Molecular characterization of HIV-1 drug resistance mutations among ARV-treated female commercial sex workers in a Nairobi cohort

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DECLARATION

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

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DEDICATION

I dedicate this work to my parents Harrison and Enilice Meeme and my siblings

Eddie, Ken and Betty.

ACKNOWLEDGEMENTS

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ABBREVIATIONS AND ACRONYMS

3TC   Lamivudine

ABC   Abicavir

ART   Antiretroviral therapy

ARV   Antiretroviral

AZT   Zidovudine (also ZDV)

CRF   Circulating Recombinant Forms

CRI   Co-receptor Inhibitor

d4T   Stavudine

ddI   Didanosine

DLV   Delavirdine

EFV   Efavirenz

ETR   Etravirine

FTC   Emtricitabine

HAART   Highly Active Antiretroviral Therapy

HIVDR   HIV Drug Resistance

HIVResNet   HIV Drug Resistance Surveillance Network

IAVI   International AIDS Vaccine Initiative

JKUAT   Jomo KenyattaUniversity of Agriculture and Technology**.**

KEMRI   Kenya Medical Research Institute.

MOH   Ministry of Health

NIAID   National Institute of Allergy and Infectious Diseases

NNRTI   Non-Nucleoside Reverse Transcriptase Inhibitor

NRTI	Nucleoside Reverse Transcriptase Inhibitor
NVP	Nevirapine
PEPFAR	The United States President's Emergency Plan for AIDS Relief
PI	Protease Inhibitor
RTI	Reverse Transcriptase Inhibitor
TDF	Tenofovir Disoproxil Fumarate
UNAIDS	United Nations' Programme on HIV/AIDS
URF	Unique Recombinant Forms
WHO	World Health Organization

ABSTRACT

Antiretroviral drug resistance is a major contributing factor in treatment failure. Resistance to drugs develops as a result of mutations within the proteins targeted by drugs. Different HIV-1 subtypes follow different paths to resistance, differ in codon sites critical to resistance and also differ in viral fitness and sequence analysis of an antiretroviral-target region provides information on presence of resistance and genetic divergence from conventionally used reference sequences. The objective of this study was to determine the pattern and prevalence of HIV-1 subtypes and HIV-1 drug resistance among ARV-treated women from the Pumwani cohort based in Nairobi, Kenya.

This was a cross-sectional, laboratory-based retrospective study involving genotypic characterization of HIV-1 RNA from stored plasma samples obtained from persons enrolled in an ART programme for over six months. Viral RNA was extracted from 84 archived plasma samples. A region of the reverse transcriptase gene (697bp) was amplified by nested polymerase chain reaction (PCR) and sequenced using v3.1 BigDye® Terminator™ technology. Sequence alignment and phylogenetic analyses were performed in Genetyx® and Treeview® using ClustalW and referenced to The Los Alamos and Stanford HIV databases.

Sixty sequences were available for analyses. Sequence alignment and phylogenetic analysis showed 58 isolates (96.7%) to be subtype A1 and 2 isolates (3.3%) to be subtype D. Five isolates (8.3%) had virus population with reverse transcriptase

inhibitor-associated resistance mutations. Of the 5, 3 were infected with subtype A1 virus and 2 with subtype D virus. Complete class resistance was identified in 2 isolates (3.3%). Minor mutations and polymorphisms were detected in all the 60 isolates that were analysed. HIV-1 subtype A1 dominates in this population while ARV- associated resistance mutations occurred in 8.3% of the patients, a low prevalence for a treated population. As sex workers are classified as a high risk group in HIV transmission, there is an urgent need to incorporate antiretroviral resistance monitoring protocols among HIV prevention programmes at both cohort and national level.

CHAPTER ONE

INTRODUCTION

In the early 1980s, a number of gay men in New York and California suddenly began to develop rare opportunistic infections and cancers such as Kaposi's sarcoma and *Pneumocystis carinii* pneunomia that seemed stubbornly resistant to any treatment (Hymes *et al.*, 1981). Signs of the syndrome that would later be named Acquired Immune Deficiency Syndrome (AIDS) had however been recorded as early as 1978 among gay men in the US and Sweden and heterosexuals in Tanzania and Haiti (Aegis 1998).In 1983, Luc Montagnier's research team in Paris provided evidence linking a retrovirus to AIDS. The following year, further data from Robert Gallo's group in the United States provided convincing evidence that this retrovirus, later named Human Immunodeficiency Virus (HIV) was the cause of AIDS (Fauci, 2008).

HIV belongs to the *lentivirus* genus of the family *Retroviridae*. It is transmitted as single-stranded, positive-sense, enveloped RNA virus. There are two strains of HIV known to exist: HIV-1 and HIV-2. HIV-1 is the virus that was initially discovered and termed lymphadenopathy-associated virus (LAV). It is more virulent, has a higher transmissibility rate, and is the cause of the majority of HIV infections globally. HIV-2 is less transmissible and is largely confined to West Africa. (Reeves and Doms, 2002) The purported origin of HIV-1 is the Common Chimpanzee while HIV-2 is thought to have originated from the Sooty Mangabey (Gao *et al.*, 1999).

1.1 HIV-1 structure

HIV is roughly spherical with a diameter of approximately 120 nm (McGovern *et al.*, 2002). It is composed of two copies of positive single-stranded RNA that codes for

the virus's nine genes enclosed by a conical capsid composed of 2,000 copies of the viral protein p24 (Kuiken *et al*., 2008). The single-stranded RNA is tightly bound to nucleocapsid proteins, p7 and the enzymes reverse transcriptase, protease, ribonuclease and integrase. A matrix composed of the viral protein p17 surrounds the capsid. This is in turn, surrounded by the viral envelope composed of two layers of phospholipids. Embedded in the viral envelope are proteins from the host cell and about 70 copies of a complex HIV protein that protrudes through the surface of the virus particle (Kuiken *et al*., 2008).This protein, known as Env, consists of a cap made of three glycoprotein (gp) 120 molecules, and a stem consisting of three gp41 molecules that anchor the structure into the viral envelope (Chan *et al*., 1997). This glycoprotein complex enables the virus to attach to and fuse with target cells to initiate the infectious cycle.

The RNA genome consists of at least 7 structural landmarks (LTR, TAR, RRE, PE, SLIP, CRS, INS) and nine genes encoding 19 proteins. *Gag*, *pol*, and *env* are structural genes while *tat*, *rev*, *nef*, *vif*, *vpr*, and *vpu* are regulatory genes for proteins that control the ability of HIV to infect cells, replicate and cause disease (Kuiken *et al*., 2008). In some strains of HIV, a mutation causes the production of an alternate accessory protein, *tev*, from the fusion of *tat*, *rev*, and *env* (Peterlin and Trono, 2003).

1.2 HIV-1 replication cycle

The replication cycle of HIV-1 involves five major steps; a) attachment and fusion, b) reverse transcription, c) integration, d) transcription and translation, e) assembly, maturation and budding (Fig. 1.1).

Viral entry to the host cell begins with attachment of the HIV envelope glycoprotein (gp120) to the CD4 T-cell receptor (Briz *et al.*, 2006). This interaction produces a conformational change in gp120, which allows it to bind to the chemokine co-receptor, either CCR5 or CXCR4. CCR5 and CXCR4 bind the V3 region of HIV gp120. Further conformational changes in gp120 occur after CCR5 or CXCR4 binding, allowing the HIV envelope gp41 fusion peptide to insert into the cellular membrane, causing the virion to fuse with the host membrane (Lewis *et al*., 2008).

Once the HIV virion enters the cell cytoplasm, it is uncoated and the viral RNA genome undergoes reverse transcription into proviral DNA. Viral uncoating involves cellular factors and the viral proteins matrix protein p17, Nef and Vif (Sierra *et al*., 2005). The proviral DNA becomes part of the pre-integration complex, which enters the nucleus (Este and Telenti, 2007) through the nuclear pore (Le Rouzic and Benichou, 2005).

The proviral DNA is then incorporated (integrated) into the host-cell genome using the HIV integrase enzyme (Savarino, 2006). This process involves recognition of specific sequences within the long terminal repeats (LTRs) of viral cDNA by the integrase protein, followed by colinear insertion into chromosomal DNA, resulting in the formation of the provirus (Ferguson *et al*., 2002). Following integration into the host chromosome, the integrated provirus serves as the template for the synthesis of the viral RNAs that ultimately encode the full complement of structural, regulatory, and accessory proteins used to direct virus replication (Freed, 2001).

The integrated DNA provirus is transcribed into mRNA, which is then spliced into smaller pieces.Transcription of viral RNAs is achieved through the RNA polymerase II enzyme. The smaller spliced units are exported from the nucleus into the cytoplasm, where they are translated into the regulatory proteins Tat and Rev. As the newly produced Rev protein accumulates in the nucleus, it binds to viral mRNAs and allows unspliced RNAs to leave the nucleus, where they are otherwise retained until spliced (Pollard and Malim, 1998). HIV viral polyproteins are then expressed using host cell machinery. At this stage, the structural proteins Gag and Env are produced from the full-length mRNA.

Assembly of new HIV-1 virions begins at the plasma membrane of the host cell. The *env* polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The *gag* (p55) and *gag-pol* (p160) polyproteins associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell (McNicholl and McNicholl, 2006). Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion (Gelderblom, 1997).
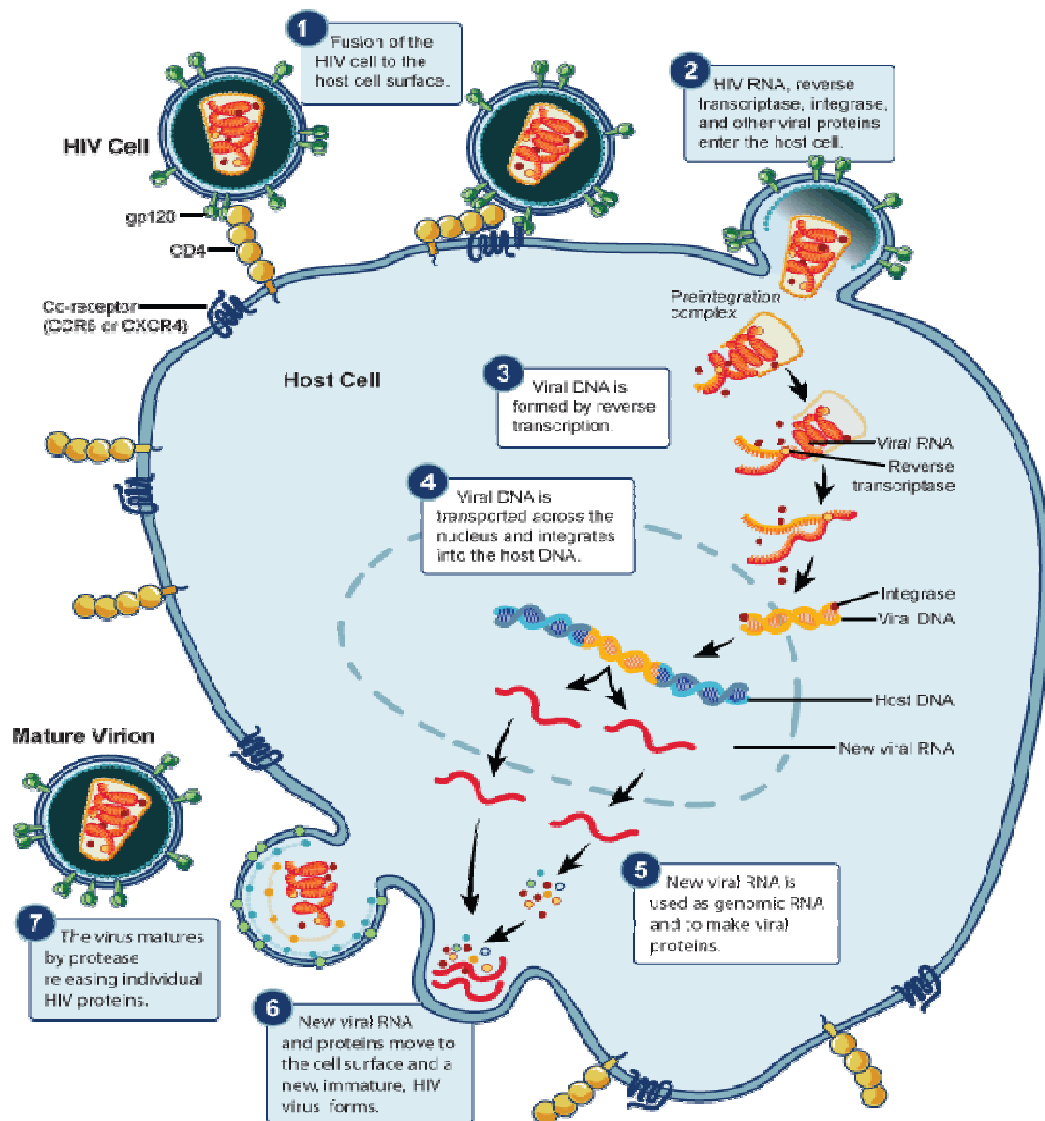
Figure 1.1. The HIV-1 replication cycle. (Source: NIAID, 2009)

1.3 Antiretroviral drugs

Currently available antiretroviral classes include nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, CCR5 inhibitors and integrase inhibitors.

These agents target such major steps in the HIV life cycle as attachment, entry, reverse transcription, integration, protein processing and maturation (Dau and Holodniy, 2009).

1.3.1 Nucleoside Reverse Transcriptase Inhibitors

Nucleoside Reverse Transcriptase Inhibitors (NRTIs) also known as nucleoside analogs, target the HIV enzyme reverse transcriptase. They act as alternative substrates competing with physiological nucleosides from which differ by only a minor modification in the ribose molecule (Hoffmann and Mulcahy, 2007). The hydroxy (-OH) group in the 3′ position is replaced by another group that is unable to form the 5′ to 3′ phosphodiester linkage essential for DNA elongation thus acting as chain terminators in the synthesis of proviral DNA (Mitsuya and Broder, 1986). NRTIs are absorbed unchanged and are only activated when three phosphates are attached by intracellular phosphorylation in a stepwise process. It is the triphosphate derivative that is efficacious (Hoffmann and Mulcahy, 2007). Nucleoside analogs were the first drugs to be used in HIV treatment and include such agents as zidovudine (AZT/ZDV), emtricitabine (FTC), lamivudine (3TC), abacavir (ABC) and tenofovir (TDF).Side effects in NRTIs are rare when using individual drugs. Fatigue, nausea, vomiting, diarrhea, headache, insomnia, myalgia are common in to AZT and ABC therapy. Peripheral polyneuropathy, very rarely pancreatitis, lactic acidosis and anaemia are associated with 3TC. Hypersensitivity reactions (HSR) in 2 to 8 %, usually within the first six weeks have been seen in ABC.

1.3.2   Non-Nucleoside Reverse Transcriptase Inhibitors

Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs) were first described in 1990 and target the reverse transcriptase enzyme. NNRTIs bind directly and noncompetitively to the enzyme at a position in close proximity to the substrate binding site for nucleosides (Grob, 1992; Merluzzi, 1990). The resulting complex blocks the catalyst activated binding site of the reverse transcriptase. This, in turn, can bind fewer nucleosides, slowing polymerization down significantly (Warnke *et al.*, 2007). In contrast to NRTIs, NNRTIs do not require activation within the cell. Agents in this group include nevirapine, delavirdine and efavirenz. A rash, usually occurring within the first six weeks is common in NNRTI and further treatment is normally possible. Headache, diarrhoea have also been recorded. Central nervous system symptoms; nightmares, confusion, dizziness, somnolence, abnormal thinking, depression, impaired concentration, insomnia, and depersonalization occur frequently in EFV therapy. These symptoms usually resolve after a few weeks.

