# Predictive value of gene mutations as a diagnostic tool for ART resistance in a Zambian population

BY

Thabiso Maseko Phiri

Thesis presented in partial fulfillment of the requirements for the degree
Master of Physiological Sciences at Stellenbosch University
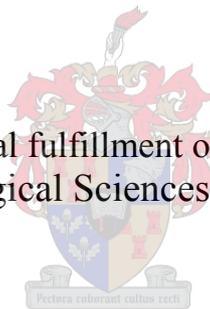
Supervisor Professor Carine Smith & Dr. Rob Smith
Faculty of Science
Department of Physiological Sciences

December 2012

Declaration

By submitting this thesis electronically, I declare that the entirety of the work contained therein is my own, original work, that I am the sole author thereof (save to the extent explicitly otherwise stated), that reproduction and publication thereof by Stellenbosch University will not infringe any third party rights and that I have not previously in its entirety or in part submitted it for obtaining any qualification.

December 2012

ABSTRACT

Background: While Selection of reverse transcriptase (RT) mutation has been reported frequently, protease (PR) mutations on antiretroviral therapy (ART) including boosted Protease inhibitor (PI) have not been reported as much in Zambia. Affordable in-house genotyping assays can been used to expand the number of patients receiving drug resistance geno-typing, which can aid in determining prevalence of RT/PI emerging mutations.

Methods: A previously published drug resistance genotyping assay was modified and used to genotype RT and PR genes. 19 patients virologically failing first-line regimen and 24 failing second-line regimen were studied to determine resistance patterns. Virological failure was defined as failing to maintain <1000 copies/mL during ART. Only major and minor RT and PR mutations (IAS-USA 2010) were considered for analysis. The in-house assay was validated by comparing sequence data of 7 previously ViroSeq tested samples and 5 randomly selected samples to determine reproducibility.

Results: The in-house assay efficiently amplified all 12 validation samples with the lowest sample scoring 99.4% sequence homology. The most common RT mutation was M184V (79% n=19) and (71% n=24) first and second-line respectively. No significant differences were reported in all the other RT mutations between first-line and second-line regimens. Drug resistant PI mutations (I54V, M46I and V82A all present 20.8%) were only found in the second-line regimen and were insignificant, p= 0.0562.

Conclusion: The in-house assays can be used as alternatives for commercial kits to genotype HIV-1C in Zambia without compromising test quality. The insignificant PI drug resistant mutations which were found, despite virological failure in patients, could indicate a possibility of other mutations within the HIV-1 genome that could reduce PI susceptibility.

ACKNOWLEDGEMENTS

LIST OF ABBREVIATIONS

ART	Antiretroviral Therapy
CCR-5	Chemokine receptor type 5
CD (4,8,or 38)	Cluster of Differentiation
cDNA	Complementary DNA
CIDRZ	Center for Infectious Disease Research in Zambia
CRF	Circulating Recombinant Form
CTL	Cytotoxic T Lymphocyte
CXC-4	Chemokine receptor type 4
ddNTP	Dideoxynucleotide tri-phosphate
DNA	Deoxyribonucleic acid
dNTP	Dideoxynucleotide tri-phosphate
FDA	Food and Drug Administration
HAART	Highly Active Antiretro Therapy
HIV-1	Human Immune Virus
HLA-DR	Human Leukocyte Antigen-DR
IAS-USA	The International AIDS Society-United States of America
IUPAC	International Union of Pure and Applied Chemistry
LTR	Long Term Repeat
NNIBP	Non-Nucleotide Binding Pocket
NNRTI	Non-Nucleotide Reverse Transcriptase Inhibitor
NRTI	Nucleotide Reverse Transcriptase Inhibitor
PI	Protease Inhibitor
RNA	Ribonucleic Acid
SIV	Simian Immune Virus
TNF	Tumor Necrotic Factor
TNFR-2	Tumor Necrotic Factor Receptor type 2
TRAIL	Tumor Necrotic Factor Related Apoptosis Inducing Ligand
tRNA	Transfer Ribonucleic acid
VQA	Virology Quality Assurance
WHO	World Health Organization

LIST OF TABLE

LIST OF FIGURES

TABLE OF CONTENTS

1.0 CHAPTER 1: INTRODUCTION

1.1 INTRODUCTION

Human Immunodeficiency Type-1 (HIV-1) and Acquired Immunodeficiency Syndrome (AIDS) are the major causes of mortality and morbidity in Sub-Saharan Africa [1]. According to the World Health Organization (WHO), by the end of 2010, about 34 million people were living with HIV and a total of 2.7 million were infected that year. A total of 1.8 million people died of AIDS during the course of that same year. Regional statistics indicated that about 22.5 million (about 67%) HIV positive individuals were from Sub-Sahara Africa and 1.9 million (about 69%) new infections were recorded from the same region. About 1.3 million (72%) deaths were recorded in 2009 in sub-Saharan Africa. Over 7,000 people are estimated to be infected by HIV each day with over 97% of these infections occurring in low and middle income countries [2].

For some time now, effective Highly Active Antiretroviral Therapy (HAART) has been available in developed and some third world countries which has prolonged the quality of life of those infected with type 1 HIV. Measurements of both absolute CD4 (cluster of differentiation) count and plasma HIV-1 viral load (VL) have been used as important parameters in patient management for both initiation of treatment and during Antiretroviral Therapy (ART) [3]. Numerous evidence has shown that a combination therapy with HAART, that inhibits the viral enzymes

such as protease and reverse transcriptase, significantly reduces HIV-1 replication. This has resulted in the reduction of mortality and morbidity associated with HIV-1/AIDS in the past decade [4-6].

Figure 11a below shows the world statistics for the selected region for the total populations on ART as compiled by the World Health Organization (WHO). World regions are identified by the color coded bar chart from the end of 2002 to the end for 2010 cumulatively. The figure was drawn based on WHO global HIV/AIDS progress report 2011.
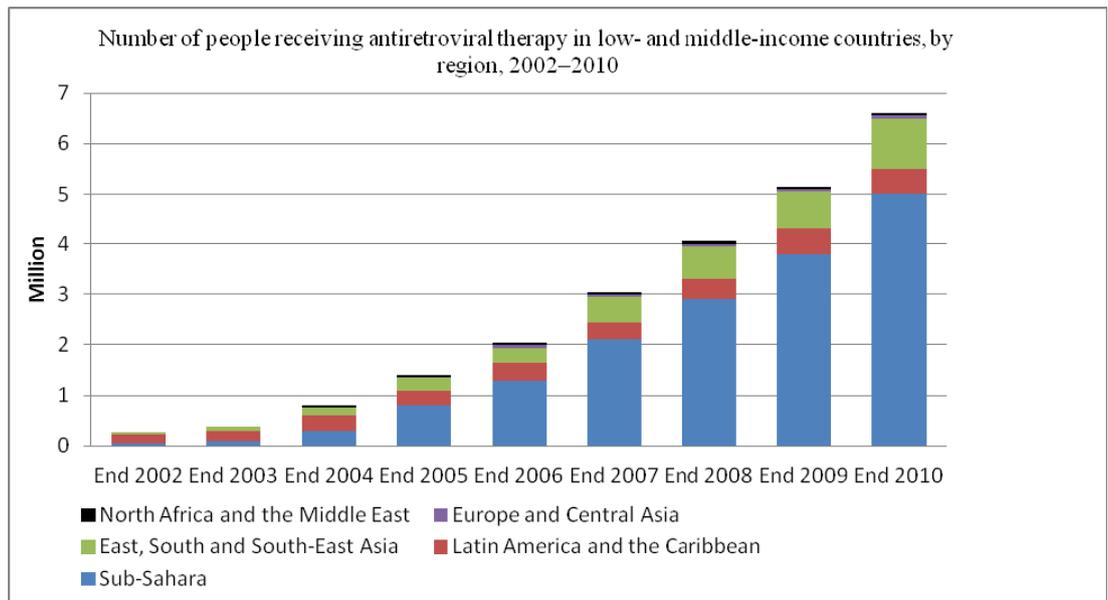
Figure 1.1a. shows an upward trend in the access to ART between 2002 and 2010 according WHO global HIV/AIDS progress report 2011 [7].

As can be seen in figure 1.1a, the WHO reported a rise of antiretroviral (ART) coverage from 7% in 2003 to 47% by end of 2010 with eastern and southern Africa having had the highest coverage (48%). [1]

Zambia has been reported to be amongst the countries in sub-Sahara with the highest HIV prevalence. By the end of 2009, the WHO reported that approximately 980,000 (about 2.8% of the global epidemic) people were living with HIV/AIDS in Zambia alone. The adult (aged 15-49) HIV prevalence in the same year was 13.5% and approximately 45,000 HIV related deaths were reported. The number of people receiving antiretroviral therapy in Zambia by the end of 2009 was estimated to be around 284,000, with about 70-79% needing therapy. Figure 1.1b below summarizes the strides accomplished in the last decade in preventing HIV related deaths using ART in Zambia [7]
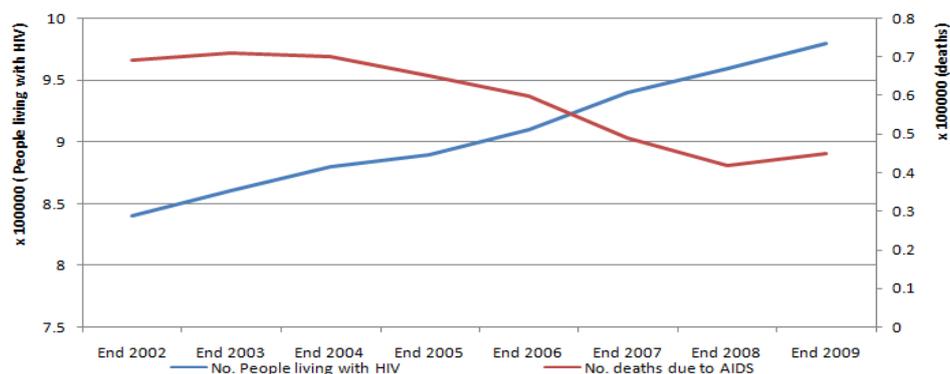
Figure 1.1b. Data of people living with HIV (blue) and deaths (red) due to AIDS between 2002 and 2009 in Zambia [7].

While HAART is a positive achievement in one sense, a long-term obstacle to it is the emergence of resistant HIV-1 variants which are less susceptible to ART drugs [8, 9]. Some of the attributed causes of this include mono-therapy, inadequate suppression of the virus replication e.g. due to lack of adherence to treatment, late initiation of HAART, and high viral replication rates coupled with high mutation errors [10-12].

3

Drug resistance testing has therefore become increasingly important in the identification of viral mutants that confer with drug resistance in patient clinical management. A number of guidelines that recommend these tests and interpretation of results have since been published [13-15].

In the next section I will give an overview of the HIV-1 genetic classification, replication cycle, host immune response and genetic diversity as a basis for understanding the HIV mechanism of resistance to currently used antiretroviral therapy during drug resistance.

1.2   THE HUMAN IMMUNOVIRUS TYPE I (HIV-1)

1.2.1 Introduction

Human Immunodeficiency Virus (HIV-1) is the main etiological agent of Acquired Immunodeficiency Syndrome AIDS. The virus was first isolated in the early 1980s from the blood of AIDS patients [16, 17]. HIV is transmitted from one infected individual to another through exposure of bodily fluids such as semen, blood, breast milk, amniotic fluids and vaginal fluids [17]. Common modes of transmission include unprotected anal or vaginal sex with an infected person, blood transfusions, transmission from mother to child during pregnancy, childbirth or breastfeeding, and through contaminated hypodermic needles [18-21]. HIV infection is characterized by long periods of clinical latency as well as weakened acquired immune responses and persistent viremia without medical intervention, patients eventually develop AIDS [22].

1.2.2 Classification

HIV is the prototype of the lentivirus genus, subfamily of retrovirus. An HIV-1 particle possesses identical single-stranded ribonucleic acid (RNA) genome, approximately

4

9.2kb long that are packaged in a cone-shaped capsid core particle [23]. Retroviruses contain internal structures which are important for viral replication and invasion of the host cell's defense. Some of the other viruses of the lentivirinae include the simian immunodeficiency virus (SIV), which causes an AIDS like disease in Asian monkeys, and equine infectious anemia virus (EIAV) that causes anemia in horses [22].

There are currently two major groups that HIV isolates have been classified as, namely, HIV type 1 (HIV-1) and HIV-type-2 (HIV-2). Classification depends on the genetic variation [24]. HIV-1 variants are either grouped as major (M), outlier (O), or new or non-M (N). Group M is the major causative agent for AIDS and accounts for more than 90% of infections worldwide [25]. Group M has further been divided into subtypes A, B, C, D, F, G, H, and K [24]. Individuals with dual or multiple infections usually have different strain recombination forming a sub-subtype designated CRFs (circulation recombinant forms) and there are more than 40 CRFs worldwide [26]. HIV-1 subtyping has often been determined using the V3 (variable-3) serotyping, *env*heterodulex mobility assay [27] or PR and RT nucleotide sequencing [28].

1.2.3 Genomic Structure

The mature HIV virion is generally characterized by having an outer lipid bilayer envelope of the host cell it was derived from. Studded within the envelope are several proteins from the host cell including major histocompatibility class 1 and 2 (MHC-I/MHC-II) antigen, actin, and ubiquitin. These proteins have been isolated from purified samples of both HIV-1 and HIV-2 as well as in SIV [29]. High resolution microscopy has reviewed the HIV-1 virion to be icosahedral in structure with 72 external spikes formed from glycoprotein 120 (gp120) anchored to the surface through

5

a trans-membrane protein, glycoprotein 41 (gp41) [30]. These envelope proteins are encoded by the *env* gene. Most retroviruses that replicate possess only three genes, *gag*, *pol* and *env* but the HIV-1 also contains six additional regulatory genes namely *vif, vpu, vpr, tat, rev* and *nef* which have been shown to be important in the replication process. HIV-1 mutants that lack functional regulatory genes are less adaptive as compared with the wild type [31-34]. The gag polyprotein sequence encodes HIV core structural proteins which include matrix (MA), capsid (CA) and nucleocapsid (NC) proteins [35]. The *pol* gene encodes reverse transcriptase (RT), integrase, and protease (PR) enzymes. HIV genomic RNA can be primary seen as a coding of the above nine reading frames that encode for 15 proteins [35] , see figure 1.2
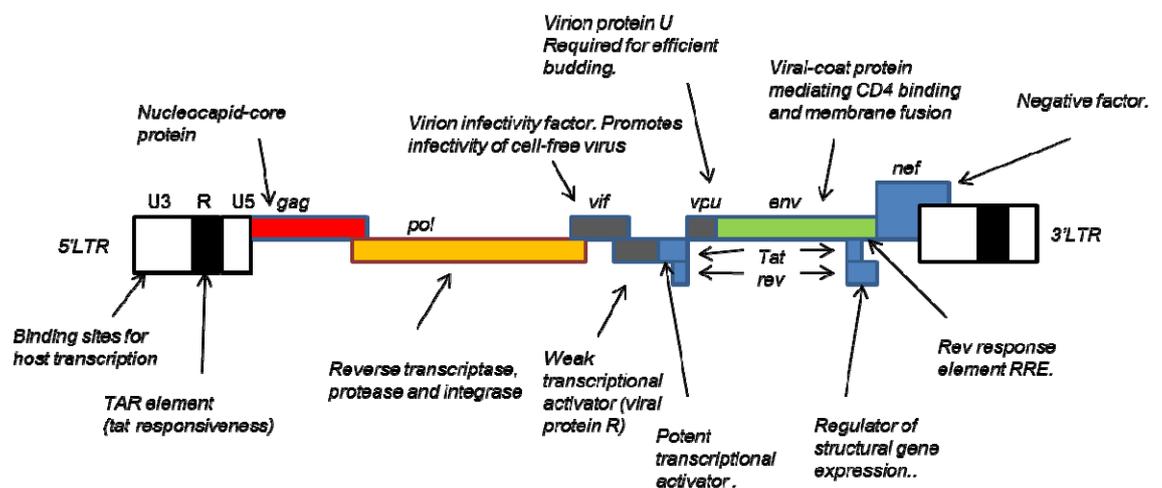
Figure 1.2 is an illustration of the HIV-1 genome showing the nine known genes and their summarized functions. Also depicted are the 5'and 3' long terminal repeats (LTRs) and the regulatory sequences recognized by various host transcription factors. Figure drawn based on online published structures [36, 37].

1.3 HIV-1 REPLICATION CYCLE

In vivo, the HIV-1 life cycle can be looked at from the time the virus enters the host cell to the time it produces new viral particles. The process has been divided into several stages: entry, reverse transcription, integration, and budding.

