes you will not be exposed to any physical risks associated with the taking of a DNA sample. There are risks of discrimination against persons who have a genetic medical disorder or at risk of a medical disorder or condition in their family. Discrimination may include barrier to obtaining life or health insurance and employment. All efforts shall be made to protect our research subjects from prejudice or use of this information that may adversely affect them. Specifically clinical and research information specific to this study will be maintained in a separate location from your hospital medical records and will not be shared or placed in your medical file in the hospitals that you attend.

Confidentiality

All information obtained about you and the results of the research will be treated confidentially. This information will be coded and kept under a password protected database. The study files will be kept electronically at the Centre. Your participation and your genetic results of the research will not appear in your medical record nor will it be shared with other medical personnel with your identifying information. The results of this study maybe published, deposited on a public database or communicated in other ways but it will be impossible to identify you. You may also choose not to know your genetic results. In this regard, we will not return the results to you. Disclosure of potential economic gain

The analysis of your sample may contribute to creation of commercial products from which you will receive no financial benefit.

Basis of participation

You are free to consent or refuse to give consent for your participation in this study. You are also free to withdraw your consent to participate in the study at any given point in time. Your choice to consent or not consent to this study will in no way affect your relationship with AMPATH, MTRH or the Universities involved in this project.

Obtaining additional Information

You are free to seek clarity or ask any questions at any point in time in the course of the study. If you desire to get more information concerning the study, feel free to call or sms Winfrida Cheriro at +254725739782 or Dr Wilfred Emonyi at 0724152908 or visit any AMPATH offices

Appendix iii: Consent

MOLECULAR CHARACTERIZATION OF PROTEASE AND REVERSE TRANSCRIPTASE HIV DRMS IN TREATMENT NAÏVE AND EXPERIENCED PATIENTS IN CARE IN ELDORET, KENYA

I have read the information stated above and have had the opportunity to ask questions regarding the study. I therefore consent to:

- My sample to be used in this study
- My sample to be stored for periods up to 25 years after end of the study
- My sample to be analyzed abroad whenever the need arises
- My sample to be used by students for training purposes
- My sample to be used for other studies approved by the Ethical Review Board

Name……

Signature……. Date……..

I, the undersigned, have fully explained the relevant details of this study to the patient.

Name…… Signature…… Date ……...

Witness…… Signature …… Date …….

Appendix iv: HIV DNA Extraction And Reverse Transcription

room #	room name	laborarory use
1	clean room	all master mix preparation (RT-PCR, nested PCR and sequencing reactions), dispensing of master mix to PCR reaction tubes, storage of clean reagents and consumables
2	sample prep room	nucleic acid extraction, addition of template for RT-PCR or first round PCR, storage of extraction reagents, serology, specimen/extract storage
3	PCR room	thermocyclers for RT-PCR or first round PCR, addition of template for nested PCR, storage of first round products
4	high template room	gel electrophoresis, PCR clean-up, sequencing clean-up, addition of template for cycle sequencing, thermocyclers for second round PCR & sequencing, ABI sequencing

CONTROLS

Negative control

1. Negative Human plasma (NHP) is included with the isolation of nucleic acids. The same sample is processed in parallel with test specimens up to the point of sequencing

2. Previously isolated Negative Human plasma (NHP) nucleic acid is included during the amplification of nucleic acids to monitor processes downstream of nucleic acid isolation

- test passes if both negative controls show no signal on electrophoresis of PCR products at any stage of the algorithm

Positive control

1. Accurun (BBI Diagnostics, cat# 5524-500) diluted to 10000 copies/ml in negative human plasma, is processed from the isolation of nucleic acids through the entire algorithm including sequencing of generated PCR product

2. Previously isolated Accurun nucleic acid is included during the amplification of nucleic acids to monitor processes downstream of nucleic acid isolation

- test passes if both positive controls produce correct pedigreed sequence

ISOLATION OF NUCLEIC ACIDS

NOTES:

- handle all biological material as potentially infectious, work with plasma/serum/DBS etc is to be performed in a Biological safety cabinet (BSC) wearing a gown and double gloves, dispose of all materials in appropriate biohazard containers
- when using a chemical for the first time familiarize oneself with material safety data sheet (MSDS)
- use plugged aerosol resistant tips (ART^{TM} or similar) pipette tips throughout
- open only a single tube at any given time throughout procedure

Supplies/Reagents Required:

NucliSens automated reagents, BioMérieux:

Note: Silica, NucliSens Extraction buffer 2 & 3 are stored at 4ºC

NucliSens Lysis Buffer cat# 280134

NucliSens Extraction Buffer 1 cat# 280130

NucliSens Extraction Buffer 2 cat# 280131

NucliSens Extraction Buffer 3 cat# 280132

NucliSens easyMAG magnetic silica (48 X 0.6ml) cat# 280133

BioHit electronic multi-channel pipette cat# 180141

BioHit filter tips cat# 280146

ELISA strips Greiner (100 X 12 strips) cat# 278303

Sample vessel carrier cat# 280145

EasyMAG disposable sample vessels cat# 280135

Automated Extraction Protocol

- Perform extraction in Sample prep room, thaw serum/plasma samples at room temperature
- turn on easyMAG robot and computer as per manufacturers instructions (NucliSens easyMAG Application Training Manual, Doc. Code: GCS TM0336, version 2 Revision 2005/08/01),

briefly:

- launch easyMAG software

- define extraction request: enter sample ID, type of sample, sample volume and elution volume desired
- organize run: select on-board lysis dispensing and off board lysis incubation
- load run: load sample vessels and scan reagents
- dispense lysis
- remove sample vessels and transport in carrier provided to biological safety cabinet (BSC)
- add 400µl of serum/plasma sample to appropriate predefined well in sample vessel containing lysis buffer and 200µl of controls to appropriate predefined wells
- allow samples to incubate for 10 minutes at room temperature for complete lysis to occur
- vortex silica thoroughly, using the electronic multi-channel pipette (EMP) provided set to program 1 and a single BioHit tip, add 550µl dH2O to silica tube, vortex
- using the EMP set to program 2 and a single BioHit tip, dispense aliquots of premix silica to the premix strips
- using the EMP set to program 3 and 8 BioHit tips, transfer silica to sample vessel
- transport sample vessels containing; sample, lysis and silica to easyMAG instrument
- start easyMAG run
- label one 1.5ml RNase-free tube per sample with the specimen ID and date
- once run complete, within 30min transfer extracted nucleic acid to labeled tube and discard silica, proceed directly to RT-PCR and store eluate at -80°C in room 3178

TESTING ALGORITHMS

- each plasma/serum sample is first amplified using algorithm step#1, if a PCR product is not obtained in algorithm #1 continue on with next step in the algorithm until algorithm complete
  - Note: for Dried Blood Spots (DBS) sample is first amplified using algorithm#3
  - if PCR product obtained continue on with sequence analysis
  - all references to position on HIV are using Acc# NC_001802

Algorithm step #1

Generate one PCR product for 1682-3129

RT-PCR primers

GaGp1-PR-out.for	1	TGA ARG AIT GYA CTG ARA GRC AGG CTA AT
RT-new-out.rev	2	CCT CIT TYT TGC ATA YTT YCC TGT T

1567bp product

Second round PCR primers

GaGp6-PR-in.for	A	YTC AGA RCA GRC CRG ARC CAA CAG C
RT-new-in.rev	D	GGY TCT TGR TAA ATT TGR TAT GTC CA

1448bp product

Algorithm step #1 alternate

Generate one PCR product for 2074-3129

RT-PCR primers

GaGp1-PR-out.for	1	TGA ARG AIT GYA CTG ARA GRC AGG CTA AT
RT-new-out.rev	2	CCT CIT TYT TGC ATA YTT YCC TGT T1567bp product

Second round PCR primers

5AFPR1.for	H5A	AGA CAG GCT AAT TTT TTA GGG A
RT-new-in.rev	D	GGY TCT TGR TAA ATT TGR TAT GTC CA

1510bp product

Algorithm step #3

Generate two overlapping PCR products, Protease gene (1682-2516) and RT gene (2354-3129)

Protease gene

RT-PCR primers

GaGp1-PR-out.for	1	TGA ARG AIT GYA CTG ARA GRC AGG CTA AT
PR-new-out.rev	5	AYC TIA TYC CTG GTG TYT CAT TRT T

923bp product

Second round PCR primers

GaGp6-PR-in.for	A	YTC AGA RCA GRC CRG ARC CAA CAG C
PR-new-in.rev	B	CTG GTG TYT CAT TRT TKR TAC TAG GT

835bp product

Note: as of June 2011 alternate forward primer may be used, 5AFPR1.for (H5A) see details in specific SOP

RT gene

RT-PCR primers

RT-new-out.for	6	TTT YAG RGA RCT YAA TAA RAG AAC TCA
RT-new-out.rev	2	CCT CIT TYT TGC ATA YTT YCC TGT T

846bp product

Second round PCR primers

RT-new-in.for	C	TTY TGG GAR GTY CAR YTA GGR ATA CC
RT-new-in.rev	D	GGY TCT TGR TAA ATT TGR TAT GTC CA

776bp product

Algorithm step #4

Generate two overlapping PCR products, Protease gene (1789-2471) and RT gene (2406-3129)

Protease gene

RT-PCR primers

RT-PROT-outer.for	3	GAA CTG TAT CCT TTA RCT TCC CTC A
RT-PROT-outer.rev	7	ATC TAA TCC CTG GTG TCT CAT TGT

747bp product

Second round PCR primers

RT-PROT-inner.for	E	CTT TAR CTT CCC TCA GAT CAC TCT
RT-PROT-inner.rev	F	TCC TGA AGT CTT YAT CTA AGG GAA C

684bp product

RT gene

RT-PCR primers

RT-outer.for	8	GGA AGT TCA ATT AGG AAT ACC ACA
RT-outer.rev	4	CTC ATT CTT GCA TAY TTT CCT GTT

810bp product

Second round PCR primers

RT-inner.for	G	AAT CAG TAA CAG TAC TGG ATG TGG GT
RT-inner.rev	H	GGC TCT TGA TAA ATT TGA TAT GTC CAT

724bp product

Algorithm step #5

Optimization of a genotypic assay applicable to all human immunodeficiency virus type 1 protease and reverse transcriptase subtypes. *J. of Virological methods*, 128 (2005) 47-53.

RT-PCR primers

AV190-1.for 5’ GCTACAYTAGAAGAAATGATGACAGCAT

CR1.rev 5’ TAGAAGAAATGATGACAGCATGYCAGGGAGT

2878bp product

Nested PCR primers

AV190-2.for 5’ TAGAAGAAATGATGACAGCATGYCAGGGAGT

CR2.rev 5’ CTTTGGGGATTGTAGGGAATNCCAAATTCCTG

2853bp product

Appendix v: Primer Site Map For In-House Primers

Positions numbers based on HXB2 reference strain accession # NC_001802

Gagp1-pr-out.for	29-mer	1603-1631
Gagp6-pr-in.for	24-mer	1682-1705
Rt-prot-outer.for	25-mer	1779-1803
Rt-prot-inner.for	24-mer	1789-1812
Rt-new-out.for	27-mer	2329-2349
RT-new-in.for	26-mer	2354-2379
RT-outer.for	24-mer	2359-2382
RT-inner.for	26-mer	2406-2431
RT-PROT-inner.rev	25-mer	2447-2471

PR-new-in.rev	26-mer	2491-2516
PR-new-out.rev	25-mer	2501-2525
RT-PROT-outer.rev	24-mer	2502-2525
RT-inner.rev	27-mer	3103-3129
RT-new-in.rev	26-mer	3104-3129
RT-outer.rev	24-mer	3145-3168
RT-new-out.rev	25-mer	3145-3169

Appendix vi: **Nucleic Acid Amplification**

Reagents/Supplies required:

OneStep RT-PCR kit, Qiagen, Cat# 210212:

RNase-free water

5X RT-PCR buffer

dNTP mix

RT-PCR enzyme mix (enzyme is a mixture of Omniscript RT, Sensiscript RT and HotStarTaq)

RNasin Ribonuclease Inhibitor (40U/µl), Promega, Cat# N251B

AccuGENE RNase-free water, Cambrex, distributed by Mandel Scientific, Cat#51200 or equivalent

0.2ml thin-walled PCR tubes, MicroAmp reaction tube with cap, ABI, Cat# N801-0540

1.5ml tube, or equivalent

DistriTip mini, 1250µl capacity, Gilson, Cat# F164140

RT-PCR Protocol

- prepare master mix in Clean room :
- avoid entering after being in higher template area (all other NLHG areas) on the same day

- ice-box designated for Rm 3174 is to remain in this room
- label 0.2ml PCR tubes, use PCR rack that has been bleached and dried
- thaw PCR reagents on ice
- prepare master mix on ice (see next page for recipe) in 1.5ml or 2.0ml RNase-free tubes

- prepare enough master mix for number of specimens plus 10% extra to account for pippette variability

Appendix vii: Gel Electrophoresis Protocol

Reagents required:

SeaKem LE agarose, distributed by Mandel, cat# 50004

10X TAE solution, Invitrogen, cat 15558-026

Ethidium Bromide stock 10 mg/ml, Invitrogen, cat# 15585-011

10X Blue Juice Gel Loading Buffer, Invitrogen, and cat # 10816-015

Low DNA mass ladder, Invitrogen, cat# 10068-013

1kb plus ladder, Invitrogen, cat# 10787-018

- Handle all solutions containing concentrated ethidium bromide (EtBr) in the fume hood and store in containers that are light protected.

Solution Preparation:

1X TAE Buffer #1

900ml ultrapure water

100ml of 10X TAE stock buffer

1X TAE Buffer #2 (with EtBr)

900ml ultrapure water

100ml of 10X TAE stock buffer

35µl of stock (10mg/ml) EtBr

Ethidium bromide working solution (1mg/ml)

100µl of stock (10mg/ml) EtBr

900µl 1X TAE running buffer

perform work on benchtop in High template room (Rm 3168)

Gel preparation:

- Prepare a 1.5% agarose gel containing EtBr (0.35µg/ml final concentration)
- Use 1X TAE buffer #1, which does NOT contain EtBr
- Small gel preparation: add 0.75g agarose powder to 50ml 1xTAE buffer #1
- Large gel preparation: add 2.25g agarose powder to 150ml 1xTAE buffer #1
- Microwave until agarose has completely dissolved
- Use heat resistant gloves to remove the solution from microwave and allow to cool
- DO NOT MICROWAVE SOLUTIONS CONTAINING EtBr

- Working in fume hood, add 17.5µl of the 1mg/ml EtBr working solution per 50ml gel, or 52.5µl for large 150ml gel
- Mix by swirling
- Pour into the electrophoresis mould with the desired comb(s)
- Once the gel has solidified, place in electrophoresis unit and submerge with 1X TAE running buffer #2 (contains EtBr)
- Load 5µl of each nested PCR product + 2µl loading dye per lane on the gel
- Include a lane for low DNA mass ladder (4µl + 2µl loading dye)
- Include a lane with 100bp or 1kb plus ladder (6µl already in loading dye) 0.5µg/6µl loaded
- Run the gel at 100V for 1hour
- Take photo of gel:
- Transfer gel to UV transilluminator, adjust camera settings and take photo of gel using UVP BioDoc-It system (Mitsubishi)
- Agarose gels containing EtBr are considered hazardous chemical waste. Place gel in container in fume hood to dehydrate. Once dehydrated, dispose of gel in designated waste containers for later incineration.
- Filter waste TAE running buffer containing EtBr with a charcoal filter (Extractor, Whatman Cat# 10448031) to remove ethidium bromide, dispose in filter in designated waste container, and pour buffer down drain.

Interpretation of gel results:

- Verify correct PCR product generated, size is dependent on primers used, see table described in PCR section for expected product sizes
- manually estimate the mass (quantity) of DNA by comparing the intensity of the band of interest to the low DNA mass ladder.

Appendix viii: Qiaxcel Protocol

Reagents required:

QIAxcel DNA Screening cartridge and reagents, Qiagen, Cat# 929004

QX Alignment Marker 15bp/5kb (1.5ml), Qiagen, Cat # 929524

QX DNA Size Marker 100bp - 3kb (50µl), Qiagen, Cat # 929553

6 x Nitrogen cylinders, Qiagen, Cat# 929705

Reference:

QIAxcel User Manual, Version 1.0 01/2008

QIAxcel DNA Handbook

Procedure

- dispense 10µl PCR product per well in 12-well strip tube or 96-well plate
- follow procedure in user manual, briefly:
- prepare alignment marker, centrifuge briefly to remove any bubbles
- launch BioCalculator software, select change buffer, load alignment marker and test samples into instrument, select desired test parameters and reference marker table, select run
- once run complete analyse data and export DNA concentration results

Appendix ix: PCR Clean-up Protocol

- perform on benchtop in High template room

Procedure reference material:
MultiScreen Separations System User Guide, P17479, Rev. E, 10/99
Montage PCR_{96} Cleanup Kit User Guide

- Note suggested amount of template for cycle sequencing: PCR product template range for 500-1000bp 5-20ng DNA, PCR product template range for 1000-2000bp 10-40ng DNA
- Use mass of DNA estimated manually or from QIAxcel (DNA concentration calculated automatically), calculate concentration of sample prior to PCR clean-up, after clean-up adjust sample concentration to 10ng/µl using water

example calculation:

- Estimate PCR product to contain 200ng DNA
- Concentration of PCR product = 40 ng/µl (ie 200ng DNA ÷ 5µl volume product loaded on the gel)
- Total mass DNA = 1600 ng DNA (ie conc. X total sample volume used in clean-up)
- Volume diluent to give final concentration of DNA 10 ng/µl = 160 µl water (ie total ng DNA ÷ 10 ng/µl)
- Suspend the dried PCR product into 160µl water during the PCR clean-up step

- Prepare a plate map of PCR positive samples to be purified
- Add 250µl RNase-free water to wells of a MultiScreen PCR 96-well plate (Millipore, cat# MSNU 03050) using a repeater pipette
- Add 40µl of PCR product to corresponding wells using a multi-channel pipette
- Place plate on vacuum manifold to dry, apply a plate sealer over the wells not being used, apply 24 inches Hg pressure for 10 minutes until the wells are dry
- Wipe excess liquid from the bottom of the plate with a large kimwipe
- Add RNase-free water to each well at appropriate volume to give final concentration 10 ng/µl DNA

- Apply plate lid
- Shake the plate on a benchtop plate shaker for 5 min (speed set to minimize risk of splatter)
- Transfer entire contents to an untreated V-bottom 96-well plate (Evergreen Scientific, cat# 290-816-01V or equivalent) using a multi-channel pipette
- Cover plate with plate sealer
- Store purified PCR product at 4°C