1.3.3   Protease Inhibitors

The HIV protease cuts the viral gag-pol polyprotein into its functional subunits thus allowing maturation of the HIV virion. This process takes place in the final stages of the HIV life cycle. Protease inhibitors (PIs) prevent proteolytic splicing resulting in release of structurally disorganized and non-infectious virus particles (Warnke *et al.*, 2007). The ten PIs presently available for the treatment of HIV infections are saquinavir, ritonavir, indinavir, nelfinavir, amprenavir, lopinavir, atazanavir, fosamprenavir, tipranavir and darunavir (De Clercq, 2009).Moderate gastrointestinal complaints such as diarrhoea and dyslipidemia are common side effects in PI

therapy. Dyslipidemia is not as pronounced in Darunavir as in other PIs. Development of a rash in 20% % of Fosamprenavir recipients has been reported in the first 2 weeks.

1.3.4   Integrase Inhibitors

Integrase inhibitors block the integrase enzyme which incorporates viral copy DNA into the cellular DNA. Raltegravir is the first integrase inhibitor to be approved for use in antiretroviral treatment–experienced adult patients with viral resistance (Cocohoba and Dong, 2008). Raltegravir blocks HIV replication by inhibiting essential strand-transfer activities of integrase. Raltegravir has also been studied in treatment-naïve patients and was found to be non-inferior to an efavirenz-based regimen (Dau and Holodniy, 2009).Anaemia, kidney damage, depression, hyperglycaemia, changes in body fat distribution, allergic reactions such as a rash, hives, itching, swelling of the mouth or throat, wheezing, or difficulty breathing have been reported as side effects in raltegravir therapy although the occur rarely.

1.3.5   Cell Entry Inhibitors

Binding of HIV to the CD4 receptor, binding to co-receptors and fusion of virus and host cell membranes are the three crucial steps for entry of HIV into the host $CD4^+$ T cell. Every step of HIV entry can theoretically be inhibited. All three possible drug classes targeting these steps, namely attachment inhibitors, co-receptor antagonists and fusion inhibitors (FIs) a therefore be summarized as entry inhibitors (Mitsuya and Broder, 1986).

i. Co-receptor Inhibitors

Co-receptor inhibitors (CRIs) interact with the co-receptors CCR5 receptors on immune cells, blocking their use as a co-receptor for cell entry by HIV (Hunt and Romanelli, 2009). The CCR5 antagonist maraviroc is the only CRI licensed presently (Hammer *et al*., 2008)**.** Ideally, CRIs should include a combination of a CCR5 antagonist and a CXCR4 antagonist so as to block both X4 and R5 HIV strains. The major problem with maraviroc is that it is only active against R5 HIV strains. In a mixed population of X4/R5 HIV strains the drug has been shown to stimulate the selection of X4 strains (De Clercq, 2009).The most common maraviroc adverse events are diarrhoea, nausea, fatigue and headache.

More severe adverse events may include CNS adverse effects, rash and aminotransferase elevations.

ii. Fusion Inhibitors

Fusion inhibitors block gp41, a viral surface protein, preventing the conformational change necessary to allow fusion of viral and cell membranes, thereby preventing entry of viral nuclear material into the cell (De Clercq, 2009). Enfuvirtide is the first, and thus far the only, fusion inhibitor to be approved by the FDA. It binds to the HR1 region of gp41 and blocks the formation of the 6-helix bundle necessary for fusion (Warnke *et al*., 2007).Common adverse drug reactions associated with enfuvirtide therapy include: injection site reactions (pain, hardening of skin, erythema, nodules, cysts, itch experienced by nearly all patients, particularly in the first week), peripheral neuropathy, insomnia, depression, cough, dyspnoea, anorexia, arthralgia, infections (including bacterial pneumonia) and/or eosinophilia. Various

hypersensitivity reactions occur infrequently (0.1–1% of patients), symptoms of which include rash, fever, nausea, vomiting, chills, rigors, hypotension, elevated hepatic transaminases (Rossi, 2006).

1.4 Statement of the problem

Antiretroviral therapy is currently the major means of delaying HIV-1 progression to AIDS and prolonging the lives of infected individuals whose $CD4^+$ T cell counts decline below 200 cells/µl of blood. Unfortunately, the HIV virion is prone to mutations that render it resistant to one or several antiretroviral drugs. It therefore becomes necessary to change the regimen with every treatment failure and with every new regimen a possibility of developing novel resistance mutations exists, narrowing a patient's options for therapy. New drugs on the other hand are not developed and/or approved for use as fast as they are rendered ineffective for some drug resistant HIV strains.

1.5 Justification

The lack of adequate information on the antiretroviral drug resistance patterns of HIV strains prevalent in Kenya may in future negatively affect the rational use of antiretroviral therapy. The current HIV treatment regimens in use in the country are modelled based on HIV-1 subtype B, a strain prevalent in Europe and America but rare in Kenya. Evidence is accumulating of different resistance patterns of non-Subtype B viruses. Similarly, there are reports of a gradual shift of the HIV-1 subtypes prevalent in Kenya from the traditional pure subtypes towards recombinant forms. The effect of such newly emerging variants on antiretroviral treatment is

unknown. A commercial sex worker population may harbour the most diverse strains of HIV in the country as the high risk lifestyle of the group presents fertile ground for virus mixing and possibly generation and transmission of unique genotypes. This study aims to map the genotypic resistance profiles of the HIV variants from patients receiving antiretroviral treatment in the Pumwani sex worker cohort. This information will contribute to the development of an in-country guideline for identification of HIV antiretroviral resistance strains to guide options for treatment of HIV/AIDS   patients.

1.6 Hypotheses

1.6.1 Null hypothesis

There is low prevalence of HIV-1 drug resistant mutations among ARV-experienced subjects in the Pumwani sex worker cohort.

1.6.2 Alternative hypothesis

There is a high prevalence of HIV-1 drug resistant mutations among ARV-experienced subjects in the Pumwani sex worker cohort.

1.7 Objectives

1.7.1 General objective

To determine HIV-1 subtype distribution and prevalence of HIV drug resistant mutations among ARV-treated patients in the Pumwani sex worker cohort.

1.7.2 Specific objectives

1. To determine HIV-1 subtypes present in the Pumwani sex worker cohort.

2. To determine prevalence of antiretroviral drug genotypic mutations in the Pumwani sex worker cohort.

CHAPTER TWO

LITERATURE REVIEW

2.1 Development of drug resistance

Antiretroviral (ARV) resistance is a major factor contributing to treatment failure (Richmann, 2001) and develops when viral replication continues in the presence of selective drug pressure. It may result from impotent regimens, suboptimal adherence, pharmacological hurdles or ineffectively treated compartments. Long-term HIV chemotherapy with persistent treatment failure and frequent antiretroviral drug changes is often associated with the accumulation of drug-resistance mutations that confer increased phenotypic resistance and lead to the clinically undesirable selection of multidrug-resistant HIV-1 strains (Richmann, 2004; Phillips *et al.,* 2005)

HIV-1 drug resistance can be acquired (developing in a person receiving antiretroviral treatment) or transmitted (occurring because a virus with drug-resistance mutations was transmitted to a drug-naive person). HIV-1 drug resistance can be transmitted both horizontally and vertically and Lwembe *et al.* (2007), while studying a group of Non-subtype B infected children found that vertically transmitted reverse transcriptase inhibitor (RTI) resistance mutations can persist for years, even in the absence of drug pressure. Acquired drug resistance results from the generation of genetic variation in the population of viruses within a person followed by the selection of drug-resistant variants during therapy. The Kisumu breastfeeding study established that even infants born to and breastfed by mothers on ARV are at risk of developing resistance to ARVs taken by their mothers. This is especially so if

commencement of treatment of the infant was delayed or ignored altogether (Thomas *et al.,* 2004). Development of such resistant strains is critical and detrimental to HIV therapy because transmission to treatment-naive populations directly translates into reduced treatment options for populations.

Resistance to drugs typically develops as a result of mutations within the genes encoding the proteins targeted by that particular drug. This enables the virus to gain the ability to replicate in the presence of the drug i.e. sufficient viral suppression is not induced. In HIV, the replication and mutation rates and the virions' ability to integrate into the host genome and remain dormant confer the ability to develop resistance to therapy very rapidly. Up to 10 billion virions may be produced daily in an untreated host, with a half life as short as 6 hours. These spontaneous mutations are attributed to HIV RNA lack of a proof-reading mechanism/activity owing to the lack of the 39-59 exonuclease activity in the reverse transcriptase gene (Havlir and Richman, 1996). With an estimated replication rate of 10^7 to 10^8 rounds per day per virion, an average of one mutation at each position in the HIV-1 genome occurs during each replication cycle (O'Neil *et al.*, 1998). Greater than 50% of the viral population is cleared and replaced daily (Fu-jie *et al.*, 2006). It is therefore suggested that at least one drug resistance mutation could occur daily to generate a distinguishable quasispecies that could emerge as a dominant strain when exposed to selective drug pressure (Paolucci *et al*., 2001). Even if a drug resistant strain accounts for only a small minority in the overall viral population, selective pressure from antiretroviral therapy will favour replication and propagation of the resistant strain.

Primary mutations in the drug target gene usually alters the binding of the drug to its target while secondary mutations increase the level of resistance by improving viral fitness of a virus carrying a primary mutation. While some mutations will result in resistance just to a single drug e.g. M184V in the gene encoding reverse transcriptase confers 100-1000-fold loss of susceptibility to lamivudine (Turner *et. al.,* 2003), other mutations may result in resistance to more than one drug or an entire class of drugs, a phenomenon referred to as cross-resistance. This has been extensively documented in non-nucleoside reverse transcriptase inhibitors (NNRTIs); the K103N mutation in the gene encoding reverse transcriptase for instance, confers resistance to all three NNRTIs (nevirapine, efavirenz and delavirdine). This mutation prevents formation of a hydrophobic binding pocket, reducing binding affinity between drug and virus (Deeks, 2001). Since the three drugs bind to the same site, cross-resistance results easily (Hsiou *et al.,* 1997). Resistance to protease inhibitors (PIs) commonly results from an accumulation of mutations and therefore takes longer to develop (Deeks, 2001); this is referred to as the genetic barrier.

2.2 The impact of antiretroviral roll-out

One of the direct results of antiretroviral roll-out has been the tremendous expansion of ART in Africa and other low income nations in recent years. Marked efforts to scale up ART have been documented in countries such as Kenya, Uganda, Rwanda, Zambia, Malawi, Botswana, Namibia and South Africa (UNAIDS, 2006; Libamba *et al.*, 2005).

On December 1st 2003, the World Health Organization (WHO) and the Joint United Nations Program On HIV/AIDS (UNAIDS) unveiled the 3 by 5 plan to avail antiretroviral drugs to 3 million HIV positive persons by the end of 2005 in developing countries with the ultimate goal of providing access to ART to all those that need it. The United States President's Emergency Plan for AIDS Relief (PEPFAR) was also launched in 2003 to combat HIV/AIDS through support of treatment, prevention and care programs in 15 countries namely Botswana, Cote d'Ivore, Ethiopia, Guyana, Haiti, Kenya, Mozambique, Namibia, Nigeria, Rwanda, South Africa, Tanzania, Uganda, Vietnam and Zambia. These countries collectively represent approximately 15% of HIV infection worldwide (PEPFAR, 2008). By September of 2008, over 2 million persons in the focus nations had received ART through the PEPFAR programme. The UNAIDS reports that the number of people receiving antiretroviral drugs in low- and middle-income countries has increased ten-fold in only six years, reaching 3 million people (2.1 million of these in Sub-Saharan Africa) by end of 2007 (UNAIDS, 2008). In Kenya, ART coverage rose progressively from under 5% (2004), 12% (2005), 28% (2006) to nearly 40% by end of 2007 (UNAIDS, 2008). By the end of September 2008, 229,700 people were on ART through the PEPFAR programme in Kenya.

The primary goals of antiretroviral therapy include maximal and durable suppression of viral load, preservation of immunologic function as well as reduction of morbidity and mortality as a result if HIV. By lowering viral load, antiretroviral drugs have an important role in lowering risk of transmission as it is well established that the lower the viral load, the less infectious a patient is (Kamps and Hoffmann, 2007). A prospective study of 415 discordant couples in Uganda showed that 90 new

infections occurred over a period of 30 months, none from an infected partner with viral load below 1500 cells/ml. the risk of infection increased with every log of viral load by a factor of 2.45 (Qiunn *et al.*, 2000). Patients are most infective therefore during the acute HIV infection stage, when the virus replicates extensively in the absence of detectable immune response (Kamps and Hoffmann, 2007).

Mathematical models have been used to demonstrate that while the above goals would be achieved there would be a downside to ART-rollout in Africa, that is, an increase in development, transmission and prevalence of drug resistant HIV strains (Blower and Farmer, 2003). It has been well established that over time ART fails to suppress HIV-1 replication in 15-50 % of individuals (Starr *et al.*, 1999; DeGruttola *et al.,* 2000; Demeter *et al.*, 2001; Krogtad *et al.*, 2002; Fletcher *et al.*, 2000).

2.3 HIV drug resistance in Africa

As far back as 2000, studies carried out as part of the UNAIDS Drug Access Initiative revealed the development of high levels of resistance in African countries that had pioneered antiretroviral use in the continent. Seventy eight percent of Ugandans on 3TC had virus resistant to the drug while 57% of Ivorians treated displayed resistance to one or more drugs (PRNewswire, 2000). A study by Eshlemann *et al.* (2001) associated the emergence of resistance to Non-Nucleoside Reverse Transcriptase Inhibitors with single dose Nevirapine for prevention of vertical transmission of HIV, in Uganda. Drug resistance has also been demonstrated on evaluation of small cohorts in Cote d' Ivoire (Adje *et al.*, 2001), Zimbabwe (Kantor *et al.*, 2002) and in Uganda (Wiedle *et al.*, 2001). In Uganda, Richard *et al.*,

(2004) demonstrated a 52% prevalence rate in 50 subjects through a cross sectional study.

Studies in the early years of the ART roll-out that showed a rise HIV-1 drug resistance among drug-naïve patients indicated a trend that would potentially threaten any advances brought about by ART. In Botswana where ART is available to persons with CD4$^+$ T counts below 300 cells/ml, development of mutations conferring resistance to protease inhibitors among drug naïve patients was estimated at 4% (Bussmann *et al.*, 2002). A study in Nigeria of subtypes G and A/G recombinants however showed absence of primary mutations in isolates of treatment-naïve individuals but showed a high percentage of background secondary mutations (Lar *et al.*, 2007). Although use of NRTIs, NNRTIs and PIs was still a feasible option with this group, there was an increased risk of development and transmission of resistance especially where adherence and proper administration were disregarded.

In Kenya drug resistance in drug naïve patients rose from 1% in 2002 to over 5% in 2003 following ART coverage of only 12-17% of the population that required therapy (Ndembi *et al.* 2008). A more recent study on Kenyan children infected with non-subtype B HIV-1 revealed major drug resistance mutations in 33.3% of the population, before treatment (Lwembe *et al.*, 2007) suggesting a rising prevalence in drug resistance among drug naïve Kenyans. A study on nevirapine treated mothers in Kenya's Rift Valley revealed 27.8% ARV-associated drug resistance mutations, 11.1% of which were specific to nevirapine (Kiptoo *et al.*, 2008) again suggesting

transmission within drug naïve populations. This study also found that 8.3% of these mothers transmitted HIV-1 infection to their infants.

2.4 ARV treatment guidelines

The public health approach to ART scale-up in resource-limited settings is aimed at supporting the development of treatment programmes that can reach as many people as possible providing a wider access to ART. Among the key tenets of this approach are the standardization and simplification of ARV regimens as documented in the 2006 revision of the World Health Organization ART guidelines. The latest revision to the guidelines (2008) also aims to achieve maximum suppression of virus with minimal toxicity and maximum simplicity, all with a goal of promoting adherence and minimizing resistance (Roehr, 2008). As a result of such HIV treatment guidelines, a 2006 evaluation has noted that almost all high-burden countries had adopted or adapted the WHO treatment guidelines to frame their respective national recommendations (Beck *et al*., 2006). Consequently, almost all the 3 million people currently on ART are started on WHO-recommended first-line regimens delivered in accordance with a public health approach. In accordance to this guideline, the cohort that involved in this study was started on two NRTIs and one NNRTI usually lamivudine (3TC) / stavudine (d4T) / zidovudine (AZT) and nevirapine (NVP) / efavirenz (EFV), of course with minor adjustments to suit each individual.