1.3.1 HIV-1 Entry

The viral envelope protein glycoprotein surface unit, SU, gp120 initiates the host cell (lymphocytes and monocytes) infection for HIV-1 through cognate recognition of the amino-terminal immunoglobulin domain of CD4 [38, 39]. HIV-1 has a strikingly high affinity for the cellular receptor (CD4) which is considered the sole high-affinity receptor for this retrovirus. The interaction of the gp120 and CD4+ receptor is however, only adequate for viral-cellular primary binding and not fusion. Viral fusion is triggered by one of the several chemokine receptors which include CXCR4 and CCR5 [40-42].   The CD4-gp120 interaction induces a conformational change that unfurls the hydrophobic N terminus of the second envelope protein, gp41, that drives the fusion of the viral particle with the cellular membrane via interaction of any of the above mentioned chemokines [40, 43].   On the basis of tropism, HIV can be categorized as either macrophage-tropic (M-tropic) or T-cell-tropic (T-tropic). Viruses (R5 viruses) that are M-tropic use CCR5 as co-receptors while X5 viruses (T-tropic) use CXCR4 as co-receptors. R5 viruses poorly infect CD4+ T-cell lines as compared to macrophages and primary T cells [44] while X4 viruses have been reported to effectively infect CD4+ T-cells as they highly express the CXCR4 co-receptor [45].

The CCR5 receptor mutation can protect the cells from HIV infection but overall cell benefits do not seem significant as other chemokine receptors are thought to replace their functions. R5 stains predominate during the early course of infection before both strains can be recovered [42]. Figure 1.3 below shows a summary of the HIV-1 life cycle in a CD4/CXCR4 or CCR5 positive T cell (see arrow 2).
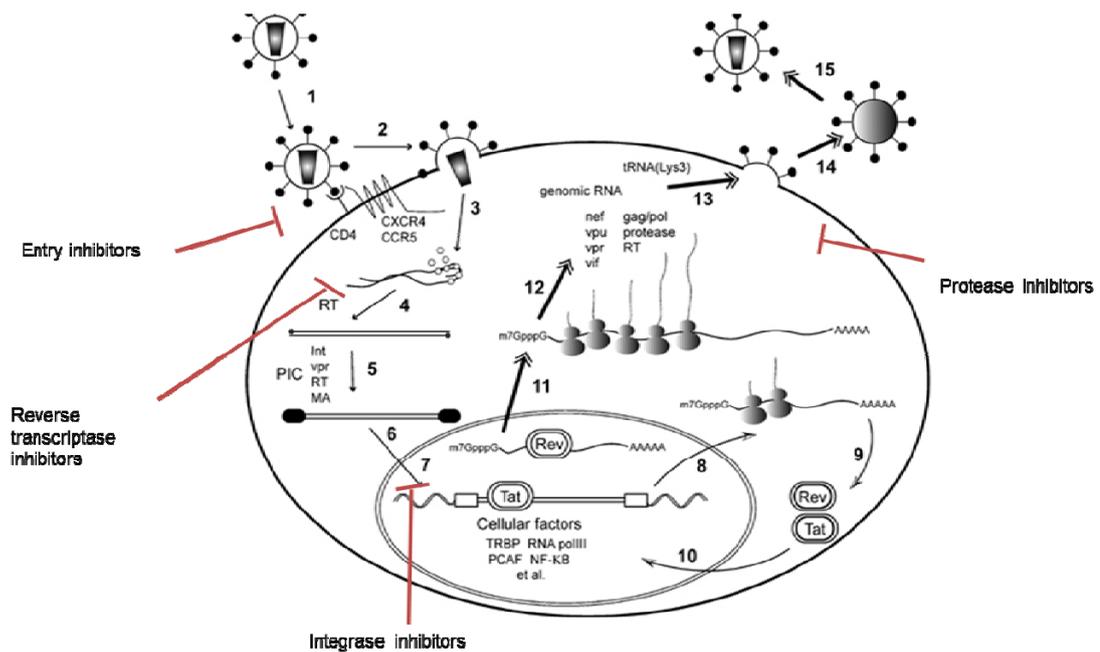
Figure 1.3 Small arrowheads (1-7), viral entry to integration. Curved arrows, early replication 8-10; double headed arrows (11-15), late replication. Graph adapted from Scherer et al [46]. Red arrows depict varied positions that drug inhibiting viral replication are targeted on.

1.3.2 Reverse Transcription

Once the HIV viral particle enters the cell, the viral capsid releases the viral reverse transcription complex. Reverse Transcriptase (RT), an enzyme that comes pre-packed in the mature HIV particle, catalyzes the reverse transcription of the viral single stranded RNA genome to form double stranded complimentary deoxyribonucleic acid, cDNA. Host cellular lysine transfer-RNA (tRNA) is used as a primer in this process

8

[47]. Vif, a 192 residue protein, has been shown to play an important role in viral replication as Vif mutant viruses have shown significantly reduced levels of viral complementary DNA (cDNA) synthesis and produce highly unstable replication intermediates [48, 49]. The translation process of the viral RNA genome is error prone [50-52] and increases four-folds in the absence of HIV-1 regulatory gene vpr [53, 54]. A number of Nucleotide Reverse Transcriptase (NRTIs) and Non-Nucleotide Reverse Transcriptase (NNRTIs), as was previously stated, have been developed to inhibit reverse transcriptase.

1.3.3 Integration

The end product after reverse transcription of the HIV-1 genome is cDNA pre-integration complex which is shuttled into the nucleus, a process vpr and vif are reported to participate [55, 56]. Integration cDNA into the host genome occurs randomly in reactions catalyzed by the viral integrase enzyme. The first integrase inhibitors were reported about 20 years ago [57-59] but currently only one drug, Raltegravir, is FDA approved [60].

1.3.4 Maturation and HIV-1 release

Cellular transcription factors activate a low level production of regulatory short multiply spliced genes, Rev, Tat and Nef, that can then amplify the viral transcription rate up to a 1000-folds [61, 62]. Rev, another gene which was mention earlier, has been reported to facilitate the exportation of unspliced viral mRNA into the cytoplasm [63-68]. Tat, Nef and Rev have been targeted in HIV-1 at the level of pre-mRNA splicing to significantly retard viral replication. This approach in combination with other antiviral strategies may be a useful tool in the fight against HIV/AIDS [69].

Pre-protein, pr55^{gag} translated from singly spliced gag mRNA and Gag-pol are synthesized as precursor non-infectious polyproteins and Gag cleaved into the mature proteins p17 matrix (MA),p24 capsid (CA), p7 nucleocapsid (NC), and p6 during viral maturation by the viral protease enzyme [70-72]. An intermediate-length mRNA pre-protein encodes glycoprotein gp160 that is cleaved by proteases to originate Vpr, Vpu, gp41 and gp120 [73]. Gag-pol pre-protein encodes viral enzymes protease (PR), integrase and RT. Inhibition of PR markedly suppresses the viral replication [74].

1.4 IMMUNE RESPONSE AND DISEASE PROGRESSION OF HIV-1 INFECTION

Following infection, HIV-1 has a tropism towards CD4$^+$ cells as has been mentioned before, and is disseminated throughout the lymphoid system and rapidly replicated within the infected cells. Within two weeks of infection, the viremia reaches peak levels, up to 10^7 copies/ml, before dropping to a set-point in the subsequent months. The set-point is predictive of how rapidly an individual will progress to developing AIDS [75]. The set-point varies by individual.

The clinical course of HIV infection can be divided into three stages: (a) primary infection (Acute HIV infection), (b) clinical latency (Chronic HIV infection), and (c) development of AIDS.

1.4.1 Acute HIV-1 Infection (Primary infection)

Acute infection is the period between infections of HIV-1 to the time the HIV-1 specific antibodies are detected 3 to 4 weeks later [76]. Following sexual transmission, the most common form of transmission, HIV-1 has been suggested to replicate locally in the genital tract or rectal mucosa cells that express CD4 and CCR5 receptors before

10

it can be detected in the plasma [77, 78]. This period is also referred to as the eclipse phase, and offers a small window of opportunity for the immune system to eliminate the virus before it archives into host genome [79]. Infection symptoms include: common cold or flu, fever, fatigue, headache, sore throat swollen lymph nodes, and, often, rash. The severity of symptoms differ for patients while some don't experience any [80].

Single genome amplification and sequencing has shown that up to 80% of mucosal infection occurs from a single virus, the funder virus, which has been shown to infect CD4+ T cells more efficiently than monocytes or macrophages [81]. The innate immune activation at this stage can contribute negatively by recruiting a number of immune cells such as lymphocytes, macrophages, and granulocytes. The lymphocytes become the target for more infections later, while the other two cell types engulf the virus and together with the infected lymphocytes disseminate the virus at other sites such as the lymph nodes [82].

During acute infection, most of the HIV-1 fails to produce productive viral particles due to the high error rate of the viral replication process as well as the intervention by the antiviral activities of the host's catalytic Apolipoprotein B mRNA-editing polypeptide-like cytidine deaminases-3G APOBEC3G, which increase the rate of defective viruses being produced. This allows for deaminases resistant HIV-1 mutation variants to be selected [83].

At the end of the eclipse phase, the virus is drained into the lymph nodes mainly by the dendritic cells where they meet more activated CD4+CCR5+ T cells which act as targets for further infection. Eventually the viruses are spread to other parts of the body

including lymphoid tissues such as the gut associated lymphoid tissue (GALT). These tissues house a great number of activated CD4+CCR5+ memory T cells which a study has shown that up to 20% can get infected during the acute phase and 60% are depleted through Fas-mediated apoptosis of both infected and uninfected cells within three weeks. Mucosal depletion of CD4+CCR5+ memory T cells are the most affected during this phase [84]. During this phase of infection the patient experiences an exponential burst of viremia to a peak of up to 10^7 copies/ml and a drop in CD4+ T cells count [84].

After reaching peak viral load, the host immune system eventually decreases the viral load over a period of 12-20 weeks to a stable level known as set point. This period correlates with the maintenance of robust T-helper $CD4^+$ T-cell and (cytotoxic T-lymphocytes (CTL) responses. Recent studies have shown that primary HIV-1 specific T cell response concurrent with failing viral load during the acute phase rapidly selects escape mutations. CD8+ T cells have been shown to play a significant role in the control of infection during this phase [85-89]. This is another way that the immune response exerts selective pressure on HIV that mutates and contributes to the pool of viral variants being generated by the erroneous HIV-1 reverse transcriptase [90].

1.4.2 Chronic HIV-1 Infection (Clinical latency phase)

The chronic phase of infection is a period of clinical latency during which circulating CD4+ T cells return to near normal levels and the patient is asymptomatic for an extended period of time. A number of studies have reported the period of a median time of disease progression to be about eight to ten years for typical progressors [91, 92].

During this period, viral replication persists and is associated with a progressive depletion of CD4+ T cells and deregulation of HIV specific CTL function [93, 94].

A striking feature of the chronic stage is the activation of innate cells: B cells and T cells. This is evident in the increased activation of biomarkers such as CD38, considered the most reliable surrogate marker for immune activation, disease progression to AIDS, and death, Ki67, and HLA-DR [95, 96]. Chronic immune activation and AIDS progression are rarely observed in naturally SIV infected sooty mangabeys- an indication of the role activation plays in disease progression. This occurs despite CD4+ cells depletion [95, 97]. Associated with immune activation during the chronic stage are B and T cell early and extensive apoptosis. This in turn results in an increase in tumor necrotic factor (TNF) – related apoptosis inducers such as immune suppressors and a killing of bystander cells. Such inducers include tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL), Fas ligand, TNF receptor type 2 (TNFR-2), and plasma micro-particles [98].

Although the exact mechanism of immune activation during early HIV-1 infection is not clearly understood, implications include; (a) damage of the gut due to CD4+ memory T cell depletion which leads to bacterial product translocation (for example Lipopolysaccharide, LPS) in to circulation and (b); viral components such as gp120, nef and viral nucleic acids which during replication activates pro-inflammatory cytokines and type I interferon both IFN-alpha and -beta [99].

The ultimate result of immune activation is a depletion of CD4+ T cells, a reduced half life of both CD4+ and CD8+ cells, exhaustion of clonal T- cells, dysregulation of T – cell trafficking, and drainage of memory T cell reservoirs [100].

13

CD4+ T cell depletion corresponds with elevation of viremia and thus serves as marker of patient prognosis (Fig 1.4).
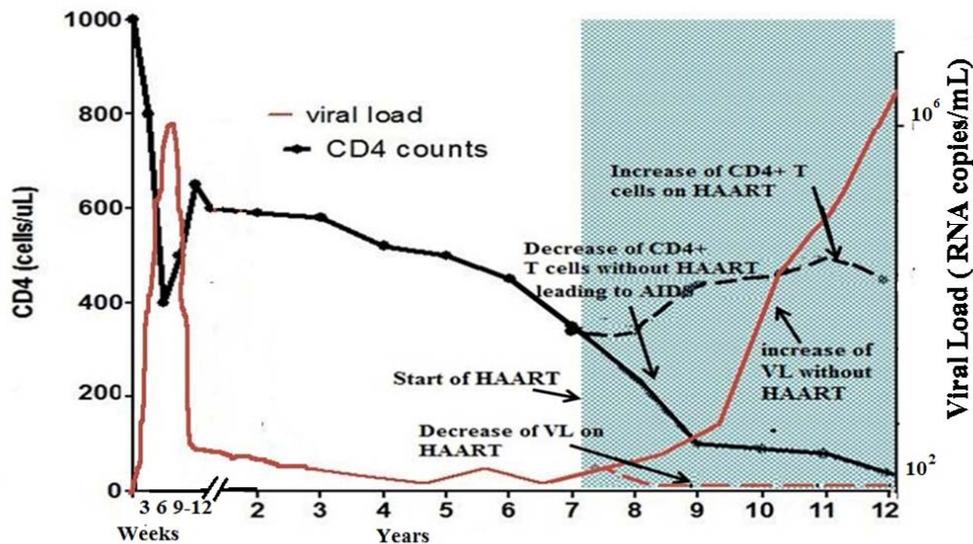
Figure 1.4 Typical course of HIV infection that depicts the relationship between the levels of HIV (viral load) and CD4+ T cell counts over the average course of untreated HIV infection shown in solid red (CD4+ T cell count) and black lines (plasma viral load). Shaded area indicates treatment period. The broken line within the shaded blue is indicating the typical trend upon HAART. Figure was drawn using Graphpad Prism5.

The introduction of HIV antiretroviral drugs over the past two decades has changed the perception of HIV infection being a death sentence. Most of these drugs however have been shown to quickly reduce in efficacy and patients develop drug resistance when used in suboptimal concentrations, which is common during mono therapy [101]. Drug resistance is a subtle change that occurs in the balance between enzymatic recognition

14

of drug inhibitors and the normal physiological substrates. Following drug pressure on the HIV-1 drug treatment, viruses with mutations are able to change the enzyme's conformation to reduce drugs from binding on the active sites that preferably get selected [102].

When several of these drugs are used in combination, the approach of treatment is known as Highly Active Antiretroviral Therapy (HAART). HAART with adherence of above 95% has been demonstrated to effectively suppress HIV-1 RNA plasma viral load to below detectable limits for a long period of time. It can enable immune reconstitution as well as avert disease progression [103], see Figure 1.4. Commonly used highly active antiretroviral therapy (HAART) typically comprises a three drug combination: two Nucleoside reverse transcriptase inhibitors (NRTIs) and one non-nucleotide reverse transcriptase inhibitor (NNRTI) or Protease inhibitor (PI)[104]. Each of the drug categories is meant to inhibit a particular process of the viral replication and if properly used with drug resistance testing, can improve virologic outcome [104].

Since drug resistance mutation arise most from the selective pressure mounted on the enzymes targeted by therapy, many of which compromise the function as was reviewed above, the mechanisms of each of these three drugs categories and Protease and Reverse Transcriptase (RT) enzymes will be reviewed below in more detail.

1.5   Genetic diversity of HIV-1

There is an enormous genetic diversity of HIV-1 both within and between infected people [105] due to the rapid replication rate of the virus, the size of the viral population within individuals, and extensive viral recombination [25, 106]. This diversity allows HIV-1 to survive and persist despite drug therapy during ART [107]. The core root of drug resistance arises from mutations occurring during viral ribonucleic acid (RNA) reverse transcription. This seems to be the cause for the extreme adaptability for the retrovirus. The reverse transcriptase (RT) do not have 3'-5' exonuclease proofreading activities making their error rates several fold higher when compared with the cellular DNA polymerase [52, 108]. Crystal structures for the reverse transcriptase and RNA polymerases have confirmed the lack of the domain responsible for 3' to 5' exonucleolytic proofreading activities. Such a domain is present in cellular enzymes, such as the DNA dependent DNA polymerase from *Escherichia coli* which is responsible for mismatch repair [108-110].