Appendix x: Sequencing Reactions

- It is preferable to perform the cycle sequencing reactions within 24 hours of sample electrophoresis (preferably on the same day)
- pol region amplified must be sequenced using 4 primers generating 2 overlapping regions

For samples amplified with algorithm step #1 or #3 use following sequence primers:

A GaGp6-PR-in.for 5' YTC AGA RCA GRC CRG ARC CAA CAG C 3'

B PR-new-in.rev 5' CTG GTG TYT CAT TRT TKR TAC TAG GT 3'

C RT-new-in.for 5' TTY TGG GAR GTY CAR YTA GGR ATA CC 3'

D RT-new-in.rev 5' GGY TCT TGR TAA ATT TGR TAT GTC CA 3'

For sample amplified with algorithm step #4 use following sequence primers:

E RT-PROT-inner.for 5' CTT TAR CTT CCC TCA GAT CAC TCT 3'

F RT-PROT-inner.rev 5' TCC TGA AGT CTT YAT CTA AGG GAA C 3'

G RT-inner.for 5' AAT CAG TAA CAG TAC TGG ATG TGG GT 3'

H RT-inner.rev 5' GGC TCT TGA TAA ATT TGA TAT GTC CAT 3'

For sample amplified with algorithm step #5 use following sequence primers:

- can use existing in-house sequencing primers since all fall within PCR product generated or can use any of the following:

AV2 5' AGTGCTTTGGTTCCCCTAAGGAGTTTACA

AV5 5' AAAGACAGCTGGACTGTCAAT

AV8 5' CATAATTTCACTAAGGGAGGGGTATT

AV9 5' CCATACAAAAGGAAACAT

AV15 5' ATAGGGGGAATTGGAGGTTTTATCAAAGT

AV36 5' CAGTACTGGATGTGGGTGATG

AV44 5' TACTAGGTATGGTAAATGCAGT

AV179mod 5' TTAAGTGTTTCAAYTGTGGCAA

OUT3 5' CATTGCTCTCCAATTACTGTGATATTTCTCATG

OUT5revmod 5' ATGTTGACAGGTGTAGGTCCTACTAATACTGTAC

Sequencing Protocol

Reagents:

BigDye Terminator v3.1 Cycle Sequencing Kit, ABI, cat# 4337456

5X Sequencing buffer v1.1 & v 3.1, ABI, cat# 4339843

- prepare a plate map of samples to be sequenced such that primer sets are in adjacent wells, for example :

	primer			
	A	B	C	D
specimen 1				
specimen 2				
specimen 3				
specimen 4				

Master Mix Preparation

- Clean room (Rm 3174) BSC
- Avoid entering after being in higher template area (all other NLHG areas) on the same day.
- Icebox is to remain in this room
- prepare master mix(s) on ice (need a master mix per primer used)
- minimize exposure to light as the dye is light-sensitive
- dispense 18µl master mix per designated well of 96-well chimney-top plate (DiaMed, cat# E212500)

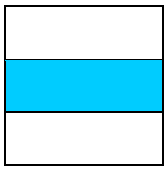
Cycle Sequencing Reaction

- benchtop high template room
- add 2µl of 10ng/µl purified PCR product to designated wells using a multi-channel pipette
- cap wells using 8-well strip caps, ensure caps are tight
- place chimney top plate into thermocycler and perform cycle sequencing reaction
- store PCR products at 4°C (short term) or –20°C (long term)

Appendix xi: Sequencing Clean-Up

Clean-up protocol

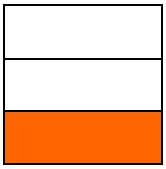
- Benchtop High template room
- Prepare Sephadex clean-up plate:
  - Pour Sephadex G50 (fine DNA grade, Sigma # S5897-100G) onto black column loader plate, use clear scraper to distribute Sephadex powder evenly across all wells, make sure all wells are completely full, then use the scraper to return any excess powder to the bottle
  - Invert the black column loader onto 96-well MultiScreen-HV plate (Millipore, Cat# MAHVN4550)
  - Add 300µl RNase-free water to each well using a non-sterile repeater pipette (pipette gently so as to not disturb the Sephadex powder)
  - Allow the Sephadex powder to hydrate for a minimum of 3 hours at room temperature
- Once the cycle sequencing reaction is complete and Sephadex is hydrated, place the Sephadex plate on top of an untreated V-bottom waste-collection plate (waste plate can be re-used)



Sephadex plate
blue frame
untreated V-bottom plate

- Centrifuge 1000 x g for 5 min at room temperature
- Remove waste-collection plate and discard water
- Transfer Sephadex plate onto optical 96-well reaction plate (MicroAmp Optical 96-well reaction plate, Cat# N801-0560) samples will be directly centrifuged into optical 96-well reaction plate
- Add sequencing reactions, entire 20µl volume, to the hydrated Sephadex wells using a multi-channel pipette - be careful not to touch the Sephadex directly

- Centrifuge 1000 x g for 5 min at room temperature



hydrated Sephadex with samples

optical plate

colored PCR rack

- protect samples from light

Appendix xii: Sequencer (ABI 3130xl Genetic Analyzer)

- High template room

Dry Cleaned Sequence Samples

- Use DNA 120 SpeedVac concentrator (ThermoElectron Corp), set to medium heat - approximate drying times are 30-45 min for one plate
- Once samples in optical plate are dry turn off speedvac
- Add 10µl HI-DI formamide (ABI, cat# 4311320) to each well, aliquots stored at -20°C, working stock at 4°C
- Quick spin the plate to remove bubbles and collect liquid
- Place a grey rubber septa on top of the optical plate
- Denature samples in the GeneAmp 9700 thermocycler for 2 min at 94°C, cool to 4°C for at least two minutes before removing the plate

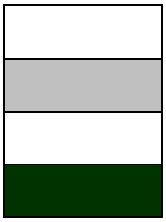
Prepare plate-map (optional)

- prepare a .txt file with the sample list of samples to be sequenced on ABI 3130
- transfer to sequencer via USB

ABI 3130 Genetic Analyzer Preparation

- Launch ABI 3130 data collection software v 3.0
- Service console will appear and icons will turn green when instrument is ready
- Select plate manager:
- Manually input sample names or import .txt file
- Import button at bottom of screen
- Locate .txt file and double-click to open
- Message will appear indicating file was successfully imported
- Open file and verify information is correct
- Clear any rows that are not to be analyzed
-
-
-
-

- Assemble optical plate into holder as shown



white plastic lid
grey rubber septa
optical plate
black plastic bottom plate

- Press the tray button on the sequencer to advance the tray to the front of the sequencer, load plate into sequencer
- Under run scheduler :
- Locate the file by searching the name or by selecting files “pending” under “find all”
- Once the plate is placed onto sequencer tray, the grey grid on the computer screen will turn yellow to acknowledge the plate
- Link the computer file with the appropriate plate (example A or B), the yellow grid will turn to green
- Press ▶ to start processing samples
- Under instrument status :
- Wait until the status message says “filling array” and “start of pre-run” before leaving.

A “run” is defined as 2 columns of 8 samples each, and each “run” takes approx. 2 hours to complete and will use approx. 50 µl of polymer.

Appendix xiii: Sequence Analysis

Sequence Assembly and Analysis

- launch Seqscape v2.5, enter password etc, select new file - enter project name, or open existing file
- import samples to project by highlighting samples and specimen# and selecting add, once samples entered select OK
- analyse samples by selecting " ▶" button, software will perform base calling and assemble sequences
- rename 'specimen' to name of sample
- alternately sequences can be loaded by defining a delimiter and using the auto add function
- look at assembly of each individual sample:

Delete any sequences that have misprimed/assembled incorrectly

- note any strands sequences that are poor in quality (QV ≤30) or have been deleted by editing sample name
- for example if sequence generated with primer A hasn't assembled or has been deleted rename the sample sample ID.XA
- for poor samples that you have not deleted the sequence name sample sample ID.xa
- next look at the project as a whole, viewing the confidence bars QV ≤30 and manually edit or reject sequence
- the first reportable mutation for Protease is at amino acid 23 and last reportable mutation for RT is amino acid 230
- Save project
- Export - project alignment - nucleotides

Sequence Editing

- Use sequence alignment editor software: BioEdit v7.0.5 (Ibis Therapeutics, Carlsbad, CA)
- Select edit mode, edit data, ie delete sequence data beyond 1260bp, remove spacing that would lead to frame shift, delete project consensus sequence

- Note: not all insertions are deleted, sequences are evaluated for insertions that lead to potential drug resistance (eg T69 insertion)
- Save file

Subtype and Drug Resistance Analysis

- Upload aligned fasta files to Stanford HIVdb sequence analysis program and / or Calibrated Population Resistance Tool (CPR)

  http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm

  http://cpr.stanford.edu/cpr/servlet/CPR

  Manually input surveillance drug resistance data, as defined in the list below, into database. Mutations not included on list below are captured within comments section of database.

  PHYLOGENETIC TREE ANALYSIS
- All sequences including HIV reference strains and controls are aligned and trimmed to same length, 1240bp
- Tree and distance analyses are performed using the Neighbour-Joining method of Saitou and Nei with Kimura 2-Parameter model as implemented in MEGA4.1.
- All specimens branching together with short branch length and/or high bootstrap values are reevaluated for geographic association in processing steps
- If specimens are phylogenetically and either temporarily or spatially related in processing, samples with be reanalyzed being with extraction of raw material
- Specimens are compared phylogenetically with the sequences of historical samples from the three months preceding and the pedigreed positive controls

  Data Reviewing and Reporting.

- After sequencing analysis is complete, data is entered
  - Data is reviewed by two individuals
  - Electropherograms are reviewed at all mixed base call sites
  - Electropherograms of all sequences reporting drug mutations are scrutinized at mutation sites and manual editing is performed if necessary
  - All non-B subtypes are analyzed using one or all of the tools described previously
  - Report drug mutations on samples that have acceptable sequence on both forward and reverse strands

- Samples that have poor quality sequence are reported as non reportable sequence (NRS) and may be tested further at a later date
- Samples that appear to contain two populations are reported as NRS and may be tested further at a later date using different primers or screened by cloning
- In some cases it is only possible to obtain sequence data from one region either protease or reverse transriptase region, if this is the case report samples as NRS
- If sample supplied has insufficient volume to perform extraction it is reported as non sufficient quantity (NSQ)
- Data entry is verified

Appendix xiv: Combined IAS / CPR Mutation List

DRUGS			
INHIBITOR TYPE	PIs	NRTIs	NNRTIs
MUTATION SITE	L23I	M41L	L100I
	L24I	K65R	K101E
	D30N	D67E	K101P
	V32I	D67G	K103N
	M46I	D67N	K103S
	M46L	T69D	V106A
	I47A	T69insertion	V106M
	I47V	69 complex (M41L, A62V, 69 insertion, K70R, L210W, T215FY, K219EQ)	V179F
	G48M	K70E	Y181C
	G48V	K70R	Y181I
	I50L	L74I	Y181V
	I50V	L74V	Y188C
	F53L	V75A	Y188H
	F53Y	V75M	Y188L
	I54A	V75S	G190A
	I54L	V75T	G190E
	I54M	F77L	G190S
	I54S	Y115F	P225H
	I54T	F116Y	M230L
	I54V	Q151M	
	G73A	151 complex (A62V, V75I, F77L, F116Y, Q151M)	

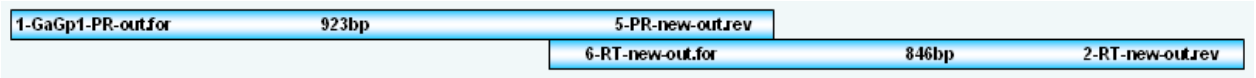
	G73C	M184I	
	G73S	M184V	
	G73T	L210W	
	L76V	T215F	
	V82A	T215Y	
	V82C	T215 revertants (T215C/D/E/I/S/V)	
	V82F	K219E	
	V82L	K219N	
	V82M	K219R	
	V82S	K219Q	
	V82T	TAM (M41L, D67N, K70R, L210W, T215FY, K219EQ)	
	N83D		
	I84A		
	I84C		
	I84V		
	I85V		
	N88D		
	N88S		
	L90M		

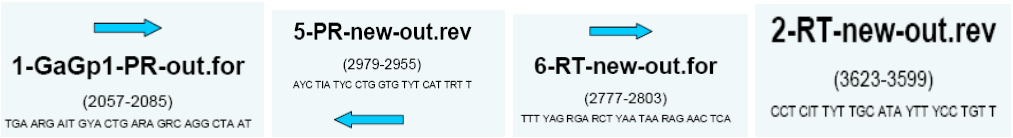
Appendix xv: Primers And Protocols For 454 Project

1. Primers for RT-PCR:

(1) for RNA extracts from Plasma:

(2) For RNA extracts from DBS:





(3) The fusion primer designed and used in this new 454 project:

Only the forward fusion primers were labelled with different MIDs and the primers are designed in the following format:

Forward primer (Primer A-Key):

5' – CGTATCGCCTCCCTCGCGCCATCAG – {MID} – {template-specific-sequence} – 3'

Reverse primer (Primer B-Key):

5' – CTATGCGCCTTGCCAGCCCGCTCAG – {template-specific-sequence} – 3'

2. Protocol for RT-PCR:

(1) Reagents/Supplies required:

OneStep RT-PCR kit, Qiagen, Cat# 210212:

RNase-free water, 5X RT-PCR buffer, dNTP mix

RT-PCR enzyme mix (enzyme is a mixture of Omniscript RT, Sensiscript RT and HotStarTaq)

RNasin Ribonuclease Inhibitor (40U/µl), Promega, Cat# N251B

HIV specific primers as indicated above

AccuGENE RNase-free water, Cambrex, distributed by Mandel Scientific, Cat#51200 or equivalent

0.2ml thin-walled PCR tubes, MicroAmp reaction tube with cap, ABI, Cat# N801-0540

1.5ml tube, or equivalent

DistriTip mini, 1250µl capacity, Gilson, Cat# F164140

II Nested PCR:

1. Primers for nested PCR: (Pre-optimized tagged primers)

454-1F-MID-X	Degr_Seg1_F	2251~2270	tccctcaRatcactctttgg	462bp	Seg 1 covers the whole PR
454-1R	Degr_Seg2_R	2692~2713	ggRttttYaggcccaatttt		
454-2F-MID-X	Degr_Seg2_F	2583~2602	tKaaag ccaggRatggatgg	390bp	Cover aa 1~140 in RT
454-2R	Degr_Seg3_R	2954-2973	tccctggtgtctcattgttt		
454-3F-MID-X	Degr_Seg3_F	2870~2890	agtactRgatgtgggWgatgc	400bp	Cover aa 107~240 in RT
454-3R	454New3270R	3249~3270	ctgtccatttRtcaggatgRa		

** For RT-PCR products from DBS: Amplicons with primer pairs 454-1F+1R and 454-2F+2R fall in the PR fragment of algorithm 3 sequencing protocol; Amplicons with primer pair 454-3F+3R falls in the RT fragment of algorithm 3 sequencing protocol;

2. Protocol for RT-PCR:

(1) Reagents/Supplies required:

Applied Biosystems AmpliTaq Gold - Buffer II and 25 mM MgCl2 kit, ABI, Cat# N808-0243

10X buffer

25mM MgCl2

AmpliTaq Gold

AccuGENE RNase-free water, Cat# 51200 or equivalent

Ultrapure dNTP set, Amersham Pharmacia Biotech, Cat# 27-2035-01

Tagged primers as indicated above

(2) Master Mix Preparation (Clean room 3)

Second Round PCR Master Mix Reagents	Volume per reaction (50µl)	Volume per reaction (50 µl)	Final concentration
RNase-free water	25.5	22.5	-
10 × buffer	5	5	1×
25 mM MgCl2	4	4	2 mM
dNTP mix (10mM each)	1	1	0.2 mM each
Forward primer (5µM)	6	6	0.6 µM
Reverse primer (5µM)	6	6	0.6 µM
AmpliTaq Gold (5U/µl)	0.5	0.5	2.5 U
TOTAL	48 Add 2µl of RT-PCR product	45 Add 5µl of RT-PCR product	

(3) PCR cycling:

Step	Number of cycles	Temperature	Time	Step Description
1	1	94°C	15 min	Activate enzyme
2	35	94°C	20 sec	Cycling (minor adjustment on Tm may be needed)
		55 °C for all 3 pairs	30 sec	
		72°C	2 min 30 sec	
3	1	72°C	10 min	Final extension
4	1	4°C	HOLD	

(4) Purification / quantification:

(1) Run agarose gel or Qiacel to check the quality of the amplicons;

(2) PCR Clean-up using MultiScreen PCR 96-well plate (Millipore, cat# MANU 03050) (Rm 3168)

Prepare a plate map of PCR positive samples to be purified

Add 250µl RNase-free water to wells of a MultiScreen PCR 96-well plate using a repeater pipette

Add entire volume of remaining PCR product (~45µl) to corresponding wells using a multi-channel pipette

Place plate on vacuum manifold to dry, apply a plate sealer over the wells not being used, and apply 24 inches Hg pressure for 10 minutes until the wells are dry;

Blot multiscreen plate with kimwipe, add proper volume of ddH_2O to each well to give final concentration 25 ng/µl DNA

Apply plate lid and shake the plate on a benchtop plate shaker for 5 min (speed set to minimize risk of splatter)

transfer 30 ul contents to an untreated V-bottom 96-well plate (Evergreen Scientific, cat# 290-816-01V or equivalent) using a multi-channel pipette; cover plate with plate sealer; store purified PCR product at 4°C for future shipment;

transfer the remaining to a new V-bottom 96-well plate (Evergreen Scientific, cat# 290-816-01V or equivalent) using a multi-channel pipette; cover plate with plate sealer; store purified PCR product at 4°C for archiving (backup) .

Positive control (for accuracy evaluation use): PCR products from plasmid templates: Pol 2, 4, 5 7, 8, 9 or 10.

----- AccuRun –RT-PCR—Nested PCR with tagged 454 primers—QiaCel screening—purification –sample shipment.