Development of current guidelines incorporated considerations for newly approved drugs (since the last ARV guideline review in 2006) and the greater understanding for disease pathology achieved in the last few years. These new drugs are

1. Maraviroc, the first drug that targets the CCR5 co-receptor,

2. Raltegravir, the first integrase inhibitor that supports the change in recommendations that emphasizes full virologic suppression and

3. Etravirine, a second generation NNRTI that has shown clear activity against NNRTI-resistant viruses.

Recommendations to start therapy in any patient with symptomatic HIV disease regardless of CD4$^+$ T cell count and /or viral load and in asymptomatic patients with CD4$^+$ T cell count less than 200 cells/ µl remain unchanged from the previous revision. The current guidelines however move more aggressively toward earlier initiation of therapy prior to decline in CD4$^+$ T cell count to less than 350 cells/ µl. Therapy may be considered in patients with CD4$^+$ T cell counts of 500 cells/ µl and above where there are comorbidities (correlates of faster HIV disease progression) e.g. High viral load or rapid decline in CD4$^+$ T cell counts, in which case the decision should be individualized, high risk of cardiovascular disease, co-infection with hepatitis B or C and occurrence of HIV- associated neuropathy (Hammer *et al*., 2008).

Current guidelines strongly recommend that resistance testing be conducted on all patients as part of their baseline work-up. There is however little change in recommendations for initial regimen in patients not infected with resistant virus although there is a stronger emphasis to individualize regimen especially in the presence of comorbid conditions (Roehr, 2008). These recommendations require that the first-line regimen for adults and adolescents contain two NRTIs plus one NNRTI

or a ritonavir-boosted protease inhibitor. This recommendation is based on available evidence, clinical experience and programmatic feasibility for the wider introduction of ART in resource-limited settings. Regimens based on combination of two NRTIs plus one NNRTI are efficacious, are generally less expensive than other regimens, have generic formulations (Calmy *et al*., 2006), are often available as fixed-dose combinations and do not require a cold chain. There are however disadvantages to these which include different drug half-lives which complicate ART stopping procedures, the fact that a single mutation is associated with resistance to some drugs (3TC and the NNRTIs), and cross-resistance within the NNRTI class.

The thiacytadine analogues (3TC or FTC) are pivotal to first-line regimens. 3TC or FTC should be used with a companion nucleoside or nucleotide analogue, the choices here being AZT, TDF, ABC or d4T.

Abacavir (ABC) should however be used in regimen with caution due to recent findings that show hypersensitivity to the drug , lower efficacy in patients with high viral load (100,000 copies/ml) and increased risk of cardiovascular disease (Rauch *et al*., 2006; Mallal *et al*., 2008; Smith *et al*., 2008). The preferred NRTI backbone is composed of AZT or TDF combined with either 3TC or FTC. Didanosine (ddI) is an adenosine analogue NRTI recommended to be reserved for second-line regimens and finally an NNRTI, either EFV or NVP, should be added (WHO, 2006).

An additional benefit of the two NRTIs plus one NNRTI combination is that they preserve the more potent class (protease inhibitors [PIs]) for second-line treatments. The second-line regimen has to involve drugs that retain activity against the patient's

virus strain and should ideally include a minimum of three active drugs, one of them drawn from at least one new class, in order to increase the likelihood of treatment success and minimize the risk of cross-resistance. The PI class is therefore most frequently reserved for second-line treatments, preferably supported by two new NRTIs.

Regimen may be changed to decrease toxicity, avoid adverse drug interactions and/or improve convenience and adherence, as long as potency of the regimen is maintained and subsequent drug interactions are managed appropriately. As such, first line failure of NNRTI-based regimen should be treated with two active NRTIs plus a ritonavir-boosted PI or the newly introduced second generation NNRTI, etravirine (Hammer *et al*., 2008). Change resulting from failure in PI-based regimen is complicated by genetic barriers such as the number of accumulated resistance points necessary for a patient to resist a drug. In early resistance, changing the NRTI component to two active drugs is sufficient to save regimen but in later resistance, darunavir (a second generation PI) and tipranavir are more effective for salvage therapy (Hammer *et al*., 2008). For heavily treated populations, enfurvitide remains an important option but it is generally and gradually being substituted with maraviroc and raltegravir.

2.5 Indicators of drug resistance

The two initial indicators of development of resistance to ART are rising plasma viral RNA concentrations (viral load) and declining $CD4^+$ T cell counts (Collazos *et al*., 2006). Ideally, viral suppression is considered successful if less than 50copies/ml of

HIV RNA (considered undetectable) occur in blood after six months of treatment while CD4$^+$ T cell counts above 200 cells/µl or 500 cells/ µl (depending on study), are defined as an indicator of success. The CD4$^+$ T cell count remains the strongest predictor of HIV-related complications, even after the initiation of therapy. The baseline pretreatment value is informative: lower CD4$^+$ T cell counts are associated with smaller and slower improvements in counts. However, precise thresholds that define treatment failure in patients starting at various CD4$^+$ T cell levels are not yet established (Garcia *et al*., 2004). As a general rule, new and progressive severe immunodeficiency is demonstrated by declining longitudinal CD4$^+$ T cell counts

Reasonable working definitions of immunological failure according to the 2006 Revision of the *WHO ART guidelines* are:

 (1) CD4$^+$ T cell count below 100 cells /mm^3 after six months of therapy

 (2) A return to, or a fall below, the pre-therapy CD4$^+$ T cell baseline after six months of therapy or

 (3) A 50% decline from the on-treatment peak CD4$^+$ T cell value (if known).

The first six months on ART are therefore very critical because clinical and immunological improvement should manifest but are not always apparent and drug toxicities may emerge. Some patients fail to respond as expected or may even exhibit clinical deterioration initially. These issues combine to present specific challenges for clinical management and it is important to allow sufficient time on therapy before judging effectiveness of ART. Numerous studies such as Garcia *et al*., 2004 and The Plato Collaboration, 2004 have used six months as minimal time after which to assess ART failure / success.

In most patients, CD4$^+$ T cell counts rise with the initiation of therapy and immune recovery. This may continue for many years into effective therapy, although this may be blunted if the baseline CD4$^+$ T cell count is very low. However, even patients with CD4$^+$ T cell counts below 10 cells/ mm^3 can achieve an effective CD4 recovery, given sufficient time after the initiation of ART. Some patients may never have CD4$^+$ T cell counts that exceed 200 cells /mm^3 and thus never leave the zone of severe immunosuppression; however, they may have drastic clinical improvement. In those who achieve a substantial peak response, a subsequent progressive decline in CD4$^+$ T cell counts in the absence of intercurrent illness indicates immunological failure. The baseline CD4$^+$ T cell count and the trend of the CD4 response assessed by regular six-monthly CD4$^+$ T cell counts are thus needed to best characterize and define immunological failure. In a minority of patients with advanced disease and low CD4$^+$ T cell counts when therapy is initiated, the CD4$^+$ T cell counts may not rise or may fall slightly, even with clinical improvement.

Usually with increasing viral load, CD4$^+$ T cell counts fall concurrently indicating virological and immunological failure respectively. A large cohort study has shown however, that CD4$^+$ T cell counts do not drop as long as the viral load remains above 10000 copies/ml or at least 1.5 logs below the individual set point (Ledergerber *et al.*, 2004). Maintenance of viral load below 50copies/ml has however been shown in numerous studies not to necessarily hinder development of resistance mutations in the virus, as replication is not halted (Hoffman and Mulcahy, 2006). Resistance mutations albeit fewer and minor ones, may thus be detectable in patients on ART and without any observable virological and / or immunological failure.

2.6 Viral fitness and antiretroviral resistance

Resistance mutations in such treated patients may be of consequence to eventual clinical progression of the patient and possible development of full resistance owing to such key factors as i.) the fact that mutations alter viral fitness ii.) viral fitness is consequential to virological and immunological fitness and iii.) there are mutations that have been shown to promote the phenomenon of cross-resistance.

Mutations alter viral fitness or viral replicative capacity and viral pathogenicity (Deeks *et al*., 2001; Prado *et* al., 2005). It was reported that virus with the protease inhibitor D30N mutation replicated 30% slower *in vivo*, than wildtype virus whereas mutants with L90M are less fit than wild -type but usually outgrow D30N mutants (Martinez-Picado, 1999). The emtricitabine (3TC) resistance-associated mutations at codon 184 are clearly associated with reduced fitness (Miller *et al*., 2003).

HIV-1 fitness may be useful for predicting clinical outcome in both drug-naive and in drug-experienced patients (De Luca, 2006). Reduced replicative capacity has been clearly linked to long-term improvements in $CD4^+$ T cell counts even in the presence of detectable viral load. A study of 191 drug-naive subjects recently diagnosed with HIV-1 infection revealed that viral replication capacity was associated with $CD4^+$ T cell count at study entry and over time, both before and after treatment initiation (Barbour *et al*., 2002).In a study of patients with low-level (less than 5000 copies/ml) virological failure being treated with protease inhibitor-based therapy, a significantly greater replication capacity was found among patients with declining $CD4^+$ T cell counts than among patients with stable or increasing $CD4^+$ T cell counts (Sufka *et al*., 2003) suggesting an inverse relationship between replication capacity and $CD4^+$

T cell count. The baseline replication capacity was the strongest predictor of an increase in $CD4^+$ T cell count from pre-study nadir to study baseline in multivariate models of an analysis of 207 patients (Haubrich *et al*., 2002) enrolled in the California Collaborative Treatment Group (CCTG 575) trial. The fitness cost associated with the development of antiretroviral resistance is probably responsible for the sustained immunological benefit that is often observed in patients with failure of HAART and virological breakthrough ((De Luca, 2006).

The decline in HIV-1 fitness is however not a short-term phenomenon. A number of mutations have been found to restore viral fitness of drug-resistant mutants. In the reverse transcriptase gene, S68G (Garcia-Lerma *et al*., 2000) and M230I (Matsumi *et al*., 2003) have been associated with recovery of viral fitness in the presence of other mutations that impair fitness while several mutations in the protease gene, including substitutions at codons 10I, 63P, 71V and 77I (Markowitz *et al*., 1995; Nijhuis *et al*.; 1999) compensate for the fitness loss associated with major protease-resistance mutations.

There is a high degree of cross resistance among nucleoside analogs. Some mutations such as the thymidine analog mutations M41L, L210W, and T215Y selected for by the NRTIs zidovudine (ZDV) and stavudine (D4T) confer cross resistance to all NRTIs (Fu-jie *et al.*, 2006). The NNRTI single mutation, K103N confers high level cross-resistance to this drug class (Brenner, 2000) while the mutations V82A/F/T/S, I84V, and L90M have been shown to confer broad cross-resistance to most PIs (Vandamme, 2004).Cross resistance limits the future of any rescue intervention(s) in the event of development of full resistance.

2.7 Drug specific resistance mutations

Gonzales *et al*. (2003), Rhee *et al.* (2005) and Svicher *et al.* (2006) have described reverse transcriptase inhibitor (RTI) resistance-associated mutations and have contributed to data available for mutations known to be concentrated on codons 1–240 of the reverse transcriptase gene. Cane *et al.* (2007) extended the dataset to include the region encompassing codons 240–400 in their study aimed at identifying accessory mutations associated with high levels of resistance to reverse transcriptase inhibitors (RTI). Cane *et al.* (2007) however acknowledged that it is unlikely that the accessory mutations they described have a significant influence on the degree of susceptibility to particular drugs compared with the primary resistance mutations (occurring on codons 1-240), so their relevance to algorithms for predicting the drug resistance phenotype will likely be limited. Analysis of secondary mutations could however play a future role in the prediction of the replicative capacity of the virus.

Resistance mutations to protease inhibitors (PIs) emerge rapidly when these drugs are administered at inadequate doses or as part of suboptimal regimens. Generally, high-level resistance to PIs results from the sequential accumulation of amino acid substitutions in the viral protease gene, along pathways that usually vary between different PI drugs (Kaplan *et al.*, 1994; Ridky and Leis, 1995). The selective advantages conferred by PI resistance mutations depend upon the nature of the drug, its local concentration and the impact of the mutation on infectivity. Due to the observation that drug concentrations *in vivo* are subject to uneven tissue distribution and can vary overtime, parameters leading to the emergence of PI-resistant strains remain poorly defined (Mammano *et al.*, 2000).

Interpretation of Protease mutants is complicated by the extensive polymorphisms found in the protease gene of HIV-I isolates from untreated patients. In one study, variation was noted in nearly 48 per cent of Protease codons compared with the consensus (wildtype) sequence (Kozal *et al*., 1996). The significance of these polymorphisms in determining treatment outcome remains uncertain, since most studies have not found any correlation between the presence of these polymorphisms and virologic response, or the rate at which PI resistance emerges. Some HIV-I subtypes have naturally occurring polymorphisms or mutations that are associated with resistance. For example, M36I is very common in subtype C and other non-subtype B isolates. Accumulation of multiple mutations can contribute to cross-resistance, and an understanding of the mechanisms involved is important for establishing effective treatment strategies in patients who stop responding to regimen containing PIs (Roberts *et al*., 1998; Winters *et al.*, 1998). In one report, four amino acid substitutions associated with indinavir therapy, M46I, L63P, V82T and I84V, were required for cross-resistance to other PIs, including saquinavir and amprenavir (Harrigan *et al.,* 2005). Another study involving more than 6000 clinical samples showed phenotypic cross-resistance in 59-80 per cent of samples with HIV-I resistance to at least one PI (Hertogs *et al.*, 2000). The predominant genotypic change in viruses with resistance to at least one PI involved codons 10, 36,46, 54, 71, 77, 82 and 90 of the protease gene. Viruses that were cross-resistant to all four PIs displayed higher frequencies of changes at these positions, and also at positions 48 and 84 (Hertogs *et al.*, 2000). Thus, along with single amino acid changes coding for drug resistance, cross-resistance with protease and RT inhibitors presents a complex challenge to antiretroviral therapy (Potter *et al*., 2004).

enfuvirtide (Fuzeon or T-20), a fusion inhibitor approved by FDA in 2003 functions by binding a region of the HIV envelope glycoprotein gp41 and preventing viral fusion with the target cell membrane (Wolf, 2006).The gp41 genome bears regions of high variability as well as highly conserved regions. Resistance mutations develop rapidly against T-20 but they seem to reduce viral fitness (Lu, 2002; Menzo, 2004). Loss of efficacy is accompanied by appearance of mutations at the T-20 binding site, the Heptad Repeat 1 (HR1) region of gp41. Mutations particularly in positions 36-46 emerge most frequently with substitutions at positions 36, 38, 40, 42, 43 and 45. Decrease in susceptibility is higher where double mutations such as G36S + L44M occur, as opposed to single mutations (Wolf, 2006)

No clear evidence of primary resistance to HIV-1 fusion inhibitors have been shown so far. Peuchant *et al.,* (2007) thus investigated the possibility of genotypic resistance in an antiretroviral–naïve French cohort and were able to show beginnings of incidence of primary resistance toT-20. The group found two individuals with T-20 resistance out of a study population of 55. While one of these individuals bore a mutation (G36D HR1) only to T-20, the second bore an N24D HR1 mutation as well as multiple reverse transcriptase and protease mutations.

Several studies have attempted to determine the prevalence of HIV drug resistance to the different antiretroviral drug classes. Richman *et al.* (2004) while basing their results on sensitivity assays found that 72-80% of patients receiving Highly Active Antiretroviral Therapy (HAART) exhibited resistance to one or more classes. Resistance to any two classes was put at 48% and at 13% to any other ARV classes. Prevalence of drug resistance with ARV class seems to be influenced by the

availability and distribution of that class of ARV as evidenced by a 7 year surveillance study conducted in Japan. Prevalence of NRTI resistance was 44% before start of HAART and rose to 54% the year that HAART was introduced while protease inhibitor resistance increased from 0% to 41% in the same period. In the final two years of the study, PI resistance frequency decreased while prevalence of NNRTI resistance rose from 5% to 18%. In these final two years, PIs in HAART were being increasingly replaced with efavirenz, an NNRTI (Sugiura *et al.*, 2004). A similar finding was reported in Kampala by Spacek *et al.*, (2006). The study was carried out in an urban clinic where patients paid for their own medicine likely resulting in poor compliance. A high frequency of NNRTI drug resistance was detected relative to resistance to PI because most patients had their regimens designed around an NNRTI backbone as it was more affordable than a PI based regimen.