The exonuclease activities of DNA polymerase are essential for the removal of mismatched nucleotides during replication. DNA Polymerase has a proofreading mechanism and has been reported to enhance substitution accuracy in eukaryotic replication up to 10^{-7} to 10^{-8}[111]. For example, the human genome has about six billion nucleotides and is replicated within a few hours with an error rate of less than one mutation per genome in one cell cycle. The proofreading activity of the polymerase is reported to play a significant role in this high fidelity [112].

16

In addition to lacking a proofreading mechanism, the RT also has an intrinsic ability to jump between templates during DNA synthesis [113, 114]. The result is an extremely high rate for recombination between the cellular and the viral RNA, between co-packaged genomes and between various regions of the viral genome [115].

Three main ways mutations can occur during HIV-1 retroviral replication include:

(i) when the provirus DNA integrated with the infected host cell is being replicated by the DNA dependant DNA polymerase during cell replication [113-115]

(ii) during viral transcription from the provirus by the DNA dependant RNA transcriptase [113-115] and

(iii) during conversion of single stranded HIV-1 viral RNA into double stranded DNA by the reverse transcriptase [113-115].

Studies have estimated mis-insertion errors during replication and retro-transcription of the HIV genome (approximate 10 kb) to be in the range of 10^{-3} and 10^{-5} per nucleotide site in one replication cycle. This translates to each progeny RNA or DNA molecule including an average of 0.1-10 mutations [50-52]. In other words, a newly infected cell can thus be said to contain a provirus that differs from the previous infected cell by about one mutation. A potential thus exists for every possible point-mutation to be generated thousands of times a day given that about a billion cells are infected each day during a chronic infection [116].

 As has already been alluded to above, such a high mutation rate is mainly attributed to the low efficiency of proofreading repair activities associated with RNA replicases and transcriptases [108].

The adaptations mentioned above summarize to form what has been referred to as a quasispecies complex. A theoretical quasispecies complex was originally defined by Eigen and colleagues as dynamic distributions of non-identical but related replicons that can result in a steady-state of organized distribution of error copies to the master sequence [117]. The mutant complexity increases as the fidelity of replication decreases, consequently increasing the viral fitness [117, 118]. However, this theoretical concept differs with replication occurring in the real and ever changing viral environment in that a steady state of equilibrium distribution in viral populations infecting host organism and cell culture is difficult to attain [118]. During HIV antiviral therapy, many studies have shown drug pressure to result in a selection favoring the genotypes, in association with mutation, that seem to have the highest replication rates [106, 117, 118]. Quasispecies complexes therefore allow a broad spectrum of mutants to be generated and constantly compete with one another. If the environment remains constant, a 'steady-state' is formed where each mutant is represented according to its fitness [106].

Within the quasispecies exists mutants that would have evolved to enhance their physiological function to easily escape drug pressure. One such function is the mutations that enable the RT to have structural changes which results in a high affinity of physiological dNTP as opposed to analogue drugs [119-121].

18

1.6   STRUCTURAL FUNCTION OF HIV-REVERSE TRANSCRIPTION

Reverse transcriptase is a hetro-dimer enzyme consisting of a 560 amino acid subunit (p66) and a 440 amino acid subunit (p51) both derived from the *pol* polyprotein [35].

The first reverse transcriptase crystal structure (3.5A), in relation with function, was determined by Kohlstaedt et al. [122] and has set the basis of understanding how any change in this conformation might result in a functional modification of the enzyme. In this structure, the N terminal 440 amino acid of p66 forms the polymerase part of the enzyme while the C-terminal 120 amino acids form the Rnase sub-domain. The Rnase and the polymerase are connected by sub-domain named connecting domain [122]. The polymerase units themselves are comprised of three sub-domains whose conformation anatomically resembles the right hand leading to the naming, palm, fingers and thumb [122]. The finger, palm, and thumb form a cleft that resembles that of Klenow fragment E. coli DNA polymerase 1 and comprises the DNA polymerase catalytic sites which harbors a triad of aspartate asp110 (D110) Asp185 (D185) and Asp186 (D186) residues. D185 and D186 are part of highly conserved tyrosine-methionine-aspartate-aspartate (YMDD) motifs, common with Klenow fragment E. coli polymerase with which any mutations and any of the aspartate in those motifs severely reduces pol activity [123]. Together the palm, finger and thumb act in synchrony as a clamp that positions template primers relative to the polymerase site [124].

19

The p51 domain of the HIV-1 RT lacks the entire 120 amino acid Rnase sub-domain. Despite its sequence also being identical with the N terminal 440 amino acid of the p66, the conformation is different and the p51 has no polymerase activity. The conformation in this structure is in such a way that it lacks the cleft (pocket): the D110, D185 and D186 thought to be important for catalysis are buried in the structure, and the fingers fold toward the palm and the thumb away from the fingers. This makes the p66/p51 heterodimer have only pol activity within the p66 [122].

Studies of RT polymerase ternary complex with dNTP in the nucleotide pocket suggest that the binding of a correct nucleotide (substrate) forms a closed structure that allows residues of the fingers in the sub-domain to become part of a complete functional binding pocket essential for nucleic acid polymerization [125]. Mutations of a number of residues within the palm and fingers of the RT are thus not surprisingly associated with most of the drug resistance of nucleoside-analogs that occur during ART as they alter normal conformation that defines the catalytic site [125].

The role that specific amino acids have in the specific positions of the HIV-1 RT has been studied by many scientists across the globe both *in vitro* and *in vivo*. *In vitro* tests are done usually by passage experiment in which HIV-1 viruses are cultured in increasing drug concentration which selects the resistant strains. HIV-1 constructs that contain known mutations can be passaged in such a test enabling the researcher to correlate mutation occurrence with enzyme function. HIV-1 mutations can also be characterized by studying viruses from patients failing therapy. This, as opposed to *in vitro* studies, offers a much broader information that can be used to correlate mutations

with patient drug history; correlate mutations with virologic response to new drug regimens or correlate HIV-1 mutations and drug susceptibility [126].

Examples of an outcome from such studies include RT in complex with DNA double strand studies that have reviewed that the amino acid residues such as Methionine 184 (M184), glutamic acid 89 (E89), glutamine 151 (Q151), mapped in the palm play an essential role during replication. For example, the M184 residue is suggested important for the interaction with the primer 3' hydroxyl terminus and the incoming dNTP. The E89 residue provides the template DNA with a grip while the Q151 makes contact with the nucleotide [127].

Others such as Lysine residues 65 (K65) and leucine 74 (L74), are found in the distal region of the dNTP binding pockets within the polymerase fingers but are significant in making contact with the $\gamma-$phosphate of the incoming nucleotide as well as positioning the templating base respectively [125].

Evidence of other amino acid residues such as K219, T215, L210, K70, D67, and M41, found in the palm and finger domain, has shown mutations of such residues alter the conformational structure of the RT that result in reduced sensitivity to some drug e.g. AZT and 3TC [128, 129].

Figure 1.5 shows the structure of RT. In A, the enzyme is shown in complex with DNA while in B; emphasis is on the two drug binding sites, NNIBP and dNTP in relationship with surrounding amino acid residues that are the frequent point for mutations.

A
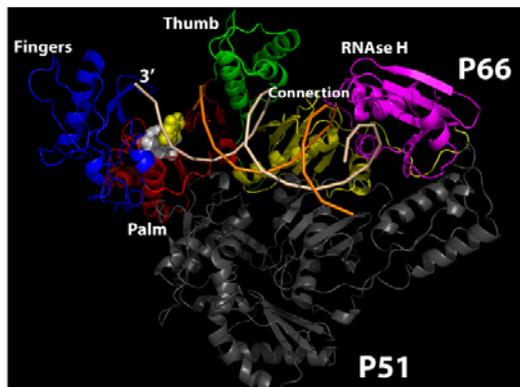
B
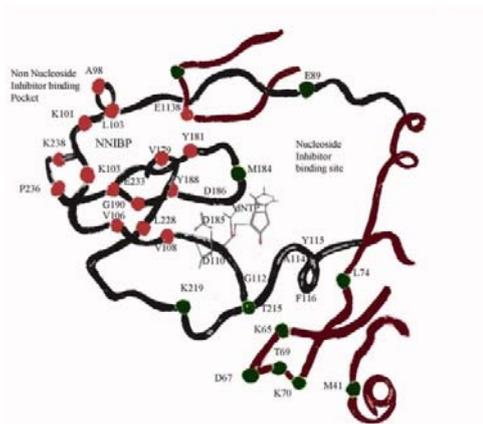
Figure 1.5.The structure of the HIV-1 RT catalytic complex. (A) Some of the RT catalytic complex depicting the RNase H domain on the right and polymerase active site on the left. For the domains of p66 all in color: fingers (blue), palm (red), thumb (green), connection (yellow), and RNase H (pink); p51 is shown in gray. The template DNA/primer complex is in white and orange threads; the NNRTI is in yellow and white spheres, figure adapted from Rhee, et al. (2003) [130]; and (B) Location of some specific NNRTI-resistance mutations (red) and NRTIs (green). NNIBP (Non-nucleoside inhibitor binding pocket.) and dNTP binding site docked with dNTP showing the location targeted by nucleoside analogues. Figure was drawn based on figure adopted from Domaoal, et al. (2004), [131].

22

1.6.1 Medical implications

Considering the role that the RT enzyme has in the viral replication cycle, a number of drugs, Reverse Transcriptase Inhibitors (RTIs), have been developed that aim to suppress its function. These drugs fall into two categories: those that mimic deoxyribonucleotide (dNTP) but terminate the elongation of the growing chain during replication called Nucleotide Reverse Transcriptase inhibitors (NRTIs), and those that inhibit the RT enzyme function in other ways than mimicking physiological dNTPs called Non-Nucleotide/Nucleoside Reverse transcriptase Inhibitors (NNRTIs).

1.6.2 Nucleoside or Nucleotide Reverse transcriptase inhibitors (NRTIs)

All drugs that have been approved by the FDA that inhibit the reverse transcriptase fall in two categories mentioned above, NRTIs or NNRTIs. The general principle of the first category, NRTIs, is that they provide the HIV-1 reverse transcriptase with nucleoside or nucleotide analogues that mimic the cellular nucleosides needed in the early stages or replication during reverse transcription. During normal HIV-1 replication, the infected host cells provide the virus with physiological nucleosides that are activated to nucleotides essential in the transcription of template viral RNA by the HIV-1 RNA dependent DNA polymerase. Nucleoside analogues are competitively incorporated in the growing HIV-1 DNA chain, which ultimately inhibit the transcription process of that DNA chain [132, 133]. Most NRTI drugs like dideoxynucleoside (ddNTPs) lack a 3' hydroxyl group on the ribose sugar allowing them to act as chain terminators that block DNA synthesis which results into an abortive DNA replication and thus a disruption of the viral life cycle [132]. Currently, the Food and Drug Administration(FDA) approved drugs are Zidovudine (1-(3-Azido-2,3-dideoxy-β-D-ribofuranosyl)-5-methylpyrimidine-2,4-1H,3H)-dione   commonly

called AZT) Lamivudine, 4-amino-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one commonly called 3TC), Didanosine (9-[(2R,5S)-5-(hydroxymethyl)oxolan-2-yl]-6,9-dihydro-3H-purin-6-one, commonly called ddI), Zalcitabine (4-amino-1-[(2R,5S)-5-(hydroxymethyl)oxolan-2-yl]-1,2-dihydropyrimidin-2-one commonly called ddC), Stavudine (1-[(2R,5S)-5-(hydroxymethyl)-2,5-dihydrofuran-2-yl]-5-methyl-1,2,3,4-tetrahydropyrimidine-2,4-dione commonly called d4T), Abacavir ([[(1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-yl]cyclopent-2-en-1-yl]methanol commonly called ABC)), and Emtricitabine (4-amino-5-fluoro-1-[(2R,5S)-2-(hydroxymethyl)-1,3-oxathiolan-5-yl]-1,2-dihydropyrimidin-2-one commonly called FTC) see table 1 [134].
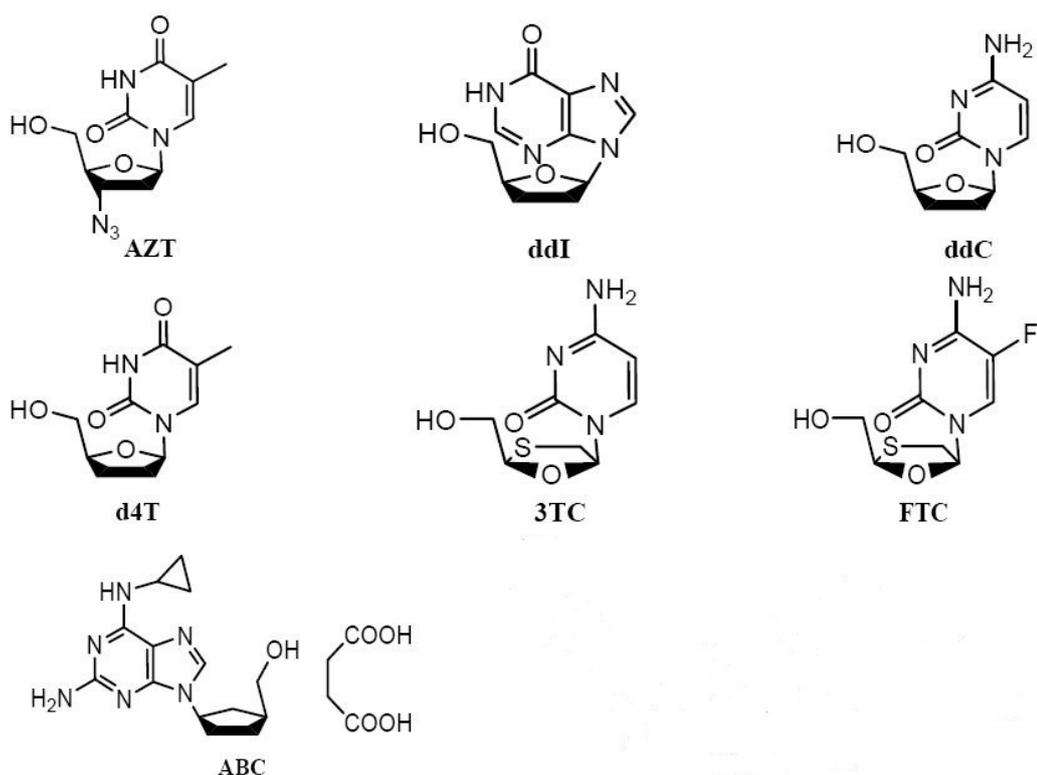
Figure 1.6 Structural formulas for some of the FDA approved NRTIs all designed to mimic physiological dNTPs.

1.6.3 Non-Nucleoside/Nucleotide Reverse Transcriptase Inhibitors (NNRTIs)

Unlike the NRTIs, NNRTIs are not incorporated into the growing HIV-1 DNA strand but instead inhibit the HIV-1 reverse transcriptase (RT) by binding the hydrophobic site pocket located near the polymerase catalytic site in the p66 subunit of the RT. Drug incorporation results in conformation changes of the RT that confers a significant reduction or complete halting of the polymerase activity [122, 135]. The limitation with this class of drugs is that slight variations in binding site (e.g. point mutation) can result in a significant impact of the drug's sensitivity on the virus, giving these drugs a low genetic barrier. They also exhibit a high level of cross resistance due to the narrowness of the binding pocket. Naturally, high drug resistance can easily and quickly develop for this category of drugs [136]. The current FDA approved NNRTIs are, Delavirdine (N-[2-({4-[3-ylamino)pyridin-2-yl]piperazin-1}carbonyl)1*H*-indol-

5yl]methanesulfornamide, commonly called DLV) Efavirenz ((4*S*)-6-chloro-4-(2-cycloro-4-(2-cyclopropylethynyl)-4-(trifluoromethynyl)-4-(trifluromethyl)-2,4-dihydro-1*H*-3,1-benzoxazin-2-one, commonly called EFV), Etravirine (4-[6-Amino-5-bromo-2-[(4-cyanophenyl)amino]pyrinmidin-4yl] oxy- 3,5-dimethylbenzonitrile, commonly called TMC125 and Nevirapine (11(-cyclopropyl-4methyl-5,11dihydro-6*H*-dipyrodo[3,2-b:2',3'-3e][1,4]diazepin-6-one, commonly called NVP) all of which target the hydrophobic pocket of the HIV-1 reverse transcriptase.