*** Use Tris-buffered water (pH8.0) (10 mM Tris, pH8.0) to elute and dilute DNA in the last two steps, (NO EDTA!!!)

** 30 µl samples at 25ng/ µl (Minimum 750ng total amplicon is needed) will be needed for 454 analysis

III Samples to prepare:

Sample map for RT-PCR experiment.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Patient 1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	36	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

Sample MID map (Applicable for all sample plate)

	1	2	3	4	5	6	7	8	9	10	11	12
A	MID -1	MID -2	MID -5	MID -6	MID -8	MID -11	MID -14	MID -15	MID -16	MID -19	MID -20	MID -21
B	MID -23	MID -25	MID -26	MID -27	MID -30	MID -31	MID -32	MID -33	MID -36	MID -37	MID -38	MID -42
C	MID -1	MID -2	MID -5	MID -6	MID -8	MID -11	MID -14	MID -15	MID -16	MID -19	MID -20	MID -21
D	MID -23	MID -25	MID -26	MID -27	MID -30	MID -31	MID -32	MID -33	MID -36	MID -37	MID -38	MID -42
E	MID -1	MID -2	MID -5	MID -6	MID -8	MID -11	MID -14	MID -15	MID -16	MID -19	MID -20	MID -21
F	MID -23	MID -25	MID -26	MID -27	MID -30	MID -31	MID -32	MID -33	MID -36	MID -37	MID -38	MID -42
G	MID -1	MID -2	MID -5	MID -6	MID -8	MID -11	MID -14	MID -15	MID -16	MID -19	MID -20	MID -21
H	MID -23	MID -25	MID -26	MID -27	MID -30	MID -31	MID -32	MID -33	MID -36	MID -37	MID -38	MID -42

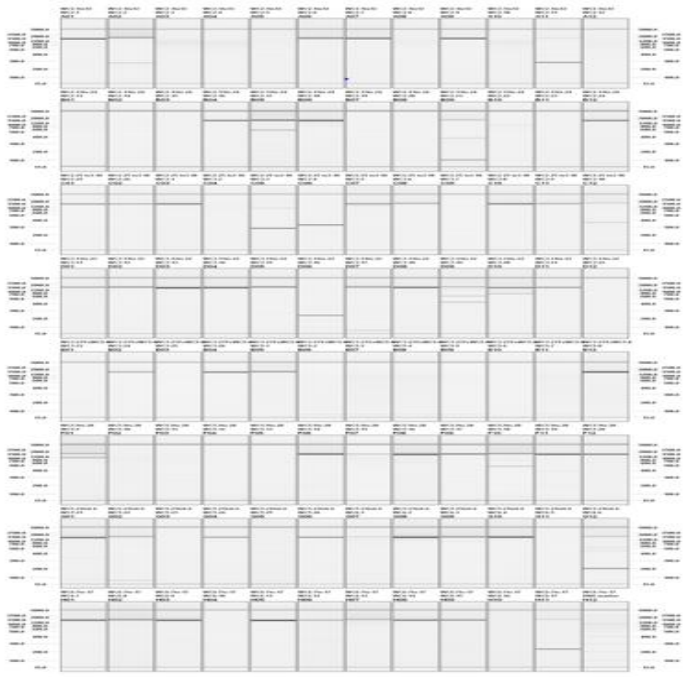
1. Sample set one (4 regions) (Plate 1: 454-1F+1R; Plate 2: 454-3F+3R; Plate 3: 454-3F+3R)

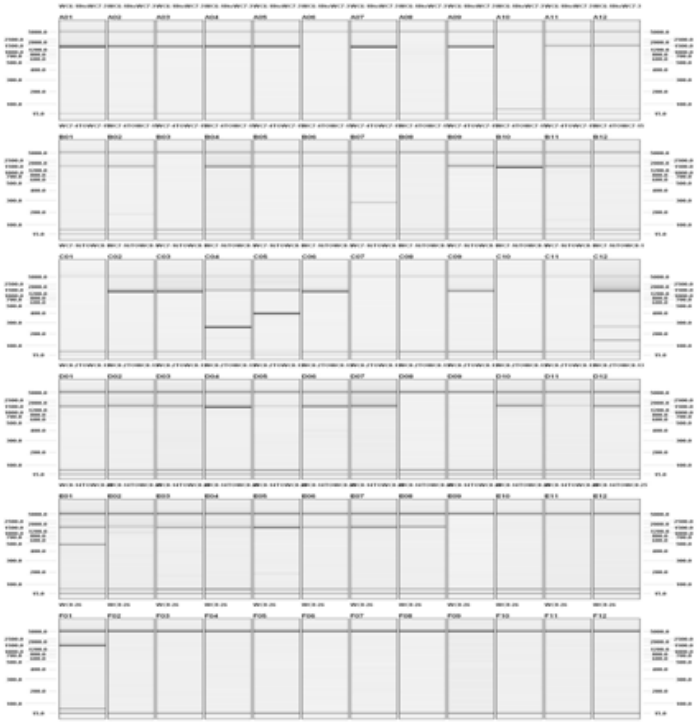
	1	2	3	4	5	6	7	8	9	10	11	12
A	Patient 1	2	3	4	5	6	7	8	9	10	11	12
B	13	14	15	16	17	18	19	20	21	22	23	24
C	25	26	27	28	29	30	31	32	33	36	35	36
D	37	38	39	40	41	42	43	44	45	46	47	48
E	49	50	51	52	53	54	55	56	57	58	59	60
F	61	62	63	64	65	66	67	68	69	70	71	72
G	73	74	75	76	77	78	79	80	81	82	83	84
H	85	86	87	88	89	90	91	92	93	94	95	96

1. Sample set one (4 regions) (Plate 1: 454-1F+1R; Plate 2: 454-3F+3R; Plate 3: 454-3F+3R) (Corresponding RT-PCR product names)

	1	2	3	4	5	6	7	8	9	10	11	12
A	Pol 2	D39-1	D39-2	D39-4	D39-5	D39-6	D39-7	D39-8	D39-9	D39-10	D39-11	D39-12
B	D39-13	D39-15	D39-16	D39-17	D39-18	D39-19	D39-20	D39-21	D39-22	D40-1	D40-2	D40-3
C	D40-4	D40-5	D40-6	D40-7	D40-8	D40-9	D40-10	D40-11	D40-12	D40-13	D40-14	D40-15
D	D40-16	D40-19	D40-21	D40-22	DR126-6	DR130-7	DR130-17	DR132-18	DR135-7	DR135-9	DR135-11	DR137-3
E	DR137-4	DR137-11	DR139-5	DR139-14	DR139-20	D22-6	D22-7	D22-21	D23-3	D23-46	D23-50	D23-52
F	D23-59	D23-68	D23-74	D23-90	IJBC09-9	IJBC09-21	IJBC09-22	IJBC09-25	IJBC09-39	IJBC09-104	Pol 4	Pol 5
G	2P+R	4P+R	7P+R	9P+R	15P+R	18P+R	19P+R	21P+R	22P+R	27P+R	29P+R	30P+R
H	31P+R	33P+R	34P+R	35P+R	36P+R	38P+R	39P+R	41P+R	42P+R	43P+R	44P+R	46P+R

Appendix xvi: QIAxcel Output





Appendix xvii: Proportion, characteristics, drms from arv naive (SS)

PID	Sex	Age	WHO	CD4	Pi.	Nrti.	Nnrti.
KE12-045	Female	39	0	79	none	None	none
KE12-061	Male	51	1	285	none	None	none
KE12-063	Female	28	1	681	none	None	none
KE12-065	Male	33	0	499	none	None	none
KE12-067	Female	27	0	511	none	None	none
KE12-068	Male	45	1	356	none	None	none
KE12-069	Male	36	0	14	none	None	none
KE12-070	Female	46	1	232	none	None	none
KE12-073	Male	30	1	319	none	None	none
KE12-074	Male	33	0	77	none	None	none
KE12-075	Female	29	0	59	none	None	none
KE12-078	Female	54	3	326	none	None	none
KE12-079	Female	44	0	45	none	None	none
KE12-080	Female	27	0	392	none	None	none
KE12-084	Female	30	3	439	none	None	none
KE12-085	Female	52	1	306	none	None	none
KE12-086	Female	45	1	395	I50V	M184I	none
KE12-088	Female	40	1	618	none	None	none
KE12-090	Female	39	0	430	none	None	none
KE12-091	Male	40	0	64	none	None	none
KE12-092	Female	39	2	39	none	None	none
KE12-095	Male	63	0	37	none	None	none
KE12-096	Female	37	1	652	none	None	none
KE12-097	Female	58	1	326	none	None	none
KE12-100	Female	25	4	15	none	None	none
KE12-101	Female	25	1	647	none	None	none
KE12-102	Male	48	0	7	none	None	none
KE12--105	Female	24	1	417	none	None	
KE12-106	Male	39	1	525	none	None	none
KE12-109	Female	39	0	537	none	None	none
KE12-112	Female	36	1	341	none	T215I	none
KE12-113	Male	43	1	15	none	None	none
KE12-114	Male	30	3	309	none	None	none
KE12-115	Female	26	1	663	none	None	none
KE12-116	Male	36	1	256	none	None	none
KE12-118	Female	35	0	205	none	None	none
KE12-123	Female	33	2	719	none	None	none
KE12-128	Female	66	1	270	none	None	K103N
KE12-129	Female	49	0	165	none	None	none
KE12-130	Female	8	0	1260	none	None	none
KE12-260	Male	46	0	301	none	None	none
KE12-275	Female	29	2	593	none	None	none

Appendix xviii: Proportion And Characteristics Of Arv- Experienced Patients Failing Therapy (SS)

PID	Sex	Age	WHO	ARV	CD4	Vload	ARV 16 months	pi	nrti	nnrti
KE12-006	M	50	3	TDF-3TC-EFV	307	252		N	M41L,K70R, M184V,T215SY	Y181C, G190S
KE12-007	F	28	2	TDF-3TC-NVP	128	26419	TDF-3TC-NVP	N	K65R, M184V	Y181C
KE12-009	M	55	4	ALUVIA	277	3920	LPV,3TC,TDF,ABC	N	none	K103N
KE12-016	F	33	1	TDF-3TC-NVP	226	1151	TDF-3TC-NVP	N	none	none
KE12-018	M	15	3	ALUVIA	420	211082	3TC,LPV,RTV,D4T	N	F116Y,Q151M, M184V	K103N,Y181I, P225H
KE12-023	M	26	2	TDF-3TC-EFV	134	22022	LPV,RTV,3TC,TDF	N	K70R, M184V, T215FIS	K103N, P225H
KE12-027	M	15	3	D4T-3TC-NVP	657	6259	AZT-3TC-NVP	N	M184V	K103N, Y188L
KE12-028	M	63	3	AZT-3TC-NVP	689	212923	ALUVIA,3TC,TDF	N	M184V	G190A
KE12-030	F	38	3	AZT-3TC-NVP	518	130152	ALUVIA,3TC,TDF	N	M184V, T215SY	K103N
KE12-031	F	39	1	AZT-3TC-NVP	118	225028	ALUVIA,3TC,TDF	N	M184V, T215Y, K219Q	K103N, M230L
KE12-034	F	26	1	D4T-3TC-NVP	186	969867	d4T-3TC-NVP	N	none	none
KE12-035		38	3	D4T-3TC-NVP	174	4829	ALUVIA,3TC,TDF	N	K70R, M184V	Y181C
KE12-036	M	37	2	D4T-3TC-NVP	117	36388	d4T-3TC-NVP	N	M184V	G190A
KE12-037	M	36	3	D4T-3TC-NVP	531	109970	d4T-3TC-NVP	N	M184V	K103N

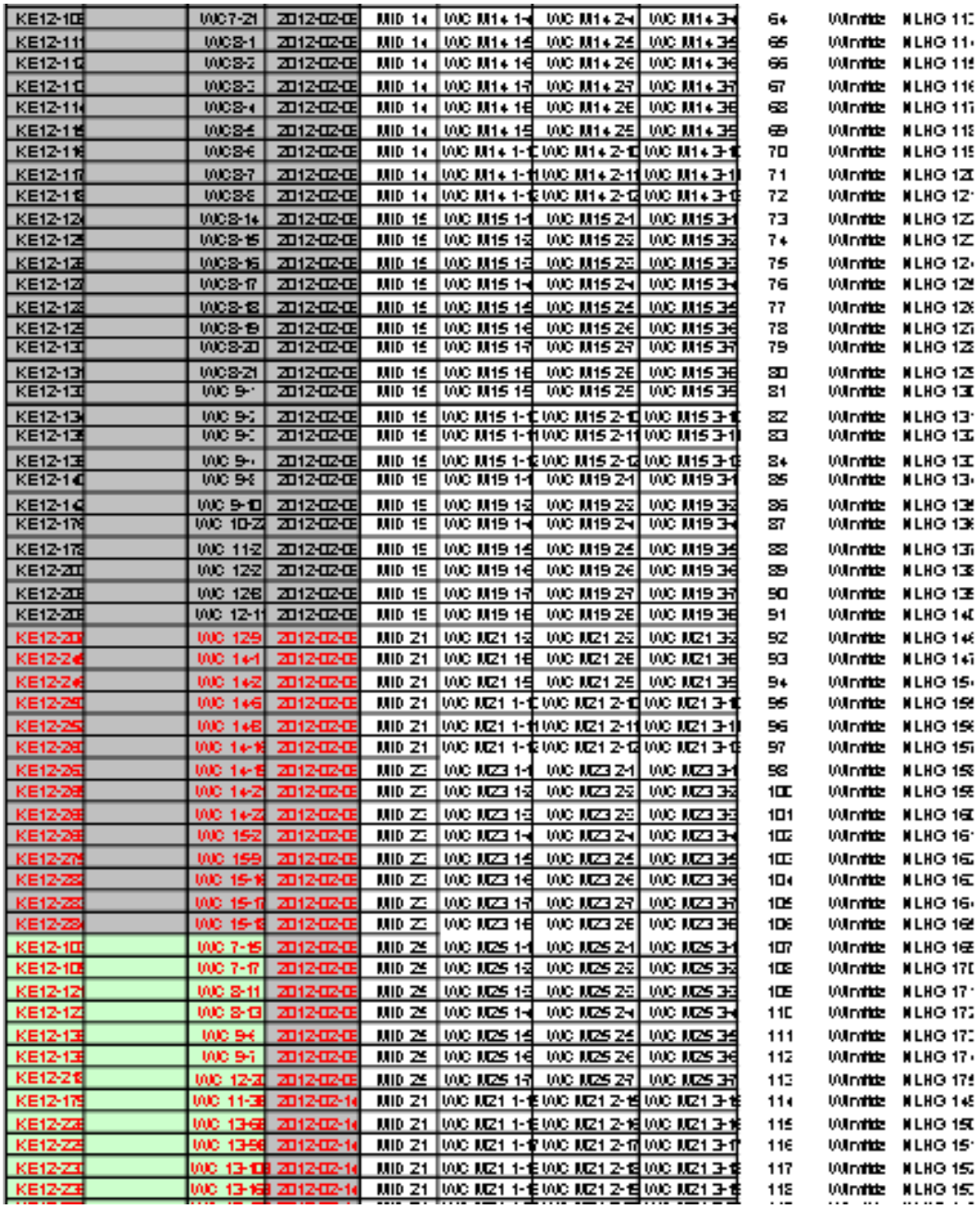
KE12-039	M	17	2	D4T-3TC-NVP	110	59901	d4T-3TC-NVP	N	D67N,M184V,L210W,T215Y, K219Q	Y181V
KE12-040	M	17	3	D4T-3TC-NVP	304	13349	d4T-3TC-NVP	N	M184V, T215Y	Y181C
KE12-043	F	36	3	D4T-3TC-NVP	129	3692	TDF-3TC-NVP	N	M184V, T215F	G190A
KE12-052	M	46	3	TDF-3TC-NVP	213	496	d4T-3TC-NVP	N	D67N, K70R, M184V, K219Q	K103N
KE12-058	F	39	4	D4T-3TC-NVP	56	151982	ALUVIA,3TC,TDF	N	M184V, T215Y	Y181C
KE12-059	F	30	3	ALU VIA	119	776		N	K70R, M184V, K219Q	Y181C
KE12-060	M	15	4	ABC-3TC-EFV	191	114339	ABC-3TC-EFV	N	K65R,D67N,Y115F,F116Y, K219E	G190E
KE12-081	F	56	0	D4T-3TC-EFV	713	11339	TDF-3TC-NVP	N	K70R, M184V, K219Q	K103N
KE12-093	F	32	2	D4T-3TC-NVP	39	48872		N	none	none
KE12-245	F	30	3	AZT-3TC-NVP	70	11439	AZT-3TC-EFV	N	M41L, K70R, V75M, M184V, L210W, T215F	G190A
KE12-246	M	54	3	AZT-3TC-NVP	301	50	AZT-3TC-NVP	N	M184V	K103S, G190A
KE12-250	F	35	3	AZT-3TC-NVP	1	11433		N	none	K103N
KE12-252	F	35	1	D4T-3TC-NVP	12	873	ALUVIA,3TC,TDF	N	D67N	K103N
KE12-282	F	51	1	D4T-3TC-NVP	114	3208		N	D67N, M184V, T215I	Y181C, G190A
KE12-300	F	36	3	AZT-3TC-NVP	261	2409		N	M184V	K103N
KE12-307	M	64	3	AZT-3TC-NVP	518	2778	ALUVIA	N	M184V	G190A
KE12-309	M	18	4	D4T-3TC-EFV	309	2896		N	M184V	K103N,V106M

KE12-316	M	50	3	D4T-3TC-NVP	193	31859	ALUVIA	N	D67N, K70R, M184V, K219Q	K101E, G190A
KE12-324	M	35	4	AZT-3TC-NVP	22	170384		N	K70R, M184V	Y181C
KE12-326	M	46	2	D4T-3TC-NVP	187	39		N	M184V	G190A

PID, patient identification, ARV; ARV, EFV; efavirenz, 3TC; lamivudine, NVP; nevirapine, ZDV; zidovudine; AZT; stavudine, TDF; tenofovir, ALUVIA;

