DETERMINATION OF ANTIRETROVIRAL DRUG RESISTANCE MUTATIONS AMONG HIV-1 INFECTED PARTICIPANTS FROM SELECTED CARE CENTRES IN KENYA

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2017

Determination of antiretroviral drug resistance mutations among HIV-1 infected participants from selected care centres in Kenya

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A thesis submitted in partial fulfillment for the degree of Master of Science in Medical Virology in the Jomo Kenyatta University of Agriculture and Technology

2017

DECLARATION

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

Signature……. Date……..

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This thesis has been submitted to the University for Examination with our approval as supervisors:

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DEDICATION

This work is dedicated my loving parents Roselyn and Fredrick Nzomo for the love, courage and inspiration that I drew from them during the entire course of my study. This work is also dedicated to my brother Mark from whom I drew strength to move on

AKNOWLEDGEMENT

I am eternally grateful to the Lord God Almighty for His grace and loving-kindness that saw me through the project.

I wish to thank Prof. Washingtone Ochieng, who was my supervisor and sponsor of the study, for his unwavering support throughout the study period.

I also wish to thank Prof. Anne Muigai for her insightful supervision and assistance in the development of this research project.

I extend my sincere gratitude to the medical and laboratory personnel at Kiambu District Hospital, among others and Centre for Virus Research at Kenya Medical Research Institute.

Special thanks to my uncle Alex, for the love and support that I drew from him each moment of the way.

Last but not least, I am grateful to the study participants who gave consent for samples to be collected for this study.

May God bless you all.

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AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretriviral Therapy
ARVs	Antiretrovirals
CCCs	Comprehensive Care Centres
CDC	Centres for Disease Control and Prevention
DNA	Deoxyribonucleic acid
DRAMs	Drug resistance –associated mutations
EID	Early Infant Diagnosis
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
KEMRI	Kenya Medical Research Institute
PCR	Poymerase Chain Reaction
RNA	Ribonucleic Acid
UNAIDS	The Joint United Nations Programme on HIV/AIDS
USA	United States of America
VL	Viral Load
VF	Virological failure
WHO	World Health Organization

ABSTRACT

Majority of people infected with HIV-1 in the developing countries continue to benefit from scaled-up access to antiretroviral medications. But the frequent appearance of drug resistance mutations poses significant threats to success of treatment. This study aimed to determine HIV drug resistance-associated mutations (DRAMs) among HIV-1 infected patients on treatment in Kenya. A total of 83 patients on highly active antiretroviral therapy (HAART) meeting the inclusion criteria were enrolled from care and treatment centres in Kiambu, Kilifi, Kajiado, Nakuru, Kisumu and Homabay counties. Viral RNA and DNA were amplified from plasma and peripheral blood mononuclear cells (PBMCs) respectively, and the *pol-RT* region of HIV-1 sequenced in 54 patients. Sequences were analyzed for DRAMS and interpreted using the Stanford HIV Drug Resistance Interpretation algorithm. Viral loads and CD4 counts were also determined using m2000 Abbot real-time assay and FACs respectively. The median participant age and duration of HAART were 34 years and 33 months respectively. The median CD4 T cell counts and viral load were 399 (range, 12-1954) cells/mm^3 and 3.51 (range, 1.59-5.96) log_{10} HIV-1 RNA copies respectively. In total, 27.8% of the patients harboured reverse transcriptase inhibitor DRAMS, with 73.3% of the mutations conferring resistance to both nucleoside RTIs (NRTIs) and non-nucleoside RTIs (NNRTIs). Mutation frequency of all DRAMS was 25 (47.2%) and 52.8% for NRTI and NNRTI types respectively. The most common of NRTI and NNRTI mutations were M184V/I and K103N occurring respectively in 73.3% and 60% of the patients with DRAMs, appearing at a mutation frequency of 44% and 32.1% respectively. Most of the subjects were infected by viruses of subtypes A (57.4%), with the rest being recombinant (22.2%), D (14.8%) and C (5.6%) strains. Recombinant viruses had the highest ratio of average DRAMs per subtype at 4.5, followed by subtypes A (3.6), C (3) and D (2.3). No residual DRAMs were observed in patients with viral load less than 1,000 RNA copies/ml. A significant proportion of patients receiving anti-HIV treatment in Kenya have developed multiple drug resistance. Viral load and Drug-resistance testing should therefore be integrated to monitoring programs to improve treatment efficacy.

CHAPTER ONE

INTRODUCTION

1.1 Background

Antiretroviral treatment (ART) has led to significant decrease in the morbidity and mortality associated with human immunodeficiency virus type 1 (HIV-1), the causative agent of acquired immunodeficiency syndrome (AIDS) (Nester et al., 2004). The AIDS epidemic is of major public health concern globally, with the WHO estimating the number of people infected with the virus to be over of 36 million (UNAIDS, 2016). The national adult HIV prevalence rate in Kenya stands at 5.6% and approximately 50,000 deaths were reported in 2011 (NASCOP, 2012). Yearly mortality figures may fluctuate, but cumulative figures are expected to rise in Kenya (CDC, 2013). Although there is currently no known cure or a vaccine for HIV, the introduction of ART in Kenya in 2003 has led to the improvement of the quality of life and health of infected individuals (NACC and NASCOP, 2012). Mortality and prevalence rates associated with the HIV-1 epidemic have been reduced significantly (CDC, 2013). Kenya continues to make significant progress in the provision of care and treatment to individuals with known HIV sero-status, and by 2007, 90% of newly diagnosed patients were eligible for ART (NACC & NASCOP, 2012). AIDS related mortality also dropped from 120,000 in 2003 to 85,000 in 2010, to coincide with introduction of ART (NASCOP, 2012).

1.2 HIV prevalence and mortality rates

The United Nations estimates about 35.3 million people to have been living with HIV infection by 2012 (UNAIDS, 2013). This represents an increase from previous years, a consequence of more people gaining access to antiretroviral therapy (Murray et al., 2014). There were 2.3 (1.9–2.7) million new HIV infections globally, showing a 33% decline in the number of new infections from 3.4 (3.1–3.7) million in 2001 (UNAIDS, 2011). At the same time the number of AIDS-related deaths is also declining with 1.6

(1.4–1.9) million AIDS deaths in 2012, down from 2.3 (2.1–2.6) million in 2005 as shown in figure 1 below (UNAIDS, 2013)

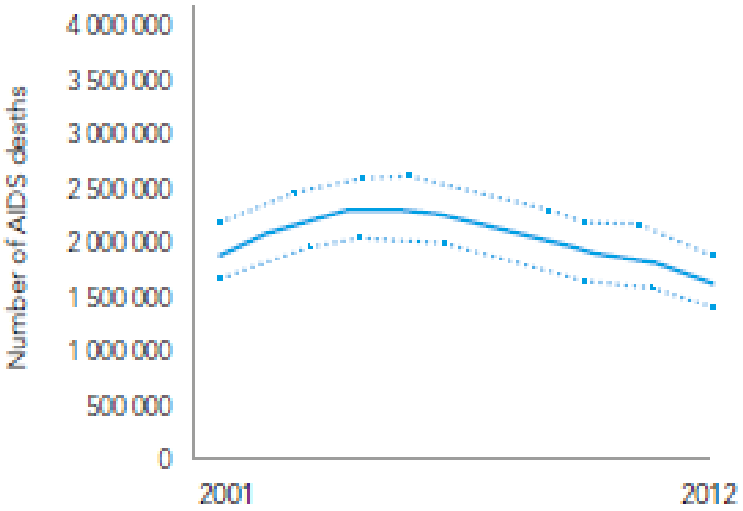

Figure 1.1 UN 2012 estimates of mortalities associated with HIV, showing a decline in AIDS-related deaths from 2.3 million in 2005 to 1.6 million in 2012

In Kenya, the Kenya Aids Indicator Survey report of 2012 puts the national prevalence of HIV among adults and adolescents at an all time low of 5.6%, representing about 1.2 million people (NASCOP, 2014). Of these, women were demonstrated to be more likely to be infected at 6.9% as opposed to men at 4.4%. In addition, the prevalence rates have been shown to vary geographically ranging from 2.1% in North Eastern region to 15.1% in Nyanza region (NACC & NASCOP, 2012). Mortalities associated with the epidemic have also fallen dramatically in Kenya, with NASCOP reporting an estimated 49,126 deaths from AIDS in 2011 (NASCOP, 2014). These figures reflect a nearly two-thirds fall from the peak in AIDS deaths in the 2002-2004 period during which an estimated 130,000 people died every year (NASCOP, 2012). Table 1.1 shows the summaries of the number of HIV-related deaths between 2010 and 2012.

Table 1.1 AIDS-related mortalities as reported by NASCOP in 2012

YEAR	NUMBER OF DEATHS
2000	170,000
2002	170,000
2004	150,000
2006	120,000
2008	85,000
2010	70,000
2012	49,100
Total	**814,100**

(Source: NACC and NASCOP AIDS epidemic report, 2012)

1.3. ART scale-up in Kenya

In Kenya, the rollout of ART has continued since the medication became accessible in Public sector in 2003, and the number of people needing treatment steadily increases every year as shown in table 1.2 below (NASCOP, 2012). By 2010, more than 400,000 HIV/AIDS patients were receiving treatment, including adults and children (CDC, 2013). With ART scale up, there has been a need for monitoring for development of HIV drug resistance at a population level (Hassan et al., 2014).

Table 1.2 Need for ART, Coverage and the ART target for adults in Kenya (NASCOP)

Year	2009/10	2010/11	2011/12	2012/13	2013/14
No. of patients in need of ARVs CD4 (<350)	694,024	738,350	771,810	807,860	844,795
Target: No. on ART (CD4<350)	360,000	500,000	620,000	702,838	760,316
Percent coverage	**52%**	**68%**	**80%**	**87%**	**90%**

(Source: NASCOP, 2012)

1.4 Antiretroviral treatment guidelines for Kenya

The optimum time for commencing ART in HIV-infected individuals has been a subject of debate for a long time (Siegfried et al., 2013). However, it is clear that initiation of treatment should be done before irreversible damage has occurred to the immune system (Rutstein et al., 2015). Early initiation of treatment is important as it is easier to control viral replication, there is lower risk of resistance (diminished latent reservoir) with complete viral suppression being achieved in many cases and this also decreases the risk of HIV transmission (Rutstein et al., 2015).

Kenya has adopted WHO recommendations for early initiation of ART at CD4 T-cell count of 350/mm^3, or the progression of disease to WHO stage 3 and 4 regardless of Immunological data, and also among HIV-1 patients co-infected with tuberculosis with or without CD4 data, as shown in table 1.3 (Ministry of Health, 2014). While the decision to start ART is based on medical criteria, other factors may impact on the patient's capacity to adhere to treatment such as social circumstances and support

systems. As a result, counselling is important to ensure patient understanding, acceptance and readiness to start and continue long-term treatment (NASCOP, 2012).

Table 1.3 Guidelines on when to start ART in therapy-naïve patients in Kenya (NASCOP)

Clinical event	CD4 not available	CD4 $\geq$ 350/mm^3	CD4 $\leq$ 350/mm^3
WHO stage 1 &2	Defer ART	Defer ART	Start ART
WHO stage 3 & 4	Start ART	Start ART	Start ART
Active TB with HIV	Start ART	Start ART	Start ART

The highly active antiretroviral treatment (HAART) guidelines as recommended by the world health organization (WHO) for Kenya stipulate that treatment combinations shall include 2 nucleoside reverse-transcriptase inhibitors (NRTIs) containing Lamivudine (3TC) and Azidovudine/Tenofovir/Stavudine (AZT/ TDF/d4T respectively) and one non nucleoside reverse-transcriptase inhibitor (NNRTI) of either Efavirenz or Nevirapine (EFV or NVP respectively) for the first line therapy (NASCOP, 2012). The guidelines further recommend particular treatment options for different types of patients. Table 1.4 details the possible combinations for treatment of HIV in Kenya

Table 1.4 ARV treatment guidelines for Kenya (NASCOP)

What ART to start in ART naïve PLHIVs	**ART Options**
adults and adolescents in Kenya	Preffered-TDF+3TC+EFV or NVP
HIV+ ARV naive adults and adolescents	Alternative- TDF+3TC+EFV or NVP
HIV pregnant women	AZT+3TC+EFV or NVP
HIV/TB co-infection	TDF+3TC+EFV or NVP
HIV/HBV co-infection	(AZT preferred but TDF acceptable; do not use EFV during first trimester) AZT+3TC+EFV TDF+3TC+EFV TDF+3TC+EFV

(Source: NASCOP; Kenya HIV drug resistance country report, 2011)

1.5 Antiretroviral drug resistance

Despite the remarkable success of ART, the gains made by the increased availability of ARV drugs in sub-Saharan Africa is threatened by the emergence of drug resistant viral strains leading to ARV treatment failure or virological failure, which further limits the treatment options available to patients (Steegen et al., 2009). Another important setback to HIV treatment and prevention efforts has been the demonstration that drug resistant HIV can be transmitted between individuals even in the absence of treatment (Lihana et al., 2009). Available data show that transmitted drug resistance (TDR) to a single class of antiretroviral therapy occurs in about 10-15% of all new cases (Hurt et al., 2009), a phenomenon that complicates treatment in the ART naïve population.