2.8 Monitoring and measuring HIV drug resistance

It is in the background of the continually mutating strains with increased adaptability and transmissibility to drug naïve populations especially due to increased overall drug pressure in the treated population that The World Health Organization (WHO) and the International AIDS Society (IAS) came together to develop the Global HIV Drug Resistance Surveillance Network (HIVResNet). The mandate of this network is to track HIV drug susceptibility, provide information necessary to develop effective treatment strategies, support and complement programs aimed at increasing antiretroviral therapy availability and assist in implementing strategies to prevent drug resistance. The collaboration also aims to promote the transfer of technology of drug resistance testing methodologies to developing countries.

WHO through HIVResNet has also put in place recommendations for measures to monitor HIV Drug Resistance (HIVDR) transmission as well as emergence in treated populations. For the latter, the organization recommends selection of national ART sentinel sites that serve the purpose of evaluating the success of ART programmes in minimizing the emergence of HIVDR in the first year of ART through monitoring of viral loads and CD4$^+$ T cell counts and genotyping of any HIVDR mutations that may arise. These sentinel sites also serve to evaluate ART programme factors associated with HIVDR prevention. WHO also suggests that countries also routinely measure relevant variables associated with HIVDR prevention, including prescribing practices, adherence, appointment-keeping and ARV drug pick-up by patients, and drug supply continuity. These serve as early warning indicators for both acquired HIVDR and by extension transmission into treatment-naïve populations (WHO, 2006).

Phenotypic resistance, genotypic resistance and clinical resistance are useful terms when measuring HIV drug resistance. Phenotypic resistance is the ability of HIV to grow in spite of the presence of ARV, genotypic resistance refers to mutations in the HIV genome resulting in diminished susceptibility or response to ARV and clinical resistance refers to diminished clinical response despite application of antiretroviral therapy (Sen *et al.*, 2006). Currently established assays available address phenotypic and genotypic resistance. Phenotypic assays currently available involve direct quantification of drug sensitivity. They involve generation of a recombinant HIV-1 with the patients HIV-1 protease and reverse transcriptase region being incorporated into an HIV-1 backbone replacing the corresponding wildtype regions (Kellam and

Larder, 1995; Sen *et al.*, 2006). Selective drug pressure is applied by increasing ARV concentration and viral replication of the recombinant is compared to that of the wildtype. Phenotypic tests that are currently commercially available include PhenoScript™, Antivirogram™ and PhenoSense™.

Genotypic assays involve analysis of mutations associated with resistance. These are determined either by direct sequencing of amplified HIV genome or through hybridization methods. Tests currently available commercially include Viroseq ™, HIV-1 TrueGene™ and GenoSure™. These assays are about half as costly as the phenotypic assays and also less lengthy. A disadvantage of both phenotypic and genotypic assays however is that a minimum amount of virus (usually 1000copies/ml) is necessary to perform the test (Wolf, 2006).These tests can also detect resistance only if 10-20% of an individual's virus population is resistant (Cairns, 2008) and therefore fail to detect drug resistance in one third or more of all newly diagnosed patients. These commercial based kit assays can also not detect minor quasispecies that are 20-30% less than the circulating population (Lecossier *et al.*, 2005; Palmer, 2005). This can however be rectified by use of newer more sensitive laboratory-based assays that are however much more expensive and less readily available in a clinical setting e.g. real-time PCR (Palmer *et al*., 2005), LigAmp assay (Metzner *at al.*, 2005) and single genome sequencing (Shi *et al*., 2004; Flys *et al*., 2005).

These tests are critical in guiding a clinician when designing start-up regimen or salvage regimens. Several clinical studies have been conducted determine the utility

of such tests with conflicting outcome. In a study conducted within the French trial group '*Agence Nationale de Recherche sur le SIDA*' (ANRS 088-NARVAL), genotypic and phenotypic tests were compared and revealed no significant differences (Meynard *et al*., 2002). The Clinical Efficacy of Resistance Testing (CERT) study on the other hand, found that the phenotypic test was beneficial while genotypic tests were of no benefit (Wegner *et al.*, 2004). The general consensus however is that genotypic assay data in clinical management shows great benefit in virologic and immunologic response (Sen *et al.*, 2006).

In addition to being expensive and largely unavailable in developing countries, these assays for measuring and monitoring HIV-1 drug resistance are developed for HIV-1 subtype B and are often inaccurate in measuring resistance in HIV-1 Non-B subtypes. Some secondary protease inhibitor mutations for instance, are more common in Non-B viruses while M36I a wildtype for subtype C would be interpreted as a resistance mutation for subtype B (Lar *et al*., 2007).

HIV drug resistance is a very dynamic phenomenon both influenced and complicated by numerous factors of the host, the virus and the interaction between the host and the virus. HIV-1 subtype diversity and dual infection with HIV are among key issues that have generated great interest in research.

2.9 HIV-1 drug resistance and HIV-1 subtype divergence

A notable hallmark of HIV-1 virus is its genetic diversity owing to its capacity to evolve rapidly. This results in an expansion of various distinct subtypes that tend to

show geographical clustering (Fig. 2). Three main groups of HIV-1 (Major, New and Outlier), have developed worldwide. M accounts for 90% of all HIV/AIDS cases reported (Renfijo *et al.*, 2002). This group has further been classified into 9 clades / subtypes A-D, F-H, J, K and currently, 43 circulating recombinant forms [CRFs] (Los Alamos, 2009), while numerous intersubtype unique recombinant forms (URFs) either in one patient or a cluster of epidemiologically closely related patients are increasingly being detected (Camacho and Vandamme, 2007).

The B subtype is found in Europe and North America most frequently though due to migration, by 2004 at least 25% of all new infections were Non-subtype B African and Asian variants (Wainberg, 2004). While the A subtype is distributed in Eastern Africa, the C subtype occurs in Eastern and Southern Africa and has created recent epicentres of the HIV-1 pandemic by uncontrolled spread in Southern Africa, the Indian sub-continent and China (Wainberg, 2004; Janssens *et al.,* 1997; Piot and Bartos, 2002; Weniger *et al.,* 1994). The D subtype is limited to Eastern and Central Africa with sporadic cases in Southern and Western Africa. The E subtype has never materialized alone but has been found as an A/E mosaic in Central Africa, Thailand, China and The Philippines while subtype H is found only in West Africa and subtype J also extensively in Western Africa (Wainberg, 2004; Piot *et al.,* 2002). Additional subtypes, CRFs and URFs are continually being discovered and migration among populations continually shapes patterns of subtype distribution (Irwin *et al.,* 1997).
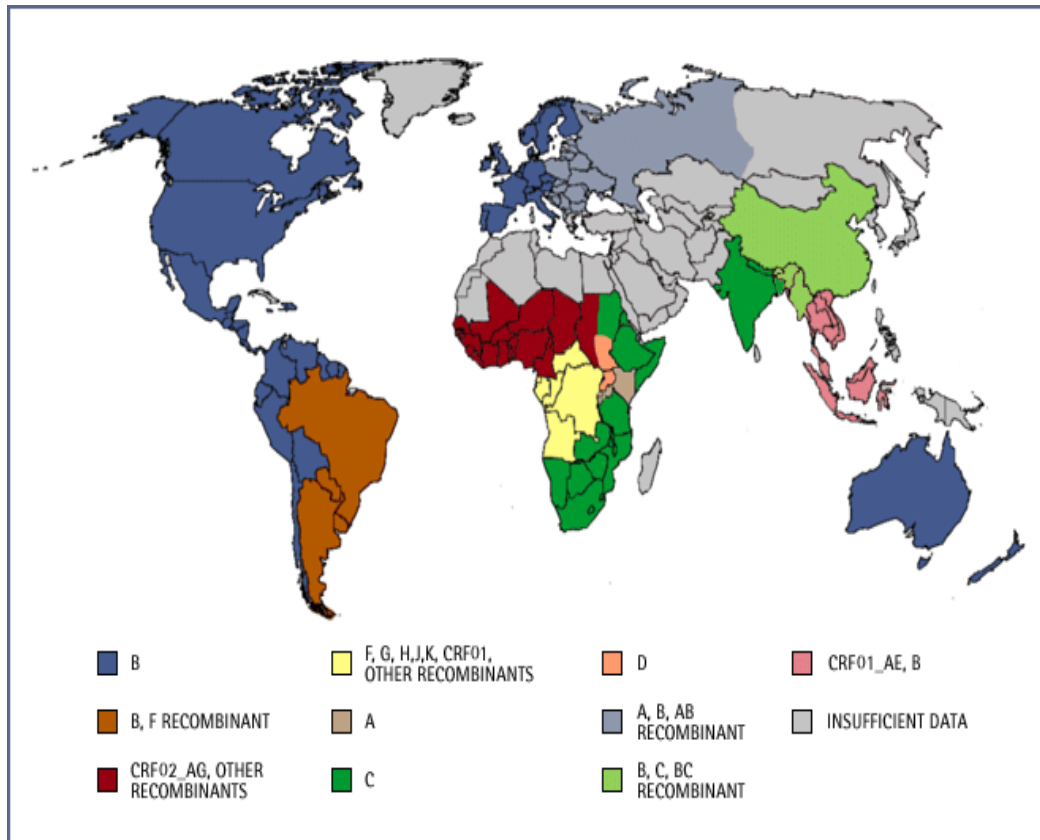
Figure 2.1   Global HIV-1 subtype distribution (Source: IAVI, 2006)

Viral diversity is greatest in Sub-Saharan Africa (Louwagie *et al.,* 1995) with all the clades and their attending CRFs and URFs being represented as well the HIV-2 strain. In Kenya, subtype A1 is dominant in Central, Western and Coast provinces while in the Northern border region subtypes occur in different proportions. Subtype C represents 42%, subtype A1 represents 43% and subtype D represents 15% of circulating HIV-1(Khamadi *et al.,* 2004). Belda *et al.* (1997), reported 73% subtype A distribution and 6.7% for subtypes C and D, each within Nairobi.

Non-B subtypes, CRFs and URFs show some differences from subtype B variants. They follow different pathways to resistance, differ in codon sites critical to resistance e.g. positions 106 and 210 on reverse transcriptase and 82 in protease, differ in binding affinities for PIs *in vitro* and viral fitness (Camacho, 2005; Frater *et al.,* 2002; De Wit *et al.,* 2004). Mutations in Non-B subtypes appear in sites not linked to resistance on subtype B e.g. M89I/V and D35N/G (Abecasis *et al.,* 2005; Al-Hawajri *et al.,* 2005).

Available evidence however suggests that virtually all HIV-1 subtypes display similar sensitivity to ARVs, at least in the short term (Spira *et al.,* 2003) but there are studies that show that viruses from some subtypes or geographical clusters may occasionally have greater propensity to develop resistance against certain drugs. For instance, the Y181C mutation in HIV-1 group O and Y181I in HIV-2 render these variants resistant to all NNRTIs (Quinones-Mateu *et al.,* 1998; Descamps *et al.,* 1995; Descamps *et al.,* 1997; Tantillo *et al.,* 1994). Subtype F in a less absolute manner shows resistance to TIBO, a non-commercialized NNRTI but is sensitive to other NNRTI (Apetrei *et al.,* 1998). In a Zimbabwean cohort, while subtypes C and B appeared similar in ARV sensitivity, some subtype C isolates showed natural resistance due to a G190A polymorphism in the reverse transcriptase gene (Loemba *et al.,* 2002; Brenner *et al.,* 2003). One of the best known subtype–dependent mutation quirks involves frequent selection of D30N mutation by nelfinavir- treated subtype B variants but a preference for L90M by Non-B variants (Grossman, 2004). Palmer *et al.* (1998) reported clade D isolates with diminished drug sensitivity

compared to clades A, B, C and D which demonstrated comparable levels of sensitivity.

Camacho (2005) suggested that response to entry inhibitors may differ greatly from one subtype to another because the gene encoding HIV-1 envelope varies by 25% from subtype to subtype. Conversely, HIV-1 *pol* is 2-3 times less divergent mainly because it encodes crucial replication enzymes; integrase, reverse transcriptase and protease which if excessively mutated would render the virus non-infectious. HIV-1 *pol* divergence is important in HIV-1 drug resistance because most ARVs target this region (Wainberg, 2004). It follows that variations in this region may affect susceptibility to ARVs and influence development of resistance.

Genetic diversity is clearly a critical issue in addressing HIV-1 drug resistance considering that HIV-1 diagnostic tools and drugs currently in use target subtype B and are used even in Non-subtype B variants where effectiveness has been shown to differ.

2.10 HIV-1 drug resistance and HIV-1 superinfection

The past few years have seen several reports of people infected with more than one HIV-1 strain. Dual infection is used as a blanket term for superinfection and coinfection. Superinfection refers to infection with a second strain of HIV after seroconversion following initial infection with a different HIV strain. Coinfection refers to infection prior to seroconversion, with two or more differing strains of HIV-1 at the same or at nearly the same time (Edmondson and Pearson, 2005).

Superinfection/ re-infection is a concern in HIV-1 drug resistance due to the possibility of viral recombination between a drug resistant strain and a drug sensitive strain which could hinder therapy or result in recombination between two or more drug resistant strains leaving the patient with even fewer options for therapy. Such dual infections result in improved viral fitness of the infecting population as manifest by increased viral load, lower $CD4^+$ T cell counts and faster progression to AIDS (Gottlieb *et al.,* 2004; Smith *et al.,* 2004). Smith *et al.* (2004) reported an individual infected with a drug sensitive clade B virus and later superinfected with another clade B virus resistant to two classes of ARVs whose viral load increased by approximately 10000 copies/ml with a 150 times decrease in $CD4^+$ T cell counts within 6 months.

HIV-1 triple infections are possible and four cases had been reported globally by end of 2007 (van der Kuyl and Cornelissen, 2007). A Cameroonian woman with a group O virus, subtype D and an A/G recombinant was reported by Takehisa *et al.* (1997; 1999). Gerhardt *et al.* (2005) reported a Tanzanian woman with two strains that were subtype A and a subtype C strain while Pernas *et al.* (2006) reported a Spanish man infected with three different subtype B strains. A Dutch man who was infected with two subtype B strains and a CR10-AE recombinant over a period of three years post seroconversion was reported by Gottlieb *et al.,* (2007). Though these cases are few, they serve as an indicator that such events are not entirely impossible especially in a high risk population such as the one being studied in this research.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This study was a cross-sectional laboratory-based retrospective study involving genotypic characterization of HIV RNA from archived (stored) plasma samples. This plasma was obtained during routine examination of persons enrolled in the "Comprehensive Studies of Mechanisms of HIV Resistance in Nairobi, Kenya".

3.2 Study site

The study was carried out in both the Centre for Virus Research (CVR)-KEMRI, the University of Nairobi/University of Manitoba collaborative group laboratories (Nairobi) and Medical Microbiology laboratories, University of Manitoba (Winnipeg).

3.3 Study population

This study involved individuals drawn from a commercial sex worker cohort based in the Majengo slum, Nairobi and managed by the University of Nairobi/University of Manitoba collaborative HIV group. The cohort was established well over two decades ago to study the epidemiology and biology of various sexually transmitted infections.