25

Table 1.1 Summary of FDA approved antiretroviral drugs arranged by classification [137]

FDA Approved drug for HIV-1 treatment	Year of FDA approval
Nucleoside reverse transcriptase inhibitors (NRTIs) Year approved	
Abacavir (ABC)	1998
Didanosine (ddI)	2000
Lamivudine (3TC)	1995
Stavudine (d4T)	1994
Zalcitabine (ddC)	1987
Zidovudine (AZT, ZDV)	1987
Emtricitabine (FTC)	2003
Tenofovir (TDF)	2001
Nonnucleoside reverse transcriptase inhibitors (NNRTIs)	
Delavirdine (DLV)	1997
Efavirenz (EFV)	1998
Nevirapine (NVP)	1996
Etravirine (ETR)	2008
Rilpivirine	2008
Protease inhibitors (PIs)	
Amprenavir (APV)	1999
Indinavir (IDV)	1996
Lopinavir (LPV)	2000
Nelfinavir (NFV)	1997
Ritonavir (RTV)	1996
Saquinavir (SQV)	1995
Atazanavir(ATV)	2003
Darunavir (DRV)	2006
Tipranavir (TPV)	2005
Integrase inhibitors	
Raltegravir (RAL)	2007
Fusion entry inhibitors	
Enfuvirtide (T-20)	2003
Maraviroc (MVC)	2007

1.6.4 Reverse Transcriptase Inhibitor (RTI) Drug resistance.

The polymerase activity of the RT enzyme is in the p66 cleft of the p66/51 hetero-dimer during transcription, the fingers and the thumb all work in unison to form a closed functional structure. Mutation in a number of the amino acid residues, most frequently in the palm and fingers, may result in a conformational change of the polymerase active site and consequently enzymes that can function better under drug pressure [123, 124]. Two types of drug resistance mutations have been categorized, 1) those that confer mutations that select for normal dNTP substrate incorporation against nucleotide analogs and, 2) those that confer mutations that enable the reverse transcriptase to excise nucleotide analogs.

Examples of the first category include M184V, M184I (confer 3TC resistance), and E89G which have been reported to increase dNTP insertion fidelity as opposed to the nucleotide analogs [119-121]. Others including Q151M mutation have also been linked to multi–dideoxynucleoside resistance, [138] and L74V ddI that confers to ddI resistance [139]. M184 and Q151 are located within the 3' hydroxyl terminus vicinity of incoming dNTPs and within the contact distance respectively. Mutation in these positions can alter the DNA conformation which in turn changes the dNTP geometry pocket and enables the RT to have increased fidelity for physiological dNTP as opposed to NRTI analogues [140]. This principle, confirmed in other studies, has shown mutations such as E89G to result in an increased nucleoside insertion fidelity by 2-23 folds and 2-6 for the L74V associated mutation [139]. These findings considered together show that such mutations are able to alter the active site of the

27

reverse transcriptase in such a way that it discriminates between the normal dNTPs and the nucleoside analogs.

Zidovudine (AZT) is probably the most well studied nucleoside analog in the second category above. Cellular enzymes convert AZT into AZT triphosphate (AZTTP), an analog product of deoxy-thymidine triphosphate (dTTP). During DNA replication, the reverse transcription gets aborted when AZTTP instead of dTTP is incorporated into the growing chain. AZT mutants however are able to discriminate against AZTTP by reversal polymerization of the terminal AZTMP to pyrophosphate (PPi) [141, 142]. Polymerase residues that are affected during AZT resistance include positions L210W, T215Y/F and K219N of the palm and M41L, D67N and K70R of the fingers. All of these mutations are located further from the pol active site and are thought to exert their effect via alteration of the enzyme from a long range of conformational changes that reposition the active site aspartate residues [128, 143].

1.7   STRUCTURAL FUNCTION OF HIV-PROTEASE

HIV-1 protease is a homo-dimeric enzyme that consists of two identical ninety-nine amino acid monomers derived from protease cleavage of gag-pol polyproteins. The interaction of the two 99 amino acids form a tunnel in the interior that consists of two conserved sequence Asp-Thr-Gly that make up the enzyme active site. Primarily, the active site is formed by amino acid residues 25-32, 47-53 and 80-84. HIV-1 Proteases facilitate the viral particle's maturation by cleavage of gag and gag-pol polyprotein at nine specific sites [144] to yield functional enzymes and structural proteins necessary for the viral life cycle [145].   These enzymes, including integrase and reverse transcriptase, were discussed earlier [146-148].

In order for the gag-pol protein segment to access the protease active site for proteolytic cleavage, the protease flaps (two flexible domains thought to function as flaps) must open and undergo a dramatic conformational change to bind and close over the substrate sequence for cleavage [149]. Two Asp's (D25) , one from each monomer, have been identified to be responsible for hydrolytically cleaving polyproteins that bind in the tunnel, through activation of a water molecule [149]. HIV proteases like plant Para retroviruses have aspartate proteinases similar to animal proteases such as pepsin, gastrin and renin and thus have been identified as aspartyl proteases [150]. See Figure 1.7 for an illustration of HIV-1 protease structure.
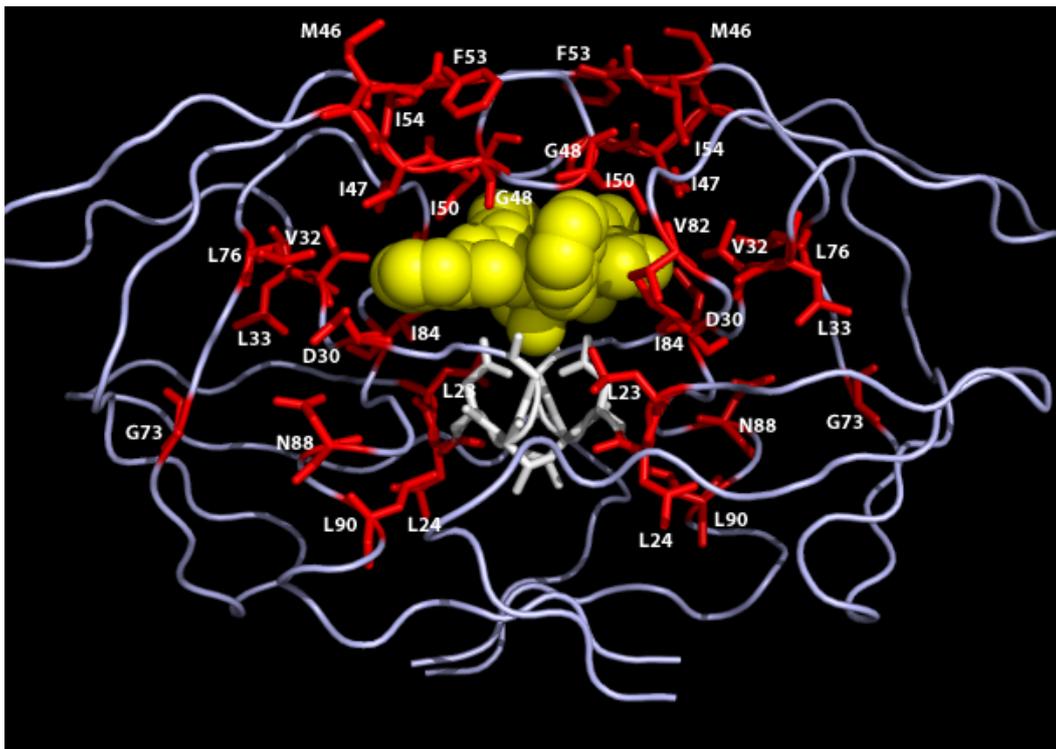
Figure 1.7 above shows an inhibitor bound wild type HIV-1, three dimensional structure of   protease with lopinavir. The white sticks show the active sites of the enzymes that comprise a conserved triad Asp-Thr-Gly at positions 25 to 27. The flexible flaps of the enzyme that close down on the active site once the gag and gag-pol polyprotein is bound is located in residues 46-56 [151]. Figure adopted from Rhee, et al. [130].

Studies have shown that more than half of the residues in the protease can mutate in different combinations that could either reduce or improve the HIV-1 fitness against protease inhibitors [9, 152]. Protease mutations that occur within the substrate's active sites have been termed "primary mutation" and their occurrence can directly reduce drug susceptibility. On the other hand, the terms "secondary mutation" contribute to drug resistance by improving the HIV-1 replication fitness with a primary mutation [153].

30

In one cohort that had 475 treatment naïve patients, researchers reported up to 69% conservation in five functionally important regions of protease which includes; the N terminal site (P1-P9), the C-terminal end (G94-F99), the catalytic site (E21-V32), the protease flap (P44-V56), and the fifth contained in region G78-N88 [154]. In the same study, the researcher identified amino acids, L89, N88, I84, V82, T74, G73, H69, C67, Q61, D60, K55, I54, G48, M46, K43, M36, E35, D30, K20, and L10 as common polymorphism and drug treatment associated mutations in 639 drug treatment patients. Most of these mutations are located in peripheral areas of the enzyme preserving the active site region [154].

Under PI drug pressure, most of the mutations initially selected are within the active site within the substrate cleft: R8, L23, D25-37, D29, D30, V32, I47, G48, I50, V82, and I84. In figure 1.7, this area is immediately around the pale yellow substrate. Mutations in this region have been termed primary mutations. Typical primary mutations include D30N, I50L/V, V82F/A/T, I84V and L90M [152]. Upon binding to the substrate or PI, the protease residue has been reported to rearrange residues either locally, such as in the flap regions or P1 loop, or in the entire protease to form a complete functional enzyme [155]. As such, a number of mutations outside the primary active sites have also been associated with the development of drug resistance during ART, most of which are in the sites mentioned before [154].

1.7.1 Medical Implications

Considering the important role that the protease enzyme has in the maturity of the HIV-1 virus, a number of FDA approved drugs have been developed that aim to halt its functions.   Most of these drugs mimic the tetrahedral structures for the viral polyprotein intermediates of the hydrolytic reactions.   A drug interaction at the catalytic protease site   results in an inhibition of gag-pol polyprotein access to the active site and consequently viral maturity [156].

The main drawback, as was reviewed earlier, is that HIV-1, like other retroviruses, replicates and mutates extremely rapidly, resulting in mutants that deny protease inhibitors access to the active site. As was mentioned above, researchers have identified some significant amino acids in the wild genome and drug naïve patients, as common polymorphism sites during drug selective pressure. Accumulation in a few numbers of such mutations on the functional parts of the enzyme could easily lead to drug failure [154].

At the time we conducted this study, Lopinavir/Ritonavir (Kaletra) was the most commonly used drug during second-line HAART in combination with two NRTIs in Zambia. This was in line with the recommendation by the Zambian Ministry of Health (MoH) guideline of 2007 for use of Lopinavir/Ritonavir, boosted Fosamprenavir, or boosted Atazanavir as anchor drugs in second-line HAART [157].   Lopinavir chemically designated (2S)-N-[(2S,4S,5S)-5-[2-(2,6-dimethylphenoxy)acetamido]-4-hydroxy-1,6-diphenylhexan-2-yl]-3-methyl-2-(2-oxo-1,3-diazinan-1-yl)butanamide and molecular formula $C_{36}H_{48}N_4O_5$ see figure 1.8, is about 3 to 4 times more active than

Ritonavir but has poor bioavailability. Small doses of Ritonavir 1,3-thiazol-5-ylmethyl N-[(2S,3S,5S)-3-hydroxy-5-[(2S)-3-methyl-2-{[methyl({[2-(propan-2-yl)-1,3-thiazol-4-yl]methyl})carbamoyl]amino}butanamido]-1,6-diphenylhexan-2-yl]carbamate ($C_{36}H_{48}N_6O_5S_2$), drastically improves its bioavailability and hence the preferred combination as Kaletra [158]. A number of studies have shown this combination to be effective treatment both in treatment naïve and treatment experienced patients. For example, in a randomized open labeled study that compared Kaletra mono-therapy with Kaletra along with AZT and 3TC as initial treatment in treatment-naïve patients with a HIV-RNA < 100, 000 copies/ml, triple therapy was reported to be superior $p < 0.02$ at 48 weeks, with patients achieving virologic suppression [159]. A similar finding supporting superiority of triple therapy of Kaletra and two NRTIs compared to Kaletra mono-therapy was reported by Pulido et al 2008 [160].

In another study, Kaletra showed greater potency when compared with other PIs, either in treatment naïve or during salvage therapy for patients that had significant exposure to all other three classes of ART with virologic failure. About 76% of patients in this study reached viral suppression within 6 months, which is defined as a plasma viral load less than 500 copies/ml [161]. See figure 1.18 (a) (b) for the structural formulas of Ritonavir and Lopinavir respectively.
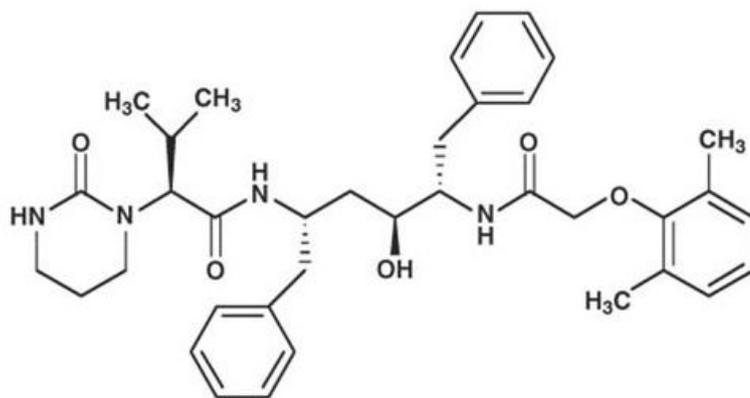
(a)
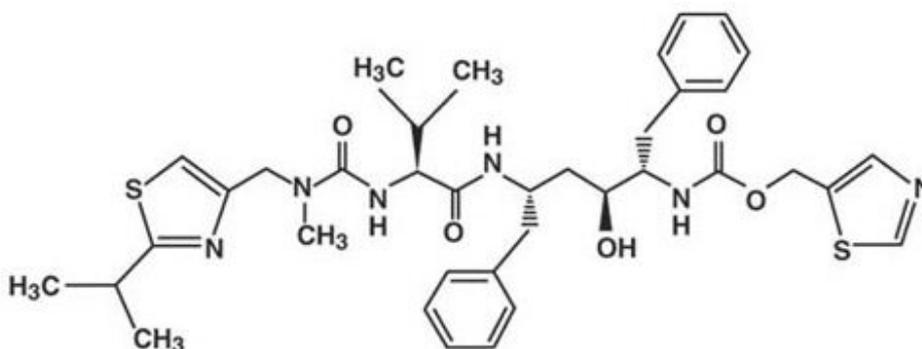
(b)

Figure 1.8 (a) Lopinavir, (b) Ritonavir, structural formula [162].

1.7.2 Drug resistance

The Lopinavir/Ritonavir combination is characterized by a high genetic barrier to drug resistance and a better tolerance to poor adherence compared with earlier unbooted PIs [163]. A number of studies have reported Lopinavir/Ritonavir's resilience against resistance during treatment. These include, one by Murphy et al, [164], in which they reported no clinical resistance in ART naïve patients treated with the

Lopinavir/Ritonavir based regimen for seven years. Saez-Llorens et al, 2003, reported similar results in pediatrics following treatment for 48 weeks [165].

Studies of Lopinavir resistance patterns conducted by Kempf et al. 2001 [166] , in patients failing treatment with other PIs, identified 11 amino acid position associated with reduced susceptibility. The positions included L10F/I/R/V, K20M/R, L24I, M46I/L, F53L, I54L/T/V, L63P, A71I/L/T/V, V82A/F/T, I84V, and L90M. Accumulation of more than 6 mutations showed a reduced virologic response, for example, mutations at positions 10, 54, 63, 71,82 and 84 were associated with relatively modest resistance (4 -10 fold) while a 20-40 fold phenotypic change was observed with mutation K20M/R and F53L in association with any other one of the 11 mutations identified earlier. Mutations at position 10, 54, 63, and 82 and/or 84, together with an average of three mutations at residues 20, 24, 46, 53, 71, and 90 showed a 20 fold reduced susceptibility in the same study [166] and confirmed in another study [161]. Specific mutations, particularly I47A/V and V32I, have recently been associated with high levels of drug resistance to Kaletra [167]. The IAS-USA keeps updated lists of major and minor mutation associated with drug resistance. Mutations associated with Kaletra resistance are shown in table 1.2 b.

In the current study, we will determine the PI and RT mutation patterns in the Zambian population at the point of drug virologic failure which is defined as viral load greater than > 1000 copies during HAART in which Kaletra was used and compare them with PI naïve patients.