1.6 Statement of the problem

A considerable number of studies on HIV-1 drug resistance mutations have been done globally. However, majority of these studies have centred on HIV B-subtype which predominates in North America, Australia and Western Europe and Japan (Taylor et al., 2008). Non-B subtypes are predominant in Africa and Asia, two of the world's regions worst hit by the epidemic and these subtypes vary significantly from the B-subtypes

(Taylor et al., 2008). Despite the high burden of HIV in sub-Saharan Africa and Asia, there is a paucity of data on drug resistance mutations among the non-B subtypes in these regions. Non synonymous point mutations in the genes that code for antiretroviral target proteins have been demonstrated in the B and non-B subtypes, where they confer resistance to antiretroviral medication (Kantor et al., 2005). These mutations are of importance to therapy as they influence responsiveness to treatment and also disease progression.

In one study on ARV naïve Kenyan children, drug failure was observed in majority of the children after treatment was initiated (Lwembe et al., 2007). Point mutations such as Y181C and K103N affecting both the NRTIs and NNRTI respectively were observed in Nevirapine and AZT recipients (Lwembe et al., 2007). Interestingly, drug resistant mutants were also present in several ARV naïve patients, implying the presence of transmitted drug resistance mutations circulating in the population even before initiation of therapy. These findings are consistent with data from another study that examined a possible emergence of drug resistance mutations among infants born to HIV infected mothers, where emergence of drug resistant mutations occurring between 2 weeks and 6 months of post-partum was attributable to maternal ARV drug exposure (Zeh et al., 2011).

1.7 Justification

Access to ART in Kenya has escalated since the first available drug to the general population in the country in 2003. In 2010, more than 400,000 HIV-infected adults and children were receiving treatment (NASCOP, 2012). However, few countrywide studies have been conducted to monitor treatment responsiveness, and particularly development of drug resistant strains of HIV-1. Considering the genetic heterogeneity of the non-sub type B HIV-1 strains in Kenya, extensive treatment is bound to result in increased incidences and prevalence of mutant viruses that will fail treatment. Without organized population-level study, such data will remain elusive to the detriment of offering evidence-based clinical management. Knowledge of drug resistance genotypes can

become more useful if such data is used to guide therapy or change in treatment strategies. This study aimed to determine the known HIV-1 drug resistance associated mutations among treated and untreated patients from selected counties in Kenya. Detailed information of type of known drug resistance associated mutations will enable set up a drug resistance database for Kenya.

1.8 Study questions

1. What HIV genotypes and antiretroviral drug resistance mutations are found in the treatment naïve and experienced subjects in the selected counties
2. What is the correlation between virological, immunological and genotype outcomes with the emergent drug resistance profiles

1.9 General objective

To evaluate the patterns of antiretroviral drug resistance mutations among subjects infected with HIV-1 in selected counties and relate those patterns with treatment responsiveness.

1.9.1 Specific objectives

1. To collate CD4 counts and viral loads (VL) data in HIV-1 infected treated and untreated subjects.
2. To determine the HIV-1 genotypes and antiretroviral drug resistance mutations through sequence analysis in treatment naïve and experienced subjects.
3. To correlate virological (viral load), immunological (CD4 counts) and HIV-1 subtype outcomes with emergent drug resistance profiles

CHAPTER TWO

LITERATURE REVIEW

2.1 HIV and AIDS

HIV/AIDS is a disease of the human immune system caused by infection with human immunodeficiency virus (HIV) (Carter & Saunders, 2007). The term HIV/AIDS represents the entire range of disease caused by the human immunodeficiency virus from early infection to late stage symptoms (Hel, McGhee & Mestecky, 2007). During the initial infection, a person may experience a brief period of influenza-like illness that is typically followed by a prolonged period without symptoms (Nester et al., 2004). As the illness progresses, it interferes more and more with the immune system, making the person much more likely to acquire infections, including opportunistic infections and tumours that do not usually affect people who have working immune systems (Nester et al., 2004).

2.2 Classification of HIV

HIV belongs to the genus *Lentivirus* and family *Retroviridae* (ICTV, 2002). Lentiviruses share many morphological and biological characteristics such as genetic make-up (single stranded RNA) molecular mechanisms of replication and biological interactions with their host (Narayan & Clements, 1989). Many species of mammals are infected by lentiviruses with the host spectrum ranging from fish to humans, and are characteristically responsible for long-duration illnesses with a long incubation periods (Carter & Saunders, 2007). Two types of HIV have been characterized: HIV-1 and HIV-2 (Narayan & Clements, 1989). HIV-1 is the virus that was originally discovered, and initially referred to also as lymphadenopathy-associated virus (LAV) or human T-lymphotropic virus type III (HTLV-III). It is more virulent, more infective, and is the cause of the majority of HIV infections globally (German Advisory Committee on Blood, 2016). There are distinct differences in the structure, function and occurrence of HIV-1 and HIV-2. Structurally, the *vpu* gene in HIV-1 is replaced by *vpx* in HIV-2,

while there are differences in the structure of the protease enzymes and their subsequent functions (Azevedo-Pereira & Santos-Costa, 2016). HIV-2 is also less infectious based on its antigenic configuration, than HIV-1 implying that fewer people exposed to HIV-2 will be infected per exposure (Carter & Saunders, 2007). Because of its relatively poor capacity for transmission, HIV-2 is largely confined to West Africa unlike HIV-1 which is responsible for over 95% of the global pandemic (Reeves, 2002).

2.3 HIV structure

HIV is a single-stranded, positive-sense, enveloped RNA virus (Nester et al., 2004). The viral envelope contains a lipid bilayer and viral spikes made of glycoproteins as shown in **Figure 2.1** below (Carter & Saunders, 2007). The gp120 the antigen is partly responsible for attachment to host cell and functions in receptor detection while gp41 aids in viral entry into host cell by triggering viral and host membrane fusion (Carter & Saunders, 2007). The lipid bilayer encloses various proteins and enzymes necessary for viral replication, assembly and release (Nester et al., 2004).

2.4 The HIV genome

The genome of HIV consists of 3 structural genes i.e *env, gag* and *pol*, that contain information necessary for synthesizing structural proteins (Nester et al., 2004). It also has 6 regulatory genes - *tat, rev, nef, vif, vpr,* and *vpu*- that contain information needed to produce proteins which control the ability of HIV to infect a host, produce new copies of virus, or cause disease (Carter & Saunders, 2007).

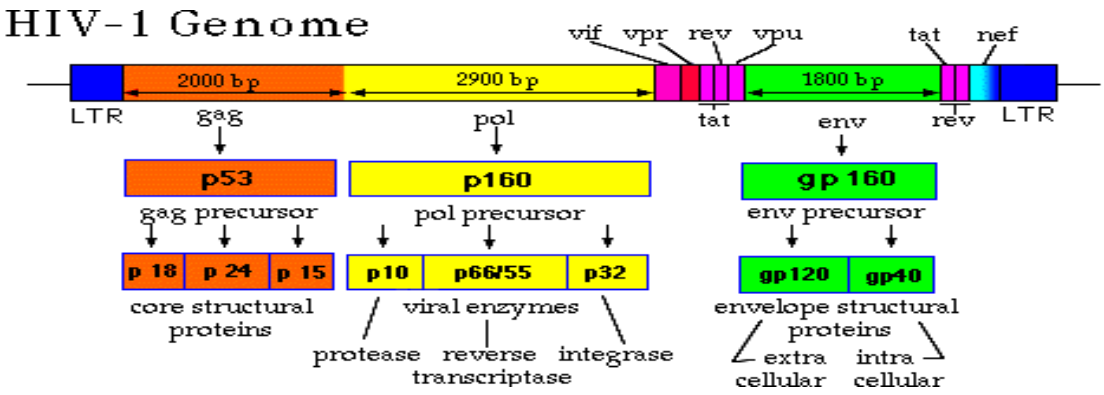

Figure 2.1 HIV-1 genome map**, showing the *gag*, *pol* and *env* genes. Protein *p53* forms the *gag* precursor, while *p160* forms the pol precursor with *gp160* forming the *env* precursor. The 3 main segments are further subdivided into specific proteins to make the core structural proteins, viral enzymes and envelope structural proteins. (Source: Principles and practice of clinical virology, 5th edition, 2007)**

2.5 HIV subtypes

HIV is a highly variable virus with two known types: HIV-1 and 2 (German Advisory Committee on Blood, 2016). HIV-1 is known to be the major cause for the AIDS epidemic while HIV-2 remains restricted largely to West Africa, with little known disease association among humans (Reeves, 2002). There are up to 9 different subtypes of HIV-1 so far identified (Hemelaar et al., 2011). These are characterized based on the genetic variability of the viruses and include subtypes A, B, C, D, F, G, H, J, and K (Taylor et al., 2008). In addition, there are 55 circulating recombinant forms (CRFs) and several unique recombinant forms (URFs) of HIV-1 (Thomsom & Najera, 2005). Subtypes A and F are further classified into A1, A2, A3, A4, A5, F1 and F2 based on genetic variation of between 15-20% (Taylor et al., 2008). This extensive genetic variation has many implications on anti-retroviral drug response, viral transmission, vaccine and diagnostics design and development (Lihana et al, 2012; Worobey, 2007).

2.6 Signs and symptoms of HIV

HIV infection is characterized by an initial acute viral illness followed by a chronic, asymptomatic phase of disease associated with active viral replication and dissemination, and lasting as long as 5–10 years. Ultimately, immune destruction results in end-stage disease (AIDS) associated with opportunistic infections, malignancies and neurological disorders. Theseclinical events correlate with virological and immunological changesThere are three main stages of HIV infection: acute infection, clinical latency and AIDS (Zuckerman et al., 2007).

2.6.1 Acute infection of HIV

The initial period following the contraction of HIV is called acute HIV, primary HIV or acute retroviral syndrome (WHO, 2007). Many individuals develop an influenza-like illness or a mononucleosis-like illness two to four weeks post exposure while others have no significant symptoms (Carter & Saunders, 2007). Symptoms occur in 40–90% of cases and the most common ones include; fever, large tender lymph nodes, throat inflammation, a rash, headache, and/or sores of the mouth and genitals (Nester et al., 2004). The rash, which occurs in 20–50% of cases, presents itself on the trunk and is maculopapular, classically (Zuckerman et al., 2007). Some people also develop opportunistic infections at this stage (WHO, 2007). Gastrointestinal symptoms such as nausea, vomiting or diarrhea may occur, as may neurological symptoms of peripheral neuropathy or Guillain-Barre syndrome (Vogel, 2010). The duration of the symptoms varies, but is usually one or two weeks (Zuckerman et al., 2007). Due to their nonspecific character, these symptoms are not often recognized as signs of HIV infection. Thus, it is recommended that HIV testing be considered in people presenting an unexplained fever who may have risk factors for the infection (Vogel, 2010).

2.6.2 Clinical latency of HIV

The initial symptoms are followed by a stage called clinical latency, asymptomatic HIV, or chronic HIV (Nester et al., 2004). In the absence of treatment, this second stage of the natural history of HIV infection can last from about three years (Clive, 2006) to over 20 years (Hicks et al., 2001). Despite there being few or no symptoms at first, near the end of this stage many people experience fever, weight loss, gastrointestinal problems and muscle pains (Evian, 2006). Between 50 and 70% of people also develop persistent generalized lymphadenopathy, characterized by unexplained, non-painful enlargement of more than one group of lymph nodes (other than in the groin) for over three to six months (Carter and Saunders, 2007). Although most HIV-1 infected individuals have a detectable viral load and in the absence of treatment will eventually progress to AIDS, a small proportion (about 5%) retain high levels of $CD4^+$ T cells (T helper cells) without antiretroviral therapy for more than 5 years (Blankson, 2010). These individuals are classified as HIV controllers or long-term non-progressors (LTNP) (Blankson, 2010). Another group is capable of maintaining low or undetectable viral load without anti-retroviral treatment and are known as "elite controllers" or "elite suppressors". These represent approximately 1 in 300 infected persons (Walker, 2007).