Appendix xix: 454 Pyrosequencing MID map

Sample ID		RT-PCR ID	Date 454 PCR	MID	amplicon #1	amplicon #2	amplicon #3	count	project	454 ID
KE12-00[illegible]		VOC 2-[illegible]	2012-02-0[illegible]	MID 1	VOC AM1 1-[illegible]	VOC AM1 2-[illegible]	VOC AM1 3-[illegible]	1	VAriette	NLHG 5[illegible]
KE12-00[illegible]		VOC 2-[illegible]	2012-02-0[illegible]	MID 1	VOC AM1 1-[illegible]	VOC AM1 2-[illegible]	VOC AM1 3-[illegible]	2	VAriette	NLHG 51
KE12-00[illegible]		VOC 2-[illegible]	2012-02-0[illegible]	MID 1	VOC AM1 1-[illegible]	VOC AM1 2-[illegible]	VOC AM1 3-[illegible]	3	VAriette	NLHG 52
KE12-00[illegible]		VOC 2-[illegible]	2012-02-0[illegible]	MID 1	VOC AM1 1-[illegible]	VOC AM1 2-[illegible]	VOC AM1 3-[illegible]	4	VAriette	NLHG 53
KE12-011		VOC 2-11	2012-02-0[illegible]	MID 1	VOC AM1 1-7	VOC AM1 2-7	VOC AM1 3-7	5	VAriette	NLHG 5[illegible]
KE12-01[illegible]		VOC 2-16	2012-02-0[illegible]	MID 1	VOC AM1 1-[illegible]	VOC AM1 2-[illegible]	VOC AM1 3-[illegible]	6	VAriette	NLHG 5[illegible]
KE12-01[illegible]		VOC 2-17	2012-02-0[illegible]	MID 1	VOC AM1 1-[illegible]	VOC AM1 2-[illegible]	VOC AM1 3-[illegible]	7	VAriette	NLHG 5[illegible]
KE12-01[illegible]		VOC 2-18	2012-02-0[illegible]	MID 1	VOC AM1 1-10	VOC AM1 2-10	VOC AM1 3-10	8	VAriette	NLHG 57
KE12-02[illegible]		VOC 2-21	2012-02-0[illegible]	MID 1	VOC AM1 1-11	VOC AM1 2-11	VOC AM1 3-11	9	VAriette	NLHG 5[illegible]
KE12-02[illegible]		VOC3-1	2012-02-0[illegible]	MID 1	VOC AM1 1-12	VOC AM1 2-12	VOC AM1 3-12	10	VAriette	NLHG 5[illegible]
KE12-02[illegible]		VOC3-[illegible]	2012-02-0[illegible]	MID 1	VOC AM1 1-[illegible]	VOC AM1 2-[illegible]	VOC AM1 3-[illegible]	11	VAriette	NLHG 6[illegible]
KE12-02[illegible]		VOC3-[illegible]	2012-02-04	MID 1	VOC AM1 1-14	VOC AM1 2-14	VOC AM1 3-14	12	VAriette	NLHG 61
KE12-03[illegible]		VOC3-[illegible]	2012-02-0[illegible]	MID 5	VOC AM5 1-1	VOC AM5 2-1	VOC AM5 3-1	13	VAriette	NLHG 62
KE12-03[illegible]		VOC3-[illegible]	2012-02-0[illegible]	MID 5	VOC AM5 1-2	VOC AM5 2-2	VOC AM5 3-2	14	VAriette	NLHG 63
KE12-03[illegible]		VOC3-11	2012-02-0[illegible]	MID 5	VOC AM5 1-3	VOC AM5 2-3	VOC AM5 3-3	15	VAriette	NLHG 64
KE12-03[illegible]		VOC3-12	2012-02-0[illegible]	MID 5	VOC AM5 1-4	VOC AM5 2-4	VOC AM5 3-4	16	VAriette	NLHG 65
KE12-03[illegible]		VOC3-14	2012-02-0[illegible]	MID 5	VOC AM5 1-[illegible]	VOC AM5 2-[illegible]	VOC AM5 3-[illegible]	17	VAriette	NLHG 66
KE12-03[illegible]		VOC3-15	2012-02-0[illegible]	MID 5	VOC AM5 1-7	VOC AM5 2-7	VOC AM5 3-7	18	VAriette	NLHG 67
KE12-03[illegible]		VOC3-17	2012-02-0[illegible]	MID 5	VOC AM5 1-[illegible]	VOC AM5 2-[illegible]	VOC AM5 3-[illegible]	19	VAriette	NLHG 68
KE12-04[illegible]		VOC3-18	2012-02-0[illegible]	MID 5	VOC AM5 1-[illegible]	VOC AM5 2-[illegible]	VOC AM5 3-[illegible]	20	VAriette	NLHG 69
KE12-04[illegible]		VOC3-[illegible]	2012-02-0[illegible]	MID 5	VOC AM5 1-10	VOC AM5 2-10	VOC AM5 3-10	21	VAriette	NLHG 7[illegible]
KE12-04[illegible]		VOC3-21	2012-02-0[illegible]	MID 5	VOC AM5 1-11	VOC AM5 2-11	VOC AM5 3-11	22	VAriette	NLHG 71
KE12-04[illegible]		VOC5-1	2012-02-0[illegible]	MID 5	VOC AM5 1-12	VOC AM5 2-12	VOC AM5 3-12	23	VAriette	NLHG 72
KE12-05[illegible]		VOC5-[illegible]	2012-02-0[illegible]	MID 5	VOC AM5 1-14	VOC AM5 2-14	VOC AM5 3-14	24	VAriette	NLHG 73
KE12-05[illegible]		VOC5-[illegible]	2012-02-0[illegible]	MID 6	VOC AM6 1-1	VOC AM6 2-1	VOC AM6 3-1	25	VAriette	NLHG 74
KE12-05[illegible]		VOC5-14	2012-02-0[illegible]	MID 6	VOC AM6 1-2	VOC AM6 2-2	VOC AM6 3-2	26	VAriette	NLHG 75
KE12-06[illegible]		VOC5-16	2012-02-0[illegible]	MID 6	VOC AM6 1-4	VOC AM6 2-4	VOC AM6 3-4	27	VAriette	NLHG 76
KE12-06[illegible]		VOC5-17	2012-02-0[illegible]	MID 6	VOC AM6 1-[illegible]	VOC AM6 2-[illegible]	VOC AM6 3-[illegible]	28	VAriette	NLHG 77
KE12-06[illegible]		VOC5-18	2012-02-0[illegible]	MID 6	VOC AM6 1-[illegible]	VOC AM6 2-[illegible]	VOC AM6 3-[illegible]	29	VAriette	NLHG 78
KE12-06[illegible]		VOC5-[illegible]	2012-02-0[illegible]	MID 6	VOC AM6 1-7	VOC AM6 2-7	VOC AM6 3-7	30	VAriette	NLHG 79
KE12-06[illegible]		VOC5-21	2012-02-0[illegible]	MID 6	VOC AM6 1-[illegible]	VOC AM6 2-[illegible]	VOC AM6 3-[illegible]	31	VAriette	NLHG 8[illegible]
KE12-06[illegible]		VOC5-22	2012-02-0[illegible]	MID 6	VOC AM6 1-10	VOC AM6 2-10	VOC AM6 3-10	32	VAriette	NLHG 81
KE12-06[illegible]		VOC6-1	2012-02-0[illegible]	MID 6	VOC AM6 1-11	VOC AM6 2-11	VOC AM6 3-11	33	VAriette	NLHG 82
KE12-06[illegible]		VOC6-2	2012-02-0[illegible]	MID 6	VOC AM6 1-12	VOC AM6 2-12	VOC AM6 3-12	34	VAriette	NLHG 83
KE12-06[illegible]		VOC6-3	2012-02-0[illegible]	MID 6	VOC AM6 1-13	VOC AM6 2-13	VOC AM6 3-13	35	VAriette	NLHG 84
KE12-070		VOC6-4	2012-02-0[illegible]	MID 6	VOC AM6 1-14	VOC AM6 2-14	VOC AM6 3-14	36	VAriette	NLHG 85
KE12-07[illegible]		VOC6-[illegible]	2012-02-0[illegible]	MID 8	VOC AM8 1-1	VOC AM8 2-1	VOC AM8 3-1	37	VAriette	NLHG 86
KE12-07[illegible]		VOC6-7	2012-02-0[illegible]	MID 8	VOC AM8 1-2	VOC AM8 2-2	VOC AM8 3-2	38	VAriette	NLHG 87
KE12-07[illegible]		VOC6-[illegible]	2012-02-0[illegible]	MID 8	VOC AM8 1-3	VOC AM8 2-3	VOC AM8 3-3	39	VAriette	NLHG 88
KE12-07[illegible]		VOC6-[illegible]	2012-02-0[illegible]	MID 8	VOC AM8 1-4	VOC AM8 2-4	VOC AM8 3-4	40	VAriette	NLHG 89
KE12-07[illegible]		VOC6-12	2012-02-0[illegible]	MID 8	VOC AM8 1-[illegible]	VOC AM8 2-[illegible]	VOC AM8 3-[illegible]	41	VAriette	NLHG 9[illegible]
KE12-07[illegible]		VOC6-13	2012-02-0[illegible]	MID 8	VOC AM8 1-7	VOC AM8 2-7	VOC AM8 3-7	42	VAriette	NLHG 91
KE12-08[illegible]		VOC6-14	2012-02-0[illegible]	MID 8	VOC AM8 1-[illegible]	VOC AM8 2-[illegible]	VOC AM8 3-[illegible]	43	VAriette	NLHG 92
KE12-08[illegible]		VOC6-15	2012-02-0[illegible]	MID 8	VOC AM8 1-[illegible]	VOC AM8 2-[illegible]	VOC AM8 3-[illegible]	44	VAriette	NLHG 93
KE12-08[illegible]		VOC6-17	2012-02-0[illegible]	MID 8	VOC AM8 1-10	VOC AM8 2-10	VOC AM8 3-10	45	VAriette	NLHG 94
KE12-08[illegible]		VOC6-18	2012-02-0[illegible]	MID 8	VOC AM8 1-11	VOC AM8 2-11	VOC AM8 3-11	46	VAriette	NLHG 95
KE12-08[illegible]		VOC6-19	2012-02-0[illegible]	MID 8	VOC AM8 1-12	VOC AM8 2-12	VOC AM8 3-12	47	VAriette	NLHG 96
KE12-08[illegible]		VOC6-20	2012-02-0[illegible]	MID 8	VOC AM8 1-13	VOC AM8 2-13	VOC AM8 3-13	48	VAriette	NLHG 97
KE12-08[illegible]		VOC6-22	2012-02-0[illegible]	MID 11	VOC AM11 1-1	VOC AM11 2-1	VOC AM11 3-1	49	VAriette	NLHG 98
KE12-09[illegible]		VOC7-2	2012-02-0[illegible]	MID 11	VOC AM11 1-2	VOC AM11 2-2	VOC AM11 3-2	50	VAriette	NLHG 99
KE12-09[illegible]		VOC7-3	2012-02-0[illegible]	MID 11	VOC AM11 1-3	VOC AM11 2-3	VOC AM11 3-3	51	VAriette	NLHG 10[illegible]
KE12-09[illegible]		VOC7-4	2012-02-0[illegible]	MID 11	VOC AM11 1-4	VOC AM11 2-4	VOC AM11 3-4	52	VAriette	NLHG 10[illegible]
KE12-09[illegible]		VOC7-5	2012-02-0[illegible]	MID 11	VOC AM11 1-5	VOC AM11 2-5	VOC AM11 3-5	53	VAriette	NLHG 10[illegible]
KE12-09[illegible]		VOC7-7	2012-02-0[illegible]	MID 11	VOC AM11 1-6	VOC AM11 2-6	VOC AM11 3-6	54	VAriette	NLHG 10[illegible]
KE12-09[illegible]		VOC7-8	2012-02-0[illegible]	MID 11	VOC AM11 1-7	VOC AM11 2-7	VOC AM11 3-7	55	VAriette	NLHG 10[illegible]
KE12-09[illegible]		VOC7-9	2012-02-0[illegible]	MID 11	VOC AM11 1-8	VOC AM11 2-8	VOC AM11 3-8	56	VAriette	NLHG 10[illegible]
KE12-09[illegible]		VOC7-10	2012-02-0[illegible]	MID 11	VOC AM11 1-9	VOC AM11 2-9	VOC AM11 3-9	57	VAriette	NLHG 10[illegible]
KE12-10[illegible]		VOC7-12	2012-02-0[illegible]	MID 11	VOC AM11 1-10	VOC AM11 2-10	VOC AM11 3-10	58	VAriette	NLHG 10[illegible]
KE12-10[illegible]		VOC7-13	2012-02-0[illegible]	MID 11	VOC AM11 1-11	VOC AM11 2-11	VOC AM11 3-11	59	VAriette	NLHG 10[illegible]
KE12-10[illegible]		VOC7-14	2012-02-0[illegible]	MID 11	VOC AM11 1-12	VOC AM11 2-12	VOC AM11 3-12	60	VAriette	NLHG 10[illegible]
KE12-10[illegible]		VOC7-16	2012-02-0[illegible]	MID 14	VOC AM14 1-1	VOC AM14 2-1	VOC AM14 3-1	61	VAriette	NLHG 11[illegible]
KE12-10[illegible]		VOC7-19	2012-02-0[illegible]	MID 14	VOC AM14 1-2	VOC AM14 2-2	VOC AM14 3-2	62	VAriette	NLHG 11[illegible]
KE12-10[illegible]		VOC7-20	2012-02-0[illegible]	MID 14	VOC AM14 1-3	VOC AM14 2-3	VOC AM14 3-3	63	VAriette	NLHG 11[illegible]



Appendix xx: 454 Pyrosequencing Phylogenetic Tree

Appendix xxi: Drug Resistance Mutation Frequencies Observed In The Arv Drug Naïve Through 454 Analysis

SAMPLE ID	GENDER	AGE	MUTATIONS	SUB-TYPE
NLHG-72	Female	39	V82A	D
NLHG-77	Male	51	M184V, N88D	A1
NLHG-78	Female	28	K103N	A1
NLHG-79	Female	28	K219R, L24I, M184V, G190E	C
NLHG-80	Male	33	Y188H, I54T, K219Q, K219E	D
NLHG-83	Male	45	K219R, N83D, K219E	A1
NLHG-84	Male	36	K219R, F77L, G190E, K219Q, K219E	AE/D
NLHG-85	Female	46	L100I, L24I, K101E	A2/D
NLHG-88	Male	30	M46L, Y188H, V82A, F53Y,G73S	A1
NLHG-89	Female	29	M184V, G190E	A1
NLHG-90	Female	54	I54T, M184V	AE/D
NLHG-91	Female	44	Y188H, L24I, N88S, I84V, I50V, Y181C	D
NLHG-92	Female	27	K101E, K219Q	B
NLHG-94	Female	41	K101E	A/AE
NLHG-95	Female	30	K65R, I47V	A1
NLHG-96	Female	52	F77L, D67G	A1
NLHG-97	Female	30	K219R, L100I, K65R, V32I, K219Q, K219E	A1
NLHG-99	Female	39	M184I, N83D,N88S, I50V, I54T, D67G, V82A	A1
NLHG-100	Male	40	K219Q	A1
NLHG-101	Female	39	K101E	A1
NLHG-103	Male	63	M184V, F53L	A1
NLHG-105	Female	58	D67N, T69D	A1
NLHG-107	Female	25	K101E, N83D	A1
NLHG-110	Male	39	D30N, I85V	D
NLHG-111	Male	32	G190E, K101E, N88D, K70R, T215Y	A1
NLHG-114	Female	28	N88D, K219Q, L74V	A1
NLHG-115	Female	36	K219Q	A1/B/D
NLHG-117	Male	30	K219Q	A1
NLHG-118	Female	26	K219Q	D
NLHG-119	Male	36	F77L, K103N	A1
NLHG-121	Female	30	K219Q	D
NLHG-172	Female	33	G73S	A1
NLHG-122	Female	36	I54T, I47V	D
NLHG-126	Female	66	K103N	A1

NLHG-128	Female	8	N83D, K219Q, M46I	D
NLHG-129	Female	33	D67G, N88D	A1
NLHG-133	Female	29	L100I, K219Q	A1/D
NLHG-173	Female	25	K219Q	A1/D
1NLHG-35	Male	33	M41L, M184I, L210W, T215Y	A1
NLHG-149	Female	38	K219R, K65R, K101E,K70R	A1
NLHG-175	Female	27	K103S, M184V, F53L, V106M, K70E	C
NLHG-150	Female	32	F77L, K101E	D
NLHG-157	Male	46	K103S, N88D, K219Q, K103N, V82A, G73S, I47V	A1/D
NLHG-159	Female	34	M184V, Y181V, N88D	A1
NLHG-162	Female	29	F53L, N88D, K103N	A1
NLHG-164	Female	30	F53L, N88D, I47V, M46I	A1

Appendix xxii: Sanger Sequencing Versus 454 Pyrosequencing DRMS

PID	sanger pi.drms	sanger nrti.drms	sanger nnrti.drms	454 pyrosequencing drug resistance mutations													
72	none	none	none	V82A	1.03												
77	none	none	none	M184V	1.75	N88D	1.14										
79	none	none	none	K219R	2.22	L24I	5.88	M184V	1.79	G190E	2.22						
80	none	none	none	Y188H	1.21	I54T	1.07	K219Q	2.13	K219E	1.06						
82	none	none	none														
83	none	none	none	K219R	1.05	N83D	1.66	K219E	1.05								
84	none	none	none	K219R	1.72	F77L	2.21	G190E	36.29	K219Q	3.86	K219E	1.72				
85	none	none	none	L100I	4.93	L24I	1.75	K101E	1.75								
86	none	none	none														
87	none	none	none														
88	none	none	none	M46L	1.66	Y188H	1.22	V82A	1.07	F53Y	1.34	G73S	1.07				
89	none	none	none	M184V	1.08	G190E	1.08										
90	none	none	none	I54T	1.27	M184V	1.95										
91	none	none	none	Y188H	1.64	L24I	2	N88S	1.92	I84V	1.92	I50V	1.89	Y181C	1.61		
92	none	none	none	K101E	2.04	K219Q	7.06										
95	none	none	none	K65R	1.12	I47V	1.47										
96	none	none	none	F77L	2.59	D67G	1.72										
97	I50V	M184I	none	K219R	1.49	L100I	4.23	K65R	1.11	V32I	4.17	K219Q	7.46	K219E	2.99		
98	none	none	none														
99	none	none	none	M184I	3.28	N83D	1.39	N88S	1.39	I50V	2.78	I54T	1.39	D67G	16.67	V82A	1.39
100	none	none	none	K219Q	1.33												
101	none	none	none	K101E	4.86												
103	none	none	none	M184V	3.7	F53L	1.54										
104	none	none	none														
105	none	none	none	D67N	8.33	T69D	8.33										
107	none	none	none	K101E	4.44	N83D	2.86										
108	none	none	none														
169	none	none	none														
170	none	none	none														
110	none	none	none	D30N	7.14	I85V	7.14										
111	none	none	none	G190E	1.48	K101E	11.11	N88D	3.33	K70R	11.11	T215Y	1.19				
113	none	none	none														
115	none	T215I	none	K219Q	9.09												
116	none	none	none														
117	none	none	none	K219Q	3.03												
118	none	none	none	K219Q	5.1												
119	none	none	none	F77L	4.35	K103N	14.29										
121	none	none	none	K219Q	1.23												
172	none	none	none	G73S	1.37												
126	none	none	K103N	K103N	71.43												
127	none	none	none														
128	none	none	none	N83D	1.42	K219Q	13.64	M46I	1.41								
157	none	none	none	K103S	1.9	N88D	4.41	K219Q	9.92	K103N	84.81	V82A	1.43	G73S	1.43	I47V	1.43
159	none	none	none	M184V	100	Y181V	100	N88D	5								

Appendix xxiii: Presentation At The 3rd KEMRI Annual Scientific & Health Conference

VENUE: KEMRI HQ, NAIROBI

Theme: Improving Public Health and National Development through Research and Innovation

Time	Abstract	Day 2:THURSDAY FEBRUARY 7, 2013
14.00-		Topic: HIV
16.00hrs		Session Chair: Dr. Raphael Lwembe
		Rapporteur: Dr. Raphael Lihana
1430-	11	Drug Resistance among HIV Infected Patients Attending the Moi
1445hrs		Teaching and Referral Hospital (MTRH), Eldoret, Kenya

Background / Introduction: Access to ARV therapy (ART) is increasing in resource-limited settings (RLS) and can successfully reduce HIV-related morbidity and mortality. However, due to the high mutation rate of HIV and the lifelong treatment, it is expected that HIV drug resistance will occur in persons not on treatment due to transmitted DRMs and those on treatment in Kenya as well even if appropriate regimens are provided and good adherence is supported.