1.8   HIGHLY ACTIVE ANTIRETROVIRAL THERAPY (HAART) RELATED RESISTANCE

1.8.1 HAART treatment regimen

According to the Zambian MoH antiretroviral therapy guide-line,[157] the following are the drug regimens for ART naïve adolescents: AZT + 3TC + EFV; AZT + 3TC + NVP; TDF + 3TC or FTC + EFV and TDF + 3TC or FTC + NVP for first-line therapy. For second-line therapy, if d4T or AZT had been used in first-line, TDF + 3TC or FTC boosted protease inhibitor (LPV/r and ATV/r) is used as the NRTI backbone in second-line and if TDF was used in first–line, d4T or TDF + 3TC or FTC boosted protease inhibitor (LPV/r and ATV/r) is used as NRTI backbone in second-line. Each of the drug categories is meant to inhibit a particular process of the viral replication and if properly used with drug resistance testing, can improve virologic outcome [104].

1.8.2 Drug failure associated mutation patterns during HAART.

Mutations that are able to resist drug pressure inhibition are selected, and with time become dominant in the quasispecies population and continue crippling the host immune system by mainly deleting CD4+ T cells [84, 168]. The evolution of mutant viral population acquiring sufficient numbers of drug resistance mutations adequate to overcome antiretroviral activity has been referred to as "genetic drug barrier [169, 170]. Drugs that require multi mutations to occur on their target proteins before losing susceptibility are referred to as having a "high genetic barrier". On the contrary, for some drugs, a single point mutation or two might be enough to alter susceptibility of HIV-1 ART. The latter is referred to as "low genetic barrier" [169].

All NNRTIs generally have a low genetic barrier, for example, selection for the RT mutation K103N results in NVP and EFV loss of susceptibility while RT mutation Y181C/I causes loss of susceptibility of all the currently available NNRTIs. NRTIs and PI drug combination however generally have a higher genetic barrier requiring multiple accumulation of mutation to render drug activity ineffective [15].

1.8.3 Genetic barrier in association with drug combination

The likelihood of developing drug resistance also varies according to the drug combination used during therapy. Therefore a genetic barrier can also be defined in relationship with the drug combination rather than a single drug used during treatment.

1.8.3.1 Low genetic barrier drug combinations with high virologic failure.

There are number of drug combinations in this category. For example, Farthing and colleagues [171, 172] were able to associate the TDF/3TC/ABC drug combination with early drug failure in an HIV-infected naïve patient pilot study. The mutation selected in this regimen mainly was M184V/I with 58% also containing nucleoside cross-resistant K65R viral isolates and no patient had K65R alone. The causes for earlier drug failure have been explored by other researchers as possibly being a result of: (1) possible negative drug-drug interactions between ABC and TDF, (2) a low genetic barrier to resistance posed by the regimen and (3) inadequate intracellular pharmacokinetic properties of 3TC and/or ABC when dosed once daily [173].

Other drug regimens with a low genetic barrier and high virologic failure include TDF/3TC/ddI, and TDF/ddI/EFV when used as initial treatment in HIV-1 antiretroviral naïve patients. The mutation selection for TDF/3TC/ddI combination seems to be similar with that described above for TDF/3TC/ABC with virologic failure observed by

12 weeks in HIV-1 antiretroviral naïve patients [172]. Single drug agent use of 3TC has been reported to select the M184V mutation of the RT that confers with high resistance capable of out populating the wild HIV strains in a few weeks [174] and almost always the earliest mutation to emerge when it is used in HAART [175, 176].

On the contrary, TDF/ddI/EFV treated HIV-1 antiretroviral naïve patients show a somewhat different mutation pattern than those described in the drug combinations above. A study by Leon and collegues [177] accessing the use TDF/ddI plus EFV or nevirapine (NVP) detected K65R, L74V, L100I, K103N/R/T, Y181C and G190E/Q/S mutations at six months, arguing against the use of TDF and ddl when the third drug is an NNRTI.

1.8.3.2 Low genetic barrier drug combinations with high virologic success

While a number of studies have reported on how some multi-drug combinations with low genetic barrier to resistance have a high drug failure [171, 172, 177], other studies have demonstrated higher rates of virologic success for the similar category of drugs. Some examples include drug combinations TDF + 3TC + EFV, ZDV + 3TC + EFV, and ABC + 3TC + EFV.

In one particular study, Pozniak and colleagues [178], in a randomized, open-label, non-inferiority trial, reported a 62% viral suppression, that is, <400 copies/ml in the antiretroviral-naive patient group that received ZDV/3TC + EFV after 96 weeks of follow-up. ZDV has a wider genetic barrier and the use of thymine analogues with 3TC or FTC has been shown to delay emergency of the TAMs in the presence of M184V, 3TC and FTC associated mutations. Similary D4T+3TC and NVP or EFV have been shown to be effective during first-line therapy in treatment naïve patients with no

statistical difference between the groups with M184V being the most prevalent mutation selecting for the NRTIs [179, 180]. Most of first-line ART regimens used in Zambia are from this category of drug combination.

1.8.3.3 HAART Regimens with High Genetic Barriers to Resistance

Most PIs meet criteria of this group of drugs. Most of them have poor bioavailability and are used in combination with Ritonavir as was reviewed earlier to boost their efficiency.

In HAART, the most used PIs in Zambia is boosted LPV (LPV/r) combined with two NRTIs. An example of such combination is d4T + 3TC + LPV/r. The combination was evaluated in treatment naive HIV-1 positive patients for a period of 24 to 108 weeks. This study showed no PI mutation upon treatment failure [181]. Another similar study was the SOLO study in which the researchers reported no PI mutation at 48 weeks after patient treatment with ABC + 3TC + FPV/r [182]. The tables 1.2 (a) (b) and (c) below summaries all currently known FDA drugs and associated resistance mutations.

Table 1.2 (a) Summary of current HIV-1 mutations associated with drug resistance to FDA approved NRTI and NNRTI antiretroviral drugs.

NRTI

Multi-nRTI Resistance : 69 insertion complex (all affect nRTIs approved by USA FDA)

M41L	**A62V**		**69-inset**	**K70R**		**L210W**	**T215YF**	**K219QE**

Multi-nRTI Resistance : 151 insertion complex (all affect nRTIs approved by USA FDA except for Tenofovir)

	A62V			**V75I**	**F77L**	Y116F	Q151M	

Muilt-nRTI Resistance: Thymidine Analogue-associated Mutations (TAM; affect all nRTIs currently approved by the US FDA)

M41L		**D67N**	**K70R**			**L210W**	**T215YF**	**K219QE**

Abacavir	**K65R**		**L74V**	Y115F	M184V			
Didanosine	**K65R**		**L74V**					
Emtricitabine	**K65R**				M184VI			
Lamivudine	**K65R**				M184VI			
Stavudine	**M41L**	**D67N**	**K70R**			**L210W**	**T215YF**	**K219QE**
Tenofovir			**K70R**					
Zidovudine	**M41L**		**K70R**			**L210W**	**T215YF**	**K219QE**

NNRTI

Efavirenz			**L100I**	**K101P**	**K103 N/S**	**V106M**	**V108I**	**Y181CI**	**Y188L**	**G190 S/A**	**P225H**	
Etravirine	V90I	A98G	**L100I**	**K101 E/H/P**		V106I	E138A G/K/Q	V179DFT	**Y181CIV**		G190SA	M230L
Nevirapine			**L100I**	**K101P**	**K103 N/S**	**V106 A/M**	**V108I**	**Y181CI**	**Y188C L/H**	**G190A**		
Rilpivitrine				**K101 E/P**		E138AGK QR	V179L	**Y181C I/V**	H221Y		F227C	**M230IL**

Table 1.2 (b) Summary of current HIV-1 mutations associated with drug resistance to FDA approved PI antiretroviral drugs

Drug	L10	V11I	G16E	K20	L24I	D30N	V32I	L33	E34Q	M36	M46	I47	G48	I50
Atazanavir /ritonavir	L10I F/V/C		G16E	K20R M/I/T/V	L24I		V32I	L33I F/V	E34Q	M36I/L/V	M46 I/L		G48V	I50 L
Darunavir /ritonavir		V11I					V32I	L33F				I47V		I50 L
Fosamprenavir /ritonavir	L10F /I/R/V						V32I				M46 I/L	I47V		I50 L
Indinavir /ritonavir	L10I R/V			K20 M/R	L24I		V32I			M36I	M46 I/L			
Lopinavir /ritonavir	L10F /I/R/V			K20 M/R	L24I		V32 I	L33F			M46 I/L	I47 V/A		I50V
Nelfinavir	L10F I					D30 N				M36I	M46 I/L			
Saquinavir /ritonavir	L10F /I/R/V				L24I								G48 V	

Table 1.2 (b) Continued below, summarizes current HIV-1 mutations associated with FDA approved NNRTI antiretroviral drugs

Drug	D60E	I62V	L63P	I64	H69	A71	G73	T74	L76	V77	V82	N83	I84	I85	N88	L90 / I89	I93
Atazanavir /ritonavir	D60E	I62V		I64 L/M/V		A71V/IT/L	G73C S/T/A				V82A T/F/I		I84V	I85V	N88 S	L90M	I93L/ M
Darunavir /ritonavir									L76 V				I84 V			I89V	
Fosamprenavir /ritonavir							G73S		L76V		V82A F/T/S		I84 V			L90M	
Indinavir /ritonavir						A71V/T	G73 S/A		L76V	V77I	V82 A F/T/S		I84 V			L90M	
Lopinavir /ritonavir			L63P			A71V/T	G73S		L76 V		V82 A F/T/S		I84V			L90M	
Nelfinavir						A71V/T				V77I	V82A F/T/S		I84V		N88S /S	L90 M	
Saquinavir /ritonavir		I62V				A71V/T	G73S			V77I	V82A F/T/S		I84V			L90 M	
Tipranavir /ritonavir					H69 K/R			T74P			V82 L/T	N83 D	I84 V			I89 I/M/V	

Tables 1.2 (a) (b) Shows a summary for HIV-1 drug related mutations Amino acid abbreviations: A, alanine; C, cysteine; D, aspartate; E, glutamate; F, phenylalanine; G, glycine; H, histidine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; P, proline; Q, glutamine; R, arginine; S, serine; T, threonine;V, valine; W, tryptophan; Y, tyrosine. Amino acids of the wild type are shown to the left of the amino acid position number and mutations on the right. Amino acids substitutions conferring resistance are shown in bold on the right of the amino acid position [183].

1.9   HIV DRUG RESISTANCE ASSAYS

1.9.1 Introduction

There are currently two types of drug resistance assays used for clinical practice. They are (1) genotypic assays which involve HIV-1 gene sequencing that identifies drug related mutation patterns and (2) phenotypic assays which are cell culture based in which viral replication is accessed in the presence or absence of drugs. The most preferred assays are population genotyping assays as they identify novel mutations as well as known mutations [184].   Most of these assays detect HIV resistance by targeted sequencing of the protease (PR, amino acids 1-99) and reverse transcriptase (RT, amino acids 1-320) in the *pol* gene of HIV as drugs that target these proteins as the most abundant of the FDA approved. The drawback is that currently the only two FDA approved commercially available assays, ViroSeqTM HIV-1 Genotyping Version 2.7 (Applied Biosystems, Foster City, CA, USA) and TruGene (Siemens, Deerfield, IL USA), are too costly for most developing countries where HIV prevalence is highest [185].

1.9.2 In-house HIV-1 genotyping assay.

In-house assays, which can be up to three times cheaper than commercial kits, offer a more feasible approach to drug resistance testing in resource constrained countries. Some researchers have also found the in-house assay to be more sensitive than the two commercially available drug resistance genotyping assays mentioned earlier. These reports have been particularly so when using "in-house" assays, designed for non-subtype B HIV-1 testing in non-subtype B populations [185, 186]. When evaluated

43

against commercial kits, blinded quality assurance programs have indicated high concordance between in-house methods and kits [187, 188].

Findings from studies such as the ones described above show that HIV-1 subtype B strains represented only 12% of the global infections.   Subtype C accounted for 50% (most prevalent strain in sub-Sahara) of all infections worldwide while Subtypes A, D and G accounted for 12%, 3% and 6%, respectively [25]. Other subtypes F, H, J and K together only accounted for 0.94% of infections by the end of the same year. The recombinant forms of the virus all together accounted for about 18% of the worldwide infections [25]. HIV-1 subtype C is the most prevalent subtype in Zambia [189].

Studies have shown that about 80% of patients who fail ART have drug resistance due to HIV-1 mutation [190]. It is therefore important that genotyping assays used to monitor these changes have high sensitivity. Both TRUGEN and ViroSeq genotyping assays do not genotype the HIV-1 subtype C as well as they genotype the HIV-1 subtype B as they have been optimized for genetic subtype B strains [191, 192]. In one study, investigators were only able to successfully sequence 69% of the samples and only 64% in another study with these commercial assays on non-subtype B strains. An HIV-1 subtype C specific genotyping assay with a sequencing rate greater than 80% needs be used to monitor viral mutation to help in clinical management [184]. An epidemic of HIV drug resistant strains can easily develop in patients with better access to antiretroviral drugs but with inadequate monitoring due to a widespread of resistant strains [186]. In a VIRAD APT randomized controlled trial carried out in France, researchers were able to show the benefit of genotypic-resistance testing in clinical monitoring of treatment [193].

Many researchers have thus developed affordable assays costing up to four times less, but with an equivalent or higher sensitivity than commercial kits in an attempt to increase accessibility of drug resistance assays in resource limited countries [184, 186].

Finding from studies such as the ones described show that due to the high rates of replication errors, and development of drug resistance during therapy, it is important that HIV-1 genotypes are determined before or during ART in patients for better clinical management. Knowing the prevalence of such mutations within a population could equally be helpful to clinicians in deciding a drug regimen for patients without an HIV genotype result. Such data is limited in the Zambian population mainly due to the high cost of performing HIV-1 genotyping in infected patients.

1.10 AIMS OF THE STUDY

The aims of the current thesis were to:

1) Develop in-house assay as an economical alternative to commercial assays.

2) Validate the in-house assay using a clinically relevant population for the mutations and resistance.

3) Describe the frequency of reverse transcriptase and protease mutation among patients failing first-line and second-line therapy in Zambia.

Hypothesis

We hypothesize, in a Zambian population, patients on second line HAART who are treated exclusively with the Lopinavir/Rotinavir Protease Inhibitor would not have selected for Lopinavir/Rotinavir drug resistance mutations at treatment failure.

2.0 CHAPTER 2: MATERIALS AND METHODS

2.1 STUDY DESIGN

2.1.1 Patient sample and testing.

Patient's whole blood, n=43, (19 were on first-line regimen and 24 on second-line regimen) was collected from those who were failing HAART, defined as having a viral load > 1000 copies/ml despite treatment. Clinicians in various clinics in Lusaka determined which patients met the criteria. The clinics included, Kamwala, Matero, Chipata, Kabwata, Makeni, Chelstone, Kanyama, Chawama, Kalingalinga, and Mutendele. We separated plasma from whole blood by centrifugation (eppendorf 5810, Germany), within 4 hours of collection. Plasma was aliquoted into Rnase-free 2ml vials and stored at -65°C to 80°C until use.

Viral load was re-tested using Abbott m2000 real time PCR technology to confirm virologic failure. Samples with a viral load greater than 1,000 copies/ml were selected for HIV drug resistance testing.

Unless requested by the clinician at the time of drug resistance testing, CD4 results used in the study was the most recent test on the patient's file. CD4+ lymphocytes from peripheral blood was counted by flow cytometry (Coulter XL; Coulter Co., Hialeah, FL), using a pan leucocyte gating (PLG) method [194].

HIV-1 viral RNA was manually isolated using QiaAmp Viral RNA mini kit. Roche expand reverse transcriptase and Expand DNA polymerase were used to perform

47

reverse transcription and PCR respectively. Chain termination cycle sequencing PCR was done using Applied Biosystem BigdyeTeminator kit and sample sequencing in an ABI 3101xl genetic analyzer.

Sequence analysis was done using sequencer software version 4.10.1 and drug resistance determined by submitting sample sequences to the online Stanford HIV data. Figure 2.1 illustrates the study design.

Ethical clearance for the use of patient sample material was obtained through the University of the Zambia Biomedical Research Ethical committee (ethics clearance number: FWA00000338, IRB00001131 of IORG000774.

Samples drawn from a patient's failing HAART,

N= 43

EDTA plasma separated from cells within four hours of blood draw

In few cases, sample could be processed without repeat VL (if patient had VL test >10 000 copies within a 2 weeks period)

Manual RNA extraction (QiaAmp KIT), Reverse Transcription (RT) and PCR done on samples with VL equal or greater than 1000cps/ml

- Viral load test repeated using the AbbotM2000 RT-PCR
- Plasma aliquot stored at -70 ^{o}C until genotyping is performed.