2.6.3 Acquired immune deficiency syndrome (AIDS)

Acquired immunodeficiency syndrome (AIDS) is defined in terms of either a $CD4^+$ T cell count below 200 cells per µL or the occurrence of specific diseases in association with an HIV infection (Carter and Saunders, 2007). In the absence of specific treatment, about half of people infected with HIV develop AIDS within ten years (Nester et al., 2004). The most common initial conditions that could signify AIDS are pneumocystis pneumonia (40%), cachexia in the form of HIV wasting syndrome (20%) and esophageal candidiasis. Other common signs include recurring respiratory tract infections (Holmes et al, 2003). Opportunistic infections may be caused by bacteria, viruses, fungi and parasites that are normally controlled by the immune system. The nature of infections occurring partly depends on what organisms are

common in the person's environment. These infections may affect nearly every organ system (Chu & Selwyn, 2011).

People with AIDS have an increased risk of developing various viral induced cancers including Kaposi's sarcoma, Burkitt's lymphoma, primary central nervous system lymphoma, and cervical cancer (Vogel et al, 2011). Kaposi's sarcoma is the most common cancer occurring in 10 to 20% of people with HIV (Nester et al., 2004). The second most common form of cancer is lymphoma which is the cause of death of nearly 16% of people with AIDS and is the initial sign of AIDS in 3 to 4% of HIV-infected individuals (Carter and Saunders, 2007). Both these cancers are associated with human herpesvirus 8. Cervical cancer occurs more frequently in those with AIDS due to its association with human papillomavirus (Vogel et al., 2011).

Additionally, people with AIDS frequently have systemic symptoms such as prolonged fevers, sweats (particularly at night), swollen lymph nodes, chills, weakness, and weight loss (Carter and Saunders, 2007). Diarrhea is another common symptom present in about 90% of people with AIDS. They can also be affected by diverse psychiatric and neurological symptoms independent of opportunistic infections and cancers (Sestak, 2005).

2.7 Pathogenesis of HIV

In the event of viral attachment and entry into the human body, there is a period of rapid viral replication, leading to an abundance of virus in the peripheral blood (Zuckerman et al., 2007). During primary infection, the level of HIV may reach several million virus particles per millilitre of blood (Nester et al., 2004). This response is accompanied by a marked drop in the number of circulating $CD4^+$ T cells (Carter and Saunders, 2007). The acute viremia is almost invariably associated with activation of $CD8^+$ T cells, which kill HIV-infected cells, and subsequently with antibody production, or seroconversion (Zuckerman et al., 2007). The $CD8^+$ T cell response is thought to be important in controlling virus levels, which peak and then decline, as the $CD4^+$ T cell counts recover. A good $CD8^+$ T cell response has been linked to slower

disease progression and a better prognosis, though it does not eliminate the virus (Carter and Saunders, 2007).

HIV causes AIDS by depleting $CD4^+$ T cells (Zuckerman et al., 2007). This weakens the host's immune system and the patient becomes vulnerable to opportunistic infections. T cells are essential to the immune response and without them, the body cannot fight infections or kill cancerous cells. The mechanism of $CD4^+$ T cell depletion differs in the acute and chronic phases of HIV infection (Hel et al., 2008). During the acute phase, HIV-induced cell lysis and killing of infected cells by cytotoxic T cells accounts for $CD4^+$ T cell depletion, although apoptosis may also be a factor. During the chronic phase, the consequences of generalized immune activation coupled with the gradual loss of the ability of the immune system to generate new T cells appear to account for the slow decline in $CD4^+$ T cell numbers (Arrivie et al., 2007).

Although the symptoms of immune deficiency characteristic of AIDS do not appear for years after infection, the bulk of $CD4^+$ T cell loss occurs during the first weeks of infection, especially in the intestinal mucosa, which harbours the majority of the lymphocytes found in the body (Mehandru et al., 2004). The reason for the preferential loss of mucosal $CD4^+$ T cells is that the majority of mucosal $CD4^+$ T cells express the CCR5 protein which HIV uses as a co-receptor to gain access to the cells, whereas only a small fraction of $CD4^+$ T cells in the bloodstream do so. A specific genetic change that alters the CCR5 protein when present in both chromosomes (XX or XY) has been demonstrated to effectively prevent HIV-1 infection (Olson & Jacobson, 2009).

HIV actively tracks and destroys CCR5 expressing $CD4^+$ T cells during acute infection (Carter and Saunders, 2007). A vigorous immune response eventually controls the infection and initiates the clinically latent phase (Olson & Jacobson, 2009). $CD4^+$ T cells in mucosal tissues remain particularly affected (Jacobson, 2009). Continuous HIV replication causes a state of generalized immune activation persisting throughout the chronic phase (Carter and Saunders, 2007). Immune activation, which is reflected by the increased activation state of immune cells and release of pro-inflammatory cytokines,

results from the activity of several HIV gene products and the immune response to ongoing HIV replication (Carter and Saunders, 2007). It is also linked to the breakdown of the immune surveillance system of the gastrointestinal mucosal barrier caused by the depletion of mucosal $CD4^+$ T cells during the acute phase of disease (Appay and Sauce, 2008).

2.8 HIV replication

The replication of HIV is defined by two events; one which is early and the other one late events (Zuckerman et al., 2007). In the early events phase, the virion binds to a CD4 molecule and the process is mediated by a CCR5 or CXCR4 co-receptor molecules common on the cell surface before the virus fuses with host's cell (Carter and Saunders, 2007). Infection then follows when the virus penetrates the cell membrane and empties its contents which include the genome, into the cell (Nester et al., 2004). Reverse transcription is the next step, whereby the viral RNA is converted into double stranded DNA by the reverse transcriptase enzyme. In the late events stage, viral DNA is integrated into host cell's DNA by the integrase enzyme. When the infected cell divides, the viral DNA is transcribed into long chains of proteins which are later assembled in the cytoplasm. The virus at this stage is still immature after assembly but buds off the infected cell, taking along some of the cells membrane with it (Zuckerman et al., 2007). Viral maturation occurs outside the cell where the viral protein chains are cleaved by the protease enzyme into the constituent proteins that make a complete infectious virus (Nester et al., 2004). A step-by-step explanation of all events in HIV replication is outlined in the figure below.

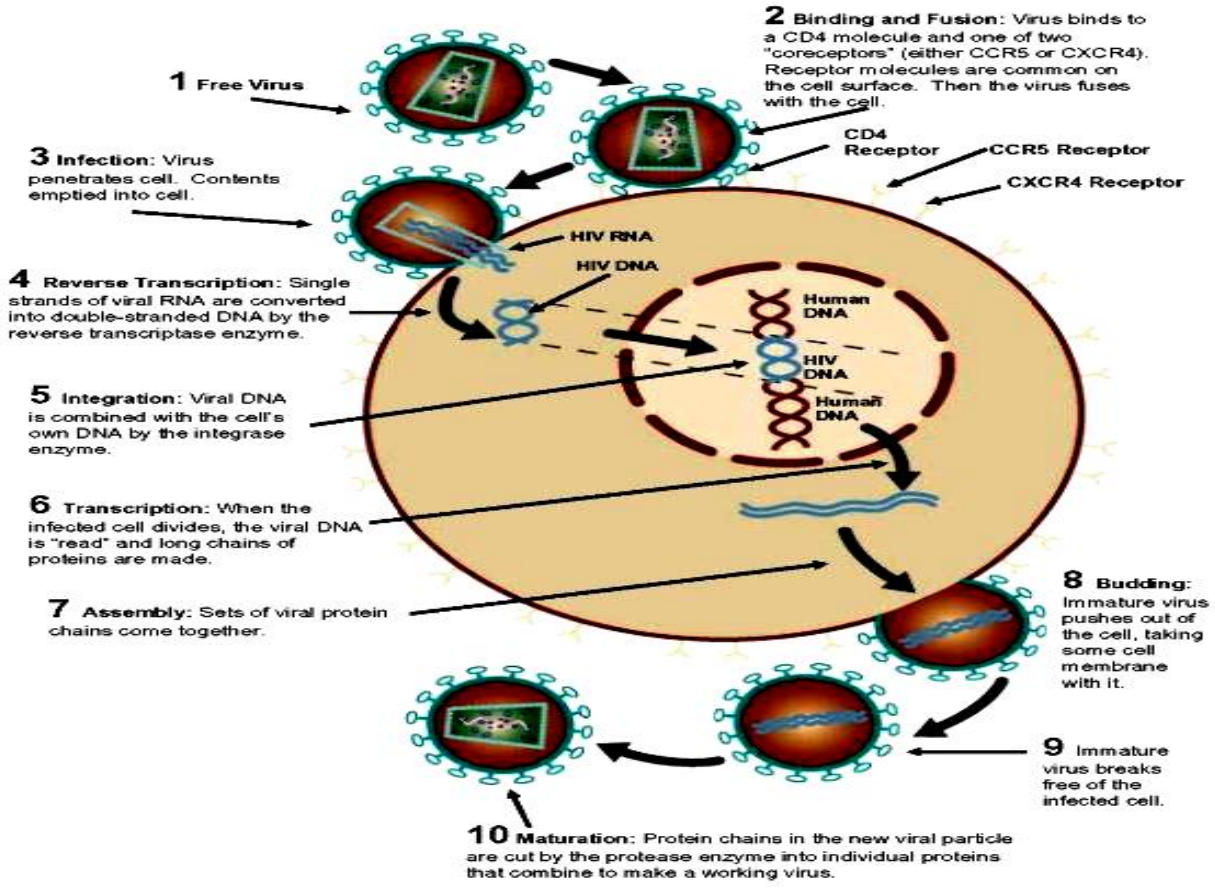

Figure 2.2 A schematic representation of the HIV replication cycle**, detailing each step involved in HIV replication from virus attachment and binding, host cell penetration, reverse-transcription, integration, transcription, assembly and maturation ((Source: Principles and practice of clinical virology, 5th edition, 2007)**

2.9 Diagnosis of HIV

HIV/AIDS is diagnosed through laboratory testing and then staged based on the presence of certain signs or symptoms (Zuckerman et al., 2007). Most people infected with HIV develop specific antibodies (i.e. seroconvert) within three to twelve weeks of the initial infection (Haaheim et al., 2002). Diagnosis of primary HIV before

seroconversion is done by measuring HIV-RNA or p24 antigen. Positive results obtained by antibody or PCR testing are confirmed either by a different antibody or by PCR (WHO, 2007).

Antibody tests in children younger than 18 months are typically inaccurate due to the continued presence of maternal antibodies (Kellerman and Essajee, 2010). Thus HIV infection can only be diagnosed by PCR testing for HIV RNA or DNA, or via testing for the p24 antigen (WHO, 2007). Much of the world lacks access to reliable PCR testing and many places simply wait until either symptoms develop or the child is old enough for accurate antibody testing (Kellerman and Essajee, 2010). In sub-Saharan Africa as of 2007–2009 between 30 and 70% of the population was aware of their HIV status (UNAIDS, 2011). In 2009, between 3.6 and 42% of men and women in Sub-Saharan countries were tested which represented a significant increase compared to previous years (UNAIDS, 2011).

2.10 Treatment of HIV infection

There is currently no cure or effective HIV vaccine, therefore treatment consists of highly active antiretroviral therapy (HAART) which slows progression of the disease (Archin et al., 2014). Treatment also includes preventive and active treatment of opportunistic infections (Haaheim et al., 2002). Current HAART options are combinations (or "cocktails") consisting of at least three medications belonging to at least two types, or "classes," of antiretroviral agents (WHO, 2010). These classes are discussed in the following section.

2.10.1 Classes of antiretroviral drugs and their mode of action

The commonly used anti-HIV drugs are classified into three groups; Protease inhibitors (PIs), Nucleoside reverse transcriptase inhibitors (NRTIs) and Non nucleoside/nucleotide reverse transcriptase inhibitors (NNRTIs) (WHO, 2013). These drugs work by interfering with the conversion of HIV-1 RNA to DNA, a reverse transcription process that must precede proviral integration into host genome and

subsequent replication of viral genetic material (Pennings, 2012). The NRTIs include; Lamivudine (3TC), Zidovudine (AZT), Stavudine (d4T), Didanosine, Emtricitabine, Zalcitabine and Abacavir while the NNRTI group is composed of Nevirapine (NVP), Efavirenz (EFV), Etravirine and Delavirdine . The third class of anti HIV-1 agents is the PIs. These include; Saquinavir, Atazanavir (ATV), Ritonavir, Amprenavir, Indinavir, Lopinavir (LPV) and Nelfinavir (WHO, 2013). Outside the three classes of ARVs is another category of antiviral agents referred to as acyclic nucleoside phosphonates (Pennings, 2012). These drugs target the DNA polymerase activity and are used in the treatment of co-infecting viral agents like cytomegalovirus or CMV (Menendez-Arias, 2010). Acyclic nucleoside phosphonates include tenofovir (TDF), administered as a prodrug which requires an esterase for full activity. NRTIs and PIs act by competitive inhibition of the binding sites as they are phosphorylated to their triphosphate forms. NNRTIs on the other hand act by binding at ahydrophobic pocket next to the active site of the polymerase in the p66 subunit of the enzyme (Menendez-Arias, 2010).