Objective: The main objective was to evaluate inter subtype reverse transcriptase, and protease gene mutations of viral isolates obtained from HIV infected patients attending Moi Teaching and referral Hospital (MTRH) clinics

Materials & Methods: In 2009, we consecutively collected plasma samples from patients attending the study site who were ARV naïve according to chart review and those who were on ART for more than 12 months and were failing therapy according to WHO guidelines. We performed genotypic drug resistance using well established in-house population based Sanger sequencing methods.

Results: We successfully extracted and sequenced 83 samples. Median age was 36.7 years. Majority were women 50/83. ARV naïve patients were forty nine and experienced group were thirty four. Three out of forty nine naïve patients had DRMs (DRMS). Out of the 34 ARV experienced group who were failing therapy according to WHO guidelines, only three did not harbor any DRMS, twenty seven harbored resistance mutations toNRTI (NRTI), thirty harbored resistance mutations to NNRTI (NNRTI), and two harbored resistance mutations to NNRTI only

Discussion: Transmitted drug resistance exists in ARV naïve patients. The majority of patients who were on ART and were failing, had DRMs resistant to at least 2 of the 3 drugs from their treatment.

Conclusion & Recommendation: Assessment of the proportion of HIV-infected persons who are naive and those who have developed ARV resistance and characterization of the causes and factors associated with resistance development are critical steps in modifying treatment guidelines and regimens to improve their effectiveness.

Appendix xxiv: Presentation At The ASLM 2014 International Conference

Drug Resistance Testing in HIV Infected on Treatment and Naïve: Implications on Treatment Outcome Background:

Abstract
Background: Access to ART is increasing in resource-limited settings (RLS) and can successfully reduce HIV related morbidity and mortality. However, due to the high mutation rate of HIV and the lifelong treatment, it is expected that HIV drug resistance will occur in persons not on treatment due to transmitted drug resistance mutation and those on treatment in Kenya as well even if appropriate regimens are provided and good adherence is supported. The main objective was to evaluate inter subtype reverse transcriptase, and protease gene mutations of viral isolates obtained from HIV infected patients attending Moi Teaching and referral Hospital (MTRH) clinics and to determine the proportion and characteristics of patients who develop resistance to drugs in ARV naïve and in ARV experienced patients failing therapy.

Methods: In 2009, we consecutively collected plasma samples from patients attending the study site who were ARV naïve according to chart review and those who were on ART for more than 12 months and were failing therapy according to WHO guidelines. We performed genotypic drug resistance using well established in-house population based Sanger sequencing methods.

Results: We successfully extracted and sequenced 83 samples. Median age was 36.7 years. Majority were women 50/83. ARV naïve patients were 49 and experienced group were 34. 3 out of 49 naïve patients had DRMs (DRMS). Out of the 34 ARV experienced group who were failing therapy according to WHO guidelines, only 3 did not harbor any DRMS, 27 harbored resistance mutations toNRTI (NRTI), thirty harbored resistance mutations to NNRTI (NNRTI), and two harbored resistance mutations to NNRTI only. Conclusion: The information observed in our study can serve as an indicator of ARV program efficiency in patients still on treatment, those who are to start treatment and those who are to be changed therapy due to failure. Drug resistance testing would be necessary before initiating ART in order to achieve a better clinical outcome. Therefore, assessment of the proportion of HIV-infected persons who are naive and those who have developed ARV resistance and characterization of the causes and factors associated with resistance development are critical steps in modifying treatment guidelines and regimens to improve their effectiveness.

Appendix xxv: Presentation At The Kenya National Aids Control Council Conference MAY 2013.

HIV Drug resistance Surveillance using 454 Pyrosequencing at the Moi Teaching and referral Hospital (MTRH), Eldoret, Kenya

***Winfrida Cheriro*[1], *M Kiptoo* [2], *G Kikuvi* [3], *S Mining* [4], *W Emonyi* [4,5], *J Koech*[5], *R Lihana* [2], *J Brooks* [6], B Liang[7], Hezhao Ji [6], *E Songok* [2,7]**

Moi Teaching and Referral Hospital (MTRH) [1], Kenya Medical Research Institute (KEMRI) [2], Jomo Kenyatta University of Agriculture and Technology (JKUAT) [3], Moi University School of Medicine (MUSOM) [4], USAID-AMPATH Partnership[5], National HIV and retro virology labs (NHRL) [6], University of Manitoba, Winnipeg – Canada[7]

BACKGROUND

Access to antiretroviral therapy (ART) in Kenya has significantly increased since the start of WHO's 3 by 5 initiative. Since the introduction of generic antiretroviral (ARVs), there has been a steep increase in the number of individuals initiating antiretroviral therapy (ART) primarily due to the government initiative in accessing free ARVs through the government run HIV comprehensive HIV clinics.

Highly active antiretroviral therapy (HAART) has dramatically improved survival and quality of life in people living with HIV and AIDS. The Kenya AIDS indicator survey of 2007 showed that of the estimated 392,000 Kenyan adults in need of ART, 138,000 (35%) had received the treatment by September 2007, which increased to 212,000 (54%) by June 2008. The increase in ART coverage is expected to lead to an increase in drug-resistant strains among drug naïve and experienced patients.

However, drug resistance surveillance remains highly expensive and mostly unavailable in many resource-limited settings (RLS). By reducing the cost of drug resistance surveillance its implementation will be enhanced in RLS. The Genome Sequencer FLX System from Roche Applied Science and 454 Life Sciences takes DNA sequencing into revolutionary new opportunities.

Here we present the drug resistance mutations identified through 454 pyrosequencing approach to study drug resistance in a cohort of HIV-positive individuals enrolled in a study through the Moi Teaching and referral Hospital, Kenya care clinics.

OBJECTIVES

The main objective was to evaluate inter subtype reverse transcriptase, and protease gene mutations of viral isolates obtained from HIV infected patients attending Moi Teaching and referral Hospital (MTRH) clinics.

Specific objectives were to determine the proportion and characteristics of patients who develop resistance to drugs in ARV naïve and in ARV experienced patients failing therapy using next-generation sequencing-based genotyping method to monitor drug resistance and

Compare the frequencies with those identified using traditional genotyping

METHODS

Study Subjects

During September 2009 and October 2011, patients who were ARV naïve and those who were receiving ARV therapy for at least 12 months and were failing according to WHO guidelines were consecutively enrolled. After informed consent was obtained, a standardized questionnaire was administered to assess demographic, epidemiologic, clinical, and treatment information.

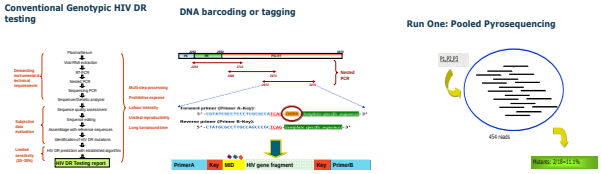
With informed consent, remnant samples from CD4 analysis were utilized. Samples from patients who met the inclusion criteria were analyzed. 3 ml of whole blood was collected on EDTA tubes.

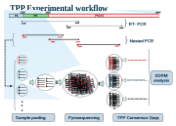
After centrifugation, plasma aliquots were frozen at −80° C. The Kenya AIDS control program defines failure as patients who have been on ARVs for more than six months with evidence of adherence with a decline of CD4+ counts of more than 25% and a viral load of greater than 10,000 copies per ml. (NASCOP 2008)

We extracted, amplified and sequenced samples with the 454 pyrosequencing. We specifically designed primers to amplify protease and reverse transcriptase. We characterized drug resistance from plasma in 100 HIV infected individuals either exposed and failing or not exposed to antiretroviral therapy collected in Eldoret, Kenya.

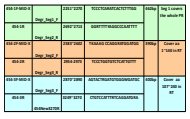
Ethics statement

The study was approved by the Institutional Research and Ethics Committee of the Moi University School of Medicine and Moi Teaching and Referral Hospital and the Institutional Review Board (IREC).) : (IREC/2010/06), (RES/STUD/17/2010). Informed consent was signed by those who met study criteria





Primers for nested PCR: (Pre-optimized tagged primers)



RESULTS

101 samples were analyzed. 59% (60/101) were ARV naïve and 67% (68/101) were women. Mean age, 35.82 sd (12.35) Male 33 sd (32.7) Female 68 sd (67.3) Naïve 60 sd (59.4) ARV Failures 41 sd (40.6)

From this data, we identified drug resistance mutations in both groups of our cohort, which was 25.6% more than frequencies identified using traditional genotyping. Majority 71% from those with DRMs were women among the ARV naïve.

Thirteen of the drug naive patients with drug resistance mutations were sequenced from samples at the time of diagnosis (visit 1). These mutations may therefore exemplify transmitted drug resistance. The remaining six treatment naï¨ve patients with drug resistance mutations were sampled at different time points after diagnosis that ranged from visit 2 to visit 9 and therefore there is less certainty about whether these mutations were transmitted or a result of spontaneous mutation.

Multiple clinical DR mutations present at higher frequencies were concordantly identified using both methods.

The two most common drug resistance mutations found in individuals after failing treatment were G190A and M184V. M184V mutations were found prior to treatment and in drug failure, found in 6 individuals after failing treatment.

Overall, prior to treatment, eight patients had resistance to NRTIs only, six patients had resistance to NNRTIs only one patient had resistance to protease inhibitor (PI) only, and one patient showed resistance to a PI + NRTI and one patient showed resistance to a PI + NNRTI

LESSONS LEARNED

We have described a method to sequence together 384 patient samples to simultaneously test for HIV drug resistance using Roche/454 pyrosequencing in a RLS.

Successful DR surveillance programs typically acquire sequencing results from large numbers of antiretroviral naï¨ve and experienced subjects and produce an estimate of the percentage of drug resistance based upon the aggregate results. The number of individuals on treatment that were sequenced in this study is very small and therefore frequencies of drug resistance presented here in treatment failures are not generalizable to the population in Kenya. The two most common drug resistance mutations found prior to treatment were K219Q(n=6) and K219R(n=5) appearing as minor mutations at frequencies <10% . These mutations known to be selected by thymidine analogues also confer reduced susceptibility to all approved nRTIs. The percentage resistance to protease and reverse transcriptase inhibitors is not described in the context of the individual but instead is attributed to the population under study. Thus far, pyrosequencing of HIV has been used to explore HIV DR in a population of viruses within an individual. However, in this proof of concept study, we use pyrosequencing on pooled specimens in order to survey for protease (PR) and reverse transcriptase (RT) DRM contained in viruses within a population.

The findings presented here are strongly supportive of analyzing pooled specimens for the determination of the prevalence of HIV DR in HIV PR and RT.

In our previous work to further evaluate the application potential of pyrosequencing in HIV DR Surveillance, we calculated and compared the material and labor costs for Sanger sequencing and pyrosequencing. For these calculations it was assumed that the respective instruments were embedded, as components of an institutional core facility, as would be expected for a laboratory capable of performing specialized HIV DR testing.

The combined labor and material costs of HIV genotyping using our in-house Sanger sequencing method was $82/specimen which is less than half the cost of commercial genotyping. Due to the common steps involved, the cost of determining drug resistance in protease is not halved but falls to $52/specimen. In comparison, the total cost for the equivalent analysis of protease using pyrosequencing was $32/specimen. For pooled pyrosequencing based surveillance of TDR in PR, assuming sequencing of three overlapping regions, costs were predicted to be $53/specimen. These calculations included costs for the additional labor required for pyrosequencing. Pyrosequencing costs may, in fact, continue to fall due to competition and the increased market penetrance of these newer platforms.

With competitive costs and the existing scaling, the pooled pyrosequencing approach may be useful in global DR surveillance through its implementation at specialized HIV DR laboratories (Hezhao Ji *et al* 2010).This finding emphasizes the importance of surveying for HIV drug resistance and highlights the significance of developing tools such as those provided here to perform these surveys faster and cheaper to choose optimal first-line/second-line or salvage therapies.

CONCLUSION

The 454 pyrosequencing method is able to run more patient samples than the current genotyping method. This method is also 4-fold more sensitive (5% minimal detection frequency vs. 20%) than the traditional Sanger-based genotyping method and can be more easily implemented in low-resource settings.

This data shows that next-generation HIV drug resistance genotyping is a better alternative to current genotyping methods and may be particularly beneficial for in-country surveillance of transmitted drug resistance

REFERENCES

Identifying and quantifying low abundance DRMs *(Simen BB, et al, JID 2009; Wang C, et al. Genome Res 2007; Hoffmann C, et al. Nucleic Acids Res 2007;Le T, et al. PLoS One 2009; Varghese V, et al. JAIDS 2009; Mitsuya Y, et al. J Virol 2008);*

Evaluating HIV quasispecies and co-receptor usage *(Rozera G, et al. Retrovirology 2009, Archer J, et al. AIDS 2009)*

Gastrointestinal and genital Microbiota analysis *(Spear GT, et al. JID 2008; Mckenna P, et al. PLoS Pathog 2007, Schellenberg J, et al. Appl Environ Microbiol. 2009)*

Genome-wide monitoring of integration site selection *(Wang GP, et al. Genome Res 2007, Molecular Therapy 2009)*

Evaluating HIV quasispecies and co-receptor usage *(Rozera G, et al. Retrovirology 2009, Archer J, et al. AIDS 2009)*

Appendix xxvi: Presentation At The 16th ICID

Abstract 42.015

Title: High prevalence of minor HIV drug resistant strains in a treatment naive population in Kenya

Author(s): W. Chelangat Cheriro[1], B. Liang[2], J. Brooks[3], H. Ji[3], M. Kiptoo[4], R. Lihana[4], S. Mining[1], E. Songok[4]; [1]Moi Teaching and Referal Hospital, Eldoret/KE, [2]Public Health Agency of Canada, Winnipeg/CA, [3]Public Health Agency of Canada, Ottawa/CA, [4]Kenya Medical Research Institute, Nairobi/KE

Abstract: Background: The advent of ARV treatment (ART) has resulted in dramatic reduction in AIDS related morbidity and mortality. However the emergence and spread of ARV drug resistance (DR) threatens to negatively impact on treatment regimens and compromise efforts to control the epidemic. It is recommended that surveillance of drug resistance occur in conjunction with scale-up efforts to ensure appropriate first-line therapy is offered relative to the resistance that exists. However standard resistance testing methods used in Subsahara Africa rely on techniques that miss out on low abundance DR variants (LADRVs) which have been documented to contribute to treatment failure. The use of next generation sequencing (NGS) has been shown to be more sensitive for LADRVS. We have carried out a preliminary investigation using NGS to determine the prevalence of LDRVS among a drug naïve population.
Methods & Materials: ARV naïve patients attending a care clinic at Moi Teaching and Referral Hospital (MTRH) in Eldoret, Kenya were requested and with consent provided blood samples for DR analysis. DNA was extracted, amplified and nested PCR conducted on pol RT region with primers tagged with multiplex identifiers (MID). Resulting PCR amplicons were purified, quantified and pyrosequenced using a GS FLX Titanium PicoTiterPlate (Roche). Valid pyrosequencing reads were aligned with HXB-2 and the frequency and distribution of nucleotide and amino acid changes determined using an in-house Perl script. DR mutations were identified using the IAS-HIV.
Results: Sixty samples were successfully sequence of which 25 were subtype A, 11 subtypes D, 1 Subtype C and the remaining were recombinants. Forty six (76.6%) had at least one drug resistance mutation; with 25 (41.6%) indicated as major and the rest 21 (35%) indicated as minor. The most prevalent mutation was NRTI position K219Q/R (11 of 46, 24%) followed by NRTI M184V (5 of 46, 11%) and NNRTI K103N(4/46).
Conclusion: Our use of NGS technology revealed a high prevalence of LADRVs among drug naive populations in Kenya. The impact of these mutations on clinical outcome on ART can only be ascertained through a long term follow-up.

PRESENTATION AT THE 16TH INTERNATIONAL CONGRESS ON INFECTIOUS DISEASES CAPE TOWN • SOUTH AFRICA APRIL 2~5, 2014

High Prevalence of HIV Minor Drug Resistant variants in a treatment naïve population in North Rift Kenya

Cheriro Winfrida[1], Brooks J[2], Liang B[2], Hezhao J[2], Lihana R[1], Kiptoo M[2], Mining S[2], Emonyi W[1] and SongokEM[1,3]

Moi Teaching and Referral Hospital (MTRH)[1], National HIV and retro virology labs (NHRL)[2], Kenya Medical Research Institute (KEMRI)[3], Jomo Kenyatta University of Agriculture and Technology (JKUAT)[4], Moi University School of Medicine (MUSOM)[5], University of Manitoba, Winnipeg – Canada[6]

Background:

- Access to antiretroviral therapy (ART) in Kenya has significantly increased since the start of WHO 3 by 5 initiative. Since the introduction of generic antiretroviral (ARVs), there has been a steep increase in the number of individuals initiating antiretroviral therapy (ART) through the government run HIV Comprehensive Care Clinics (CCCs).
- Though this has resulted in dramatic reduction in AIDS related morbidity and mortality. the emergence and spread of antiretroviral drug resistance (DR) threatens to negatively impact on treatment regimens and compromise efforts to control the epidemic.
- It is hence recommended that surveillance of drug resistance occur in conjunction with scale-up efforts to ensure appropriate first-line therapy is offered relative to the resistance that exists.
- However standard resistance testing methods rely n techniques that are expensive and miss out on ow abundance DR variants (LADRVs) which have been documented to contribute to treatment
- The use of next generation sequencing (NGS) has been shown to be cheaper and more sensitive for LADRVS.
- We have carried out a preliminary investigation using NGS to determine the prevalence of LDRVS among a drug nae population in North Rift Kenya.