3.3.1 Sample - size determination

The prevalence of HIV drug resistance strains among drug naïve patients is estimated at 5% in Kenya (Ndembi *et al.*, 2008). The minimum size of the sample required was therefore determined by the formula below (Cochran, 1963);

$$n = \frac{z^2\alpha_{/2}.pq}{e^2}$$

Whereby;

n is the minimum sample size required

p is the estimated prevalence (5 %)

q is 1 less p (1-p)

e is the degree of precision(sampling error), which is 5 %

α is the level of significance (95%)

z is the standard normal deviate that corresponds to 95% confidence interval

$$\text{Therefore, n} = \frac{(1.96)^2 \times 0.95/2 \times 0.05(0.95)}{(0.05)^2}$$

$$\frac{3.8416 \times 0.475 \times 0.475}{0.0025} = 35$$

3.3.2 Inclusion criteria

− Samples obtained from individuals enrolled in the study "Comprehensive Studies of Mechanisms of HIV Resistance in Nairobi, Kenya".

− Samples obtained from individuals in the cohort, undergoing ARV treatment for six months or more, upon collection of the plasma sample.

3.3.3 Exclusion criteria

− Samples obtained from individuals in the cohort, undergoing ARV treatment for less than six months upon collection of the plasma sample.

One hundred and thirty eight (138) samples were available before applying the exclusion and inclusion criteria. After application of the exclusion and inclusion criteria, 84 samples were available for study. A census sampling method (Israel, 1992) was applied so that all the 84 samples were included in this study.

3.4 Laboratory procedures

3.4.1 RNA isolation from plasma

RNA isolation was carried out using plasma previously archived / stored at -80 ° C. Plasma was concentrated using Microcon® centrifugal filter devices (Millipore Corporation, Bedford, MA), according to the manufacturer's instructions. This concentrate was then resuspended in 140 µl PBS and RNA the isolated using The QIAamp® Viral RNA Mini Kit (Qiagen Inc., Cahtsworth, CA), in accordance to the manufacturer's protocol. The RNA so obtained was stored at -80 ° C.

3.4.2 Complementary DNA synthesis

Complementary DNA (cDNA) was synthesised using The High Capacity cDNA Reverse Transcription kit (Applied Biosystems, foster City, CA) following the manufacturer's guidelines. The maximum amount of RNA was used for each reaction. The reverse transcription product was stored at -20 ° C until use.

3.4.3 Polymerase chain reaction (PCR)

PCR reactions were carried out using The High Specificity PCR system (Invitrogen Corporation, Carlsbad, CA). A region of the pol-RT gene corresponding to nt 2480–nt 3180 of HIV-1 HXB2 was amplified by nested PCR with primer pairs, RT18 (5'-GGAAACCAAAAAT GATAGGGGGAATTGGAGG-3') and KS104 (5'-TGACTTGCCCAATTTAGTTTTCCCACTAA-3')in the first round, and KS101 (5'-GTAGGACCTACACCTGTTCAACATAATTGGAAG-3') and KS102 (5'-CCCATCCAAAGAAATGGAGGAGGTTCTTTCTGATG-3') in the second round (Songok et *al.,* 2004). Amplification was done with a hold at of 95 ° C for 2 min., 35 cycles of 95 ° C for 30 sec., 55 ° C for 30 sec., and 72 ° C for 1 min. and a final extension at 72 ° C for 10 min. Confirmation of amplification of the 697bp long segment was carried out by ethidium bromide staining of samples electrophoresed on 1.5 % agarose gel. Visualization and pictures were taken using The Gel Doc™ system (Bio-Rad Laboratories, Hercules, CA).

3.4.4 Sequencing

Sequencing PCR was performed using BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, foster City, CA) following the manufacturer's instructions.

Purification of the PCR product was performed using BigDye® Xterminator^{TM} purification kit (Applied Biosystems, foster City, CA) also following the manufacturer's guidelines.

All sequencing was done using BigDye$^®$ v3.1 sequencing technology (Applied Biosystems, foster City, CA) in a 16 capillary 3130xl Genetic Analyzer (Applied Biosystems, foster City, CA)

3.4.5 Experimental controls

Distilled water was used as a blank control, an HIV- free sample as a negative control, and an RNA sample isolated from cultured HIV-1 Subtype B infected cells as a positive control to monitor cross-contamination.

3.5 Analyses

3.5.1 General sequence analysis

Sequence Scanner® version 1.0 (Applied Biosystems, Foster City, CA) was used for initial visualisation of resultant sequences. Visual inspection of the sequence peaks was done and inadequate sequences were discarded to remove any discrepancies. Genetyx™ software was used to align sequences so generated.

3.5.2 HIV-1 resistance mutation analysis

RT nucleotide sequences generated from the procedures elucidated above (697bps) were translated into the corresponding amino acids and submitted to the Stanford HIV drug resistance database (http://hivdb.stanford.edu) and in the National Centre

for Biotechnology Information- Genbank Database (http://www.ncbi.nlm.nih.gov/Genbank/) for evaluation and identification mutations as well as interpretation of resistance. Resistance mutations so identified were also manually compared to those in the International AIDS Society-USA (IAS-USA), 2008 list (Johnson *et al.*, 2008).

3.5.3 Subtype analysis

The National Centre for Biotechnology Information (NCBI) subtyping program (http://www.ncbi.nih.gov/projects/genotyping/formpage.cgi), the HIV Blast tool (http://www.hiv.lanl.gov/content.hiv-db/BASIC_BLAST/basic_blast.html) and the REGA HIV-1 Subtyping tool (http://www.bioafrica.net/subtypetool/) were used for initial rapid subtype screening before confirmation by phylogenetic analysis using reference sequences from The Los Alamos website (http://www.hiv.lanl.gov/content.hiv-db/SUBTYPE_REF/align.html) . The subsequent detailed phylogenetic analysis was done in Treeview® and Molecular Evolutionary Genetics Analysis (MEGA) version 4.0 softwares, using Clustal W. A Neighbour Joining (NJ) tree based on the bootstrapped Kimura 2-parameter method was generated.

3.6 Ethical Considerations

Informed consent for demographic and behavioural surveys, as well as biological sampling was previously obtained from all study participants in the cohort. A consent form so used appears in the appendix. This study did not involve direct contact with subjects as archived plasma samples obtained from persons enrolled in the

"Comprehensive Studies of Mechanisms of HIV Resistance in Nairobi, Kenya" were used. Clearance to carry out this study was sought from and granted by the KEMRI Scientific Steering Committee (Ref: ESACIPAC/SCC/2873) and the KEMRI Ethical Review Committee (Ref: KEMRI/RES/7/3/1). All clinical investigations were conducted according to the Principles of Helsinki Declaration.

3.7 Data storage

All data besides being recorded in hard copy was stored in a computer hard drive, portable disks (USB stick$_{(s)}$, CD-ROM). The University of Nairobi / University of Manitoba HIV collaborative group also manages a database for all ongoing and previous studies to which access is highly restricted and data generated from this research was archived in this database.

CHAPTER FOUR

RESULTS

4.1 Patient treatment history

Following laboratory procedures 60 samples were available for analyses. All patients studied were on a NRTI/ NNRTI combination therapy except for ML2572 who was on a NRTI/ NNRTI/ PI combination therapy (Appendix II). CD4$^+$ T cell count taken at sample point ranged from 43 cells/ml to 704 cells/ml (Fig. 4.1). The length of time each patient was on ARV treatment ranged from 24 weeks to 149 weeks; an average of 88 weeks(Fig. 4.2).

4.2 Subtype distribution

Sequence alignment and phylogenetic analysis showed 58 isolates (97%) to be HIV-1 subtype A1 while 2 isolates (3%) were HIV-1 subtype D (Table 4.1). Isolates that were found to be HIV-1 subtype AI showed further diverse sub-clustering in the Neighbour-Joining tree (Fig. 4.3) indicating divergence within the HIV-1 subtype A1 among the patients.

Table 4.1   Summary of subtype and genotype distribution

	Subtype A1	Subtype D	
Wildtype	55	0	55 (91.6%)
Mutant	3	2	5 (8.3%)
	58 (96.7%)	2 (3.3%)	60
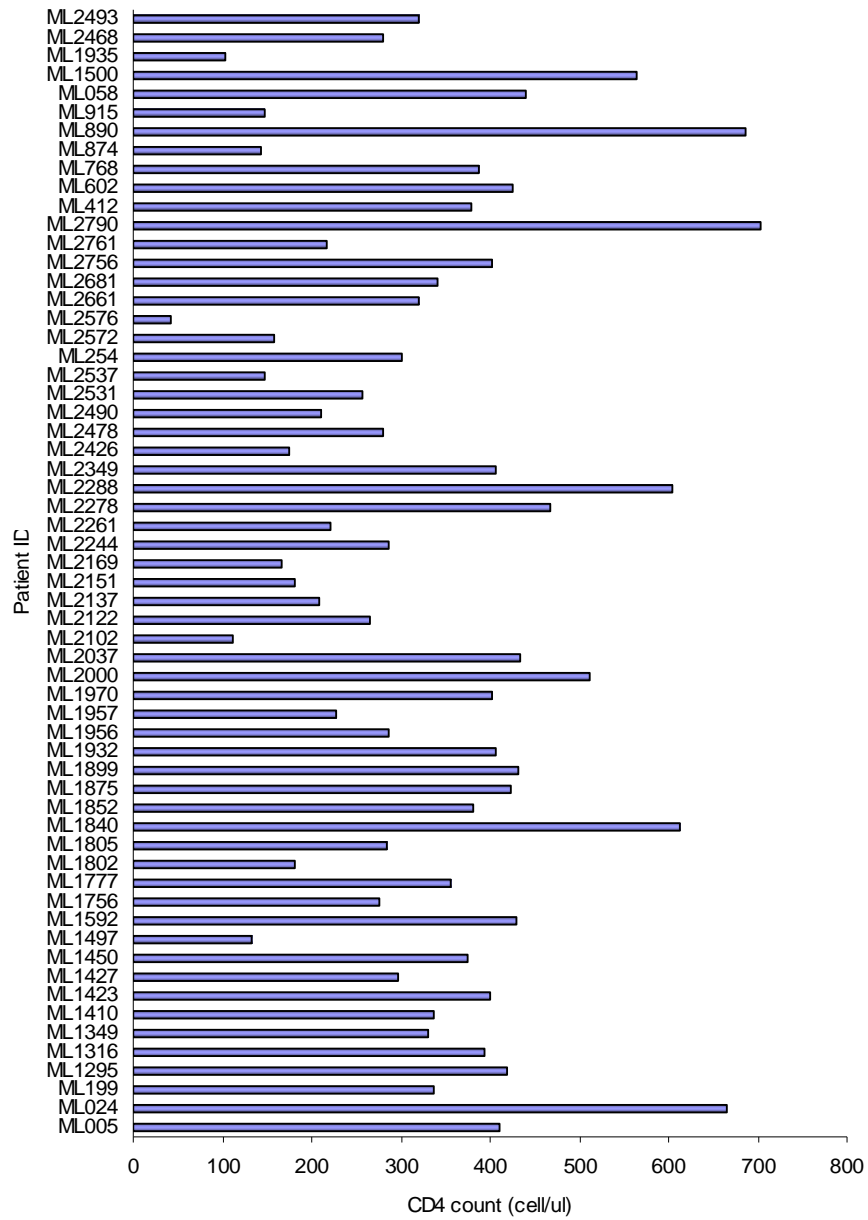
Figure 4.1   CD4$^+$ T cell counts for patients at sample collection
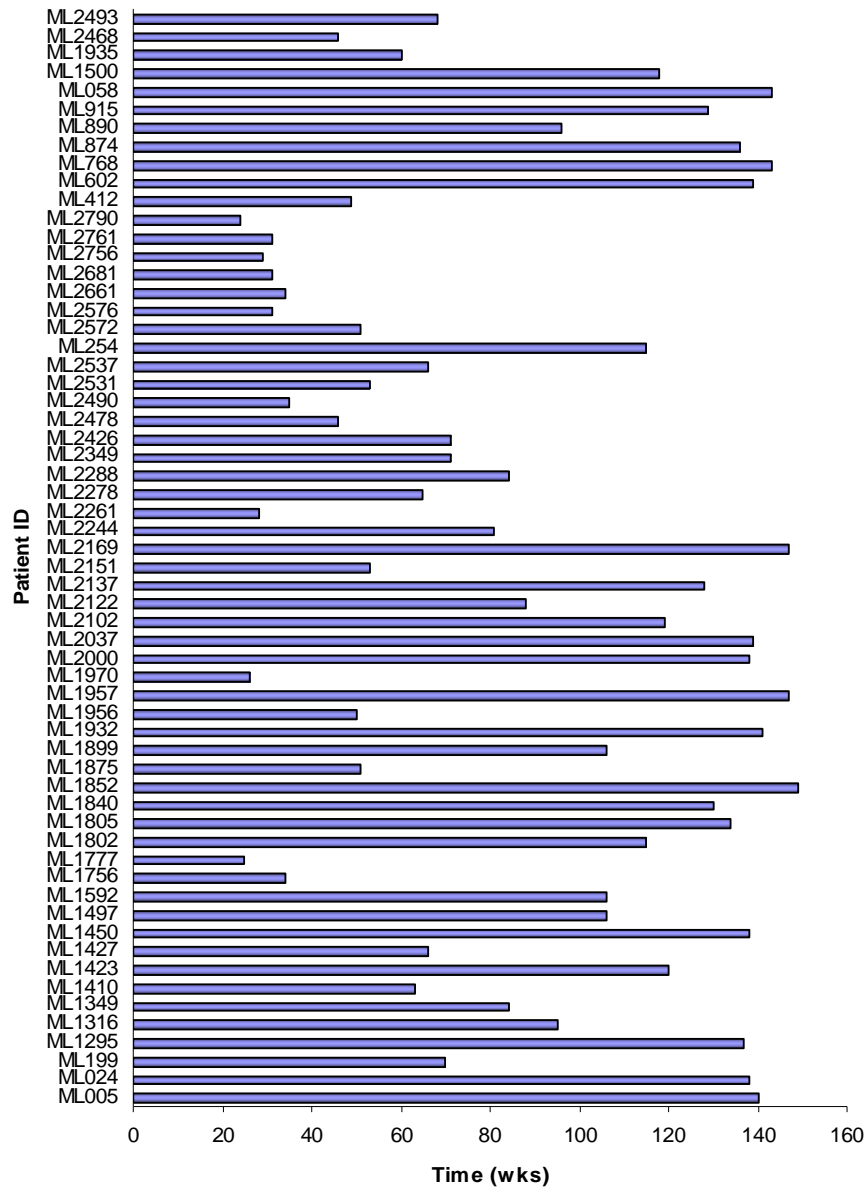
Figure 4.2   Length of time on ARV treatment for each patient at the point of sample collection.
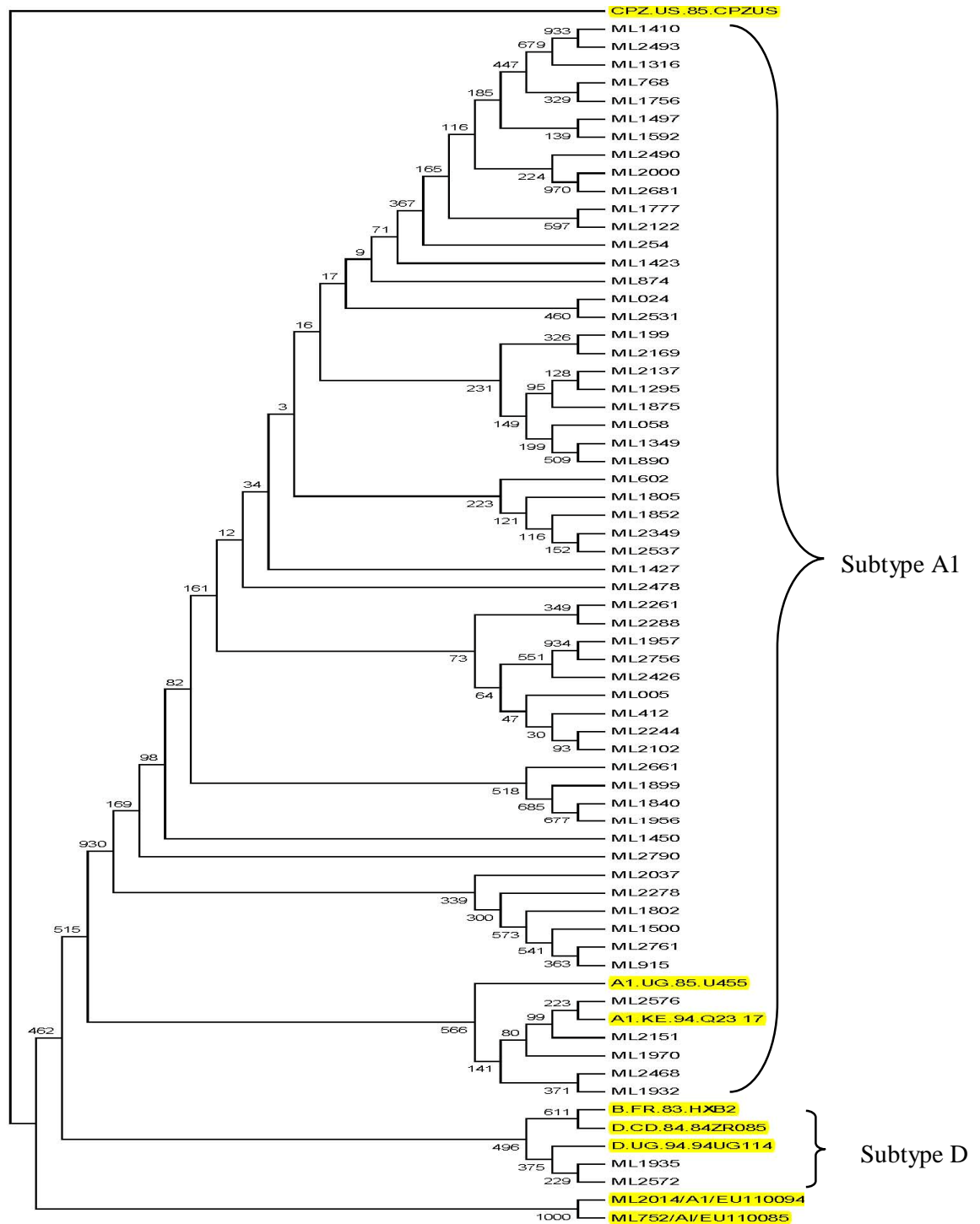
Figure 4.3 Neighbour-Joining tree of the pol-RT gene corresponding to nt 2480–

nt 3180 of HIV-1 HXB2 of 60 HIV-1 sequences. Study samples are indicated by an

ML number. The reference sequences are highlighted.