2.11 Correlates of antiretroviral treatment failure

Treatment failure can be categorized as virologic failure, immunologic failure, and clinical failure, or in certain cases, combinations of the three (Zuckerman et al., 2007). Laboratory results must be confirmed with repeat testing before a final assessment of virologic or immunologic treatment failure is made (Althoff et al., 2010).

2.11.1 Virologic failure

Virologic failure occurs as an incomplete initial response to therapy or as a viral rebound after virologic suppression is achieved (Aleman et al., 2002). Virologic suppression is defined as having plasma HIV RNA below the level of quantification using the most sensitive assay (<20 to 75 a confirmed HIV RNA level below the lower limit of detection (Althoff et al., 2010). Virologic failure on the other hand refers to the inability to achieve or maintain suppression of viral replication to an HIV RNA level below this limit (Carter and Saunders, 2007). Virologic failure is defined for all children as a plasma HIV RNA >200 copies/mL after six months of therapy or repeated plasma HIV

RNA greater than the level of quantification using the most sensitive assay after 12 months of therapy (Ribaudo et al., 2009). Occasionally, infants with high plasma HIV RNA levels at initiation of therapy have HIV RNA levels that are declining but remain >200 copies/mL after 6 months of therapy (Eshleman et al., 2001). Among many of those receiving Ritonavir-boosted lopinavir, suppression can be achieved without regimen change if efforts are made to improve adherence. However, ongoing non-suppression especially with non-nucleoside reverse transcription inhibitor (NNRTI)-based regimens increases risk of drug resistance (Eshleman et al., 2001). HIV-infected adults with detectable HIV RNA and a quantified result <200 copies/mL after 6 months of combination antiretroviral therapy (cART) often ultimately achieve virologic suppression without regimen change (Aleman et al, 2002). "Blips," defined as isolated episodes of plasma HIV RNA <500 copies/mL followed by return to viral suppression, are common and not generally reflective of virologic failure (Lee et al., 2007; Grennan et al., 2012). Repeated or persistent plasma HIV RNA detection above the level of quantification (especially if >500 copies/mL) after having achieved virologic suppression usually represents virologic failure (Grennan et al., 2012).

2.11.2 Immunologic failure

Immunologic failure is defined as an incomplete immunologic response to therapy or an immunologic decline while on therapy (Medscape Education, 2009). Despite there being no standardized definition, many experts would consider as incomplete immunologic response to therapy the failure to maintain or achieve a CD4 T lymphocyte (CD4) cell count/percentage that is at least above the age-specific range for severe immunodeficiency (Flynn et al., 2004). Evaluation of immune response in children is complicated by the normal age-related changes in CD4 cell count. Thus, the normal decline in CD4 values with age needs to be considered when evaluating declines in CD4 parameters. CD4 percentage tends to vary less with age. At about age 5 years, absolute CD4 count values in children approach those of adults; consequently, changes in absolute count can be used in children aged ≥5 years (Flynn et al., 2004).

2.11.3 Clinical failure

Clinical failure is defined as the occurrence of new opportunistic infections (OIs) and/or other clinical evidence of HIV disease progression during therapy (Medscape Education, 2009). Clinical failure represents the most urgent and concerning type of treatment failure and should prompt an immediate evaluation (Tan et al., 2008). Clinical findings should be viewed in the context of virologic and immunologic response to therapy; in patients with stable virologic and immunologic parameters, development of clinical symptoms may not represent treatment failure. Clinical events occurring in the first several months after cART initiation often do not represent cART failure. For example, the development or worsening of an OI in a patient who recently initiated cART may reflect a degree of persistent immune dysfunction in the context of early recovery, or conversely, be a result of immune reconstitution inflammatory syndrome (IRIS). However, the occurrence of significant clinical disease progression should prompt strong consideration that the current treatment regimen is failing (Saitoh et al., 2008).

2.12 Antiviral drug targets and molecular basis for resistance

Currently available antiretroviral medications target different steps of the virus life-cycle: (i) viral entry (including co-receptor antagonists and fusion inhibitors); (ii) reverse transcription [i.e. NRTIs and NNRTIs (both RT inhibitors), (iii) integration (integrase inhibitors); and (iv) viral maturation i.e protease inhibitors (Zuckerman et al., 2007). Most of the licensed drugs in circulation are RT or protease inhibitors (Menendez-Arias, 2010). These drugs constitute the backbone of highly active antiretroviral therapy (HAART). HAART regimens constitute the standard care for HIV infected persons and include at least one or two NRTIs, one NNRTI and/or one PI (NASCOP, 2016). However, the emergence of multi-drug resistant HIV mutants still raises serious clinical implications even under HAART (Menendez-Arias, 2010).

The reverse transcriptase (RT) enzyme targeted by most ARV drugs is responsible for the conversion of viral RNA to proviral DNA (Pennings, 2012). Structurally, the RT enzyme is made of two sub-units, p66 and p55 weighing 66 and 55 kDa respectively

(Menendez-Arias, 2010). The crystal structure of the RT reveals marked similarity in the two sub-units as they both have 4 similar sub-domains with identical folding. The DNA polymerase domain of the enzyme carries the active site for catalysis and is the primary target for anti-HIV-1 medications. RT inhibitors are designed to block the polymerase site, making it unavailable for viral replication (Menendez-Arias, 2010).

2.13 Emergence of HIV drug resistance

Despite the massive contribution made by antiretroviral drugs in rolling back HIV-1 mortality and morbidity, major drawbacks still abound with drug tolerability, adherence and long term ARV-toxicity being main concerns (Menendez-Arias, 2010). HIV-1 exhibits a very high rate of mutation of approximately 10^{-4} to 10^{-5} mutations per nucleotide (Grant et al., 2002). This, together with the high frequency of recombination, and rapid viral turnover in an infected person (about 109 viral particles released daily), exacerbate the appearance of drug-resistant mutants and the eventual emergence antiretroviral treatment failure (Little et al., 2002).

2.13.1 HIV-1 drug resistance genotypes

Resistance to RT inhibitors is achieved by the accumulation of one or more mutations in the RT-coding region of the virus (Menendez-Arias et al., 2003). Generally these resistance mutations arise at the expense of viral fitness (viral fitness predominates in the absence of antiretroviral drugs). Compensatory mutations may occur to improve the viral replication capacity and increase viral fitness (Wei et al., 2003). Single-nucleotide mutations that confer resistance to RT inhibitors have been demonstrated in Lamivudine treatment, and are frequently implicated in the resistance to NNRTIs (Martinez-Picado and Martinez, 2008). These mutations cause amino acid substitutions in the viral RT which decrease the ability of the enzyme to bind the inhibitor (Tang & Shafer, 2012). One such mutation is the M184V, a mutation associated with high-level resistance to Lamivudine (Tang & Shafer, 2012). Both M184V and M184I mutations are associated with diminished replicative capacity of the virus, particularly at low dNTP concentrations (Wei et al., 2003).

Other mutations, like K65R, L74V, K70E or V75T confer resistance to nucleoside analogues by way of nucleotide discrimination (Menendez-Arias, 2010). The loss of certain important interactions, either electrostatic or hydrophobic (e.g. in K103N and Y181C respectively) in the case of NNRTIs plays an important role in the acquisition of resistance to Efavirenz and Nevirapine respectively (Shulman et al., 2000).

Suboptimal treatments can accelerate the emergence of HIV drug resistant mutants by accumulating inhibitor specific mutations. However, combinational therapies select for mutations which confer multi-drug resistance (Menendez-Arias, 2010). This is the case with the resistance mutation Q151M, first isolated from patients treated with both Zidovudine and Didanosine but not those treated with either individual drug (Smith et al., 2008). Resistant mutants to the NRTIs contained the amino acid changes, V75I, F77L,A62V, F116Y and Q151M, and showed high-level resistance to Didanosine, Zidovudine and Stavudine. Low-level resistance to Lamivudine, Abacavir, Tenofovir and Emtricitabine has been demonstrated in phenotypic assays (Smith et al., 2008; Feng et al., 2005)

2.13.2 Mechanisms for NRTIs resistance

The NRTI class of anti HIV-1 drugs include; Lamivudine, Zidovudine, Stavudine, Didanosine, Emtricitabine, Zalcitabine and Abacavir. Zidovudine (or AZT) is the most studied of the NRTIs and one of the mechanisms for resistance to AZT is excision (Menendez-Arias, 2008). The drug acquires mutations in the RT that promote an ATP mediated removal of the AZT monophosphate (Menendez-Arias, 2008). The excision reaction happens at the dNTP binding site using ATP as the pyrophosphate donor and effectively unblocks the primer terminus to allow reverse transcriptase to initiate viral DNA synthesis (Tu et al., 2010). In this way, the NRTI is selectively excluded from one end of the viral DNA after incorporation by the polymerase. The excision pathway of AZT resistance results in primarily two resistance mutations, K70R and T215Y/F, which enhance the ability of the variant RT to bind the ATP (Menendez-Arias, 2010). A second mechanism by which AZT resistance emerges is called exclusion (Boyer et al.,

2012), and occurs during early treatment protocol using a combination of AZT and dideoxyinosine (ddI). This mechanism partially depends on the host cell's ability to convert ddI into ddATP. The combinational therapy used in the early protocol selected for drug-resistance mutations in the HIV-1 RT and many of them included the primary mutation Q151M (Tang & Shafer, 2012). Minor mutations like V75I, F77L, F116Y and A62V were also observed in various combinations with Q151M. In the mutant RT, binding and incorporation of AZTTP/ddATP is reduced compared to those in TTP and dATP, which are the normal substrates. It is speculated that the Q151M changes the hydrogen bond interactions between HIV-1 RT and the 3'-OH of the incoming dNTP (Menendez-Arias, 2010). This causes an increased discrimination against NRTI-TPs. The purpose of other changes in the Q151M complex is a subject of conjecture, with suggestions that they could be compensatory mutations to counteract the anticipated deleterious effects of the Q151M mutation. Other studies suggest they could enhance the capability of the mutant RT in discriminating against the NRTI-TPs (Boyer et al., 2012).

2.13.3 Mechanisms for NNRTI resistance

NNRTIs are chemically distinct from NRTIs. The approved antiviral drugs in this class include; nevirapine, Efavirenz, delavirdine and etravirine (Tang & Shafer, 2012). These drugs interfere with dNTP incorporation in the RT of HIV-1 by binding to an allosteric site in the enzyme (Xia et al., 2007). By so binding, the drugs alter the conformation of the residues near the active site as opposed to directly affecting phosphodiester bond formation (Spense et al., 1995). Single amino acid changes as are the cases in K103N, Y181C and G190A mutations are usually sufficient to confer a high level of resistance to Nevirapine, Efavirenz and other NNRTIs (Menendez-Arias, 2010).

2.13.4 Resistance mechanisms to protease inhibitors

The HIV-1 protease is another major target in antiretroviral therapy. Protease inhibitors are of major importance in combinational regimens and most of them are substrate-based (Huff and Kahn, 2001). The atomic basis for substrate-inhibitor binding has been well studied and it is from this structural understanding that drug resistance has been

elucidated in the HIV-1 protease (Ali et al., 2010). Selective mutation due to drug pressure coupled with heterogenetic diversity among the HIV-1 proteases alter the conformation of the enzyme active site, as well as causing changes in the sequences within the gag polyprotein binding sites (Louis et al., 2007). All these changes act synergistically to confer a high-level drug resistance to the protease inhibitors (Menendez-Arias, 2010; Ali et al., 2010).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study design

This was a laboratory based cross-sectional study. Subjects were recruited from Comprehensive Care Centres (CCCs) in six counties in Kenya representing high and low HIV burden areas. These counties (county population in parenthesis) were; Kiambu (1,623,282), Kisumu (968,909), Nakuru (1,603,325), Kajiado (687,312), Homabay (963,794) and Kilifi (1,109,735) Counties. Recruitment was done within the existing framework of partnering local HIV comprehensive care centres (CCCs) which were used as study sites. Blood processing was carried out at investigating laboratories including the Centre for Virus Research at the Kenya Medical Research Institute (KEMRI) and the Centre for Research in Therapeutic Sciences (CREATES), Strathmore University in Nairobi.

3.2 Study population

Demographic and other non-personal and non-intrusive data was obtained for the consenting subjects. Participants included adults of age 18-64 years and children and minors aged 5-17 years, as outlined in the inclusion criteria. Additionally, baseline data including CD4 cell counts, initial viral load, ARV regimen and history and demographic data were obtained immediately or shortly after consenting. Subjects were asked to participate out of free will.