- PCR product detected using agarose gel electrophoresis. Samples without product repeated once.
- Product concentration determined by nano-drop spectrophotometery.

Amplicon purified/Sequencing PCR /sequence electrophoresis using ABI 3130xl analyzer.

Genotype analysis

- Sequencher software version 4.10.1

Drug mutation profile analysis

- Online Stanford HIV database analysis tool

List of PI accessed

ATV/r	TPV/r
DRV/r	SQV/r
FPV/r	NFV
IDV/r	LPV/r

List of RTI drugs accessed for resistance

NRTI	**NNRTI**
3TC, AZT	Ritivitrine
ABC, D4T	EFV, ETR

Figure 2.1 Schematic representation of the study design

2.2 HIV-1 GENOTYPING ASSAY

2.2.1 Introduction

The HIV-1 Genotyping System is intended for use in detecting HIV genomic mutations that confer resistance to specific types of antiretroviral drugs, as an aid in monitoring and treating HIV infection. As was stated earlier, two commercial kits with FDA approval (TRUGENE and ViroSeq) can be used to genotype HIV-1 for the following purposes: (1) Detect HIV-1 Subtype B viral resistance in plasma samples collected in EDTA with a viral load ranging from 1000 to 750,000 copies/mL and (2) Genotype the entire HIV-1 protease gene from codons 1-99 and two-thirds of the reverse transcriptase (RT) gene from codons 1-321. In this study, an in-house assay adapted from, Wallis et al was used [184].

2.2.2 Assay design

Genetic analyses of the HIV-1 plasma-derived *pol* gene was done using reverse transcriptase polymerase chain reaction, polymerase chain reaction (PCR) and genotyping PR (amino acids 1-99) and RT (amino acids 1-321) [184]. As was mentioned earlier, HIV-1 replicates using the virally encoded RT which contains a catalytically active larger unit (p66) and a smaller subunit (p51). The assay was designed to capture only the PR gene and the subunit of the p66 which comprises the DNA polymerase (1-321) [124] to which most of the RT inhibitors target. The other residues of p66 include connection domain (322-440) and the ribonuclease (Rnase) H (441-560) domain [124, 195]. As was previously stated, Rnase activity is responsible for the degradation of RNA of the RNA/DNA hybrid, an important stage in the conversion of single stranded RNA to proviral DNA [196]. The figure below summarizes the assay design. In (A) the red bar shows the portion (2047-3610) of the

9719kb HIV-genome targeted by our PCR primers and in (B), is an illustration of the primer design. At the bottom of the red bar are the two PCR primers and at top are the six primers used for sequencing. Note that primer RWF2 (P21) was only used as backup primer if quality of the overlapping portions of primers CWS1, CWS2, CWS3, and CWS4 and CWS5 came out poorly.
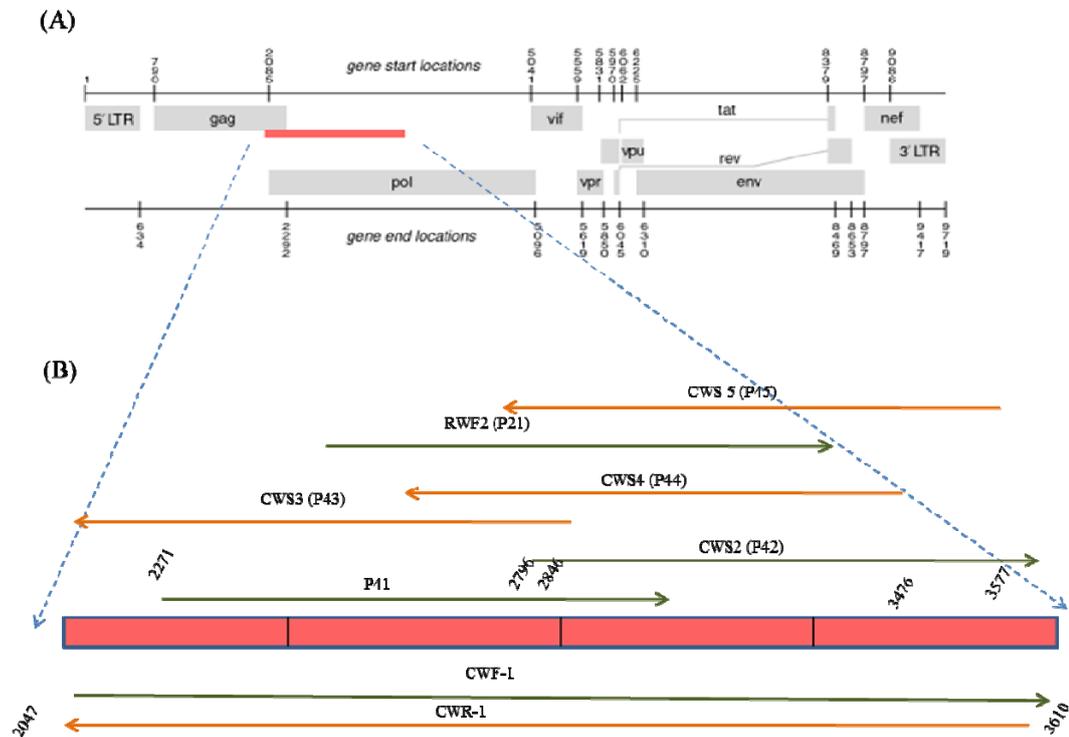
Figure 2.2 The top (A) shows the liner genome of HIV-1. The solid grey block shows regions of that genome that encodes for various proteins. The top scale shows the start position for various genes while the bottom scale shows the gene stop locations. The red blocks indicate base position 2047-3610 of the *Pol* gene (B); the solid red block is a part of the *pol* gene magnified from (A); to illustrate primer design for RT/PCR and sequencing; orange arrows show reverse or antisense primers while the green arrows depicting the forward or sense primers (B). All the arrows above the *pol* gene (red block) P21, P41, P42, P43, P44 and P45 are for sequencing while the ones below (CWF-1 and CWR-1) are for RT/PCR. Figure 2.2 (A) was generated online using a Los Alamos National Laboratory (LANL) HIV sequence locator tool.

2.2.3 RNA Isolation.

Whole blood samples were collected in EDTA containers at the clinics, and sent to the

laboratory within two hours of collection. Plasma was immediately separated from cells

52

using an Allegra^{TM} X22R Beckman centrifuge (Beckman coulter, CA Germany) at 1000xg for 15 minutes and aliquoted in 2ml Rnase free vials. Plasma was then stored between -65°C to 80°C until use.

To isolate RNA, 500µl of thawed plasma was ultra-spun at 21 000xg in an Allegra^{TM} X22R Beckman centrifuge (Beckman coulter, CA Germany), at 4°C for one hour in 2ml vials. This was done to concentrate nucleic acid to the bottom pellet that forms. 360 µl of supernatant plasma was removed from vials within 5minutes, avoiding touching the pellet which was then re-suspended in the remaining 140µl plasma. The 140µl of plasma was then treated as a start sample and RNA was extracted using QiaAmp Viral RNA mini kit (QIAGEN technology, USA) as per manufacturer's instructions.

2.2.4 HIV-1 Reverse Transcription

Extracted viral RNA was synthesized into cDNA using the reverse primer CWR1 (5'-GCA TAC TTY CCT GTTTTC AG-3"; HXB2 nucleotide position 3610to 3594) and the Expand Reverse Transcriptase (RT) kit (Roche Diagnostics, Germany). For each sample, including negative and positive controls, 8µl of extracted RNA was incubated with 2.5µl of 20µM CWR1 oligonucleotides in THERMOCYCLES at 65°C for 10 minutes. The samples were immediately transferred to ice after incubation period and 9.5 ml of reaction master mix added in each. See Table 2.1 for master mix preparation. The reaction tubes were then incubated at 42°C for one hour (Table 2.2).

Table 2.1 Reverse transcriptase reaction concentrations.

Reagent	Volume for n=1 (μl)	Concentration	Manufacturer
5X RT Buffer	4	1X	Roche, MA, German
DTT	2	-	Roche, MA, German
dNTP (10mM)	2	1mM	Roche, MA, German
RNAse Inhibitor (5000U)	0.5	125U	Roche, MA, German
Expand RT	1		Roche, MA, German
Final volume	**9.5**		

Table 2.2 Reverse transcription incubation conditions

Temperature (^{o}C)	Time
65	10 min
42	60 min
4	∞

2.2.5 Polymerase Chain Reaction. (PCR)

Amplification was performed using primers CWF1 (5'-GAA GGA CAC CAA ATG AAA GAY TG-3'; HXB2 nucleotide position 2047 to 2066) and CWR1 and 20μl of synthesized cDNA with the Expand High Fidelity^{PLUS}kit (Roche, Germany). The PCR master mix was prepared as per manufacturer's instructions (Table 2.3), [184]. The cycling conditions (GeneAmp PCR system 9700, Applied Biosystems, Foster City, USA), consisted of an initial denaturizing step of 94°C for 2 minutes, followed by 10

54

cycles of 94$^{\text{o}}$C for 30 seconds, 54.5$^{\text{o}}$C for 30 seconds and 72$^{\text{o}}$C for 2 minutes and then 35 cycles of 94$^{\text{o}}$C for 30 seconds, 55$^{\text{o}}$C for 30 seconds and 72$^{\text{o}}$C for 2 minutes increasing by 10 seconds each cycle, and a final elongation step of 72$^{\text{o}}$C for 10 minutes [184].

Note that all PCR and sequencing primers were designed from the 2004 consensus HIV-1 subtype C sequence available on the Los Alamos Database (www.hiv.lanl.gov/).[184]

Table 2.3 PCR reaction mixture.

Reagent	Volume for 1 sample (μl)	Final concentration in 50ml	Manufacturer
5X Buffer	10	1X	Roche, USA
10mM dNTP	1	0.2μM	Roche, USA
20mM CWR-1	1	0.4μM	Roche, USA
20mM CWF-1	1	0.4μM	Roche, USA
Expand polymerase (250U)	0.5	2.5U	Roche, USA
DEPC free Rnase free Water	33.5	-	Ambion, TX, USA
Total	**47.0**	**-**	

2.2.6 Gel Electrophoresis

Successful PCR of samples was determined by electrophoresis and UV light (302nm) visualization of DNA bands on a solid 0.8% molecular grade agarose gel (Bio-Rad, USA) which was prepared in a 1X 89mM Tris, 89mM boric acid, 2mM EDTA buffer (TBE), (fermentas USA) and 0.5µg/ml ethidium bromide. To prepare a 0.8% gel, 0.8 grams of agarose was weighed on a digital scale (Mettler Toledo, Canada) and dissolved in 100mls of 1X TBE buffer. The mixture was heated to boil in a microwave and was allowed to cool to about 60°C. 5µl of 10mg/ml ethidium bromide was then added to the gel, which was poured into a molding tray containing a 1.5mm x 30 comb. The gel was allowed to solidify at room temperature between 15 and 30 minutes and then the comb was removed, creating wells.

A DNA lambda DNA/HindIII marker, 2 (thermo fisher, USA) was prepared as per manufacturer's instruction and 5ml loading in the first well. 5 ml amplicon for the test samples was mixed with 1ml 6X loading dye and then the mixture was transferred in the gel. Electrophoresis was the performed as a constant voltage of 80 for 45 minutes. Bands were then visualized using a MultiDoc digital imaging system (Cambridge, UK). The run was considered invalid if a band was detected in the negative control sample.

2.2.7 PCR products Purification and concentration determination

All samples which showed a successful PCR on gel (approximately 1.5 kb in length) were purified and concentrated in micro columns using the GFX DNA gel purification kit (AEC Amersham, UK) to remove any unreactive substances such as oligonucleotides and DNA polymerase. A total of 60µl was eluted.

1µl to 2µl was used to determine sample concentration using a nanodrop spectrophotometer and diluted according to their concentration to 10-14ng/µl. Any sample that had a concentration below 5ng/ml was re-amplified to increase the concentration as the PCR procedure stated earlier.

2.2.8 Chain termination PCR and sequencing (Cycle sequencing)

Sequencing was performed using 3.2pmol each of five sequencing primers (CWS1: 5'CCTCAAATCACTCTTTGGC-3'; HXB2 nucleotide position 2253 to 2271; CWS2: 5'AGAACTCAAGACTTTTGGG-3'; HXB2 nucleotide position 2796 to 2814; CWS3: 5'TGCTGGGTGTGGTATTC-3'; HXB2 nucleotide position 2846 to 2830; CWS4: 5'TCCCTGTTCTCTGCCAATTC-3'; HXB2 nucleotide position 3472 to 3453; CWS5: 5'TGGTAAATTTGATATGTCCATTG-3'; HXB2 nucleotide position 3577 to 3555[184], and one additional primer not used by wallis et al. P21: 5'GGAGGTTTTATCAAAGTAAGACAGTATGA-3; HXB2 nucleotide position 2403 to 2432, 11μl (8-12ng/dl) of purified PCR product, 0.5x BigDye® Terminator v1.1/3.1 Sequencing Buffer and 0.5x Ready Reaction Premix from the ABI PRISM® BigDye® Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) in a total volume of 20μl, with standard cycle sequencing conditions as per manufacturer's instructions[184]. Unincorporated ddNTPs were then removed by precipitation with ethanol-sodium acetate, method, and then the dried pellet was re-suspended in 20μl Hi-DiTM Formamide (Applied Biosystems, UK). Sequence data was obtained using an ABI 3130xl genetic analyzer, (Applied Biosystems, Foster City, USA) and edited using the Sequencing analysis 5.3.1. Program (Applied Biosystems, Foster City, CA, USA).Sequence data was further edited using Sequencer 4.10.1 (Gene codes, cooperation, MI, USA) and primary mutations (PRMs) that are associated with drug resistance to PR and RT were detected by means of Stanford University calibrated resistance tool online.

To perform chain termination sequencing PCR (cycle sequencing), reagents were prepared as shown in table 2.4 below for each reaction.

Table 2.4 Cycle sequencing reaction concentrations.

Reagent	Volume for 1 sample (μl)	Final concentration in 50ml	Manufacturer
BigBye ready reaction Premix (2.5X)	4	0.5X	AB*
BigDye® Terminator v1.1/3.1 Sequencing Buffer	2	0.5X	AB*
3.2μM Primer	1		
DNA sample	11		INQ**
DEPC free Rnase free Water		-	Fermentas***
	2		
Total	**20μl**	**-**	

*Applied Biosystems UK. **Inqaba biotec, SA. *** Ferments MD, USA,

To purify with Ethanol/sodium acetate, 2μl of 3.0M sodium acetate, pH 5.2 was added to 50μl of 100% ethanol to mark working reagent. The total was added into each sequencing reaction and plate centrifuged 20 minutes at 2000 x g. The plate was immediately inverted without disturbing the pellet as soon as centrifuge had stopped and centrifuged at 150 x g for 1 minute to remove the supernatant. 150ml of 70% freshly prepared ethanol was added to each reaction and the plate centrifuged at 2000 x g for 5 minutes and supernatants removed as described before. Pellets were dried for about 5 minutes and re-suspended in 20μl Hi-DiTM formamide (Applied Biosystems, UK).

2.3   HIV-1 GENOTYPING ASSAY

2.3.1 Parallel testing

Seven samples were sent to the University teaching hospital in Zambia for genotyping using the commercial ViroSeq kit. We tested the same samples with the in-house kit, using the method as described above and the sequences were compared at the nucleotide and mutation level to determine the level of incongruity between the consensuses.

2.3.2 Accuracy and reliability assessment of assay.

Five samples were run on the ABI3130XL and repeated a minimum of three times. Sequences were compared at a nucleotide and mutation level to determine the level of incongruities between the consensuses.

2.4   DATA ANALYSIS:

2.4.1 Parallel testing

To define genotypic correlation between the in-house and viroseq assays, complete in-house, tested parallel sequences (*n=7),* encompassing the *pol* region of interest were assembled and manually edited using Sequencher version 4.10.1 (Genecodes, MI,USA) to generate consensus sequences which were compared with those from the parallel assay. BioEdit version 7.0.9 analysis tool software, (NC, USA) was used to generate phylogenetic trees to ensure there was no cross-contamination of samples.

Both ViroSeq and "in-house" consensus sequences were submitted to the Stanford Database (http://hivdb.stanford.edu/),   to generate an HIV-1 drug resistance mutation report,   and   also   submitted   to   the   REGA   sub-typing   tool, (http://dbpartners.stanford.edu/RegaSubtyping/) to determine HIV-1 subtypes. The

60

University of Stanford HIV drug resistance database utilizes known HIV-1-B wild type genetic sequences maintained at the Los Alamos HIV Sequence Database (hiv-web.lanl.gov) to determine base substitutions in submitted consensus sequences which acts as the non drug resistant control.