4.3 Drug-associated resistance mutations

Of the 60 isolates available for analyses, 55 (91.6%) were found to be wildtype while 5 (8.3%) showed mutations conferring resistance to reverse transcriptase inhibitors (Table 4.1) indicating an HIV-1 drug resistance prevalence rate of 8.3%. Of the 5 isolates found to have RTI-associated resistance mutations, 3 (5%) were found to be infected with HIV-1 subtype A1 while 2 (3.3%) were found to be infected with HIV-1 subtype D (Table 4.1). Genotypic evidence of mutation points, relative to a consensus HIV-1 subtype B sequence is indicated in figure 4.4.

4.4 Resistance patterns in relation treatment regimen

Complete class resistance to NNRTI was identified in 2 isolates (3.3%), ML1935 and ML2572; both of them of HIV-1 subtype D. These 2 isolates at the same time showed resistance to some drugs from the NRTI class. Complete class resistance to the NRTI class was not observed in any isolate. Two isolates (3.3%), both if HIV-1 subtype A1 (ML1756, ML2661) were susceptible to all drugs in the NNRTI class while 1 isolate (ML1932) representing 1.7% was susceptible to all NRTI (Table 4.2). All patients with RTI-associated mutations showed evidence of resistance to at least one drug in their treatment regimen (Table 4.2).

4.5 Minor mutation and polymorphisms

Minor mutations and polymorphisms were detected in all the 60 isolates that were analysed. K11T, K20R, V21I, V35T, T39R/I, V60I, K122E, D123S, K173A/V, Q174K, V179I, G196E, T200V, Q207A/E/L/D and R211S/H/K were present in over 50% of the isolates. V60I was present in all (100%) of the isolates (figure 4.5).

Table 4.2   Important characteristics of 5 isolates with ARV-associated drug resistance mutations.

Patient ID	ART at study point	Length of time on ART (wks)	HIV-1 Subtype	RTI resistance mutations	Drugs for which mutations compromise efficacy Class: NRTI	Drugs for which mutations compromise efficacy Class: NNRTI
ML1756	NVP/d4T 30/3TC	34	A1	T2I5S	ABC, AZT, **d4T**, ddI, TDF	None
ML1932	NVP/d4T 40/3TC	141	A1	G190A	None	**NVP**, ETR, EFV
ML1935	NVP/d4T 40/3TC	60	D	M184V, Y181C	**3TC**, ABC, FTC	All (DLV, EFV, ETR, **NVP**)
ML2572	TDF/KALETRA/ AZT	51	D	D67N, V118I, M184V, K101E, Y181C, G190A	3TC, ABC, **AZT**, d4T, ddI, FTC	All (DLV, EFV, ETR, NVP)
ML2661	NVP/d4T 30/ 3TC	34	A1	D67A	**d4T**, **3TC**, AZT	None

Drugs whose efficacy was compromised and were part of patient's regimen are underlined and highlighted in bold-type.

```
consensus   LNFPISPIETVPVKLKPGMDGPKVKQWPLTEEKIKALVEICTEMEKEGKISKIGPENPYN 60
ML2572      LNFPISPIETVPVKLKPGMDGPKVKQWPLTKEKIEALTEICTEMEKEGKISRIGPENPYN 60
ML1935      LNFPISPIKTVPVKLKPGMDGPKVKQWPLTEEKIKALTEICTDMEKEGKISRIGPENPYN 60
ML1756      FDFPISPIETVPVTLKPGMDGPRINQWPLTEEKIKALTEICTEMEKEGKISKIGPENPYN 60
ML2661      LNFPISPIETVPVTLKPGMDGPRIKQWPLTEEKIKALTEICREMEKEGKISKIGPENPYN 60
ML1932      LNFPISHIETVPVKLKPGMDGPKVKQWPLTEEKIKALTEICTEMEKEGKISKIGPENPYN 60
            ::**** *:****.********:::*****:***:**.*** .********:********

consensus   TPVFAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFS 120
ML2572      TPIFAIKKKNSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLEKKKSVTVLDVGDAYFS 120
ML1935      TPIFAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHRAGLKKKKSVTVLDVGDAYFS 120
ML1756      TPIFAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFS 120
ML2661      TPIFAIKKKASTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFS 120
ML1932      TPIFAIKKKDSTKWRKLVDFRELNKRTQDFWEVQLGIPHPAGLKKKKSVTVLDVGDAYFS 120
            **:******.******************************.****:**************

consensus   VPLDEDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNPD 180
ML2572      IPLDENFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNPE 180
ML1935      VPLDEDFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRKQNPE 180
ML1756      VPLDESFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRAKNPE 180
ML2661      VPLDESFRKYTAFTIPSINNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRAKNPD 180
ML1932      VPLDESFRKYTAFTIPSTNNETPGIRYQYNVLPQGWKGSPAIFQSSMTKILEPFRSKNPE 180
            :****.**********.*************************************.**:

consensus   YQYMDDLYVGIVISDLEIGQHRTKIEELRQHLLVGPTPVNIIGRNLLTQIGCT 233
ML2572      CQYVDDLYVAMVISDLEIGQHRAKIEELREHLNG---DFHTSKNNLLTQIGCT 230
ML1935      CQYVDDLYVGMVISDLEIGQHRAKIEELREHLLN--VGITTPDKNFVDSDWLS 231
ML1756      YQYMDDLYVGVIISDLEIEQHRVKIEELRAHLLT--LGIFYTRQKFVDPVLVV 231
ML2661      YQYMDDLYVGIIISDLEIEQHRVKIEELRAHLLR--WGFTTPDKNFVDPDWLS 231
ML1932      YQYMDDLYVAIIISDLEIGQHRTKVEELRDHLLS--WGLTTPDKK-VDPDWLS 230
            **:*****.::******.***.*:**** **      .     .: :
```

Figure 4.4   Genotypic evidence of mutations conferring resistance to reverse transcriptase inhibitors detected in 5 isolates.
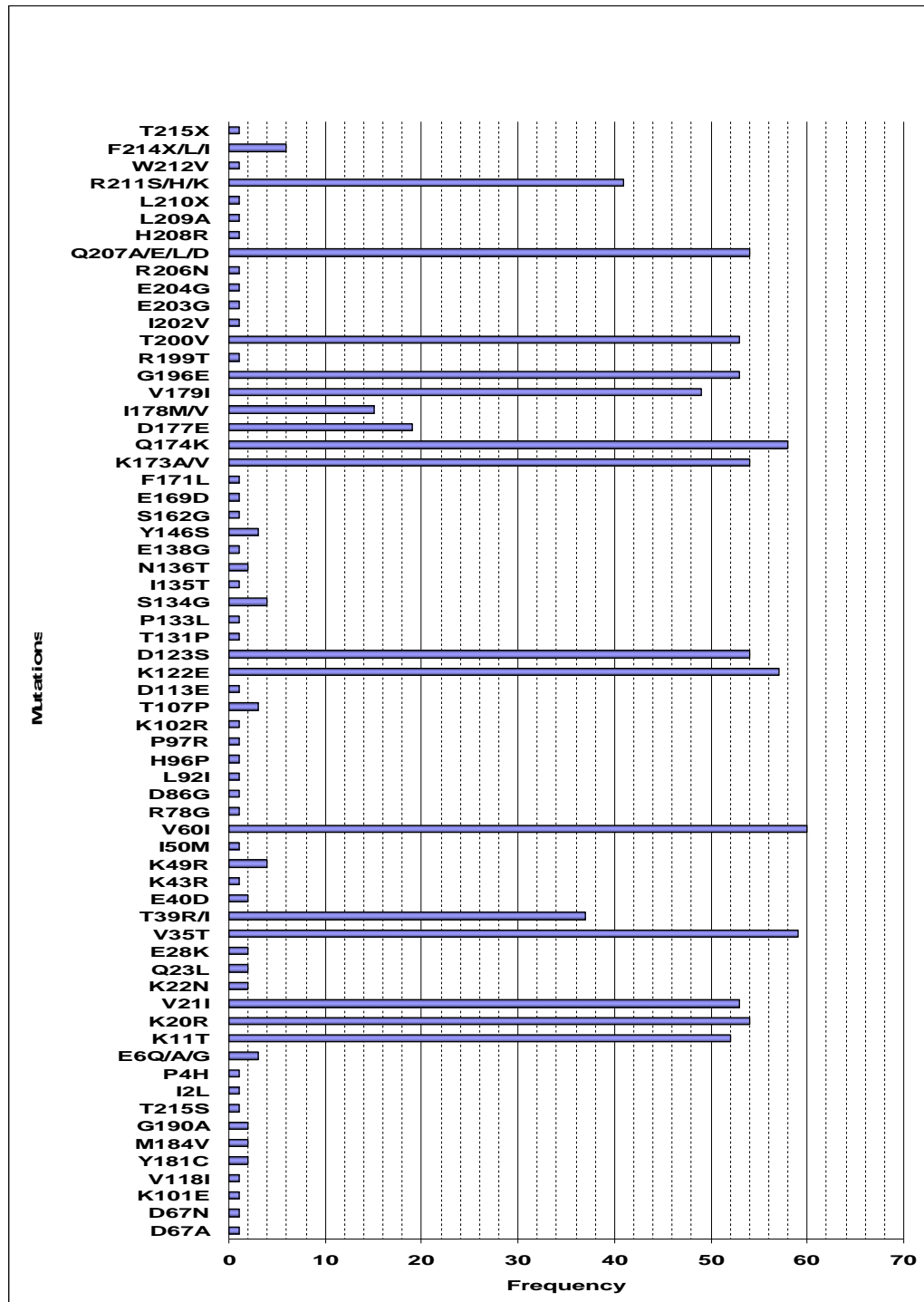
Figure 4.5 Major RTI-associated mutations, other minor mutations and polymorphisms detected in the 60 HIV-1 isolates.

CHAPTER FIVE

DISCUSSION

5.1 Subtype distribution

This study, by use of sequence subtyping and phylogenetic analyses of the pol-RT region corresponding to nt 2480 to nt 3180 of the HIV-1 HXB2 virus, explores genetic diversity in this group of women under study. HIV-1 subtype A1 was found to be predominant in the group at 97%, while HIV-1 subtype D was observed in 3% of the women (Fig.4.1). This is consistent with previous studies that have shown HIV-1 subtype A to be the predominantly circulating subtype in various regions in Kenya, and the country as a whole (Dowling *et al*., 2002; Khamadi *et al*., 2004; Lihana *et al*., 2006; Land *et al*., 2008; Khoja *et al*., 2008). Yang et *al*. in 2004 found HIV-1 subtype A1 to be most predominant in Western Kenya, followed by HIV-1 subtype D and this was when analyses were carried out on both the gag-p24 and env-gp41 regions.

There are a few studies on HIV-1 subtype distribution that have been carried out within the Pumwani cohort. Following evaluation of complete HIV-1 RNA sequences, Fang *et al*. (2001) found that 60% of the samples were purely HIV-1 subtype A, 10% were purely subtype D ad 30% were intersubtype recombinants. Land *et al.* (2008) reported 50% pure HIV-1 subtype A and 50% were unique intersubtype recombinants C/D, A2/C/D, A1/D, A1/C/G and A1/C/D. In both studies just as in this study, HIV-1 subtype A predominates. Intersubtype recombinants represent an important and significant proportion of the study population in both

studies by Fang *et al.* (2001) and Land *et al.* (2008). An examination of subtype distribution between 1986 and 2000 in Kenya showed an increase in the number of circulating recombinant viruses (Rainwater *et al.*, 2005). Recombination analyses would therefore be useful in extending the dataset in the Majengo cohort, in any follow-up studies.

5.2 Minor mutations

Additional mutations other than those designated by the International AIDS Society (IAS) have been associated with exposure to ARVs (Cane *et al.*, 2007). The number of changes in the RT gene relative to consensus increases from a median of 4 in untreated individuals to 14 in heavily treated individuals (Gonzales *et al.*, 2003). Populations with numerous minor mutations that may have influence or no influence on the main/major ART-associated mutations do occur among both drug naive and ARV-treated populations and may have either an inverse or a direct correlation to resistance (Saracino *et al.*, 2006).

Minor mutations and polymorphisms were detected in all the 60 isolates that were analysed. V60I was present in all (100%) of the isolates. K11T, K20R, V21I, V35T, T39R/I, V60I, K122E, D123S, K173A/V, Q174K, V179I, G196E, T200V, Q207A/E/L/D and R211S/H/K were present in over 50% of the isolates. Others present in lower frequencies were IL2, P4H, E6Q/A/G, K22N, Q23L, E28K, E40D, K23R, K49R, I50M, R78G, D86G, L92I, H96P, P97R, K102R, T107P, D113E, T131P, P133L, S134G, I1357, N136T, E138G, T146S, S162G, E169D, F171L,

D177E, I178M/V, R199T, I202V, E203G, E204G, R206N, H208R, L209A, L210X, W212V, F214X/L/I and T215X.

K49R, V60I, V179I, Q207A/E/K/D, R211S/K, F214Q/K/T/N/ L and F177E have been identified as subtype B-associated naturally occurring polymorphisms (Nyombi *et al.*, 2008). In non-B subtypes, mutations at positions T200, Q174, K173, D123, K122, V60, T39, V35 and K20 have been identified in both treated and drug naïve populations (Kantor and Katzenstein, 2003; Cerqueira *et al*., 2004). Mutations at these positions are therefore transmissible and this could explain the high frequency of these mutations observed in this study. V60I has also been identified as a polymorphic amino acid residue in specifically both HIV-1 subtypes A and D and this therefore could further account for its occurrence in all the isolates studied. Q207A and R211S have identified in HIV-1 subtype A and K49R in HIV-1 subtype D (Nyombi *et al.*, 2008). While K49R was identified in both subtype D isolates, it also occurred in 2 subtype A isolates. The mutation at position 207 in both subtype D isolates was Q207E while other variations Q207A//L/D occurred in the subtype A isolates.