3.3 Inclusion criteria

Consenting adults aged between 18-64 years who were either receiving or not yet receiving anti-retroviral drugs, HIV-positive children and minors aged between 5-17 years who were on ARVS or not, and whose parents or guardians gave formal consent.

3.4 Exclusion criteria

All non-consenting subjects, as well as patients with an indeterminate HIV sero-status.

3.5 Ethical considerations

Clearance to carry out the study was obtained from the Scientific Steering Committee and Ethical Review Committee of Kenya Medical Research Institute (KEMRI) under SSC/ERC Number-2477 (Appendix 3and 4). All procedures were carried out in accordance with KEMRI Biosafety guidelines. The study was also approved by the Jomo Kenyatta University of Agriculture and Technology (JKUAT) Board of Post-graduate Studies. Informed consent was obtained from all adult subjects and from parents or guardians of children and minors.

3.6 Sample size estimation

Previous studies among non-B HIV-1 subtype in East Africa have reported prevalence rates of drug resistance mutations of between **1.0%** and **7.4%** among patients on first line regimen of ARVs (Gupta et al., 2012). Although transmitted drug resistance has been documented to be as high as **17.8%** in prevalence (Hurt et al., 2009), a national estimate of post-treatment drug resistance has not been determined. This therefore being a descriptive cross-sectional study, Fisher's exact formula (Fisher, 2006) was used, based on the national HIV prevalence rate of (Ministry of Health Kenya, 2014).

$$n = \frac{z\ \propto^2 \times p \times q}{d^2}$$

z_α_= z score for (1-95% confidence interval)

p= national prevalence of HIV = 5.7%

q= 1-p

d= precision (5% precision will be used)

$$n = \frac{1.96^2 \times 0.057 \times 0.943}{0.05^2}$$

= 82.6

The minimum sample required for the study was therefore 83 patients. This study was however a sub-set of a larger study in which 546 patients were enrolled. From the larger study, 83 patients meeting the recruitment criteria and either failing or not failing treatment by virologic definition were sampled for drug resistance testing.

3.7 Clinical procedures and blood processing

About10ml of EDTA venous blood was drawn from each subject by venepuncture and by professional phlebotomists. Out of this100µL of the fresh EDTA whole blood was used for CD4 count assessment. The remaining portion was centrifuged at 2,000 x g for 5 minutes to obtain plasma used for viral RNA isolation and the subsequent resistance mutation analysis. The plasma was preserved in cryogenic vials at -80^{o}C. The remaining portion of the blood was processed for peripheral blood mononuclear cells (PBMCs) using 0.84% ammonium chloride method and used for DNA extraction

3.8 CD4 T-Cell assays

CD4+ T-Cell counts were determined from50µL of fresh EDTA blood as part of regular point-of-care ART management protocol. Three facilities (Kiambu District Hospital,

Nakuru Provincial General Hospital and Kisumu District Hospital) used FACS (Becton-Dickinson, Franklin Lakes, NJ) machines while the other three facilities (Kilifi, Kajiado and Homabay District Hospitals) used the AlerePima™ CD4 Analyzer (Waltham, MA) to enumerate CD4 T-cells. For the FACs platform, 50µL of blood was pipetted into a labelled BD Trucount tube and mixed with 20µL of BD Tritest CD3/CD4/CD45 reagent (Becton-Dickinson, Franklin Lakes, NJ). The mixture was vortexed and incubated in the dark room at room temperature for 15 minutes after which it was placed into the BD FACS Loader for analysis using BD Multiset software. For the AlerePima protocol, 25µL of blood was collected into a disposable Pima test cartridge, capped then loaded into the Pima Analyzer. Software installed in the analyser captured and analysed CD4 data. All protocols were adapted wholly from respective manufacturers' kit manuals.

3.9 Viral RNA load quantitation

Viral RNA loads (VL) were determined using an automated Abbot RealTime HIV-1 assay system (Abbot, North Chicago, IL) following manufacturer's instructions. Briefly, 200µL of plasma was thawed and internal control RNA added for simultaneous processing. The plasma samples were then transferred to the Abbot m2000sp platform for RNA extraction. RNA samples were then mixed with the Abbot RealTime master mix reagents in a 96-well microtiterplate. The plate was then sealed and transferred to the Abbot m2000rt instrument for gene amplification and quantification. The lower limit of detection for this instrument was 40 HIV-1 RNA copies, while the upper limit was 10,000,000copies/ml. All the VL assays were performed in duplicates at the Early Infant Diagnosis (EID) laboratory of the Kenya Medical Research Institute.

3.10 Nucleic acid extraction

3.10.1 RNA isolation

Viral RNA was extracted from plasma using silica membrane based procedure (RNeasy mini kit, Qiagen) following manufacturer's instructions. Two hundred microlitres of plasma was mixed with 20µL of QIAGEN protease and 200µL of buffer AL in a 1.5ml

microcentifuge tube and the mixture vortexed for 15 seconds. The mixture was then incubated at 56º C for 10 minutes then centrifuged briefly. Approximately 200µL of 96% ethanol was added to the sample then vortexed to mix and centrifuged. The mixture was then transferred to QIAmp Mini spin column and centrifuged before adding AW1 buffer. This was followed by addition of AW2 buffer and then centrifuged at 20000×g for 3 minutes. RNA was eluted in 40µL of nuclease-free water.

3.10.2 DNA isolation

DNA was extracted from PBMCs using the QiaAmp DNA Blood Minitkit (Qiagen, CA). Briefly, PBMCs were suspended in 200µL of phosphate buffered saline (PBS) and mixed with 20µL of proteinase K. About 200µL of buffer AL was added, then vortexed and incubated at 56^{o}C for 10 minutes before adding 200µL of ethanol and the mixture transferred to spin columns for centrifugation. Following centrifugation at 6000×g for 1 minute, the columns were then washed with buffer AW1 and rinsed with AW2 at 6000×g for 1 minute and 20000×g for 3 minutes respectively. DNA was then eluted in a 1.5ml microcentrifuge tube using 30µL of nuclease-free water, followed by a one minute incubation step at room temperature and centrifugation at 6000×g.

3.11 Gene amplification by PCR

For amplification where RNA was the starting material, a 701 base pair segment of HIV-1 *po*l-RT gene corresponding to nucleotides 2480–3180 of HIV-1_{HXB2} was amplified using Qiagen One-Step RT-PCR kit (Qiagen, CA) followed by a nested PCR. One-Step RT-PCR was accomplished using primers RT18 (forward) and KS104 (reverse). Primers sequences are detailed in table 1.5 below. A final PCR reaction volume of 25µl included a 1× PCR buffer, 0.4mM dNTPs, 1.5mM $MgCl_2$, 5µl of RNA template, 1µl enzyme mix, 0.6µM of each primer and 5 units of RNAseOut. Reverse transcription was performed at 50^{o}C for 30 minutes followed by the first round PCR that comprised a 95^{o}C denaturation for 15 minutes and 38 cycles of 94^{o}C for 30sec denaturation, annealing at 55^{o}C for 45sec and extension at 72^{o}C for 1 minute. A final extension step

was performed at 72^{o}C for 10 min. Nested PCR was done using HotStar Taq polymerase (New England Biolabs, Ipswich, MA) protocol in a reaction volume of 25µL comprising 1× PCR buffer, 0.2mM dNTPs, 2mM $MgCl_2$, 0.5µM forward and reverse primers, 0.625 units of the enzyme and 3µl of RT-PCR product as template. KS101 and KS102 were used as forward and reverse nested PCR primers respectively. Thermocycling conditions for nested PCR included denaturation at 95^{o}C for 5 minutes followed by 38 cycles of denaturation at 95^{o}Cfor 30seconds, annealing at 56^{o}C for 45seconds and extension at 68^{o}C for 45 seconds. A final DNA extension was done at 68^{o}C for 10 minutes.

For amplification where DNA was the starting material, PCR amplification was performed in a total volume of 25µL comprised of 5µL of DNA template, 0.6µM of unlabelled forward and reverse primers (RT18 and KS104), 0.4mM dNTPs, 1.5mM $MgCl_2$, and 1µL enzyme mix (Qiagen, CA). Amplification was carried out using GeneAMP®PCR System 9700. The PCR reactions included a five minute denaturation step at 95^{o}C followed by 38 cycles of thirty seconds at 95^{o}C, forty-five seconds at 56^{o}C annealing temperature, forty-five seconds at 68^{o}C and a final extension at 68^{o}C for 10 minutes. This procedure was repeated for nested PCR using KS101 and KS102 primers as described above.

Table 3.1 List of primers used for amplification and sequencing

HIV-1 region	Primer	Primer sequence	Purpose	Citation
Pol-RT (*nt*2480–3180 HXB2)	RT18	5'-GGAAACCAAAAATGATAGGGGGAATTGGAGG -3'	Round 1 PCR	Lwembe et al., 2007; Lihana et al., 2009
	KS104	5'-TGACTTGCCCAATTTAGTTTTCCCACTAA-3'		
	KS101	5'-GTAGGACCTACACCTGTTCAACATAATTGGAAG-3'	Nested PCR/ sequencing	Lwembe et al., 2007; Lihana et al., 2009
	KS102	5'-CCCATCCAAAGAAATGGAGGAGGTTCTTTCTGATG -3'		

3.12 Gel electrophoresis

PCR products were detected using agarose gel electrophoresis based on negatively charged DNA migrating in the agarose matrix towards anode when placed in an electrolyte under the influence of an electric current. About 1.6% (w/v) agarose gel was made using 0.64g of agarose powder (Invitrogen, USA) dissolved in 40 ml of 1X Tris-acetate-EDTA (TAE) (40 mMTris-Acetate, 1 mM EDTA at pH 8.3) (Sambrook et al., 1989) electrophoresis buffer, and then heated in a microwave oven until completely melted. The gel was stained with 2.5% ethidium bromide to facilitate visualization of DNA after electrophoresis. After cooling the solution to about 60ºC, it was poured into a casting tray containing a sample comb and allowed to polymerize at room temperature. After the gel had polymerized and the comb removed, the gel (still in its casting tray) was inserted horizontally into the electrophoresis chamber and covered with buffer. About 5µL of DNA samples were then loaded into the sample wells. In addition, 2.0 µL aliquot of PCR marker (Promega, Madison, USA) was pipetted into the first well of each lane as a ladder. The lid and power leads were placed on the apparatus, and a current of 110 volts applied for 30minutes. The gel was then visualised and the image captured using UVIpro® Version 12.4 gel image software (UVitec, Cambridge, UK).

3.13 Sequencing and Phyologenetic sequence analysis

PCR products were purified using QiaQuick PCR purification kit (Qiagen, Redwood City, CA) as per manufacturer's instructions and sequenced using Big Dye Terminator cycle sequencing kitv.3.1 (Applied Biosystems, Waltham, MA). A reaction mixture was prepared by adding the PCR products to a master mix comprising of forward and reverse primers, sequencing buffers, deionized water among other reagents and a PCR run initiated. Sequencing products were purified using Centri-Sep™ spin columns (Applied Biosystems, Waltham, MA) followed by a sequencing run. Sequence data was captured using Applied Biosystems 3500 genetic analyzer. Sequences of clinical relevance to drug resistance included positions 41- 236 of the reverse transcriptase inhibitor (RTI) -

associated mutations plus position 318 for non-nucleoside reverse transcriptase inhibitor (NNRTI)-associated resistance.

3.14 Sequence and phenotype analysis

The BioEdit sequence alignment editor version 7.2.5 (Ibis Biosciences, Carlsbad, CA) was used for all initial manual editing of sequences. The sequences were then pairwise aligned to join the forward and reverse sequences, followed by multiple sequence alignment of all query and reference sequences using ClustalW (Version 2.1; Thompson et al., 1994). Drug resistance-related mutations were inferred from the Stanford Genotypic Resistance database (http://hivdb.stanford.edu/pages/algs/HIVdb.html; accessed 12th May 2015). Genotypic drug resistance was defined as the occurrence of one or more resistance-associated mutations, as outlined in the latest consensus mutation data of the International AIDS Society. Drug resistance levels were also inferred from the database. The estimated level of resistance to a drug was determined by adding up the penalty scores associated with each of the DRAMs present in a submitted sequence. Phylogenetic analyses were done using MEGA version 6.06 (Tamura et al., 2013) and involved the Neighbour-Joining method with 1000 bootstraps. Evolutionary distances were computed using the Kimura 2-parameter method, with gaps partially deleted at 50% cut off. Phylogenetic trees were also generated using the software which was further used to assign subtypes. Alternate subtype assignment was done using the JPHMM tool for comparison.