Sequence similarity between the two corresponding samples was done by submitting their protease and RT sequences online at the bioinformatics Institute website clustalw2 tool (http://www.ebi.ac.uk/Tool/msa/clustalw2/) which aligns sequences for ease of comparing. Hamming distances of the corresponding samples were calculated manually to determine congruence. The hamming distance was calculated by computing percentage of the total nucleotide mismatches and partial mismatches (mixtures) divided by total nucleotides compared. A hamming distance of $\geq 98\%$ was considered acceptable, indicating high sequence similarity.

Samples between the two laboratories were compared at the nucleotide and mutation level to determine the level of disagreements between the consensuses: a) total number of errors in a sequence was accessed; b) number of mismatches between the two methods was compared. There are three types of mismatches: partial (R vs. G), complete (A vs. G), and data missing (N), see Table 2.5 below for IUPAC nucleotide codes. For our analysis only complete and partial mismatches were investigated; c) the mutation lists were compared.

61

Table 2.5 IUPAC Nucleotide code.

IUPAC nucleotide code	Base
A	Adenine
C	Cytosine
G	Guanine
T (or U)	Thymine (or Uracil)
R	A or G
Y	C or T
S	G or C
W	A or T
K	G or T
M	A or C
B	C or G or T
D	A or G or T
H	A or C or G
V	A or C or G
N	Any base
. or -	Gap

2.4.2 Assay specificity

All five samples used for this purpose were analyzed exactly as mentioned above in section 2.4.1, and similarly compared between runs.

2.4.3 Statistical analysis.

A contingency table in GraphPad Prism5 statistical software was used to determine the prevalence of drug resistance mutations between samples from patients on first and second-line therapy drug regimens and reported with 95% CIs based on a normal approximation of binominal distribution. The numbers of NRTI, NNRTI and PI drug resistant mutations in both groups were reported and their associations were tested using Fisher's exact test. All tests were 2-sided and *P* value was statistically significant if alpha <0.05.

3.0 CHAPTER 3: RESULTS

3.1   GEL ELECTROPHORESIS.

All sample's amplicons were visualized on a 1% gel prepared with 5μg/ml concentration ethidium bromide as was described in the Materials and Methods section. Following electrophoresis at constant voltage of 80, bands were visualized with UV light and picture taken. In figure 3.1, a representative example of one run is shown: sample migration was from top (cathode) to bottom (anode). On the far left, in the first well was the lambda DNA/HindIII marker2 mass ladder. Our bands of interest were about 1500bps in size and are seen slightly above the 2027 position of the ladder for the next 9 samples toward the right; see ruler on the far right in black and white for size reference.
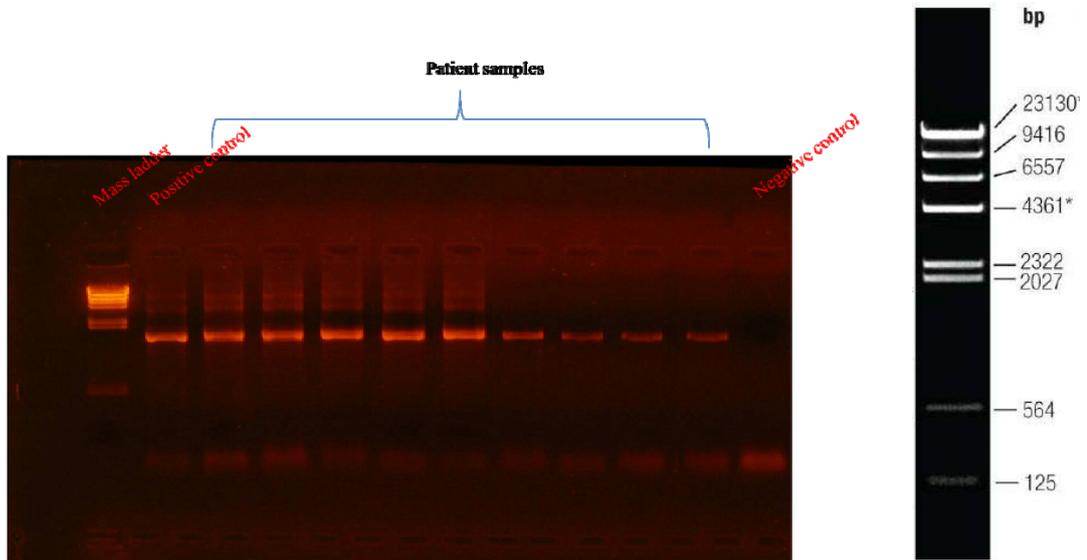
Figure 3.1 A representative example of one gel electrophoresis result of 10 samples following PCR. Gel and sample were prepared and loaded on the gel as was described in the Material and Methods section. From your left, in the first position is the Mass ladder (lambda II) followed by a Positive control sample. The interpretation of the mass ladder can be seen one the scale on the furthest right. Positions 3 to 8 indicate patients sample and in position 9, negative control.

3.2   SEQUENCING

Each sample was cycle sequenced in aliquots of five 11ml per primer as described in the material and methods section and capillary electrophoresis was performed in a 20μl Hi-DiTM Formamide suspension. Sequences were aligned and overlapping sequences connected using the Sequencher 4.10.1 software, figure 3.2. In the same figure, the deep green thick bar indicates the region of PR and RT that was adequately covered by primer sequence run in both directions. The light blue color on the same bar on both ends show regions where sequencing primers did not overlap.

Figure 3.3 is an extraction from Sequencher 4.10.1, of a picture showing electropherograms of nucleosides (bases): adenine, A, in green, thymidine, T, in red, guanine, G, in black and cytosine, C, in blue. The figure shows segments (A), (B) and (C) of regions analyzed for sample 130-08810-7 covering PR and RT gene. Two mixed bases calling in (A) and (C) for cytosine or adenine and adenine or guanine respectively are called M and R as per IUPAC codes respectively. Finalized edited sequences were used to generate a single complete consensus sequence for each sample, see appendices.

Phylogenetic trees were produced on Bioedit using consensus sequences for each sample to determine their similarity within the HIV-1 clades, see figure 3.4.

66
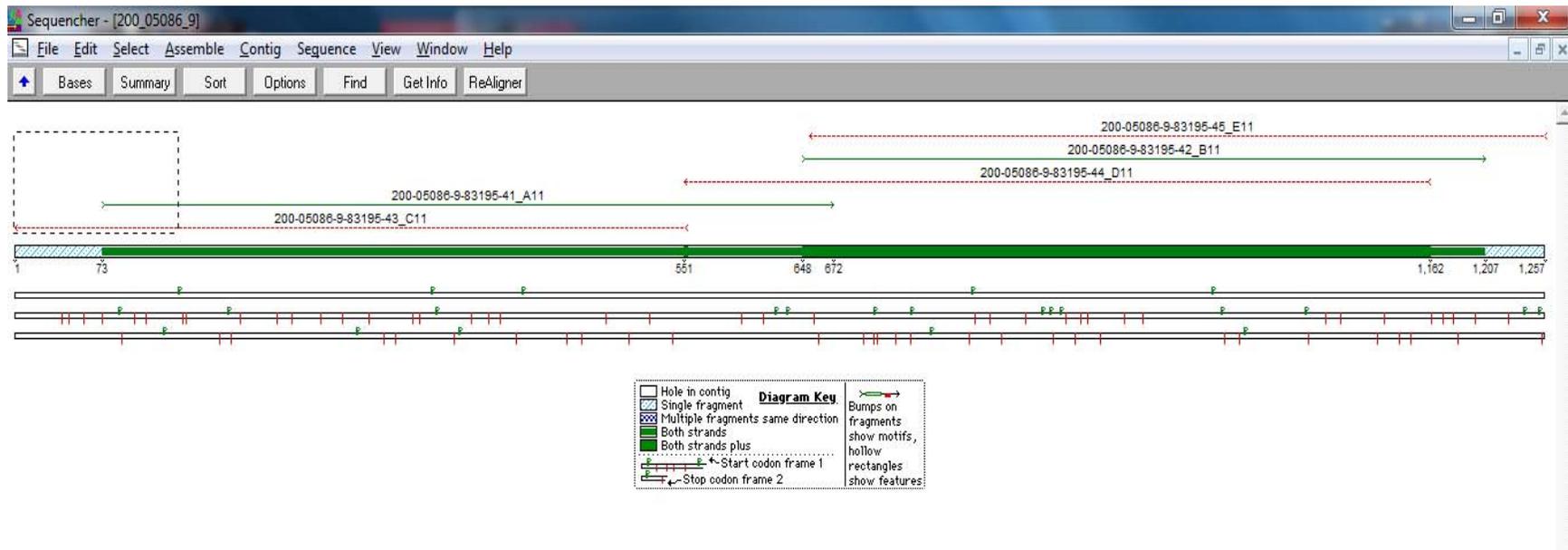
 Figure 3.2 A represatative result of one the samples tested (200-05086-9) showing muiltple primer alignment to form a continues PR/RT gene fragement. The red dotted lines show the coverage of three reverse primers, CWS3 (P43), CWS4 (P44) and CWS5 (P45); the green arrows indicate region covered by the Forward primers CWS1 (P41) and CWS2 (P41). Figure generated using sequencher 4.10.1
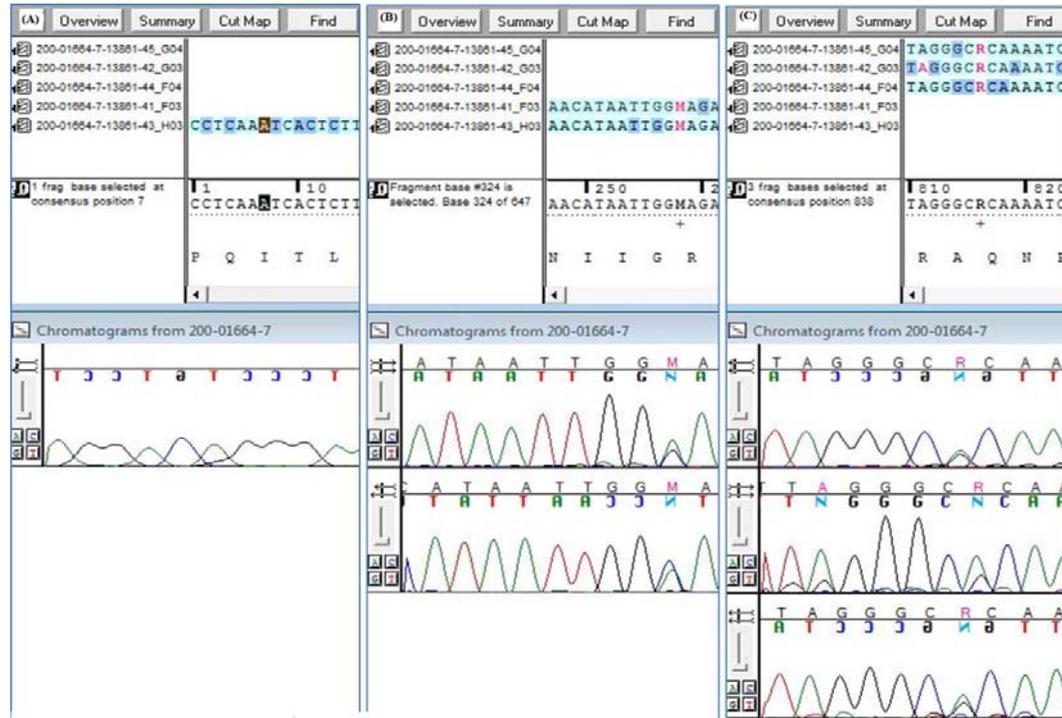
Figure3.3 A representative sample. Eletropherograms extracted at 3 different locations of the *pol* gene segments. Electropherograms for bases A, C, G and T are shown in colors green, blue, black and red respectively: (A) Shows the first 12 start sequences covered by only one primer. (B) Shows positions between 247 and 260 of the protease region. Mixture nucleotide in red (M) was manually edited representing either C or A. (C) Shows positions between 810 and 824 of the protease/RT region (position 513-527 of RT). Mixture nucleotide in red (R) was manually edited representing either A or G. The figure was generated using Sequencher s version 4.10.1 software
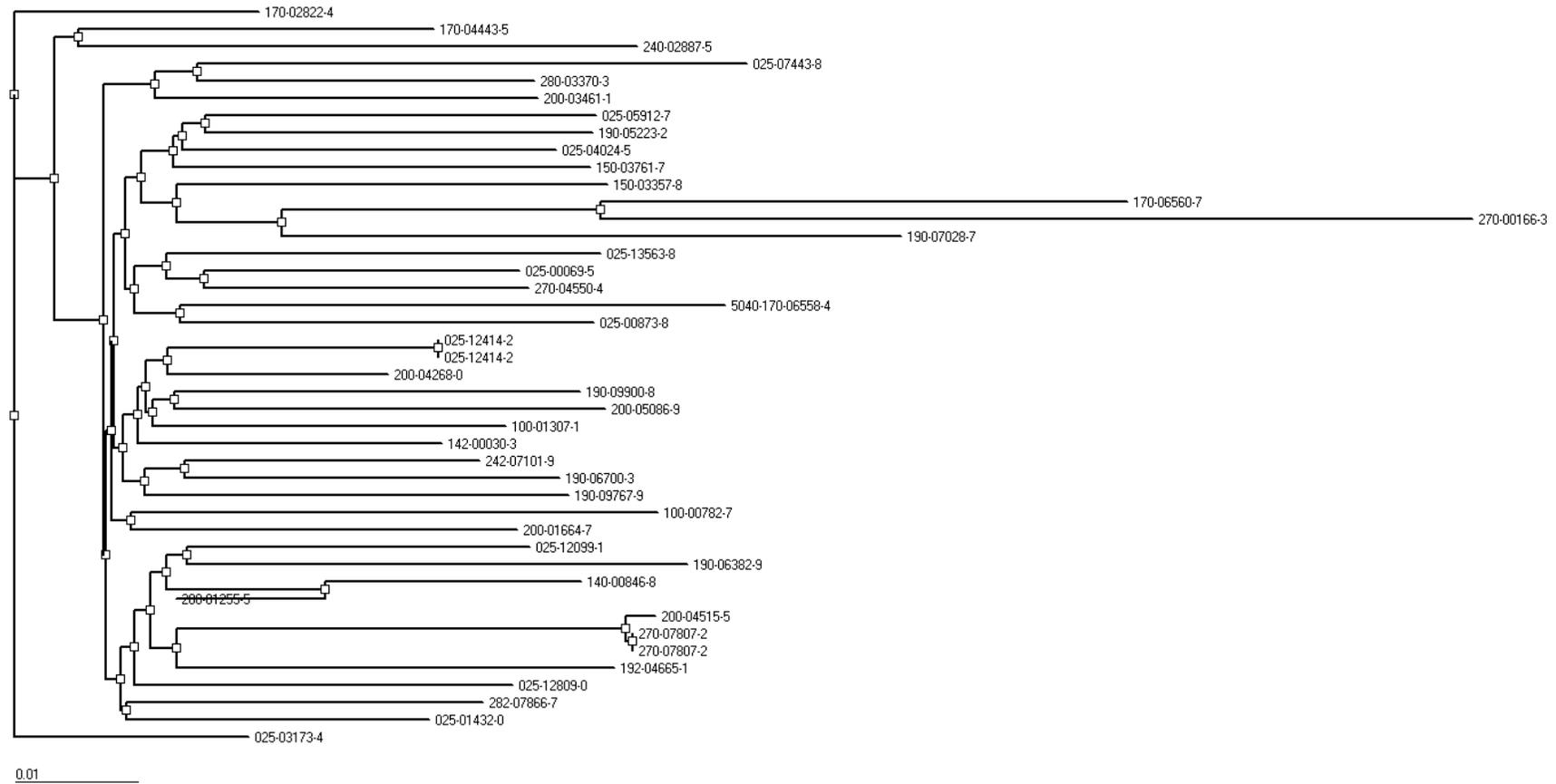
Figure 3.4 Phylogenetic tree analyses of 43 samples constructed using HIV RT and PR amino acid sequences. The alignment and the phylogenetic analysis were done using bio-edit software version 7.0.9. The scale bar indicates the evolutionary distance of 1% nucleotides per position in the sequence.