In a study to verify possible association of previously unreported RT mutations with a decrease of phenotypic susceptibility to RTIs, mutations at positions K20, T39, V35, K122, D123, K173, Q174, V179, T200, Q207 and R211 were identified (Saracino *et al*.,2006). These mutations occurred more frequently in the treated population than in the drug naïve population. Ngonzales *et al*. (2003) also identified mutations at positions K20 and K43 as being associated with previous use of RTIs. A

correlation has been found between the presence of K20R and the use of lamivudine while T39R was associated with the previous use of AZT and with the development of thymidine analog resistance (Saracino *et al*., 2006). Fifty nine of sixty patients in this study were on lamivudine and K20R occurred in 54 isolates, correlating with the findings by Saracino *et al*., (2006). Five patients in this study were on AZT and while they all had a mutation at position T39, 32 patients that were not treated with AZT also showed the same mutation.

Positions I135, D133 and K43 are traditionally conserved and are only polymorphic in the presence of drug pressure ((Florance *et al*., 2003). These positions play a role in viral enzymatic activity with which such polymorphisms may interfere. These mutations were observed only in a single isolate each. This is probably because they are naturally selected against in order to preserve viral enzymatic integrity. Conducting viral fitness studies on these isolates would therefore be useful in establishing the effect of these mutations.

5.3 Prevalence of ART-associated mutations

HIV-1 drug resistance in Kenya has been observed at 5% in drug naïve populations (Ndembi *et al*., 2008). In this study population, 8.3% drug resistance prevalence was observed. This prevalence is low relative to previous studies on treated populations in Kenya which have reported prevalence of resistance mutations that is much higher (Kiptoo *et al*., 2008; Lwembe *et al*., 2007). Antiretroviral therapy was introduced into this population only recently (late 2005, early 2006) and this could account for the much lower prevalence observed.

The isolates studied were collected at different times in the treatment courses individual patients, ranging from 34 weeks to 149 weeks on treatment. The length of time on treatment however did not appear to have an influence on the number of drug resistance mutations in the isolates (table 4.2). This is despite findings that show that prevalence of drug resistance rises with increase in the length of time of exposure to ART (Grant *et al*., 2002; Lucas *et al*., 2004; Vaclavikova *et al*., 2005; Li *et al*., 2007).

5.4 Significance of specific mutations

A total of seven mutation points (at codons 67, 101 118, 181, 184, 190 and 215) were identified in the 5 isolates. G190A, M184V and Y181C were each identified in two isolates while the rest T215S, D67N, V118I, K101E and D67A were detected in only one isolate each. G190A, Y181C and K101E compromise NNRTI efficacy while T215S, V118I, D67N, D67A and M184V compromise NRTI efficacy. Presence of the revertant mutation I/S at position 215 suggests suboptimal therapeutic regimens that are unable to prevent development of resistance (Wagner *et al*., 2000). V118I is considered a strong marker of HIV-1 disease progression (Zaccarelli *et al.*, 2007) and was observed in subject ML2572 who has since been deceased. Owing to such a small sample size however it is not possible to identify mutation(s) most selected for in this population; another reason is that this was a one point-in-time study rather than a follow-up study.

There are two resistance pathways described for NNRTI; the K103N, V106M, Y188L pathway and the L100I, V106A, Y181C/A, G190A/S, M230L pathway. All

three isolates ML1932, ML1935, ML2572 that showed NNRTI resistance in this study follow the latter pathway, the pathway most often found to be associated with nevirapine (NVP) therapy (Deforche *et al.*, 2008). Nevirapine made up part of the ART regimen for ML1932 and ML1935.

5.5 Resistance and ARV regimen

All patients with RTI-associated mutations showed evidence of resistance to at least one drug in their treatment regimen (table 4.2).

While ML1756 was on NVP, d4T and 3TC, she showed evidence of resistance to the NRTIs ABC, AZT, d4T, ddI and TDF. The RTI-associated resistance mutation T215S found in ML1756 is known to confer low level resistance to AZT and d4T and could potentially confer low level resistance to ABC and TDF.

Patient ML1932 was on NVP, d4T and 3TC and had the RTI-associated resistance mutation resistance mutation G190A which confers low level resistance to ETR, intermediate resistance to EFV and high level resistance to NVP. The patient showed compromised efficacy to NVP, ETR and EFV.

Patient ML1935 was being treated with NVP, d4T and 3TC and had the RTI-associated resistance mutations M184V and Y181C. M184V confers high level resistance to the NRTIs 3TC and FTC and low level resistance to ABC and ddI. Y181C confers high level resistance to DLV and NVP and low level resistance to

EFV. ML1935 was found to be resistant to the NRTIs 3TC, ABC, FTC and all NNRTIs (DLV, EFV, ETR and NVP).

Patient ML2572 was on TDF, AZT and the protease inhibitor, KALETRA. She had the RTI-associated resistance mutations D67N, V118I, M184V, K101E, Y181C and G190A. K101E confers low level resistance to EFV and ETR, and intermediate resistance to DLV and NVP. V118I confers low level resistance to 3TC. M184V confers high level resistance to the NRTIs 3TC and FTC and low level resistance to ABC and ddI. Y181C confers high level resistance to DLV and NVP and low level resistance to NVP. D67N confers low level resistance to ABC, AZT, d4T and ddI while G190A confers low level resistance to ETR, intermediate resistance to EFV and high level resistance to NVP. ML2572 was found to be resistant to the NRTIs 3TC, ABC, AZT, d4T, ddI and FTC and all NNRTIs (DLV, EFV, ETR and NVP). ML2572 was therefore susceptible only to TDF, among the RTIs.

Patient ML2661 as on NVP, d4T and 3TC and had the RTI-associated resistance mutations D67A. Although A is an atypical mutation at codon 67, it could potentially confer low level resistance to d4T. ML2661 showed resistance to 3TC and d4T.

Complete class resistance to NNRTI was observed in ML1935, ML2572. According to a study conducted within the REACH cohort of San Francisco, California, NNRTI resistance occurs most often at lower levels of adherence to ART (Bangsberg *et al.*, 2004; Orofino *et al.*, 2004). This, coupled with the occurrence of mutations that have

been associated with suboptimal therapeutic regimens as mentioned above, suggests

that adherence to ART in this population should be monitored closely.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

HIV-1 subtype A1 occurred in 97% of the patients studied while HIV-1 subtype D occurred in 3% of the patients studied. There was evidence high divergence observed among the subtype A1 isolates.

Resistance mutations occurred in 8.3% of patients studied. This is a low prevalence for a treated population compared to reports from previous studies. The prevalence rate in this study is however highly significant as the population studied poses a high risk of transmitting to the general population.

6.2 Recommendations

This population needs to be followed up in a longitudinal study in order to clearly map the HIV-1 drug resistance characteristics. These studies should involve detailed evaluation of host factors such as adherence as well as viral factors.

Baseline genotypic resistance testing for all persons enrolling in the cohort is recommended in order to determine the prevalence of HIV-1 drug resistance in the naïve population and also as a guide for clinicians when designing regimen.

There is an urgent need to incorporate antiretroviral resistance monitoring protocols among HIV-1 prevention programs nationally in order to monitor risk of transmission.

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Comprehensive Studies of Mechanisms of HIV Resistance in Nairobi, Kenya

Majengo Research Clinic

Resurvey: Patient Information and Consent Form

This information will be communicated orally in English, Swahili or other Kenyan dialect of potential participant's preference.

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Background information

The University of Nairobi and its collaborators from Canada have been working for many years to fight the epidemics of AIDS and other sexually transmitted infections that we are facing in Kenya. This basic science research program is conducting studies to determine the relationship between immunity and susceptibility to sexually transmitted infections (STI) with the goal of developing vaccines or treatments for STIs. You are being asked to participate in this study because you are:

        a) At a very high risk of acquiring an STI or are already infected with an STI
or

b) At a low risk of acquiring an STI; or

c) The relative of a person in group a) or b).

The purpose of this research program is to determine if there are factors that could protect individuals from acquiring sexually transmitted infections (STI) especially HIV. It is important to keep free of other sexually transmitted diseases, as the presence of these infections may increase your risk of becoming infected with HIV. If you have an STI, you should seek treatment for it as quickly as possible. However, sometimes you may have an STD and not know it, because you may not have any symptoms and thus advised to visit the clinic monthly for free check ups.

Why Is This Study Being Done?

This study is being done to find out why some people are more or less likely to get the Human Immunodeficiency Virus (HIV), the virus that causes AIDS. There is more and more evidence that the immune system in some people is able to protect them against infection with HIV. Since most people get HIV through sexual exposure to an HIV infected partner, the first contact with the virus occur in the genital tract, the vagina and cervix in women. We know from some of our previous work that some women, who seem to be protected against HIV, have a special type of immune
response that it not present in women who get HIV. The purpose of this study is to try to find out the targets of this immune response in the vagina, uterus and cervix and to try to find out what is special about the immune system of these few individuals. This work may be helpful in eventually making a vaccine for HIV.

To help you understand what is involved in the study a drawing of the vagina, cervix and uterus of the female genital tract is shown below.

Fallopian Tube

Uterus (womb)

Ovary   Cervix

Vagina
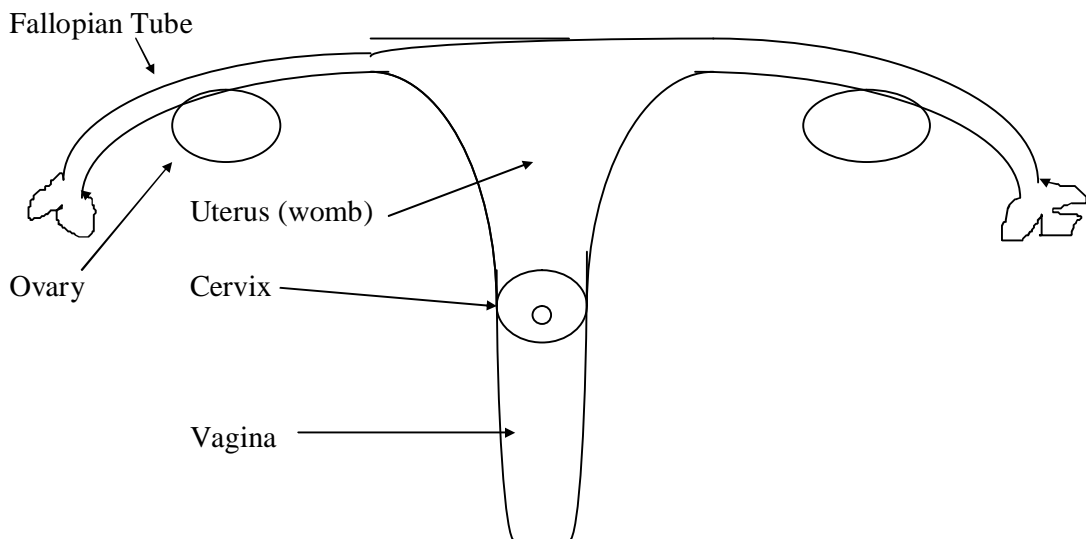
How Many People Will Take Part in the Study

About 3000 participants mainly women will take part in this study.

<u>What Is Involved in the Study?</u>

You have been invited to voluntarily participate in this study because all are at risk of becoming infected with STDs and HIV. Some sexual behavior especially among sex workers or those who use sex as an income generating activity exposes those involved or their partners to a higher risk of contracting HIV. If you agree to participate in the study, you will be given additional counseling, advised on appropriate STIs prevention strategies and requested to practice safer sex. You can also choose to leave sex work at any time but you will be asked to return to the clinic every month for free check ups. Again, the results of these tests will be ready after one week or less, and you will be informed of the results and given the correct treatment if you have an infection. You will also be encouraged to come to the clinic for examination and treatment at any other time that you feel ill. If you forget to return to the clinic for one of your scheduled visits, a clinic staff member will contact you by phone, SMS or send one of your friends to remind you of the missed appointment**. All study participants will also be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on HIV infection status every three months.** In addition, we will store specimens from your blood for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

Clinic visits
First visit and semi-annual visits (All study participants)
1. We will ask you general questions about your life, about problems you are having, and about your sexual history.
2. The doctor will examine your body, including your female parts.
3. Swab and washing from your vagina to look for germs and to collect samples for studying your immune response.
4. Swab from your cervix to look from germs and to collect samples for studying your immune response.
5. A thin plastic tube will be placed in your cervix (opening to your womb) to get some of the mucous your cervix makes.
6. Urine to look for germs.
7. Three tablespoons of blood will be taken for testing syphilis, and HIV and for studying your immune response. We will inform you of your results at your one month visit. We also will test your spouse for 0the HIV virus free of charge if he/she wishes.

Monthly visits
1. Questions will be asked about you, and what problems you are having.
2. If you have any complaints the doctor will examine your body, including your female parts.
3. Every third month all study participants will be encouraged to either retest for HIV or recheck their CD4/CD8 profiles depending on individuals HIV infection status.

Follow-up visits (All study participants)

1. You will be asked to return 3 to 7 days after every visit to be given you laboratory results.
2. You will be treated for new infections, free of charge.

How Long Will I Be in the Study?

The study will last 5 years. Although we would appreciate if you stayed in the study for the entire period you may choose to leave the study at any time without any penalty to you.

What Are the Risks of the Study?

Risk of blood and cervical collection

This study requires the use of your blood. In order to get the blood we will need to insert a needle into a vein in your arm so that the blood can be removed. There will be some pain associated with the needle stick but this will be only for a short period of time. There may be some bruising around the needle site and, although we will sterilize the site to minimize infection, there is a very minimal risk of infection at the site. There is also some discomfort associated with taking specimens from your cervix.

HIV test

Non-physical risks:

1. If you are HIV positive, learning so may cause you to become depressed. We will counsel you about your HIV test results if you are negative or positive. If you are HIV positive, we have antiretrovirals in the clinic to manage your condition. We will also test one boyfriend for HIV virus if he wants.

Risks of taking antibiotics / Antiretrovirals

If we find that you have an STD or AIDS we will provide you with the appropriate treatment. With any drug there is some potential for side effects. For the antibiotics/ antiretrovirals you might receive, the following side effects are possible.

Very likely:
1. Sick to your stomach
2. Headache
3. Metallic taste in mouth
4. Diarrhea
5. If a woman - infection of your vagina by yeast (a white discharge with itching). If this happens, we will give you medicine to put inside your vagina to treat the yeast infection.

Less likely but serious:
1. Less than 1 person in 100 will have a severe allergic reaction to one of the antibiotics/ antiretrovirals.

Are There Benefits to Taking Part in the Study?

The benefits that you will get from this study are that you will be examined regularly, and if you are found to have an STD or AIDS, you will receive appropriate and effective medication. Medical care will also be provided for other illnesses that you might have. You will also be informed about what you are suffering from, and you will be informed about the future implications of these STDs and of HIV.

What about Confidentiality?

Efforts will be made to keep your personal information confidential. We will record your information only by a special number assigned to you. The number will only be known to the clinic staff and yourself.

Organizations that may inspect and/or copy your research records for quality assurance and data analysis include groups such as: the researchers, members of the local and international ethics teams and the National Institutes of Health in the United States of America. The research results will be published, but your identity will remain secret.

What Are My Rights as a Participant?

Taking part in this study is voluntary. You may choose not to take part or may leave the study at any time. Leaving the study will not result in any penalty or loss of benefits to which you are entitled. If the participation in the study results in you becoming ill, the study team will provide you with medical care for the problem for free.

Although you will not be paid to participate in the study, you will be offered a small payment of two hundred shillings (KSh 200) for the resurvey visits only to compensate you for your transportation to the clinic.

We will also provide you with any new information and findings from the study that may affect your health, welfare, or willingness to stay in this study.

All information that is obtained will be kept strictly confidential, and your identity will not be known, except to those providing your medical care.