Objectives:

- To determine the proportion and characteristics of drug nae HIV infected patients who arbourlow abundant drug resistant mutations
- Compare the frequencies with those identified using traditional genotyping

Study site

- The study was carried out at Moi Teaching and Referral Hospital (MTRH)
- MTRH provides both routine and referral health services for Western Kenya region.
- The hospital is located in Eldoret Town in Uasin-Gishu county

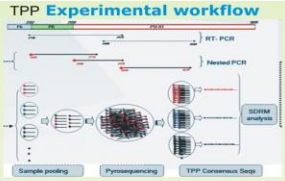
Methods

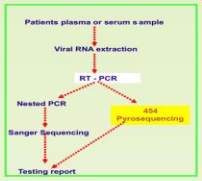
- Antiretroviral naïve patients attending a care clinic at Moi Teaching and Referral Hospital (MTRH) in Eldoret, Kenya were requested and with consent provided blood samples for DR analysis.
- DNA was extracted, amplified and nested PCR conducted on pol RT region using primers tagged with multiplex identifiers (MID).
- Resulting PCR amplicons were purified, quantified and pyrosequenced using a GS FLX Titanium PicoTiterPlate (Roche).
- Valid pyrosequencing reads were aligned with HXB-2 and the frequency and distribution of nucleotide and amino acid changes determined using an in-house Perl script.
- DR mutations were identified using the IAS-USA HIV DR mutation database.c

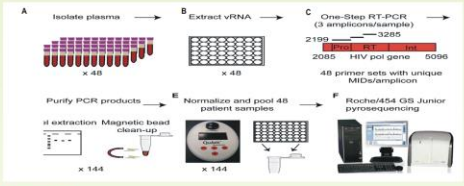
The workflow of sequencing HIV DR genotyping

- HIV-1 nucleic acid was extracted from 400µl of plasma using the Nuclisens EasyMag system (Biomerieux, Canada) following manufacturer's instructions.
- Briefly, viral RNA was reverse transcribed and amplified according to the manufacturer's directions using the QIAGEN one-step RT-PCR kit (QIAGEN,Canada).
- Products screened using agarose gel and/or Qiaxcel (Quantification)
- PCR amplicons were then purified and diluted to15ng/ml for DNA.
- Sequencing was done using ABI Prism BigDye 3.1 Cycle Sequencing System (Applied Biosystems, Carlsbad, CA) following manufacturer's instructions.
- Generated sequences, edited and sent to Stanford HIV database and DRMS reported.

TPP Experimental workflow







Data analysis

- All the data generated in this study was saved in Microsoft Excel worksheets with a detailed database established to capture all the necessary information.
- Generated sequences were edited using Bio Edit v 7.0.5B.
- Valid pyrosequencing reads were aligned with HXB-2 and the frequency and distribution of nucleotide and amino acid changes determined using an in-house Perl script.
- Aligned fasta files were uploaded to Stanford HIV Drug resistance http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm.
- All Mutations were identified using a surveillance drug resistance mutation (SDRM) list (Bennett et al 2009)

Socio-demographic characteristics

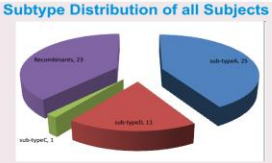
Characteristic	N, (%)
Gender	
Male	16 (26.67)
Female	44 (73.3)
Median age, (IQR)	36.7 (28.80-45.81)
Mean age (sd)	37.01 (12.50)

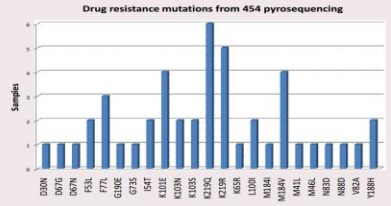
Results in summary

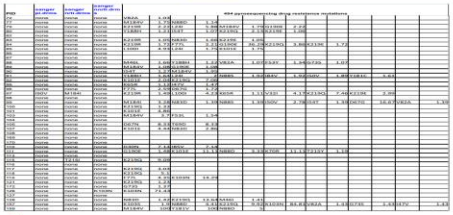
- Sixty samples were successfully sequenced of which 25 were subtype A, 11 subtypes D, 1 Subtype C and the remaining were recombinants.
- Forty six (76.6%) had at least one drug resistance mutation; with 25 (41.6%) indicated as major and the rest 21 (35%) indicated as minor.
- The most prevalent mutation was NRTI position K219Q/R (11 of 46, 24%) followed by NRTI M184V (5 of 46, 11%) and NNRTI K103N (4 of 46, 9%).
- Multiple clinical DR mutations present at higher frequencies were concordantly identified using both methods.

454 Phylogenetic tree

Subtype Distribution of all Subjects







Findings

- The 454 pyrosequencing method was able to run more patient samples than the Sanger genotyping method. The method was also 4-fold more sensitive (5% minimal detection frequency vs. 20%) than the traditional Sanger-based genotyping method.
- HIV Subtype A still the most prevalent ladein North Rift Region
- NRTI hymldineanalogue mutations (K219Q/R) was the most prevalent mutation.
- The findings strongly support analyzing pooled specimens for the determination of the prevalence of HIV DR in HIV RT as a cost effective procedure.

CONCLUSION AND WAY FORWARD

- Our use of NGS technology revealed a high prevalence of LADRVs among drug naive populations in Kenya.
- The impact of these mutations on clinical outcome on ART can only be ascertained through a long term follow-up.

Acknowledgments

•Kinyua J, Odero I

•Dr. Kimaiyo S,
•Dr. Nyandiko,
•Koech J, Kiplagat c.
•Obila J, kalekha L,
•Muhindi P, Kibwai J,mowlem P,
•Kuta L, Chepkwemoi M,

•H,Merks ,Vallee D, Joanisse J

•Canada Department of Foreign Affairs and Trade (DFAIT)

Appendix xxvii: Publication 1: High prevalence of hiv low abundance drug-resistant variants in a treatment-naïve population in north rift kenya

AIDS RESEARCH AND HUMAN RETROVIRUSES
Volume 31, Number 12, 2015

DOI: 10.1089/aid.2015.0039

High Prevalence of HIV Low Abundance Drug-Resistant Variants in a Treatment-Naive Population in North Rift Kenya

Winfrida Cheriro,[1,2] Michael Kiptoo,[1,3] Gideon Kikuvi,[1] Simeon Mining,[4] Wilfred Emonyi,[4] and Elijah Songok[1,3,5]

Abstract

The advent of antiretroviral treatment (ART) has resulted in a dramatic reduction in AIDS-related morbidity and mortality. However, the emergence and spread of antiretroviral drug resistance (DR) threaten to negatively impact treatment regimens and compromise efforts to control the epidemic. It is recommended that surveillance of drug resistance occur in conjunction with scale-up efforts to ensure that appropriate first-line therapy is offered relative to the resistance that exists. However, standard resistance testing methods used in Sub-Saharan Africa rely on techniques that do not include low abundance DR variants (LADRVs) that have been documented to contribute to treatment failure. The use of next generation sequencing (NGS) has been shown to be more sensitive to LADRVS. We have carried out a preliminary investigation using NGS to determine the prevalence of LDRVS among a drug-naive population in North Rift Kenya. Antiretroviral-naive patients attending a care clinic in North Rift Kenya were requested to provide and with consent provided blood samples for DR analysis. DNA was extracted and amplified and nested PCR was conducted on the pol RT region using primers tagged with multiplex identifiers (MID). Resulting PCR amplicons were purified, quantified, and pyrosequenced using a GS FLX Titanium PicoTiterPlate (Roche). Valid pyrosequencing reads were aligned with HXB-2 and the frequency and distribution of nucleotide and amino acid changes were determined using an in-house Perl script. DR mutations were identified using the IAS-USA HIV DR mutation database. Sixty samples were successfully sequenced of which 26 were subtype A, 9 were subtype D, 2 were subtype C, and the remaining were recombinants. Forty-six (76.6%) had at least one drug resistance mutation, with 25 (41.6%) indicated as major and the remaining 21 (35%) indicated as minor. The most prevalent mutation was NRTI position K219Q/R (11/46, 24%) followed by NRTI M184V (5/46, 11%) and NNRTI K103N (4/46, 9%). Our use of NGS technology revealed a high prevalence of LADRVs among drug-naive populations in Kenya, a region with predominantly non-B subtypes. The impact of these mutations on the clinical outcome of ART can be ascertained only through long-term follow-up.

Introduction

In Kenya the availability of antiretroviral therapy (ART) is increasing as well as in other low-income and middle-income countries. As ART use continues to be scaled up, there is mounting evidence suggesting that drug resistance (DR) will develop and increase over time.[1] A survey performed in Kampala between 2009 and 2010 showed that the prevalence of transmitted drug resistance was 8.6%.[2] It is highly recommended by the World Health Organization (WHO) that surveillance of drug resistance occurs in conjunction with scale-up efforts to ensure that appropriate first-line therapy is offered relative to the resistance that exists.[3]

It is believed that surveillance will maximize the utility of first-line therapy and help minimize the cost of providing ART, thereby sustaining current antiretroviral drug programs. However, current standard genotypic resistance testing methods used in surveillance programs rely on Sanger sequencing (SS), a method that has a detection limit in the neighborhood of 20% of the virus quasispecies.[4] Increased rates of virological failure to ART regimens, especially

[1]Institute of Tropical Medicine and Infectious Diseases, Jomo Kenyatta University of Agriculture and Technology (JKUAT), Nairobi, Kenya.
[2]Moi Teaching and Referral Hospital, Eldoret, Kenya.
[3]Kenya Medical Research Institute, Nairobi, Kenya.
[4]School of Medicine, Moi University, Eldoret, Kenya.
[5]Department of Medical Microbiology, University of Manitoba, Winnipeg, MN, Canada.

nonnucleoside reverse transcriptase inhibitors (NNRTIs), have been noticed despite no evidence of DR mutations by SS at baseline. In fact, many studies have shown a low abundance of DR variants (LADRVs) at frequencies less than 20% in both ART-naive and heavily ART-treated subjects.[5,6]

Furthermore, it has been noted that these LADRVs can increase and outcompete wild-type strains under drug selection pressure leading to treatment failure.[7] Due to limitations of Sanger sequencing and the need for low-cost genome sequencing, there has been a revolution in the large-scale genomics field. To date, three major next-generation sequencing (NGS) technologies—Roche 454 Life Science FLX (454), Illumina (Solexa), and Ion PGM (Life Technologies)—have been commercialized.[8] These technologies share the paradigm of massive, parallel, clonal analysis of DNA templates with high data throughput. One application of these technologies is ultradeep pyrosequencing (UDPS), which makes it possible to identify LADRVs not detectable by the standard Sanger sequencing genotypic technique. Various studies from North America and Europe have shown that UDPS could identify LADRVs at frequencies as low as 0.05% of the entire viral population and enable detailed coverage of rare HIV DR variants.[9]

The majority of LADRV studies have been carried in Europe and North America, regions predominantly infected with HIV subtype B viruses. Less information is available from Sub-Saharan Africa, a region in which non-B subtypes are prevalent and which reportedly has the highest projected rate of emerging transmitted HIV drug resistance.[4] The aim of our study was to survey the prevalence of LADRVS among treatment-naive populations in North Rift Kenya using NGS techniques to inform on current status of HIV DR in the country.

Materials and Methods

Between September 2009 and October 2011, patients who were ART naive according to WHO guidelines were consecutively enrolled at the Moi Teaching and Referral Hospital, Eldoret. After informed consent was obtained, a standardized questionnaire was administered to assess demographic, epidemiological, clinical, and treatment information. Remnant blood samples from clinical analyses were acquired and DNA was extracted and amplified as per the procedures previously outlined.[10] Briefly, viral RNA was reverse transcribed and amplified according to the manufacturer's directions using the QIAGEN one-step RT-PCR kit (QIAGEN, Canada). Reverse transcriptase (RT) primers were TGAARGAITGYACTGAR AGRCAGGCTAAT and CCTCATTYTTGCATAYTTYCC TGTT with cycling conditions of 50°C 40 min one cycle in the first step, 95°C 15 min one cycle in the second step, 35 cycles at 95°C 30 s, 53°C 30 s, and 72°C 2 min 30 s in the third step, one cycle at 72°C 10 min in the fourth cycle, and one cycle at 4°C as a final extension. Nested PCR was conducted using fusion primers with forward primers tagged with multiplex identifiers (MID). The sequences of the MID-tagged primers are shown in Table 1. All MID-tagged forward fusion primers consisted of a forward primer adaptor sequence (5′-CGT ATCGCCTCCCTCGCGCCA-3′) and a reverse adaptor (5′-CTATGCGCCTTGCCAGCCCGC-3′).

All nested PCR procedures were performed using common reaction conditions at an annealing temperature of 58.9°C. The resulting PCR amplicons were purified, quantified, and pyrosequenced using 1/16 the capacity of a full GS FLX Titanium PicoTiterPlate. Reads that passed the quality control software, which were of sufficient read length to cover the amplicon and could be successfully mapped to the HXB-2 reference sequence, underwent further analysis.

Sequence and phylogenetic analysis

The valid pyrosequencing reads were aligned with HXB-2 and the frequency and distribution of nucleotide and AA changes were determined using an in-house Perl script.[10] Drug resistance mutations were identified using the IAS-USA HIV DR mutation database with a threshold of 0.1% of sequences set to show the same mutation.

Phylogenetic analysis was performed on the group of pyrosequencing reads containing the TDR mutation of interest, and subtype determination was done with reference sequences.

Results

Sequence analysis and drug-resistant mutations (DRMs)

Sixty samples from ARV-naive patients were successfully sequenced of which 25 were subtypes A, 11 were subtype D, 1 was subtype C, and the remaining were recombinants. Forty-six (76.6%) had at least one drug resistance mutation, with 25 (41.6%) indicated as major and the remaining 21 (35%) indicated as minor. Thirty-one of the 60 patients (51.67%) had the RT mutations. Nineteen patients (31%) had established NRTI-resistant mutations. The most prevalent mutation was NRTI position K219Q/R (11 of 46, 24%) followed by NRTI M184V/I (5 of 46, 11%) and NNRTI K103N (4 of 46, 9%). Table 2 shows the profiles of the patients, the DRM frequencies, and the subtypes.

Discussion

Our use of NGS technology revealed a high prevalence of low abundance drug-resistant variants among the drug-naive populations of North Rift Kenya. To our knowledge this is

Table 1. Primer Sequences Used in the Analysis

454-1F-MID-X	Degr_Seg1_F	2251 ~ 2270	TCCCTCARATCACTCTTTGG	462 bp	Seg 1 covers the whole PR
454-1R	Degr_Seg2_R	2692 ~ 2713	GGRTTTTYAGGCCCAATTTT		
454-2F-MID-X	Degr_Seg2_F	2583 ~ 2602	TKAAAG CCAGGRATGGATGG	390 bp	Cover aa 1 ~ 140 in RT
454-2R	Degr_Seg3_R	2954–2973	TCCCTGGTGTCTCATTGTTT		
454-3F-MID-X	Degr_Seg3_F	2870 ~ 2890	AGTACTRGATGTGGGWGATGC	400 bp	Cover aa 107 ~ 240 in RT

Primers for nested PCR (preoptimized tagged primers).
PR, protease; RT, reverse transcriptase.

Table 2. Drug Resistance Mutation Frequencies Observed in the Antiretroviral Drug-Naive Population in North Rift Kenya

Sample ID	*Gender*	*Age*	*Mutations and percentages*	*Subtype*
NLHG-72	Female	39	V82A 1.03	D
NLHG-77	Male	51	M184V 1.75, N88D1.14	A1
NLHG-78	Female	28	K103N 7.14	A1
NLHG-79	Female	28	K219R 2.22, L24I 5.88I, M184V 1.79, G190E 2.22	C
NLHG-80	Male	33	Y188H 1.21, I54T 1.07, K219Q 2.13, K219E 1.06	D
NLHG-83	Male	45	K219R 1.05, N83D 1.66, K219E1.05	A1
NLHG-84	Male	36	K219R 1.72, F77L 2.21, G190E 36.29, K219Q 3.86, K219E 1.72	AE/D
NLHG-85	Female	46	L1004.93I, L24I1.75, K101E1.75	A2/D
NLHG-88	Male	30	M46L 1.66, Y188H 1.22, V82A 1.07, F53Y 1.34, G73S 1.07	A1
NLHG-89	Female	29	M184V 1.08, G190E 1.08	A1
NLHG-90	Female	54	I541.27 T, M184V 1.95	AE/D
NLHG-91	Female	44	Y188H 1.64, L24I 2, N88S1.92, I84V 1.92, I50V 1.89, Y181C 1.61	D
NLHG-92	Female	27	K101E 2.04, K219Q 7.06	B
NLHG-94	Female	41	K101E 50	A/AE
NLHG-95	Female	30	K65R 1.12, I47V 1.47	A1
NLHG-96	Female	52	F77L 2.59, D67G 1.72	A1
NLHG-97	Female	30	K219R 1.49, L100 4.23I, K65R 1.11, V32I 4.17, K219Q 7.46, K219E 2.99	A1
NLHG-99	Female	39	M184I3.28, N83D 1.39, N88S 1.39, I50V 2.78, I54T 2.78, D67G 16.67, V82A1.39	A1
NLHG-100	Male	40	K219Q 1.33	A1
NLHG-101	Female	39	K101E 4.86	A1
NLHG-103	Male	63	M184V 3.7, F53L 1.54	A1
NLHG-105	Female	58	D67N 8.33, T69D8.33	A1
NLHG-107	Female	25	K101E 4.44, N83D 2.86	A1
NLHG-110	Male	39	D30N 7.14, I85V 7.14	D
NLHG-111	Male	32	G190E 1.48, K101E 11.11, N88D 3.33, K70R 11.11, T215Y 1.19	A1
NLHG-114	Female	28	N88D1.36, K219Q 3.23, L74V 3.03	A1
NLHG-115	Female	36	K219Q 9.09	A1/B/D
NLHG-117	Male	30	K219Q 3.03	A1
NLHG-118	Female	26	K219Q 5.1	D
NLHG-119	Male	36	F77L 4.35, K103N 14.29	A1
NLHG-121	Female	30	K219Q1.23	D
NLHG-172	Female	33	G73S1.37	A1
NLHG-122	Female	36	I54T 7.14, I47V 7.14	D
NLHG-126	Female	66	K103N 71.43	A1
NLHG-128	Female	8	N83D 1.42, K219Q 13.64, M46I 1.41	D
NLHG-129	Female	33	D67G 3.12, N88D 1.45	A1
NLHG-133	Female	29	L100 5.26I, K219Q 10.09	A1/D
NLHG-173	Female	25	K219Q 3.33	A1/D
1NLHG-35	Male	33	M41L 2.5, M184I 1.42, L210W 2.56, T215Y 2.56	A1
NLHG-149	Female	38	K219R 8.16 K65R 1.54, K101E 1.49, K70R 1.46	A1
NLHG-175	Female	27	K103S 100, M184V 95.35, F53L 9.09, V106M 100, K70E 100	C
NLHG-150	Female	32	F77L 1.16, K101E 1.16	D
NLHG-157	Male	46	K103S 1.9, N88D 4.41, K219Q 9.92, K103N 84.81, V82A 1.43, G73S 1.43, I47V 1.43	A1/D
NLHG-159	Female	34	M184V 100, Y181V 100, N88D 5	A1
NLHG-162	Female	29	F53L1.64, N88D 1.67, K103N 3.41	A1
NLHG-164	Female	0	F53L3.77, N88D 1.85, I47V 1.85, M46I 1.85	A1

one of the rare instances in which NGS technology has been utilized to determine the status of HIV drug resistance in HIV-infected populations in the country. Our earlier studies of HIV DR on drug-naive antenatal clinic attendees in the region using direct Sanger sequencing had revealed a prevalence of transmitted drug resistance mutations of only 3.2%.[11] This high outcome with the use of NGS is in concordance with results observed elsewhere in Africa with a prevalence of 80% among drug-naive populations in Zambia infected by HIV subtype C,[12] thus confirming the high sensitivity of this technique for the detection and quantification of DRMs.