3.15 Data entry and analysis

All subject data relevant to drug resistance were captured and stored in a computerized data entry system. To ensure subject confidentiality during analysis and storage, unique subject identity codes were used. Relevant demographic information for each subject was also captured, and the subsequent corresponding laboratory data matched with the respective demographics. Data analysis was done using Statistical Package for Social Scientistsv.20 (IBM SPSS). Log-transformed VL, CD4 T-cell counts, age and ART duration were entered as scale variables and Age-group, Sex, HAART regimen, DRAMs

and HIV subtype as categorical variables. Analysis of variance (ANOVA) was used to compare means of outcome variables between independent variables. Associations between sets of independent variables were computed using Chi-Square statistic. P values ≤ 0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 Patient and treatment characteristics

Out of the 83 subjects enrolled in the study, PCR amplification was accomplished in 54 (65.9%) of the subjects, with viral RNA as the starting material from 35 patients while pro-viral DNA was isolated from 19 subjects. Non-amplification was attributed low sample volumes and other factors such as viral suppression. All the 54 subjects had been on Highly Active Antiretroviral Treatment (HAART) for a median 33 months. The median age of all subjects was 34 years (range, 18-56). The median plasma viral load (VL) and blood T cell counts were 3.51 (range 1.59-5.96) $\log_{10}$ HIV-1 copies/ml and 399 (range, 12-1954) cells/mm^3 respectively. Baseline demographic and treatment characteristics as recorded before the study are shown in **Table 4.1**. The number of female participants was twice that of male subjects, with CD4 T cell counts of males being significantly lower than that of females (p=0.031). Majority (46%) of the patients were on D4T-containing first-line treatment regimen. Tenofovir (TDF) and Zidovudine (AZT) containing regimens were being taken by 25.9% of the patients, with the rest (1.9%) on Abacavir (ABC) containing first-line regimen. Out of the 54 sequenced patient nucleic acid material, 35 (64.8%) were from plasma-derived RNA while 19 (35.2%) were from cellular DNA isolated from peripheral blood mononuclear cells (PBMC). Subsequent analysis of drug resistance was based on these two nucleic acid source materials. Patient and treatment characteristics are presented in **Table 4.1**.

Table 4.1 Demographic and treatment characteristics of the study participants

Characteristics	All	Male	Female
Age (Years)			
Median (Range)	34 (18-56)	41(28-53)	31 (18-56)
ART arm, n (%)			
AZT+3TC+NVP	14 (25.9)	5 (9.3)	9 (16.7)
D4T+3TC+NVP	25 (46.3)	7 (13)	18 (33.3)
TDF+3TC+NVP	14 (25.9)	6 (11.1)	8 (14.8)
ABC+3TC+NVP	1 (1.9)	0	1 (1.9)
Months on HAART			
Median (Range)	33 (8-102)	48.5 (13-96)	31 (8-102)
CD4 + T Cells, counts/mm^3			
[1]Median (Range)	399 (12-1954)	276 (12-568)	461 (45-1954)
Viral load, log_{10} HIV RNA copies/ml			
[2]Median (Range)	3.51 (1.59-5.96)	3.73 (1.59-5.96)	3.41 (1.59-5.95)
Total, N	**54**	**18 (33.3)**	**36 (66.7)**

[1]ANOVA p=0.031 comparing CD4 levels between gender. [2]Viral load not significantly different between genders. AZT-Zidovudine, 3TC-Lamivudine, TDF-Tenofovir, D4T-Stavudine, ABC-Abacavir, NVP-Nevirapine., EFV-Efavirenz

4.2 Occurrence, Frequency and Distribution of HIV drug resistance-associated mutations

Among the 54 sequenced specimens, 15 (27.8%) harboured reverse transcriptase inhibitor (RTI) Drug Resistance-Associated Mutations (DRAMs). All the 15 isolates with DRAMs are presented in **Table 4.2**, and were from plasma-derived RNA, corresponding to 42.9% circulating drug resistance phenotypes, while none of the 19 cell-associated viruses had any DRAMs. Eleven (73.3%) of the 15 patients with DRAMs

were resistant to both nucleoside RTI (NRTI) and non-nucleoside RTI (NNRTIs) classes of ARVs (Table 4.2). Subjects KHC093, MLD003, MLD011 and MLD191 did not harbour DRAMs. Another 4/15 (26.6%) of the viruses were resistant to NNRTIs only, while no patient was resistant only to the NRTIs.

Table 4.2 Occurrence and distribution of reverse transcriptase inhibitor resistance mutations in cell-free blood

Patient ID	Subtype	Regimen arm*	NRTI mutations	NNRTI mutations
KAH004	A1D	TDF	D67G, K70E, M184I	V90I, K101E, Y181C, G190A
KHC093	A1	D4T	None	K103N
KMB077	D	TDF	M184V	V90I, K103N
KMB160	A1	AZT	D67N, K70R, M184V, K219Q	Y188L, G190A
MLD003	A1	NR	None	E138A
MLD011	D	NR	None	K103N
MLD013	A1	AZT	D67N, K70R, M184V	G190A
MLD016	A1	AZT	M184V	K103N
MLD040	A1C	NR	M184V	V108I, Y181C, H221Y
MLD060	C	TDF	M184V	K103N, V106M
MLD183	A1	ABC	L74V, Y115F, M184V	K103N, H221Y, F227L
MLD191	D	NR	None	V90I, K101E, E138G
MLD198	A1A2	D4T	M41L, V75I, M184V, T215Y	K103N
MLD245	A2B	AZT	M184V	K103N
MLD545	A1	NR	M41L, M184V, T215F	K103S, E138Q

*The NRTI arm of the 3TC backbone of the first-line ARV regimen. AZT-Azidovudine, D4T-Stavudine, TDF- Tenofovir, ABC-Abacavir, N.R-not recorded

4.3 Frequency and distribution of DRAMs

Each DRAM was next analysed in terms of only the patients with the specified resistance phenotype. DRAM frequency is presented as the number of times a specific mutation is detected as a percentage of all NRTI mutations and also as a percentage of all DRAMS in the population of patients with resistance phenotype. There were 53 DRAMs in total from the 15 patients with resistance phenotypes. Of these mutations, 25 (47.2%) were of NRTI or thymidine analogue mutations (TAM) type, while 52.8% were of NNRTI type (Table 4.3).

4.3.1 Prevalence and frequency of NRTI mutations

The most common of NRTI mutation was M184V/I, which was detected in 11/15 (73.3%) patient samples. The M184V/I mutation occurred at a mutation frequency of 20.8% (11/53 mutations), or at 44% of all 25 NRTI DRAMs (**Table 4.3**). In six of the patients with NRTI mutations, M184V/I occurred together with at least two other major NRTI mutations (**table 4.2**). The frequencies of other TAMs are presented in **Table 4.3** Two subjects had both TAM-1 mutations T215Y/F and M41L, which represented a mutation frequency of 8% for each compared against all 25 TAMs. These TAM-1 mutations are associated with resistance to D4T and AZT, and occurred in common with M184V/I in the two patients (**Table 4.3**).

4.3.2 Prevalence and frequency of NNRTI mutations

A total of 28 of the 53 (52.8%) DRAMS were of NNRTI phenotype, where they confer resistance to NNRTI class of drugs (**Table 4.3**). Mutations at position K103 were the most common of all NNRTI DRAMs, appearing in 9/15 (60%) of the patients.K103 DRAMs occurred at a mutation frequency of 32.1% of the 28 NNRTI mutations or 17% of all 53DRAMS (**Table 4.3**). A single mutation at this position (K103) was observed in 5/15 (33.3%) subjects but appeared in combination with other major DRAMs in 4/15 (26.7%) subjects. Other major NNRTI DRAMs including G190A, E138A/Q and V90I were each found in 3/15 (20%) of the patients. Each of these DRAMs represented 10.7% (3/28) of the NNRTI mutations or 6% of all DRAMs. Mutations Y181C, V108I, K101E and H221Y appeared in 3 patients each at a frequency of 7.1% (2/28 NNRTI DRAMs). These mutations and the rest of the NNRTI DRAMs are captured in **Table 4.3.**

Table 4.3 Frequency and distribution of DRAMs

NRTI DRAM	**Selected drug**	**n (%)**	**NNRTI DRAM**	**Selected drug**	**n (%)**
M184V/I	3TC, FTC.	11 (44)	K103N/S	NVP, EFV	9 (32.1)
D67N/G	AZT, D4T, ABC, TDF, DDI	3 (12)	E138A/Q	ETR, RPV	3 (10.7)
K70R/E	TDF, ABC, DDI & 3TC, FTC	3 (12)	G190A	NVP, EFV	3 (10.7)
T215Y/F	D4T, ABC, DDI,	2 (8)	V90I	+All NNRTIs	3 (10.7)
M41L	AZT	2 (8)	H221Y	+All NNRTIs	2 (7.1)
K219Q	AZT & D4T	1 (4)	K101E	NPV, EFV, ETR, RPV	2 (7.1)
L74V	ABC, DDI	1 (4)	Y181C	NPV, EFV, ETR, RPV	2 (7.1)
Y115F	ABC	1 (4)	F227L	NVP, EFV	1 (3.6)
V75I	D4T, DDI	1 (4)	V106M	+All NNRTIs	1 (3.6)
			V108I	+All NNRTIs	1 (3.6)
			Y188L	NVP, EFV, RPV	1 (3.6)
Total		25 (100)			28 (100)

+All the non-nucleoside reverse-transcriptase inhibitors including NVP, EFV, ETR, and RPV.

NRTI-nucleoside reverse transcriptase inhibitors, NNRTI-non nucleoside reverse transcriptase inhibitors, DRAM-drug resistance-associated mutation

4.4 Degree and level of patients' resistance to specific RTI class of drugs

The Stanford drug resistance database (http://hivdb.stanford.edu/pages/algs/HIVdb.html; accessed 12th May 2015) was used to assign resistance level in each case of reported DRAM from all patients showing any resistance phenotype (**Table 4.4**). Resistance level as assigned by the Stanford drug resistance data base relies on penalty scores associated with each of the DRAMs present in a submitted sequence. Eleven patients with NRTI DRAMS had high-level resistance to at least three different ARV drugs, 7/15 (46.6%) had intermediate-level resistance to at least two drugs while 12/15 (80%) had low-level resistance to at least one ARV drug (**Table 4.4)**. Eleven of these patients had high (n=1), intermediate (n=4) and low-level (n=6) resistance to Didanosine (DDI). The DDI resistance phenotype was accompanied by a common M184V mutation without any record of DDI exposure (**Table 4.4**). Previous exposure of DDI could not however, be authoritatively verified in these patients. In the NNRTI mutations' category, 13 (81.7%) of 15 patients with NNRTI DRAMs had a high-level resistance phenotype affecting at least two drugs (**Table 4.4**). Eleven of these 13 also included high-level resistance both to Nevirapine and Efavirenz, the two common and alternately used NNRTI regimens in this population. High, intermediate and low-level resistance to Etravirine (ETR) and Rilpivirine (RPV) were detected in 8 patients who had no record of ETR or RPV exposure. One patient, MLD003, had only E138A mutation associated low-level resistance to ETR and RPV. These data are captured in **Table 4.4.**

Table 4.4 Phenotype and resistance level associated with observed mutations

Patient	DRAMs NRTI | NNRTI	High-level resistance to	Intermediate resistance to	Low- level resistance to
KAH004	D67G, K70E, M184I | V90I, K101E, Y181C, G190A	3TC, FTC, NVP, EFV, RPV	ABC, AZT, D4T, DDI, ETR	D4T, TDF
KMB160	D67N, K70R, M184V, K219Q | Y188L, G190A	3TC, FTC, EFV, NVP, RPV	ABC, AZT, D4T, DDI, ETR	TDF
KMB077	M184V | V90I, K103N	3TC, FTC, EFV, NVP		ABC, DDI
MLD245	M184V | K103N	3TC, FTC, NVP, EFV	-	ABC, DDI
MLD011	None | K103N	EFV, NVP		
MLD183	L74V, Y115F, M184V | K103N, H221Y, F227L	3TC, ABC, DDI, FTC, EFV, NVP	_	TDF, ETR, RPV
MLD191	None | V90I, K101E, E138G		NVP, RPV	EFV, ETR
MLD198	M41L, V75I, M184V, T215Y | K103N	3TC, AZT, D4T, FTC, EFV, NVP	ABC, DDI, TDF	_
MLD040	M184V | V108I, Y181C, H221Y	3TC, FTC, NVP	EFV, ETR, RPV	ABC, DDI
MLD545	M41L, M184V, T215F | K103S, E138Q	3TC, AZT, D4T, FTC, NVP	ABC, DDI, EFV	TDF, ETR, RPV
MLD060	M184V | K103N, V106M	3TC, FTC, EFV, NVP	-	ABC, DDI
KHC093	None | K103N	EFV, NVP		
MLD003	None | E138A*	-	-	ETR, RPV
MLD013	D67N, K70R, M184V | G190A	3TC, FTC, NVP	ABC, AZT, EFV	D4T, DDI, ETR, RPV
MLD016	M184V | K103N	3TC, FTC, NVP, EFV	-	ABC, DDI

*This mutation confers cross-resistance to ETR and RPV

4.5 Drug resistance mutations, viral load and CD4 counts among various HIV-1 subtypes

Majority of the patients (57.4%) were infected with HIV-1 subtype A1, while recombinant viruses constituted 22.2% of all the infections (table 4.5). Subtype A had the largest proportion of RTI-associated mutations at 47.2% of the total 53 DRAMs, followed by recombinant viruses (34%). Subtype D and C contributed 13.2% and 5.7% of all the RTI DRAMs respectively (**Table 4.5**). In terms of average mutations per subtype, recombinant viruses had the highest number at 4.5 average DRAMs while subtype D had the least (2.3). Of the recombinants, A1D had 7, A1C (4), A1A2 (5) and A2B (2) DRAMs each.