At the end of every year, we will be holding baraza's at the different clinics to give progress reports and share any new findings from the study with all members of the different clinics.

WHOM DO I CALL IF I HAVE QUESTIONS OR PROBLEMS?

For questions about the study or a research-related injury, call or contact Dr. Wachihi any one of the researchers named above at the Medical Microbiology Annex at the University of Nairobi

For questions about your rights as a research participant, contact Professor Bhatt, who is the chairperson of the Ethical Review Committee at the University of Nairobi, by calling 725452, or make an appointment to see her in the Department of Medicine, at the University of Nairobi.

Statement of Consent:

I have read the attached written information and / or received verbal information on the above study. I have been given the opportunity and time to have any questions about the research answered to my satisfaction. I consent to take part in the study and I am aware that my participation is entirely voluntary. I understand that I may withdraw at any time without giving a reason and without this affecting my future care.

By signing this information and consent form I agree that my personal data, may be used as described in this consent form and may be consulted by qualified representatives from sponsor the Ethics Committee or the health authorities.

I understand that the following (check the box only if you fully understand and agree with each statement):

☐ the goals of this research program are to study resistance and susceptibility to sexually transmitted infections

☐ enrolment is completely voluntary and I can withdraw from the study at any time

☐ blood, cervical and vaginal specimens will be required for this study and may be used for genetic studies

☐ any blood specimens previously collected may be used for this study

☐ a portion of my blood, cervical and vaginal specimens will be stored for future studies of the genes involved in resistance and susceptibility to HIV and other infections.

I am willing to participate in the study.

Name of Study Participant_____

Signature/Thumb print: _____ Date: _____

For clinic staff:

I, _____, have explained the nature and purpose of

the above study to _____

Name of Clinic Staff: _____

Signature: _____ Date: _____

Assigned Study Number / Clinic Number ____ ____ ____

Standards of Medical Care for Participants in the Research Clinics

This document outlines the standard of medical care for all participants in the Majengo, MCH Pumwani, Kindred, Kibera and Korogocho cohorts, regardless of HIV-1 serostatus. It should be emphasized that any member of the said cohorts may freely decline to take part in any cohort substudy, and that this decision will in no way affect their access to this standard of care. All care outlined is provided free of charge, thereby significantly improving health care access and outcomes for all members of the cohorts... The nature of the medical care will vary depending on HIV-1 serostatus of the participants, as outlined below.

1. **General medical care for all participants, regardless of HIV-1 status**.
    - HIV and STD prevention services: provision of the male condom, and peer-based and clinic-based counseling regarding safer sexual practices.
    - Family planning services as directed in the *Kenyan National Family Planning Guidelines*
    - Rapid and effective treatment of sexually transmitted diseases in accordance with the *Kenya National Guidelines for the Syndromic Management of Sexually Transmitted Diseases*
    - Medical care for acute and chronic illnesses, both infectious and non-infectious
    - Access to diagnostic testing in haematology, biochemistry, infectious diseases, immunology, radiology
    - Prompt referral for specialist consultation and hospitalization when indicated

2. **Management of Opportunistic Infections in HIV-1 Infected Participants**.
    - **Primary Prophylaxis**: Trimethoprim-Sulphamethoxazole (Septrin): all participants with a CD4+ T cell count $<200/mm^3$ or on Anti-TB must use Septrin for prevention of PCP, toxoplasmosis and bacterial infections (bacterial pneumonia, bacteremias, some bacterial diarrhoea), according to *National AIDS/STD Control Program (NASCOP) Guidelines.*
    However, all HIV infected individuals with CD4+ <350 should be encouraged to use Septrin according to the latest WHO guidelines (2006)
    - **Secondary Prophylaxis:** Septrin: offered to all participants regardless of CD4+ T cell count after an episode of PCP, toxoplasmosis, or severe bacterial infection. Fluconazole: provided for secondary prevention of Cryptococcus

    Treatment
    - Herpes. simplex/Herpes zoster infection: acyclovir
    - Candidiasis (oral, esophageal, vaginal): nystatin, clotrimazole, Fluconazole
    - Tuberculosis (pulmonary or extra pulmonary): referral to National TB Programme

- Toxoplasmosis: referral for inpatient therapy
- Cryptococcus: referral for inpatient therapy
- PCP: Septrin (with prednisolone, if severe)
- Kaposi's Sarcoma: ARV and referral to Clinical Oncologist

3. **Antiretroviral therapy.**

Antiretroviral therapy rollout in Kenya is supported and directed by NASCOP and The Ministry of Health. Kenya is a recipient of ARVs and infrastructure support through the *Presidents Emergency Plan for AIDS Relief (PEPFAR)* a US government international development initiative.

- ARV drugs and infrastructure support has been secured by the University of Manitoba from NASCOP/PEPFAR and CDC PEPFAR to provide HIV basic and ARV care for **all** cohorts members who are eligible as per the *"Guidelines for Antiretroviral Drug Therapy in Kenya"* (NASCOP-2005 edition). Such medical treatment and its requisite follow-up, integrated with the above standard of care, will also be provided at no cost.

APPENDIX II: PATIENT TREATMENT INFORMATION, HIV-1 CLADE AND MUTATION DATA

	Patient ID	ART at study point	Length of time on ART (wks)	CD4 at study point	HIV-1 Subtype	RTI-associated resistance mutations	Other Mutations
1.	ML005	NVP/3TC/D4T40	140	410	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V, Q207A
2.	ML024	STOCRINE/3TC/D4T30	138	665	A1	–	I2L, K11T, K20R, V21I, V35T, T39R, V60I, K122E, D123S,K173A, Q174K,V179I, G196E, T200V
3.	ML199	NVP/D4T30/3TC	70	336	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V, Q207A
4.	ML1295	STOCRINE/3TC/D4T30	137	419	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,Q207A, R211S
5.	ML1316	NVP/3TC/D4T40	95	394	A1	–	K11T, K20R, V21I, V35T, I50M, V60I, K122E,D123S, S134G,K173A, Q174K,D177E, I178M,V179I, G196E,T200V, Q207A, R211S
6.	ML1349	NVP/3TC/D4T30	84	331	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,Q207A, R211S
7.	ML1410	NVP/AZT/3TC	63	336	A1	–	K11T, K20R, V21I, V35T,

							V60I, K122E, D123S,K173A, Q174K,D177E, I178M,V179I, G196E,T200V, Q207A, R211S
8.	ML1423	NVP/3TC/AZT	120	401	A1	–	K11T, K20R, V21I, V35T, V60I, R78G, K122E,D123S, K173A,Q174K, I178M,V179I, G196E,T200V, Q207A
9.	ML1427	NVP/D4T40/3TC	66	297	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,Q207A, R211S
10.	ML1450	NVP/3TC/D4T40	138	375	A1	–	K11T, K20R, V21I, V35T, T39IKRT, V60I, K122E, D123S,K173A, Q174K,V179I, G196E,T200V, Q207A, R211S
11.	ML1497	NVP/3TC/D4T40	106	133	A1	–	E6AEGV, K11T, K20R, V21I, Q23LPQR, V35T, K43R, V60I, K122E, D123S,K173A, Q174K,D177E, I178V,V179I, G196E,T200V, R206N,Q207L, H208R, L209A
12.	ML1592	NVP/3TC/D4T40	106	429	A1	–	K11T, K20R, V21I, K22N, V35T, V60I, K122E,D123S, K173A,Q174K, D177E,I178V, V179I,G196E, T200V,Q207A, R211S
13.	ML1756	NVP/d4T 30/3TC	34	276	A1	NRTI: **T215S** NNRTI: None	K11T, K20R, V21I, K22N, V35T, V60I, K122E,D123S, K173A,Q174K, D177E,I178V, V179I,G196E, T200V, Q207A
14.	ML1777	NVP/AZT/3TC	25	355	A1	–	K11T, K20R, V21I, V35T, V60I, H96P, K122E,D123S, K173A,Q174K, I178V,V179I, G196E,T200V, Q207A,R211S, F214X
15.	ML1802	NVP/3TC/D4T30	115	181	A1	–	K11T, K20R, V21I, V35T, T39R, V60I,

							K122E,D123S, K173A,Q174K, D177E,G196E, T200V,Q207A, R211S
16.	ML1805	STOCRINE/3TC/D4T40	134	284	A1	–	E6Q, K11T, K20R, V21I, V35T, T39R, V60I, K122E, D123S,K173A, Q174K,V179I, G196E,T200V, Q207A, R211S
17.	ML1840	STOCRINE/3TC/D4T30	130	612	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V, Q207A
18.	ML1852	NVP/3TC/D4T30	149	380	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,E203G, Q207A, R211S
19.	ML1875	NVP/D4T30/3TC	51	424	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V, Q207A
20.	ML1899	NVP/3TC/VIREAD	106	431	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,Q207A, R211S
21.	ML1932	NVP/d4T 40/3TC	141	407	A1	NRTI: None NNRTI: **G190A**	P4H, V35T, V60I, K122E, D123S,I135T, K173S,Q174K, D177E,V179I, I202V,Q207D, R211S, F214L
22.	ML1956	NVP/D4T30/3TC	50	287	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, Q91, H96P, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,Q207A, R211S
23.	ML1957	NVP/3TC/D4T30	147	228	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, D123S,K173A, Q174K,I178M, V179I,G196E, T200V,Q207A, R211S
24.	ML1970	NVP/D4T30/3TC	26	402	A1	–	K20R, V35T, E40D, K49R, V60I, K122E, D123N,I135T, E169D,K173V,

							Q174K,D177E, V179I,T200A, Q207A, R211S
25.	ML2000	NVP/3TC/D4T40	138	511	A1	–	K11T, K20R, V21I, V35T, V60I, L92I, K102R,T107P, D113E,K122E, D123S,S134G, N136T,E138G, Y146S,K173A, Q174K,D177E, I178V,V179I, G196E,T200V, Q207A, R211S
26.	ML2037	NVP/3TC/D4T40	139	433	A1	–	K11T, K20R, V21I, V35T, V60I, K122E, D123S,K173A, Q174K,D177E, G196E,T200V, Q207A, R211S
27.	ML2102	NVP/3TC/D4T30	119	111	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,Q207A, R211S
28.	ML2122	NVP/3TC/D4T30	88	266	A1	–	K11T, K20R, V21I, V35T, V60I, K122E, D123S,K173A, Q174K,I178V, V179I,G196E, T200V,Q207A, R211S
29.	ML2137	3TC/TDF/STOCRINE	128	208	A1	–	K11T, K20R, V21I, V35T, V60I, K122E, D123S,K173A, Q174K,V179I, G196E,T200V, Q207A, R211S
30.	ML2151	EFV/D4T30/3TC	53	180	A1	–	V35K, V60I, K122E,D123N, I135T,K173L, Q174K,T200A, Q207A, R211H
31.	ML2169	NVP/3TC/D4T30	147	167	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V
32.	ML2244	NVP/TDF/3TC	81	286	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, V108A,K122E, D123S,K173A, Q174K,V179I, G196E, T200V
33.	ML2261	NVP/D4T30/3TC	28	222	A1	–	E6A, K11T, K20R, V21I, V35T, T39R, V60I, T107P, K122E,D123S, S134G,Y146S, K173A,Q174K, G196E,T200V,

							Q207A, R211S
34.	ML2278	NVP/D4T30/3TC	65	468	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, D177E,G196E, T200V,Q207A, R211S
35.	ML2288	NVP/3TC/TDF	84	605	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, Y146C,K173A, Q174K,V179I, G196E,T200V, Q207A, R211S
36.	ML2349	NVP/D4T40/3TC	71	406	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, I132M,K173A, Q174K,V179I, G196E,T200V, Q207A, R211S
37.	ML2426	NVP/D4T30/3TC	71	174	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, D123S,K173A, Q174K,V179I, G196E,T200V, Q207A
38.	ML2478	NVP/D4T30/3TC	46	281	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,Q207A, R211S
39.	ML2490	NVP/D4T30/3TC	35	210	A1	–	K11T, K20R, V21I, V35T, V60I, K122E, D123S,K173A, Q174K,V179I, G196E,T200V, Q207A, R211S
40.	ML2531	NVP/D4T40/3TC	53	256	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,E204G, Q207A, R211S
41.	ML2537	NVP/AZT/3TC	66	148	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, F171L,K173A, Q174K,V179I, G196E, T200V
42.	ML254	NVP/3TC/D4T30	115	302	A1	–	K11T, K20R, V21I, V35T, V60I, K122E, D123S,K173A, Q174K,V179I, G196E,T200V, Q207A, R211S
43.	ML2572	TDF/KALETRA/AZT	51	157	D	NRTI: **D67N, V118I, M184V**	E28K, K32E, V35T, K49R, V60I, K122E,

						NNRTI: **K101E, Y181C,G190A**	D123N,D177E, I178M,T200A, Q207E,L210X, R211K, T215X, D218V
44.	ML2576	NVP/D4T30/3TC	31	43	A1	–	V35T, K49R, V60I, K122E, I135T,T139K, S162G, K173S,Q174K, V179I,T200A, Q207A, R211K
45.	ML2661	NVP/d4T 30/ 3TC	34	319	A1	NRTI: **D67A** NNRTI: None	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V, Q207A
46.	ML2681	AZT/D4T30/3TC	31	342	A1	–	K11T, K20R, V21I, V35T, V60I, T107P, K122E,D123S, T131P,P133L, S134G,N136T, T139P,Y146S, K173A,Q174K, I178V,V179I, G196E,T200V, Q207A,R211S, F214X
47.	ML2756	NVP/D4T30/3TC	29	402	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, D123S,K173A, Q174K,I178M, V179I,G196E, T200V
48.	ML2761	NVP/D4T30/3TC	31	216	A1	–	E6AEGV, K11T, K20R, V21I, Q23LPQR, V35T, T39R, V60I, K122E, D123S,K173A, Q174K,D177E, G196E,T200V, Q207A
49.	ML2790	STOCRINE/D4T30/ 3TC	24	704	A1	–	K11T, K20R, V21I, V35T, T39R, E40K, V60I, K122E, D123S,T139A, K173A,Q174K, G196E,T200V, Q207D, R211S
50.	ML412	NVP/D4T30/3TC	49	379	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V, Q207A
51.	ML602	STOCRINE/3TC/D4 T40	139	426	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V, Q207A, R211S
52.	ML768	NVP/3TC/D4T40	143	388	A1	–	K11T, K20R,

No.	ID	Regimen			Subtype	Mutations	Sequence
							V21I, V35T, V60I, K122E, D123S,K173A, Q174K,D177E, V179I,G196E, T200V,Q207A, R211S
53.	ML874	NVP/3TC/D4T40	136	144	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V
54.	ML890	NVP/3TC/D4T40	96	687	A1	–	K11T, K20R, V21I, E28K, V35T, T39R, V60I, K122E, D123S,N136D, K173A,Q174K, V179I,G196E, T200V, Q207A
55.	ML915	NVP/3TC/D4T40	129	147	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, D177E,G196E, R199T,T200V, Q207A,R211S, D218A
56.	ML058	STOCRINE/3TC/D4T30	143	439	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, K122E,D123S, K173A,Q174K, V179I,G196E, T200V,Q207A, R211S
57.	ML1500	NVP/3TC/AZT	118	564	A1	–	K11T, K20R, V21I, V35T, T39R, V60I, D86G, K122E, D123S,K173A, Q174K,D177E, G196E,T200V, Q207D, R211S
58.	ML1935	NVP/d4T 40/3TC	60	103	D	NRTI: **M184V** NNRTI: **Y181C**	E6K, V35T, E40D, K49R, V60I, P97R, K122E,D177E, I178M,T200A, Q207E,R211N, W212V, F214I
59.	ML2468	NVP/D4T30/3TC	46	279	A1	–	V35T, V60I, K122E,D123N, K173S,Q174K, D177E,V179I, Q207A, R211S
60.	ML2493	NVP/D4T30/3TC	68	319	A1	–	K11T, K20R, V21I, V35T, V60I, K122E, D123S,K173A, Q174K,D177E, I178M,V179I, G196E,T200V, Q207A,R211S, F214X