The most prevalent variants observed in our study were the mutants carrying the thymidine analogue mutations (TAM) K219Q/R and M184V/I and none with TAM-K103N. Viruses with K219Q mutations have been noted to evolve rapidly to zidovudine (AZT) resistance and show high replicative fitness in the presence of AZT. However, the M184V mutation confers high-level resistance to lamivudine (3TC), a key backbone to first-line antiretroviral treatment regimens in Kenya. The M184I mutation has been noted to be the first to appear, but is quickly replaced by the M184V mutation since this mutation has a greater ability to induce a higher replicative capacity.[13]

In the WHO drug resistance reports an increase of transmitted drug-resistant variants has been observed in Sub-Saharan Africa over time with the most commonly observed DRMs being M184V and K103N.[14] The K103N mutation in particular was noted in more than half of HIV-infected patients presenting with NNRTI resistance. Despite such evidence of increasing rates of transmitted and acquired NNRTI resistance, efavirenz or nevirapine is still a key component in first-line ART in Africa.

It should be noted that our assessment of the prevalence of each mutation was population based and we did not assemble reads into variants (haplotypes). Our sequence read lengths were short (about 400 bps) and we used three amplicons to cover the whole protease and only 1–240 amino acids of the reverse transcriptase gene. Our approach could therefore not determine if multiple mutations were on the same amplicon.

Nevertheless, the outcome of our study denotes widespread low abundance drug-resistant strains in regions with non-B subtypes. Our use of NGS technology revealed a high prevalence of LADRVs among drug-naive populations in Kenya. This scenario might have been the result of the transmission of drug-resistant viruses from partners infected with the resistant virus or selection as a result of undisclosed use of ART. Nevertheless, this calls for the use of feasible next generation sequencing technologies for the surveillance of HIV drug resistance at the population level to reliably detect and monitor emerging drug resistance patterns that may impact ART. Similarly, they will be a need for a continued follow-up of persons with LADRVS to determine clinical impact and help guide therapies for drug-naive populations.

Sequence Data

The sequence data have been deposited at GenBank under accession number SRP053141.

Acknowledgments

We wish to thank the directors and staff of AMPATH clinics and laboratories in Eldoret, Kenya, the staff of the Kenya Medical Research Institute (KEMRI) in Nairobi, and HIV laboratory personnel of the National HIV and Retrovirology Laboratories (NHRL), Ottawa for their technical support and cooperation. This study was funded by the Canadian Commonwealth Scholarship Program (CCSP) of the Canada Ministry of Foreign Affairs and International Trade (DFAIT).

Author Disclosure Statement

No competing financial interests exist.

References

1. Steegen K, Stanley L, Kenny D, *et al.:* Effectiveness of antiretroviral therapy and development of drug resistance in HIV-1 infected patients in Mombasa, Kenya. AIDS Res Ther 2009;16(6):12.
2. Lihana R, Khamadi SA, Lubano K, *et al.:* HIV type 1 subtype diversity and drug RS among HIV type 1-infected Kenyan patients initiating antiretroviral therapy. AIDS Res Hum Retroviruses 2009;25(12):1211–1217.
3. Ndembi N, Hamers RL, Sigaloff KC, *et al.:* Transmitted antiretroviral drug resistance among newly HIV-1 diagnosed young individuals in Kampala. AIDS 2011;25:905–910.
4. Bennett DE, Myatt M, Bertagnolio S, *et al.:* Recommendations for surveillance of transmitted HIV drug resistance in countries scaling up antiretroviral treatment. Antivir Ther 2008;13(Suppl 2):2.
5. World Health Organization and Joint United Nations Programme on HIV/AIDS: Global HIV/AIDS response: Epidemic update and health sector progress towards universal access: progress report 2011. Available at www.who.int/hiv/pub/progress report2011/hiv. Full report 2011, pdf.
6. Paredes R, Lalama CM, Ribaudo HJ, *et al.:* Pre-existing minority drug-resistant HIV-1 variants, adherence, and risk of antiretroviral treatment failure. J Infect Dis 2010;201(5):662–671.
7. Dudley DM, Chin EN, Bimber BN, *et al.:* Low-cost ultra-wide genotyping using Roche/454 pyrosequencing for surveillance of HIV drug resistance. PLoS One 2012;7(5):e36494.
8. Bennett DE, Camacho RJ, Otelea D, *et al.:* Drug RS mutations for surveillance of transmitted HIV-1 drug-RS: 2009 update. PLoS One 2009;4:e4724.
9. Ji H, Masse N, Tyler S, *et al.:* HIV drug resistance surveillance using pooled pyrosequencing. PLoS One 2010;5:e9263.
10. Li JZ, Paredes R, Ribaudo HJ, *et al.:* Low frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: A systematic review and pooled analysis. JAMA 2011;305:1327–1335.
11. Kiptoo M, Ichimura H, Wembe RL, *et al.:* Prevalence of nevirapine-associated resistance mutations after single dose prophylactic treatment among antenatal clinic attendees in North Rift Kenya. AIDS Res Hum Retroviruses 2008;24(12):1555–1559.
12. Gonzalez S, Tully DC, Gondwe C, and Wood C: Low-abundance resistant mutations in HIV-1 subtype C antiretroviral therapy-naive individuals as revealed by pyrosequencing. Current HIV Res 2013;11:43–49.
13. García-Lerma JG, MacInnes H, Bennett D, *et al.:* Transmitted human immunodeficiency virus type 1 carrying the D67N or K219Q/E mutation evolves rapidly to zidovudine resistance in vitro and shows a high replicative fitness in the presence of zidovudine. J Virol 2004;78:7545–7552.
14. WHO Drug resistance report 2012, available as pdf-report at www.who.int.

Address correspondence to:
Elijah Songok
Center for Virus Research
P.O. Box 54840
Kenya Medical Research Institute
Nairobi 02000
Kenya

E-mail: esongok@kemri.org

Appendix xxviii: Publication 2: Drug resistance testing in hiv infected individuals on treatment and naive: implications on treatment outcome

Journal of HIV for Clinical and Scientific Research Peertechz

Winfrida Cheriro[1,2], Gideon Kikuvi[2], Simeon Mining[3], Wilfred Emonyi[3], Erick Rutto[3], Elijah Songok[4] and Michael Kiptoo[4,5]*

[1]Mot Teaching and Referral Hospital (MTRH), Kenya
[2]Jomo Kenyatta University of Agriculture and Technology (JKUAT), Kenya
[3]Mot University School of Medicine (MUSOM), Kenya
[4]Kenya Medical Research Institute (KEMRI), Kenya
[5]South Eastern Kenya University (SEKU), Kenya

Dates: Received: 05 September, 2015; Accepted: 24 September, 2015; Published: 25 September, 2015

***Corresponding author:** Prof. Michael Kiptoo, Principal Research Scientist, 5South Eastern Kenya University (SEKU), Tel: +254722756076; E-mail: mkiptoo@kemri.org

www.peertechz.com

Keywords: HIV/AIDS; Antiretroviral; Drug resistance; Drug resistance mutations

Research Article

Drug Resistance Testing in HIV Infected Individuals on Treatment and Naive: Implications on Treatment Outcome

Abstract

Background: The Government of Kenya started offering ART in the public sector since 2003. Despite the dramatic reduction in AIDS related morbidity and mortality, the emergence and spread of drug resistance (DR) threatens to negatively impact on treatment regimens and compromise efforts to control the epidemic. Therefore, there is a need for information on the situation of DR Mutations (DRMS) and their implications on treatment.

Objectives: To evaluate DRMS and their implications on treatment in HIV infected individuals attending Moi Teaching and Referral Hospital (MTRH) clinics.

Method: In 2009, we consecutively collected plasma samples from two groups of HIV infected individuals, antiretroviral (ARV) naive and ARV experienced for more than 12 months and failing therapy according to world health organisation (WHO) guidelines. We performed genotypic DR using well established in-house Sanger sequencing methods. We then followed up the patients and compared the DRMS in relation to their drug regimens at the time of sample collection and16 months later.

Results: We successfully extracted and sequenced 75 samples. Median age was 36.7 years. Out of 41 drug naive individuals only 3 had DRMS. Out of the 34 ARV experienced, 29 had DRMS to nucleoside reverse transcriptase inhibitor (NRTI), and 31 to non NRTI (NNRTI). After 16 months from sample collection date, 20/31(64%) ARV experienced patients with DRMS had not been changed therapy and only 5/20(25%) were susceptible to primary ARV while 12/14 changed were susceptible to new ARV.

Conclusion: The information obtained in our study can serve as an indicator of ARV program efficiency in patients still on treatment, those who are to start treatment and those who are to be changed therapy due to failure. DR testing would be necessary before initiating and /or changing ART in order to achieve optimal clinical outcome.

Abbreviations

AIDS: Acquired immunodeficiency syndrome; AMPATH: Academic Model Providing Access to Health care partnership clinics; ARDR: Antiretroviral Drug resistance; ART: Antiretroviral treatment; ARV: Antiretroviral; AZT: Zidovudine; CD4: Cluster of differentiation; DNA: Deoxyribonucleic acid; DR: Drug resistance; DRMs: Drug resistance mutations; EFV: Efavirenz; HAART: Highly active antiretroviral therapy; HIV: Human immunodeficiency virus; MTRH: Moi Teaching and Referral Hospital; NASCOP: National AIDS control program; NNRTI: Nucleoside reverse transcriptase; NRTI: Nucleotide reverse transcriptase; NVP: Nevirapine; PCR: Polymerase chain reaction; PI: Protease Inhibitors; PR: Protease; RT: Reverse transcriptase; SD:Standard deviation; 3TC: Lamivudine; TDF: Tenofovir; TDR: Transmitted Drug resistance mutations;

Introduction

Most data concerning Human Immunodeficiency Virus (HIV) non-B subtypes remain controversial. Highly Active Antiretroviral Therapy (HAART) has radically changed the clinical outcome of HIV, leading to decreased mortality and morbidity. Development of HIV drug resistance is inevitable in patients on ART. Increase in antiretroviral therapy (ART) in resource-limited settings (RLS) will successfully reduce HIV-related morbidity and mortality [1]. The increase in ART coverage is expected to lead to an increase in drug-resistant strains among experienced patients. Improved access to alternative combinations of antiretroviral drugs in sub-Saharan Africa is warranted [2].

As the rollout of ART in Kenya is on the rise, there is a need to monitor the patients on ART [3]. The use of Cluster of Differentiation (CD4) and viral load measurements is important in monitoring HIV patients both immunologically and virologically. Though virological and immunological monitoring is important, there is a need to provide HIVDR testing services for patients who are starting therapy and those who are suspected to be failing treatment before they are switched to a different regimen [4]. A public-health approach based on standardized, affordable drug regimens and limited laboratory monitoring is crucial in scale up efforts. The ever-expanding rollout of antiretroviral therapy in RLS without routine virological monitoring

has been accompanied with development of drug resistance that has resulted in limited treatment success. A survey performed in Kampala showed a prevalence of transmitted drug resistance at 8.6% [5].

In Kenya, availability of ART is increasing. As ART use increases there is mounting evidence suggesting that DR will increase over time [6]. A recent cross-sectional study to determine treatment failure and drug resistance mutations among adults receiving first-line (3TC_d4T/AZT_NVP/EFV) and second-line (3TC/AZT/LPV/r) in Nairobi, Kenya, concluded that the detected accumulated resistance strains due to emergence of HIV drug resistance will continue to be a big challenge [7]. Another study carried out to evaluate treatment success and development of ART drug resistance at the Coast Province General Hospital, Mombasa, Kenya, revealed a high rate of treatment success after short term ART in patients treated at a public provincial hospital detected minority complex drug resistance profiles that were predictive of resistance to currently used second-line NRTIs and NNRTIs regimens [1]. In this article we present detailed data on DRMS from patients who had not started ARV and those who were failing with their implications on therapy. Identifying the relevant DRMS among non-B subtypes will be important for monitoring the evolution and transmission of drug resistance, determination of initial treatment strategies for persons infected with HIV non-subtype B [4]. According to International AIDS Society recommendations (IAS), evaluating susceptibility patterns among non-clade B persons should be a high priority because these viruses are by far the most prevalent world-wide. It is believed that surveillance will maximize the utility of first-line therapy and help minimize the cost of providing ART thereby sustaining current antiretroviral drug programs. The HIVDR testing is important as it gives the clinician accurate information of the most appropriate drug options. With ART scale up, there has been a need for monitoring for development of HIV drug resistance at a population level. Therefore, there is a need for country information on the situation of antiretroviral drug resistance (ARDR) to inform on policy guidelines.

Materials and Methods

Setting

The study was conducted at Moi Teaching and Referral Hospital (MTRH), AMPATH (Academic Model Providing Access To Health care partnership clinics), Eldoret, Kenya clinics.

The region includes the expansive Rift Valley, Western and Nyanza provinces, a cumulative population of about 15 million. The hospital is located in Eldoret Town in Uasin-Gishu (UG) County, which forms part of the UG Plateau West of the Great Rift Valley, at an altitude of 2118m above the sea level, latitude 00°30'52"N and longitude 035°17'52"E [2].

Study subjects

The study was conducted on isolates from patients who were known HIV positive attending the study site and met the selection criteria. During September 2009 and October 2011, patients receiving ARV therapy for at least 12 months and were suspected to be failing according to WHO guidelines were consecutively enrolled. After informed consent was obtained, a standardized questionnaire was administered to assess demographic, epidemiologic, clinical, and treatment information. ART-naive patients were also enrolled during the same period at the same study clinics. Samples from patients who had no history of exposure to ARV drugs and ARV drug naïve status according to a medical chart review and personal interview were collected consecutively.

Study design

The study conducted was a hospital-based prospective utilizing isolates from HIV positive patients who met the selection criteria. The study clinics provided ART according to the national guidelines for ART scale-up as recommended by WHO surveillance and monitoring surveys.

Data Collection Tools and Procedures

Laboratory procedures

Sample collection: Remnant blood samples collected for CD4 analysis from ARV naïve were centrifuged and plasma was collected. Remnant samples collected for viral load analysis from ARV experienced patients suspected to be failing therapy clinically were also centrifuged and plasma were collected and stored at −80°C.

HIV DNA extraction: HIV-1 nucleic acid was extracted from 400 µl of plasma using the Nuclisens Easy Mag system (Biomerieux, Canada) following manufacturer's instructions.

Reverse Transcription and polymerase chain reaction: HIV-1 protease (PR) and reverse transcriptase (RT) were bidirectional sequenced with an in-house protocol (8). Briefly, viral RNA was reverse transcribed and amplified according to the manufacturer's directions using the QIAGEN one-step RT-PCR kit (QIAGEN, Canada). The primers used were GaGp1-PR-out.for with a sequence of TGA ARG AIT GYA CTG ARA GRC AGG CTA AT and RT-new-out. Rev of CCT CIT TYT TGC ATA YTT YCC TGT T with nested primers GaGp6-PR-in.for YTC AGA RCA GRC CRG ARC CAA CAG C and RT-new-in.rev GGY TCT TGR TAA ATT TGR TAT GTC CA. All reactions were carried out using standard conditions using GeneAmp PCR System 9700 (ABI) thermocycler.

PCR product purification and sequencing

The PCR products were purified using Multi Screen Separations System as previously described [9] and diluted to 15 ng/ml for DNA sequencing. Amplicons were sequenced using ABI Prism Big Dye 3.1 Cycle Sequencing System (Applied Bio systems, USA) following manufacturer's instructions.

Data analysis

All the data generated in this study was saved in Microsoft Excel worksheets with a detailed database established to capture all the necessary information. Generated sequences were edited using Bio Edit v 7.0.5B. Aligned fasta files were uploaded to Stanford HIV Drug resistance (http://hivdb6.stanford.edu/asi/deployed/hiv_central.pl?program=hivdb&action=showSequenceForm). Phylogenetic relationships of newly derived viral sequences for comparisons with those of previously reported HIV group M from the Los

Alamos database by CLUSTAL W profile alignment was utilized. To improve the accuracy of HIV-1 subtyping, the genotyping tool (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi) was used and the REGA sub typing tool (http://dbpartners.stanford.edu/RegaSubtyping/) was utilized as needed. Drug resistance mutation and subtype data collected from the Stanford HIV database sequence analysis program were manually input into appropriate excel spreadsheet file, verified and corrections made as needed. Categorical variables were presented in form of frequency tables while continuous variables were mainly summarized using means together with standard deviation and median.

To test significance of skewed continuous variables, Wilcoxon rank sum test was employed. Chi-square test was used to compare the association between categorical variables. Fisher's exact test was also used to compare categorical variables where some cells had expected value of less than 5. Level of significance was set at $p < 0.05$, with a 95 % confidence interval. All analyses were done using STATA version 11.0.

Ethical considerations

The study was approved by the Institutional Research and Ethics Committee (IREC) of the Moi University School of Medicine (MUSOM) and MTRH Review Board (IREC):(IREC/2010/06) and AMPATH (RES/STUD/17/2010).

Results

Socio-demographic characteristics

Among the 264 individuals who met the selection criteria, 128 were declared to be ART naïve and 136 had been on ARV for more than 12 months and were failing therapy clinically. Mean age was 37.01 years (SD=12.50). Majority were female 44(60.02%) (Table 1).

Polymerase Chain Reaction and Sequencing outcomes: One hundred and ten (110) samples were successfully amplified. Out of these, 75 samples were successful sequenced and analysed for the presence of drug resistance mutations. Out of 75, ARV experienced individuals failing therapy were 34 and most patients, 25 (73.5%) received 3TC + d4T/AZT + EFV/NVP as first-line treatment. Patients who reported treatment interruption or switch were 9(26.5%). Switch concerned mainly replacement of d4T or AZT by TDF or ABC and only 3(8.8%) had been switched to protease inhibitor (PI) regimens (Tables 1-3).