Comparisons were also made between viral load (VL), virologic treatment response and CD4 T-cell counts and subtypes, and between patients with or without DRAMs. Virologic failure (VF) was defined by two sequential VL measurements as VL1 above or below 1000 HIV RNA copies followed by VL2 above 1000 copies during HAART continuously sustained over at least six months. Out of 54 patients, 35.2% failed treatment by this definition, with mean VL of 4.79 log copies which was significantly higher than VL of patients not failing treatment (mean, 1.98 log copies, $p<0.001$). Patients with DRAMs had significantly higher VL than patients without DRAMs ($p=0.001$). Among the patients with treatment failure, VL did not differ between those with and those without DRAMs. VL did not also differ significantly between different HIV-1 subtypes (**Table 4.5**).

Virological failure (VF) as a percentage of the total study population was 20.4% for patients with DRAMs and 14.8% for those without DRAMs. By comparison, 5.6% and 44.4% respectively of patients with or without DRAMs did not fail treatment. Chi square test of independent associations revealed a significant correlation between virologic response (VF or lack thereof) and DRAM (presence or lack thereof, χ^2 $p<0.001$). Specifically, 57.9% of the patients with VF had DRAMs as compared to 42% who did not have DRAMs but failing treatment. By comparison, 89% of the treatment responders lacked any DRAM as compared to just 11% who had at least one DRAM but not failing

treatment. These differences were significant at the mentioned level of χ^2test (**Table 4.5**).

Proportions of VF were comparable within subtypes A and C, but were significantly higher within subtypes D and recombinants. However, patients failing treatment within any subtype group had at least 1log VL above the VL of patients not failing treatment within that group. CD4 T-cell counts did not differ significantly between groups of patients with and those without DRAMS. Taken together, VL differed significantly between patients with DRAMs and those without DRAMs, but did not differ by HIV subtype, although more patients infected with recombinant or subtype D viruses tended to fail treatment than the rest (**Table 4.5**).

Table 4.5 Viral load and CD4 T-cell counts for various genotypic and phenotypic characteristics

DRAMS distribution by HIV-1 genotype	Patients, n | %nt	N patients with | Without DRAMS	DRAMs | %nt	Average DRAMs per subtype
HIV-1 Subtype				
A	31 | 57.4	7 | 24	25 | 47.2	3.6
C	3 | 5.6	1 | 2	3 | 5.7	3
D	8 | 14.8	3 | 5	7 | 13.2	2.3
Recombinants	12 | 22.2	4 | 8	18 | 34	4.5
TOTAL, Nt	54	15 | 39	53 | 100	3.5

Treatment outcome by Subtype	n | %	[1]Viral load log_{10}copies/ml, mean | range	[2]CD4 T-cells counts/mm^3, mean | range	Treatment failure, % n (VL)
A	31 | 57.4	2.91 | 1.59-6.13	427 | 45-1954	32.3 (5.06)
C	3 | 5.6	2.5 | 1.59-3.45	412 | 321-507	33.3 (3.44)
D	8 | 14.8	3.26 | 1.59-6.39	341 | 12-800	37.5 (5.23)
Recombinants	12 | 22.2	3.04 | 1.59-5.09	362 | 137-714	41.7 (4.26)
Total	54 | 100	2.97 | 1.59-6.39	399 | 12-1954	35.2 (4.79*)

Cross-tabulation and correlation test	Number, n | VL	With DRAMs, %n | VL (Log_{10})	Without DRAMs, %n | VL (Log_{10})
Virologic failure	19 | 4.79	57.9 | 4.81	42.1 | 4.76
Responders	35 | 1.98	11.4 | 1.74	88.6 | 2.0
Total	54 | 2.97	27.8 | 3.99	72.2 | 2.57
†Pearson's Chi square			$p<0.001$

[1]ANOVA p=0.001 comparing viral load at time 2 (VL2) of patients with DRAMs and those without DRAMs. This analysis is not significant between subtypes. [2]ANOVA p-value is not significant for similar comparison of CD4 T-cell counts.*$p<0.001$ compared between failures

and log 1.98 VL of non-failures.† test of independent associations between virologic failure patients and DRAM groups.

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Heightened efforts to improve the availability and access to ART in resource-poor settings is paying off and many low and middle income countries are currently able to provide first-line ARV drugs to a significant number of HIV-1 infected persons in need of treatment (Bennet, 2006). However, HIV drug resistance testing and monitoring still remains largely unavailable in these countries (Bennet, 2006; Hirsch et al., 2008). This study sought to determine the prevalence and frequency of drug resistance-associated mutations (DRAMs). In addition, it also related virologic treatment failure (VF) and immunological treatment failure with the occurrence of DRAMs among Kenyan patients who had been on HAART for a median duration of 33 months. The prevalence of DRAMS was significantly high at 27.8%, so as virologic failure (35.2%), consistent with findings from other studies on similar populations (Lie'geois et al., 2012).

5.1.1 Immunological and virological correlates of treatment

Routine measurement of plasma HIV-1 load and CD4 T cell counts are central to management of HIV-1 infection in resource rich nations (Pillay et al., 2005). While the latter is now widely available even in limited resource settings, the availability of viral load testing and drug resistance testing remains scanty (Duda et al., 2014). CD4 T cell counts are routinely used as a point of care tool for monitoring treatment and in this group of subjects, the mean CD4 T cell was 399 cells/mm^3. While the majority of patients (64.8%) in this study had their viral load below1000 copies/ml, which was the threshold criteria for defining virological responsiveness or failure, a significant proportion (35.2%) proportion could not attain viral suppression. More recent studies have demonstrated that however, immunological criteria does not accurately predict

virological failure resulting in significant misclassification of therapeutic responses, thus calling for an urgent need to scale up viral load and drug resistance testing (Ferreyra et al., 2012; Hassan et al., 2014). It is on the evidence of these emerging data that a virological criterion was preferred when defining treatment responsiveness or failure in this study.

5.1.2 Drug resistance mutations and treatment failure

Analysis of DRAMs was done on plasma material to assess the prevalence of circulating resistance and on proviral DNA sequences to assess any residual (non-circulating) resistance. No residual resistance was detected from proviral DNA material. The prevalence of DRAMS was 27.8% for all patients, representing 42.8 % of DRAMs circulation in plasma. Virological failure rates in this population were very high but comparable with prevalence of DRAMs, with 35.2% of all the patients unable to achieve viral suppression below 1000 HIV copies/ml plasma. A study of Gabonese patients found VF rate of 41.3% while the prevalence of DRAMS was at least 56% in the same population (Lie'geois et al., 2012). From that study, it can be implied that development of DRAMs precede VF, although it is also likely that many patients may develop DRAMs and not fail treatment. While the study reported here clearly included a smaller study population, the randomness of the sampling allows the drawing of reasonable arguments. The data suggests that patients with DRAMS in this study were more likely to fail treatment and those without DRAMs were more likely to respond to treatment as predicted.

5.1.3 NRTI resistance-associated mutations

The NRTI mutation, M184V/I was detected in a vast majority of patients with DRAMs, where it occurred in combination with other TAMs. This mutation confers high-level resistance to 3TC and FTC, and low-level cross-resistance to ABC and DDI. This possibly explains why nine patients had between high and low-level resistance profiles to DDI despite having no history of exposure to the drug. The combination of low genetic barriers and the extensive use of drugs such as ABC, 3TC and DDI contributes

to the emergence and high prevalence of the M184V/I mutation among patients receiving regimens with those drugs (Tang, 2012). This was suspected to be the case with the subjects in this study receiving regimens containing the aforementioned drugs, though we could not rule out the pre-existence of the mutations through unspecified transmission mechanisms among the patients before commencement of treatment.

The M184V mutation is known to be associated with increasing susceptibility of HIV-1 to TDF and AZT, and with reduced viral fitness (Tang, 2012; Hirsch et al., 2012; Johnson et al., 2013), therefore preserving the scope of the treatment options in patients treated with 3TC. The predominance of this mutation among the patients studied could thus suggest a positive outcome in patients treated with TDF and AZT, despite their non-responsiveness to 3TC. TAM-1 and TAM-2 mutations, though selected by thymidine analogues AZT and D4T confer cross-resistance to ABC, TDF and ddI (Tang, 2012).

5.1.4 NNRTI resistance-associated mutations

DRAMs associated with NNRTIs were also very common, with mutations at position K103 occurring in 60% of all patients who had any resistance phenotype. The K103 mutation conferred a high-level resistance to Nevirapine and Efavirenz. These two drugs form the NNRTI arm of antiretroviral therapy according to the Kenyan treatment protocol (Ministry of Health, 2014).The observed high frequency of these mutations is not surprising as NNRTI class of ARV drugs are extensively used in the Kenyan population as standard first-line ART regimens (Ministry of Health, 2014). Other factors that might influence the high prevalence include the use of single-dose Nevirapine for the prevention of mother to child transmission (Arrive et al., 2007), high selection frequencies observed in treated patients (Gupta et al., 2009), low genetic barriers for developing resistance to this class of drugs (Tang, 2012), and persistence of viral replication due to restricted fitness cost (Little et al., 2008). This observation implies that majority of these patients had exhausted their NNRTI class of available regimens, an observation that points towards a generalized diminishing possibilities for treatment with these drugs in Kenya.

In three patients treated with Nevirapine, viral strains with intermediate-level resistance to Etravirine (a second generation NNRTI) were found despite lack of user experience with Etravirine, implying the emergence of cross-resistance to the drug. The Y181C mutation commonly occurring with Nevirapine-containing regimes has been associated with partial cross-resistance to Etravirine (Bunupuradah et al., 2011). Further, several other studies have demonstrated that cross-resistance to Etravirine requires the development of more than one NNRI resistance mutations (Vingerhoets et al., 2005).The occurrence of the Y181C, occurring simultaneously with other NNRTI resistance mutations such as G190A, Y188L and H221Y accounts for the cross-resistance to ETR in the three patients. High to low-levels of resistance to RPV were also detected in seven patients, despite having no exposure to these drugs. This was attributed to cross-resistance associated with the NNRTI mutations G190A, Y181C and Y188L among others (Tang, 2012).

5.1.5 HIV subtypes and drug resistance

In agreement with other studies in Kenya, A1 was the most prevalent subtype in this study, underlining the continued predominance of these viruses in the country (Lwembe et al., 2007; Lihana et al., 2009; Kiptoo et al., 2013). Nevertheless, recombinant viruses were also detected in a significant proportion of the subjects. The largest number of the DRAMs total was detected in subtype A, as expected since majority of the circulating HIV strains were of this subtype. However, recombinant viruses had the highest average number of DRAMS per subtype. Given the relative paucity of data relating to clinically significant associations between subtypes and genetic barriers to resistance (Van de Vijver et al., 2006), and clinically important DRAMs (Kantor et al., 2005), these findings may not be taken to imply that recombinant HIV strains are linked to a high prevalence of DRAMs compared to other subtypes. Nevertheless, the processes leading to viral recombination present a number of pathways for developing DRAMs (Steain et al., 2004) and multiple factors could synergistically act to cause the high number of DRAMs in recombinants compared to pure strains.