Drug resistance mutations in ARV naive

Drug resistance mutations were identified in 3/41 (7.3%) of patients and per drug class the values were as follows: 1 for PI and 2 for NRTI and 1 for NNRTI. In 1 patient, multiple mutations against 2 drug classes were seen, suggesting that they were probably not naïve (Table 4). Approximately 3/28(10.7%) of female subjects had DRMS. None of their male counterparts had mutations. There was no statistical difference when male vs. female respondents were compared (p=0.235).

Drug resistance mutations in ARV experienced

Among the ARV experienced patients who were failing therapy according to WHO guidelines, 34 samples which were successfully extracted and sequenced were studied. Mean age was 35.85years (SD=14.06). Majority were male 18(52.9%). Out of 34 samples, 27 had DRMS to nucleoside reverse transcriptase inhibitor (NRTI), 30 had DRMS to non-nucleoside reverse transcriptase inhibitor (NNRTI), and 2 had DRMS to NNRTI only (Table 5).

ARV therapy sixteen months after sample collection

Sixteen months after sample collection, 20/34 ARV experienced patients failing therapy were still on the same ARVs. Only 4/20(20%) of these patients were susceptible to the ARVs they were taking from DRMS analysis. Twelve out of the fourteen who were changed

Table 1: Socio-demographic and clinical characteristics among study subjects.

Characteristic	N, (%)
Male	31 (39.8)
Female	44 (60.2)
Median age, (IQR)	36.29 (28.80-45.81)
Mean age (sd)	37.01 (12.50)
Median CD4 count, (IQR)	287 (119.0-430.0)
Median viral load, (IQR)	10,970 (1,676.0-59,901.0)

N; number, (%); percentage, IQR; interquartile range, sd; standard deviation, CD4; cluster of differentiation.

Table 2: Patient Regimen.

Regimen	N, (%)
d4T-3TC-NVP	12 (29.4)
d4T-3TC-EFV	2 (5.9)
AZT-3TC-NVP	11 (26.5)
TDF-3TC-EFV	2 (5.9)
TDF-3TC-NVP	3 (8.8)
Alluvia	3 (8.8)
ABC-3TC-EFV	1 (14.7)

EFV; Efavirenz; 3TC; Lamivudine; NVP; Nevirapine, AZT; zidovudine, d4T; Stavudine, TDF; Tenofovir, ABC;Abacavir, LPV/r; Lopinavir, ALUVIA; Lopinavir /Ritonavir.

Table 3: Differences between Patient characteristics between the two study groups.

	ARV Naive		ARV Experienced	
Sex	F	M	F	M
Age	28	13	16	18
Mean Age	36	40	36	35
Mean Viral loads	-	-	50,669	34,527
Mean CD4 counts	405	218	178	287
HIV Subtype A	16	8	11	14
B	2	1	1	1
C	3	1	0	0
D	7	3	4	2
G	0	0	0	1

therapy were susceptible to the new drugs and 2 patients had their ARVs changed into other drugs that they were already resistant to (KE12-027, KE12043) (Table 6).

Discussion

The results present depiction of the importance of HIV DR testing in a resource limited setting and their implications on treatment in both ARV naive and ARV experienced failing therapy before starting or changing therapy. However, we noted a low rate of amplification which may have been due to integrity of sample storage and transportation. Sequences obtained from 35 samples that were successfully amplified did not meet the integrity of good sequences for final drug resistance analysis.

Drug resistance mutations in ARV naive

In our study, drug resistant mutants were detected in three patients who were ARV naïve according to chart review. The prevalence of DRMs among drug naive populations revealed in our

Table 4: DRMS in ARV Naive Patients.

PID	AGE	GENDER	NRTI drms	NNRTI drms	PI drms
KE12-086	30	FEMALE	M184I	NONE	I50V
KE12-112	36	FEMALE	T215I	NONE	NONE
KE12-128	66	FEMALE	NONE	K103N	NONE

NRTIs; nucleoside reverse transcriptase inhibitors, drms; drug resistant mutations NNRTIs; non-nucleoside reverse transcriptase inhibitors, Pi; protease inhibitors.

Table 5: DRMS and level of resistance to baseline ARVs.

PID	NRTI	NNRTI	PI	Level of RS to baseline ARV
KE12-006	M41L,K70R,M184V, T215SY	Y181C, G190S	None	HL RS to TDF,3TC
KE12-007	K65R, M184V	Y181C	None	HL RS to NVP,3TC
KE12-009	None	K103N	None	susceptible
KE12-016	None	None	None	susceptible
KE12-018	F116Y, Q151M, M184V	K103N,Y181I,P225H	None	susceptible
KE12-023	K70R, M184V,T215FIS	K103N, P225H	None	HL RS to EFV,3TC
KE12-027	M184V	K103N, Y188L	None	HLRS to NVP,3TC
KE12-028	M184V	G190A	None	HLRS to NVP,3TC
KE12-030	M184V, T215SY	K103N	None	HLRS to NVP,3TC
KE12-031	M184V,T215Y,K219Q	K103N, M230L	None	HLRS to NVP,3TC
KE12-034	None	None	None	susceptible
KE12-035	K70R, M184V	Y181C	None	HLRS to NVP,3TC
KE12-036	M184V	G190A	None	HLRS to NVP,3TC
KE12-037	M184V	K103N	None	HLRS to NVP,3TC
KE12-039	D67N,M184V,L210W,T215Y,K219Q	Y181V	None	HLRS to NVP,3TC
KE12-040	M184V, T215Y	Y181C	None	HLRS to NVP,3TC
KE12-043	M184V, T215F	G190A	None	HLRS to NVP,3TC
KE12-052	D67N, K70R, M184V, K219Q	K103N	None	HLRS to NVP,3TC
KE12-058	M184V, T215Y	Y181C	None	HLRS to NVP,3TC
KE12-059	K70R, M184V, K219Q	Y181C	None	susceptible
KE12-060	K65R, D67N,Y115F, F116Y, K219E	G190E	None	HLRS to EFV,ABC
KE12-081	K70R, M184V, K219Q	K103N	None	HLRS to NVP,EFV
KE12-093	None	None	None	susceptible
KE12-245	M41L,K70R,V75M,M184V,L210W,T215F	G190A	None	HLRS to NVP,3TC
KE12-246	M184V	K103S, G190A	None	HLRS to NVP,3TC
KE12-250	None	K103N	None	HL RS to NVP
KE12-252	D67N	K103N	None	HL RS to NVP
KE12-282	D67N, M184V, T215I	Y181C, G190A	None	HLRS to NVP,3TC
KE12-300	M184V	K103N	None	HLRS to NVP,3TC
KE12-307	M184V	G190A	None	HLRS to NVP,3TC
KE12-309	M184V	K103N, V106M	None	HLRS to NVP,EFV
KE12-316	D67N, K70R, M184V, K219Q	K101E, G190A	None	HLRS to NVP,3TC
KE12-324	K70R, M184V	Y181C	None	susceptible
KE12-326	M184V	G190A	None	HLRS to NVP,3TC

PID; patient identification, NNRTIs; non-nucleoside reverse transcriptase inhibitors, NRTIs; nucleoside reverse transcriptase inhibitors, PI; Protease inhibitors, RS; resistant, HLRS; highly resistant, ARV; antiretroviral therapy; EFV; Efavirenz; 3TC; Lamivudine; NVP; Nevirapine, AZT; zidovudine, d4T; Stavudine, TDF; Tenofovir, ABC;Abacavir.

Table 6: The ARV therapy, level of susceptibility and resistance.

PID	ARV on sample collection	RS to primary ARV	ARV at 16 months	RS to changed ARV
KE12-006	TDF-3TC-EFV	HL RS to TDF,3TC	Not changed	
KE12-007	TDF-3TC-NVP	HL RS to NVP,3TC	Not changed	
KE12-009	ALUVIA	susceptible	TDF,3TC,NVP	susceptible
KE12-016	TDF-3TC-NVP	susceptible	Not changed	
KE12-018	ALUVIA	susceptible	LPV,3TC,TDF,ABC	Susceptible
KE12-023	TDF-3TC-EFV	HLRS to EFV,3TC	LPV,3TC,TDF,ABC	Susceptible
KE12-027	d4T-3TC-NVP	HLRS to NVP,3TC	AZT-3TC-NVP	Resistant
KE12-028	AZT-3TC-NVP	HLRS to NVP,3TC	ALUVIA,3TC,TDF	Susceptible
KE12-030	AZT-3TC-NVP	HLRS to NVP,3TC	ALUVIA,3TC,TDF	Susceptible
KE12-031	AZT-3TC-NVP	HLRS to NVP,3TC	ALUVIA,3TC,TDF	Susceptible
KE12-034	d4T-3TC-NVP	susceptible	Not changed	
KE12-035	d4T-3TC-NVP	HLRS to NVP,3TC	ALUVIA,3TC,TDF	Susceptible
KE12-036	d4T-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-037	d4T-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-039	d4T-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-040	d4T-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-043	d4T-3TC-NVP	HLRS to NVP,3TC	TDF,3TC,NVP	Resistant
KE12-052	TDF-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-058	d4T-3TC-NVP	HLRS to NVP,3TC	ALUVIA,3TC,TDF	Susceptible
KE12-059	ALUVIA	susceptible	Not changed	
KE12-060	ABC-3TC-EFV	HLRS to EFV,ABC	Not changed	
KE12-081	d4T-3TC-EFV	HLRS to NVP,EFV	TDF,3TC,NVP	Susceptible
KE12-093	AZT-3TC-NVP	susceptible	Not changed	
KE12-245	AZT-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-246	AZT-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-250	AZT-3TC-NVP	HL RS to NVP	Not changed	
KE12-252	d4T-3TC-NVP	HL RS to NVP	ALUVIA,3TC,TDF	Susceptible
KE12-282	AZT-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-300	AZT-3TC-NVP	HLRS to NVP,3TC	Not changed	
KE12-307	AZT-3TC-NVP	HLRS to NVP,3TC	ALUVIA	Susceptible
KE12-309	d4T-3TC-EFV	HLRS to NVP,EFV	Not changed	
KE12-316	d4T-3TC-NVP	HLRS to NVP,3TC	ALUVIA	Susceptible
KE12-324	AZT-3TC-NVP	susceptible	Not changed	
KE12-326	d4T-3TC-NVP	HLRS to NVP,3TC	Not changed	

PID; patient identification, NNRTIs; non-nucleoside reverse transcriptase inhibitors, NRTIs; nucleoside reverse transcriptase inhibitors, RS; resistant, HLRS; highly resistant, ARV; antiretroviral therapy; EFV; Efavirenz; 3TC; Lamivudine; NVP; Nevirapine, AZT; zidovudine, d4T; Stavudine, TDF; Tenofovir, ABC;Abacavir, LPV/r; Lopinavir, ALUVIA; Lopinavir /Ritonavir.

study might have been the result of the transmission of drug-resistant viruses from partners infected with the resistant virus or selection as a result of undisclosed use of ART. One patient had multiple NRTI drug resistance mutations, an indication that the patient may have had previous drug exposure. Although mutations conferring NRTI resistance have previously been reported among drug naive patients, the possibility that our patients had previous unreported contact with antiretroviral drugs could not be excluded [8].

Drug resistance mutations in ARV experienced

From our study, we observed that due to lack of information about any existing mutations before the therapy were changed, only 4/20(20%)who had not changed therapy were susceptible to the ARVs they were taking from DRMS analysis. Twelve out the fourteen who had their therapy changed were susceptible to the new drugs and the two patients had their therapy changed into ARVs they were already

resistant to (KE12-027, KE12043). Most harboured a mutation at position M184I/V associated with 3TC and EFV resistance. The M184V on the other hand confer high level resistance to 3TC, a key backbone to first line antiretroviral treatment regimens in Kenya. The M184I mutation has been noted to be the first to appear but is quickly replaced by the M184V since this mutation has greater ability to induce higher replicative capacity [10].

Majority of the patients we reported with drug resistance mutations, were resistant to AZT and/or d4T because they harboured either mutation in the RT gene associated with resistance to RT inhibitors: multi NRTI-69 insertion complex b which affects all NRTIS (K70R (n=2), T215YF (n=5), K219QE (n=3). The presence of 3 of the following mutations M41L, D67N, L210W, T215Y/F, and K219Q/E has been associated with resistance to didanosin [6]. The presence of these mutations may improve subsequent virologic response to NNRTI-containing regimens (nevirapine or efavirenz) in NNRTI-naive individuals, although no clinical data exist for improved response to etravirine in NNRTI experienced individuals. KE12-027 had K65R, M184V while KE12043 had both M184V and T215Y. When associated with TAMs, M184V has been reported to increase abacavir resistance [11]. Studies have shown that the presence of K70R or M184V alone does not decrease virologic response to didanosine. The presence of mutations G190A had already compromised the use of next generation NNRTI etravirine. Importantly, presence of the K65R mutation compromises also the use of second-line regimens. Previous findings suggest that implementation of programs to consider the various socioeconomic and cultural barriers that may prevent successful uptake of antiretroviral prophylaxes are important [12].

Practical implications

Patients whose physicians have access to information about any existing mutations before the therapy are changed usually have more significant decreases in the viral load than patients in whom treatment is changed without knowledge of the resistance profile. Similar studies have led to development of new NRTIs, as well as new NNRTIs and PIs with different resistances profiles [3]. The options after treatment failures if improved will thereby increase the importance of resistance testing. On the other hand, simply changing national recommendations for initial ARV therapy from an NNRTI-based regimen to a protease inhibitor (PI)-based regimen would be suboptimal because PI-based regimens are more expensive and often less tolerated than NNRTI-based regimens. From our study, practical implications show that 80% (20/34) of patients whose therapy was not changed based on immunological and virological results only had DRMS to the regimens they were still taking while 85% (12/14) of those changed therapy were susceptible to the new regimens as per DRMS Stanford report. It is worth noting that M184V mutation is also associated with reduced viral replication and this may explain the reason for not changing regimen for the individuals that harbour this mutation.

Therefore, pre-therapy genotypic resistance testing would be useful to identify which patients should receive standard first-line therapy and which should receive a PI-containing regime [12].

Limitations

Viral load data was only available for ARV experienced patients who were failing therapy clinically.

Conclusion

The outcome of our study denotes high drug resistant strains in regions with non- B subtypes. The high prevalence of DRMs among drug experienced with evidence of drug failure populations revealed in our study might have been due to lack of DR analysis before start of therapy.

Acknowledgements

The authors would like to express their gratitude to the institutional research and ethics committee (IREC) and the AMPATH research office for permission to conduct this study. The authors also wish to thank the directors and staff of AMPATH clinics and laboratories, for providing logistics and technical support and for their cooperation.

Recommendations

Drug resistance testing would be necessary before initiating and /or changing ART in order to achieve an optimal clinical outcome. Similarly, there will be a need for a continued follow-up of persons with DRVS to determine clinical impact and help guide therapies for drug naïve and experienced with evidence of drug failure populations.

Authors' contributions

WC(MTRH) conceived the study, carried out field work, sample collection; sample analysis and manuscript preparation; GK(JKUAT) and SM(MUSOM) coordinated project implementation, WE(MUSOM) coordinated sample collection and analysis; ER(MUSOM) coordinated clinical oversight, ES(KEMRI) did project implementation oversight and overall supervision; MK(KEMRI/SEKU) coordinated field work, overall supervision and manuscript preparation. All authors read and approved the manuscript.

Sequence data

The sequence data has been deposited at Gen bank under accession numbers KP877888 to KP877961 and KR003380 to KR003405.

References

1. Steegen K, Luchters S, Dauwe K, Reynaerts J, Mandaliya K, et al. (2009) Effectiveness of antiretroviral therapy and development of drug resistance in HIV-1 infected patients in Mombasa, Kenya. AIDS research and therapy 6: 12.
2. Einterz RM, Kimaiyo S, Mengech HN, Khwa-Otsyula BO, Esamai F, et al. (2007) Responding to the HIV pandemic: the power of an academic medical partnership. Acad Med 82: 812-818.
3. Gilks CF, Crowley S, Ekpini R, Gove S, Perriens J, et al. (2006) The WHO public-health approach to antiretroviral treatment against HIV in resource-limited settings. Lancet 368: 505-510.
4. Lwembe R, Ochieng W, Panikulam A, Mongoina CO, Palakudy T, et al. (2007) Anti-retroviral drug resistance-associated mutations among non-subtype B HIV-1-infected Kenyan children with treatment failure. J Med Virol 79: 865-872.
5. Bennett DE, Camacho RJ, Otelea D, Kuritzkes DR, Fleury H, et al. (2009) Drug resistance mutations for surveillance of transmitted HIV-1 drug-resistance: 2009 update. PloS one 4: e4724.

6. Lihana RW, Khamadi SA, Lwembe RM, Kinyua JG, Muriuki JK, et al. (2009) HIV-1 subtype and viral tropism determination for evaluating antiretroviral therapy options: an analysis of archived Kenyan blood samples. BMC infectious diseases 9: 215.

7. Koigi P, Ngayo MO, Khamadi S, Ngugi C, Nyamache AK (2014) HIV type 1 drug resistance patterns among patients failing first and second line antiretroviral therapy in Nairobi, Kenya. BMC research notes 7: 890.

8. Lihana RW, Khamadi SA, Lubano K, Lwembe R, Kiptoo MK, et al. (2009) HIV type 1 subtype diversity and drug resistance among HIV type 1-infected Kenyan patients initiating antiretroviral therapy. AIDS res hum retroviruses 25: 1211-1217.

9. Ji H, Masse N, Tyler S, Liang B, Li Y, et al. (2010) HIV drug resistance surveillance using pooled pyrosequencing. PloS one 5: e9263.

10. Garcia-Lerma JG (2005) Diversity of thymidine analogue resistance genotypes among newly diagnosed HIV-1-infected persons. J Antimicrob Chemother 56: 265-269.

11. Johnson VA, Calvez V, Gunthard HF, Paredes R, Pillay D, et al. (2011) update of the drug resistance mutations in HIV-1. Top Antivir Med 19: 156-164.

12. Gupta RK, Goodall RL, Ranopa M, Kityo C, Munderi P, et al. (2014) High rate of HIV resuppression after viral failure on first-line antiretroviral therapy in the absence of switch to second-line therapy. Clinical infectious diseases: an official publication of the Infectious Diseases Society of America 58: 1023-1026.

Citation: *Cheriro W, Kikuvi G, Mining S, Emonyi W, Rutto E, et al. (2015) Drug Resistance Testing in HIV Infected Individuals on Treatment and Naive: Implications on Treatment Outcome. J HIV Clin Scientific Res 2(3): 062-068.*

068