Although VL did not differ significantly between subtypes, VF was higher among patients infected with recombinant and subtype D viruses than those infected with other genotypes (37.5% and 41.7% respectively) as shown in **Table 4.5**. HIV subtype D has been associated with adverse disease outcomes including a faster progression to AIDS in studies from Uganda, Kenya's neighbour to the West (Eller et al., 2014). The increasing prevalence of the D subtype and recombinant strains in Kenya may pose significant threats to the efficacy of ART scale-up in the country. In this study, there were no significant linkages between CD4 T cell-counts, subtypes or DRAMs, as has also been observed in other studies (Chaix et al., 2013; Paraskevis et al., 2013)

5.1.6 Limitations of the study

This study aimed at determining the prevalence of, and drug resistance profiles of HIV-infected individuals from selected care centres in Kenya. After the initial sample size of 83 patients was arrived at, amplification and sequencing was possible only in 54 (65.9%) of the patients. This was attributed to insufficient sample volumes and low or no viraemia in some participants, leading to amplification failures. Additionally, the limited sample size could not allow for generalization of findings on the correlation between virological failure and HIV subtypes.

5.2 Conclusions

Virological failure rates in this population were very high with 35.2% of all the patients unable to achieve viral suppression below 1000 HIV copies/ml of plasma. The mean CD4 T cell counts means was also high, at 399 (range, 12-1954) cells/mm^3, with CD4 T cell counts of males being significantly lower than that of females (p=0.031). Subtype A viruses were the most predominant (57.4%), followed by recombinants (22.2%), D (14.8%) and C (5.6%). The most common DRAMs were M184V/I (for NRTIs) and K103N (for NNRTIs) occurring respectively in 73.3% and 60% of the patients with DRAMs and at a mutation frequency of 44% and 32.1% respectively. The study revealed high prevalence rates for DRAMS (27.8%) and good correlation virological failure and DRAMS, though the data are derived from a small subset of a larger

population therefore the prevalence of drug resistance may not reflect national rates. CD4 counts did not differ significantly among patients with and without DRAMs therefore VF was a more useful correlate of treatment failure. Virologic treatment failure did not relate with subtype variations in this study, although recombinant viruses tended to have more DRAMs on average, than other subtypes. Limited sample numbers does not allow making authoritative conclusion about the predictive value of subtype variations on treatment outcome in this study.

5.3 Recommendations

To optimize treatment outcomes, viral load testing should be regularized as a tool for monitoring antiretroviral treatment responsiveness among patients on ART. In patients where virological failure is suspected, drug resistance testing should be considered as a tool for determining the presence, prevalence and types of DRAMs, all of which have implications on treatment regimen choices. In addition, care and treatment policy makers and other stakeholders should consider integrating and scaling up drug resistance genotyping and virus sub-typing as part of standard of care.

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APPENDICES

Appendix 1: Informed consent

Determination of antiretroviral drug resistance mutations among HIV-1 infected Kenyan subjects

Principal Investigator: Timothy Nzomo

Information to study participants

The Human Immunodeficiency Virus (HIV) which is responsible for AIDS has no known cure or vaccine, but the drugs available for treatment can prolong life and significantly improve the quality of life and health among infected individuals. However, long term use of medication, sub-optimal dosages, co-infection with other virus strains among other factors may lead to the emergence of drug resistance mutations and subsequent drug failure. To mitigate against the effects of drug resistance, frequent monitoring for these mutations is necessary in order to inform therapy options and general care and management of HIV. It is for this reason that we ask for your voluntary consent to collect a small amount of blood so we may analyze for drug resistance.

Procedure to be followed

In this investigation, we will take 5ml blood sample from you once now and if you agree, two more times at a later date, each time after 3 months. We will use the blood only for the reasons already explained. Because we may need to look at the samples again in the future, we will store whatever is left of your samples for future examination of similar nature without taking extra blood from you.

Benefits of the study

The study offers only indirect benefits to you, which in the long-term will help your physicians manage your conditions better. We will test if you are still responding well to the HIV drugs you are receiving through what is called drug resistance analysis. If we find that you are developing resistance, we will recommend a change in treatment. Overall, your doctors will use these test results to decide how best to treat you. The study has no other immediate benefit for you. However, the information obtained will be

used to improve the medical attention offered to you and others with HIV such as in diagnosis and finding different or alternative ways of managing the disease.

Confidentiality of your identity

The information you give us about you or your child will not be disclosed to any other parties, not even your medical records. Your name and identity is not required and you will neither be linked to the results nor your identity be made public in any forum.

Medical risks and problems.

We don't expect that this investigation will cause you any medical problems other than the reasons for which you normally visit this health facility. However, some minor problems associated with drawing your blood may occur. These include swelling at the point of blood-draw, itching and pain. In rare but plausible instances, contamination at the point of blood-draw may result in infection and other complications if appropriate care is not used to draw your blood. To minimize these risks, skilled and experienced personnel will be utilized.

Storing your blood for future studies

Part of the blood that you will give us will be stored for future studies to analyze or control for the present factor analyses. You will not be required to give extra blood for this purpose.

Basis of participation

You are free to refuse to participate in this study. If you choose to do so, your rights to be attended to in this or other hospitals now or in future will not be affected.

I have read the information stated above and have had the opportunity to ask questions all of which were answered satisfactorily. I hereby give consent for my and/or my child's participation in this study as explained to me.

Unique study No……Nationality/origin..……

Age…… …… …… Sex ……...

……..

Occupation……. …… Locality ……..

Are you on ARV medication……

I agree and consent to give my blood and participate in this study. I have received the information provided by the study staff and have not been coerced or induced to participate. I understand that I can refuse to consent and withdraw from the study at any time of my choosing.

Patient/subject's Signature or thumb print……Date……

OR

Signature of Guardian of child or minor……Date……

In case of feedback, questions or complaints contact;

Timothy Nzomo

Mobile; 0726201699

Email; **jaytim2010@gmail.com**

Appendix 2; ARV treatment profile

Determination of antiretroviral drug resistance mutations among HIV-1 infected Kenyan subjects

Principal Investigator: Timothy Nzomo

Unique study No……Nationality/origin..……..

Age…… …… …… Sex ……...

……

Date of Initiation……Regimen……..

Date of change (where applicable)……Regimen…….....

CD4 Count at baseline…….

Viral load at baseline……

CD4 count 1……..Date……..

CD4 count 2……Date…….

CD4 Count 3……Date…….

CD4 count 4……..Date……

Viral load 1…….Date……..

Viral load 2…….Date…….

Viral load 3……...Date……

Viral load 4……Date……

Appendix 3: Approval letter from Scientific Steering Committee/KEMRI

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

ESACIPAC/SSC/101264

13th December, 2012

Bernhards Ogutu

Thro'
Director, CCR
NAIROBI

17/12/2012

REF: SSC No. 2477 (Revised) – Genetic characterization of HIV-1 strains circulating in Kenya and determination of known mutations conferring resistance to antiretroviral drugs

Thank you for your letter received on 7th December, 2012 responding to the comments raised by the KEMRI SSC.

I am pleased to inform you that your protocol now has formal scientific approval from SSC.

The SSC however, advises that work on the proposed study can only start after ERC approval

Sammy Njenga, PhD
SECRETARY, SSC

Appendix 4: Approval letter from Ethical Review Committee/KEMRI

KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **April 15, 2013**

TO: **WASHINGTON OCHIENG (PRINCIPAL INVESTIGATOR)**

THROUGH : **DR. JUMA RASHID; DIRECTOR, CCR** Forwarded 16/04/2013

RE: **SSC PROTOCOL NO. 2477 – REVISED (*RE-SUBMISSION*): GENETIC CHARACTERIZATION OF HIV-1 STRAINS CIRCULATING IN KENYA AND DETERMINATION OF KNOWN MUTATIONS CONFERRING RESISTANCE TO ANTIRETROVIRAL DRUGS**

Make reference to your letter dated 4th April, 2013, Received on April 4, 2013.

We acknowledge receipt of the Revised Study Protocol.

This is to inform you that the Ethics Review Committee (ERC) reviewed the document listed above and is satisfied that the issues raised at the 211th meeting held on 26th November, 2012 have been adequately addressed.

The study is granted approval for implementation effective this **15th day of April 2013**. Please note that authorization to conduct this study will automatically expire on **April 14, 2014**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuing approval to the ERC Secretariat by **March 4, 2014**.

Any unanticipated problems resulting from the implementation of this protocol should be brought to the attention of the ERC. You are also required to submit any proposed changes to this protocol to the ERC prior to initiation and advise the ERC when the study is completed or discontinued.

You may embark on the study.

Sincerely,

EAB

Dr. Elizabeth A. Bukusi,
ACTING SECRETARY,
KEMRI/ETHICS REVIEW COMMITTEE

Appendix 5: Demographic, clinical and HIV subtype information of all study subjects

Patient ID	GeneBank Accession#	Age	Sex	HAART regimen	SUBTYPE	
					JPHMM	PHYLOGENY
KHC016	KM853131	28	M	TDF/3TC/NVP	A1	A1
KHC025	KM853132	32	F	AZT/3TC/NVP	D	D
KHC040	KM853133	29	F	TDF/3TC/NVP	C	C
KHC045	KM853134	31	F	AZT/3TC/NVP	A1	A1
KHC048	KM853135	22	F	D4T/3TC/NVP	A1	A1
KAH016	KM853136	49	M	TDF/3TC/NVP	A1C	A1
KMB039	KM853137	35	F	TDF/3TC/NVP	A2D	A2
KMB067	KM853138	50	M	D4T/3TC/NVP	A1	A1
MLD185	KM853139	52	M	AZT/3TC/NVP	A1	A1
NVS090	KM853140	46	F	AZT/3TC/NVP	A1	A1
NVS095	KM853141	28	M	D4T/3TC/NVP	A1A2	A1
NVS032	KM853142	47	M	AZT/3TC/NVP	A1	A1
NVS047	KM853143	39	F	D4T/3TC/EFV	A1	A1
NVS052	KM853144	45	M	AZT/3TC/EFV	A1C	A1
NVS056	KM853145	56	F	D4T/3TC/EFV	A1	A1
NVS069	KM853146	41	M	D4T+3TC+NVP	A1	A1
NVS085	KM853147	37	M	TDF/3TC/NVP	A1	A1
NVS122	KM853148	28	F	D4T/3TC/NVP	A1	A1
NVS152	KM853149	30	F	AZT/3TC/EFV	D	C
HND200	KM853096	42	F	NR	A1	A1
KAH004	KM853097	24	F	TDF/3TC/NVP	A1D	H
KAH010	KM853098	34	F	TDF/3TC/NVP	A1	A1
KHC106	KM853099	35	F	D4T/3TC/NVP	A1	A1
KHC160	KM853100	25	F	TDF/3TC/NVP	A1	A1

KHC163	KM853101	26	F	AZT/3TC/NVP	A1	A1
KMB145	KM853102	38	F	TDF/3TC/NVP	A1	A1
KMB160	KM853103	43	M	AZT/3TC/NVP	A1	A1
KMB077	KM853104	45	M	TDF/3TC/NVP	D	G
MLD245	KM853105	32	F	AZT/3TC/NVP	A2B	A2
MLD011	KM853106	31	F	NR	D	D
MLD183	KM853107	7	F	ABC/3TC/NVP	A1	A1
MLD191	KM853108	34	M	TDF/3TC/NVP	D	D
MLD198	KM853109	26	F	D4T/3TC/NVP	A1A2	A1
MLD021	KM853110	41	M	TDF/3TC/NVP	D	D
MLD040	KM853111	49	F	NR	A1C	A1
MLD541	KM853112	53	M	D4T/3TC/NVP	A1	A1
MLD545	KM853113	52	F	AZT/3TC/EFV	A1	A1
MLD548	KM853114	46	F	D4T/3TC/NVP	D	N.D
MLD060	KM853115	36	F	TDF/3TC/EFV	C	C
KHC093	KM853116	43	M	D4T/3TC/NVP	A1	A1
NVS058	KM853117	30	F	AZT/3TC/NVP	A1C	A1
MLD001	KM853118	30	M	AZT/3TC/NVP	A1	A1
MLD002	KM853119	29	F	AZT/3TC/NVP	A2C	A2
MLD003	KM853120	44	F	AZT/3TC/EFV	A1	A1
MLD008	KM853121	45	M	D4T/3TC/NVP	A1	A1
MLD009	KM853122	47	M	AZT/3TC/NVP	A1	A1
MLD010	KM853123	22	F	None	CD	C
MLDU011	KM853124	46	F	TDF/3TC/NVP	D	B
MLD012	KM853125	56	F	D4T/3TC/NVP	A1	A1
MLD013	KM853126	39	M	D4T/3TC/NVP	A1	A1
MLD014	KM853127	50	M	None	A1	A1
MLD016	KM853128	46	F	NR	A1	A1

MLD166	KM853129	28	M	D4T/3TC/NVP	A1C	A1
MLD167	KM853130	32	F	D4T/3TC/NVP	C	C

Key: NR, not reported. HAART, highly active antiretroviral therapy.N.D, not determined- sequence did not cluster with any of the reference sequences.