3.3　HIV-1 ASSAY VALIDATION

In order to validate the in-house genotyping assay, we compared 7 samples with results obtained using a commercial and FDA approved kit, viroseq. Sequences were aligned by the clastaw2 tool of Bio-edit version 7.0.9 software, figure 3.5 and nucleotides manually compared. Hamming distances were calculated and all samples were found comparable (Table 3.1). The minimum hamming distance was 99.54%. No complete nucleotide mismatches were found between samples 0A UTH and 0A CIDRZ, 0B UTH and 0B CIDRZ, 0C UTH and 0C CIDRZ, 0E UTH and 0E CIDRZ. Five, zero, four and four partial mismatches were observed for the same pairs respectively. Two, five and one complete mismatches were observed between samples, 0F UTH and 0F CIDRZ, 0G UTH and 0G CIDRZ, 0D UTH and 0D CIDRZ respectively. We observed three, six and one partial mismatches in the pairs respectively. All samples showed 99.2% similarities in the amino acid translation.

In order to determine assay reproducibility, 5 samples were repeated 3 times each and nucleotides compared manually and sequences analyzed as stated above. The minimum hamming distance was 99.52%. We observed no complete mismatched mutation in all the five repeated sequences and a 100% similarly in amino acid translation, see Table 3.2 for a summary.

Phylogenetic relationships of unambiguously aligned nucleotide sequences for both experiments representing the reverse transcriptase and protease enzymes of the *pol* gene are shown in figures 3.6 and 3.7. The alignment and the phylogenetic analysis were done using bio-edit version 7.0.9 software as indicated in the material and methods section. The scale bar represents nucleotide substitutions per site.
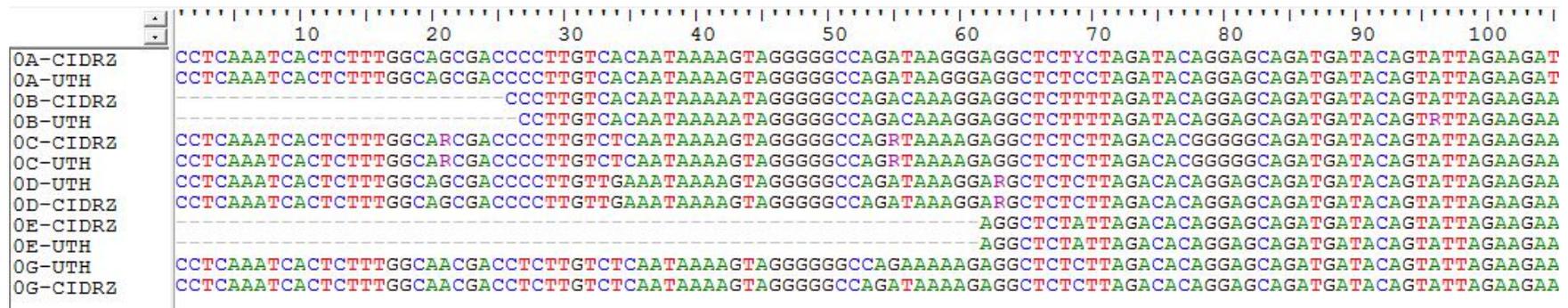
Figure 3.5 shows aligned, sample sequences used in parallel assay validation. These were used for nucleotide comparison between sequences to determine the level of similarity or incongruity between the consensuses. European Bioinformatics institute online software was used for sequence alignments.

Table 3.1 Parallel validation.

PID	Length compared	Reference sequence	complete Mismatched	Partial Mixture	Hamming distance (same; ≥98%)	Subtype
OA-CIDRZ	1173	OA-UTH	0	5	99.57%	C
OB-CIDRZ	1186	OB-UTH	0	4	99.66%	C
OC-CIDRZ	1224	OC-UTH	0	0	100.00%	C
OD-CIDRZ	1178	OD-UTH	1	6	99.41%	C
OE-CIDRZ	1205	OE-UTH	0	4	99.67%	C
OF-CIDRZ	1088	OF-UTH	2	3	99.54%	C
OG-CIDRZ	1265	OG-UTH	5	1	99.53%	C

Table 3.1 above and **3.2** below shows the results of parallel testing results for the 7 samples (CIDRZ) tested with the "in-house" genotyping assay compared with 7 (UTH) corresponding reference sequences previously genotyped using Viroseq Kit. Table 3.2 shows a summary of reproducibility results comparing a minimum of 3 sequences. Hamming distances for both experiments were calculated for the Protease and RT p66 genes and the Rega HIV-1 subtyping tool (http://hivdb.stanford.edu) was used to assign the HIV-1 subtype for the patients

Table 3.2 Reproducibility

PID	Length compared	Reference sequence	complete Mismatched	Partial Mixture	Hamming distance (same; ≥98%)	Subtype
190-05223-2	1208	190-05223-2	0	1	99.92%	C
190-05223-2	1208	190-05223-2	0	3	99.75%	C
190-05223-2	1208	190-05223-2	0	3	99.75%	C
025-12099-1	1212	025-12099-1	0	0	100.00%	C
025-12099-1	1212	025-12099-1	0	2	99.83%	C
40200-B	1166	40200A	0	0	100.00%	C
40200-C	1166	40200A	0	3	99.74%	C
ZamB	1258	ZamA	0	6	99.52%	C
ZamC	1258	ZamA	0	5	99.60%	C
200-04515-5	1190	200-04515-5	0	0	100.00%	C
200-04515-5	1190	200-04515-5	0	2	99.83%	C
200-04515-5	1190	200-04515-5	0	2	99.83%	C
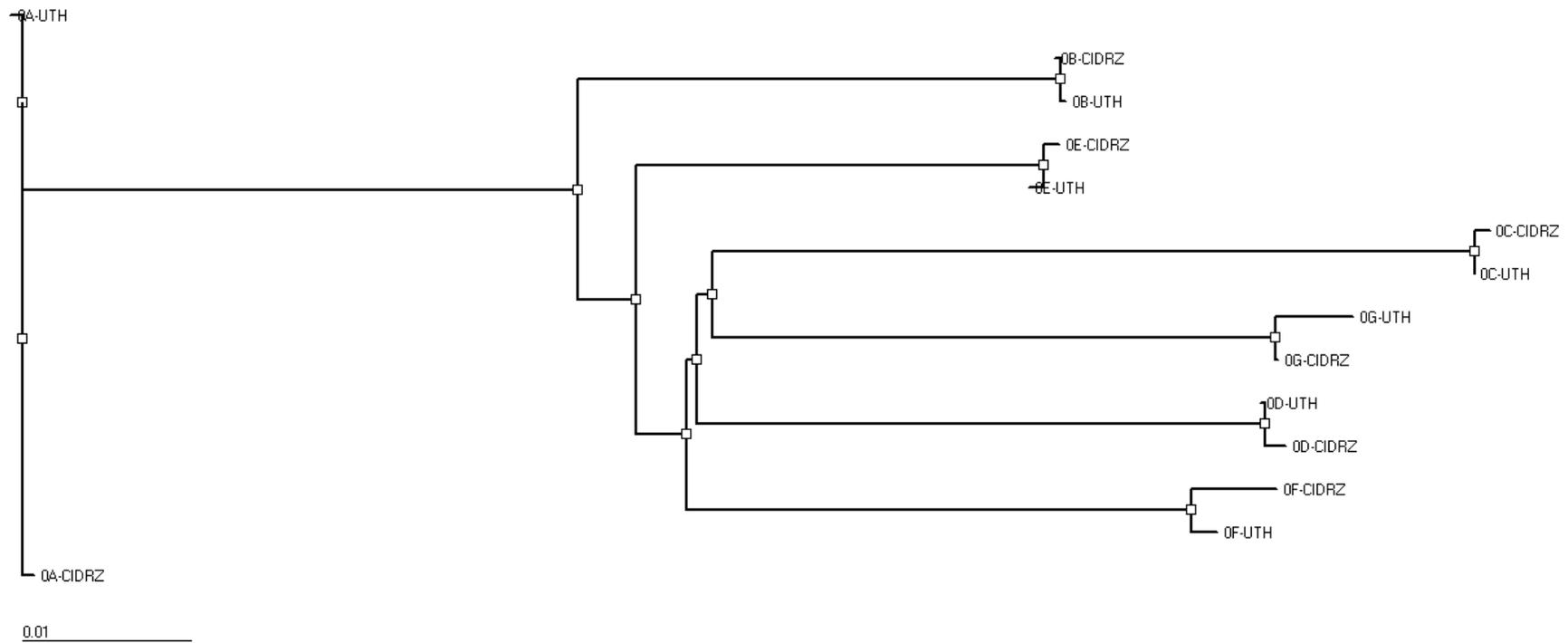
Figure 3.6 Phylogenetic relationships of 7 unambiguously aligned nucleotide sequences representing the reverse transcriptase and protease enzymes of the *pol* gene of the HIV-1C. The alignment and the phylogenetic analysis were done using bio-edit software version 7.0.9. The scale bar indicates the evolutionary distance of 1% nucleotides per position in the sequence.
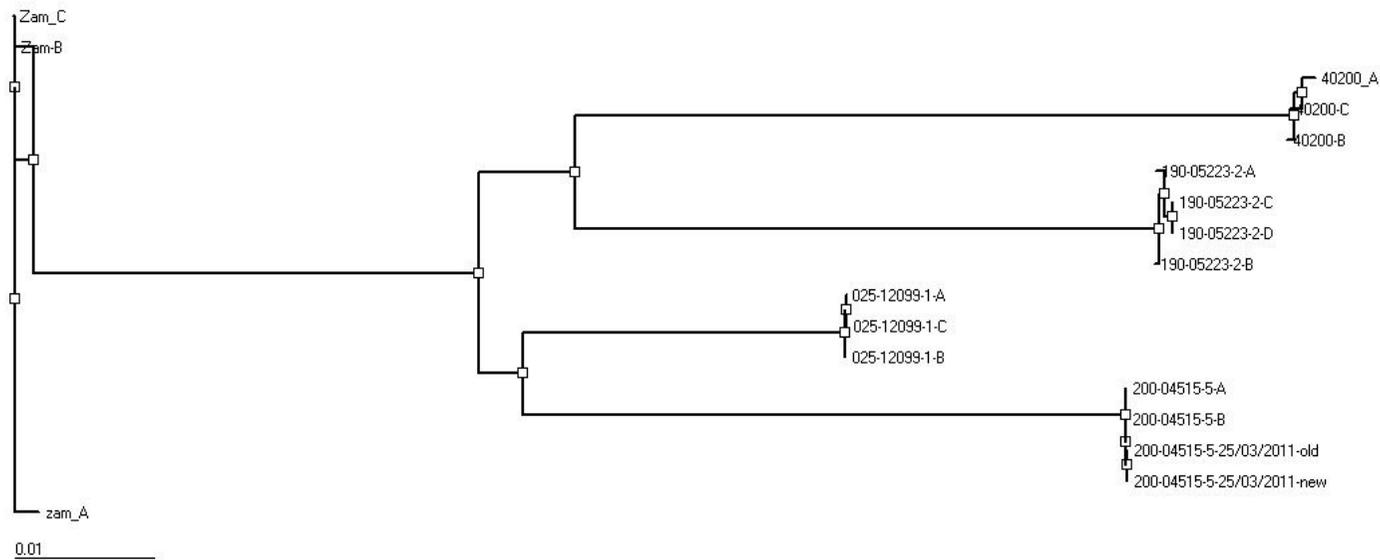
Figure 3.7 Phylogenetic relationships of 5 unambiguously aligned nucleotide sequences representing the reverse transcriptase and protease enzymes of the *pol* gene of the HIV-1C. The alignment and the phylogenetic analysis were done using bio-edit software version 7.0.9. The scale bar indicates the evolutionary distance of 1% nucleotides per position in the sequence.

3.4 GENOTYPIC DRUG RESISTANCE TEST RESULTS.

The table 3.3 below shows the characteristics of patients at the time point that the patient samples were analyzed. 37% patients in this study were male. Apart from two patients with HIV-1 subtypes K and 06_CPX, all the other patients were infected with HIV-1 subtype C. The median CD4 count within < 6 months at the time of the study was 133 cells/mm^3 (interquartale range, IQR, 2-430 cells/mm^3). The median HIV-1 RNA count within at the time point of the study was $5.3x10^4$ copies/ml (interquartale range, IQR, $1.0x10^3 – 8.7x10^5$ copies/ml.

Table 3.3 Characteristics of study population.

Characteristic	Patients n=43	First-line regimen (n=19)	Second-line Regimen (n24)	*P*
Age, median Year (IQR)	39 (11-73)			
Male sex	16(37%)			
Black race	43			
CD4 cell count, median cell/mm^3 (IQR) ≤6months	133 (2-430)			
Plasma HIV-1 RNA level, median log$_{10}$ copies/mL	5E+04 (1E+3 to 8E+5)			
Duration on HAART, median years (IQR)		42(12-72)	60(12-108)	
Duration on Second-line regimen, n=24, median months (IQR)			12(12-72)	
Regimen: First-line Regimen				
AZT+3TC+NVP		3(16%)	11(45%)	ns
AZT+3TC+EFV		1(5%)	6(25%)	ns
D4T+3TC+NVP		10(53%)	9(38%)	ns
D4T+3TC+EFV		1(5%)	1(4%)	ns
TDF+3TC+NVP		4(21%)	5(21%)	ns
TDF+3TC+EFV		2(11%)	0	ns
TDF+FTC+EFV		4(21%)	2(8%)	ns
TDF+FTC+NVP		0(0%)	3(13%)	ns
ABC+3TC+NVP		1(5%)	1(4%)	ns
DDI+D4T+NVP		1(5%)	0	ns
Second-line Regimen				N/A
DDI+3TC+LPVr+RTV			1(4%)	N/A
DDI+ABC+LPV+RTV			3(13%)	N/A
ABC+3TC+LPV+RTV			3(13%)	N/A
TDF+FTC+LPV+RTV			6(25%)	N/A
FTC+LPV+RTV+AZT			3(13%)	N/A
TDF+3TC+LPV+RTV			7(30%)	N/A
TDF+3TC+LPV+RTV+AZT			3(13%)	N/A
AZT+3TC+LPV+RTV			1(4%)	N/A

The most common NRTIs drug resistant mutations observed for the group on first-line therapy in this study were M184V (79%), K65R (37 %), D67N (21%), K16R (15%), A62V (16%), K70E/T, (5%), Y115F (5%), V75M (5%), T215Y (5%), M41L (5%) and K219E/Q (5%). Similar mutation patterns: M184V (71%), K65R (17 %), D67N (33%), K70R (29%), A62V (17%), K70E/T, (8%), Y115F (4%), V75M (13%), T215Y (13%),

T215F (17%), M41L (13%) and K219E/Q (8%) were observed for the group on second-line therapy. see Table 3.3 and figure 3.7

The most common NNRTIs drug resistant mutation observed for the groups on first-line therapy in this study were; K103N (40%), Y181C (30%), G190A/S (20%), V108I (15%), V106A/M (20%), Y181V/I (10%) and Y188C/L (10%). Drug resistance mutations; K103N (29%), Y181C (25%), G190A/S (29%), V108I (13%), V106A/M (21%), Y181V/I (4%) and Y188C/L (8%), P225H (4%) and M230L (4%) were observed for patients on second-line therapy. See Table 3.3 and figure 3.8

One or more drug RT resistance mutations were detected in 85% of patients on first-line therapy and in 83% of patients that had been switched from first-line to second-line therapy, table 3.4 (a).

The prevalence of all protease mutation observed in the study are shown in table 3.4 (b). The most common drug resistance associated major mutations (primary active site mutations) reported included M46I, I54V and V82A all at 20.8% shown in figure 3.12.

Table 3.4 (a) frequencies of selected mutation in reverse transcriptase

Mutations (NRTI)	First-line regimen No. (%)	Second-line regimen No. (%)	P
M41L	3(16)	3(13)	0.6176
A62V	7(37)	4(17)	1
K65R	4(21)	8(33.3)	0.1703
D67N	1(5)	0(0)	0.5
K70E	1(5)	2(8.3)	0.45
K70T	3(16)	7(29	1
K70R	0(0)	0(0)	0.4701
L74V	1(5)	3(13)	1
V75M	1(5)	1(4.2)	0.2425
Y115F	15(79)	17(70.8)	1
M184V	0(0)	0(0)	0.7279
L210W	0(0)	3(12.5)	1
T215Y	0(0)	4(17)	0.6176
T215F	1(5)	4(17)	0.1301
K219E	1(5)	2(8.3)	0.3626
> 1 Significant mutation (NRTI)	17(85)	18(75)	
Muiltiple (≥ 2) drug resistance (NRTIs)			
Resistance	16(80)	18(75)	
intermidiate	1(5)	0(0)	1
Mutations (NNRTIS)			
L100I	1(5)	0(0)	1
K101E	8(40)	7(29)	0.45
K103N	1(5)	2(8)	0.34
V106A	2(10)	3(13)	1
V106M	3(15)	3(13)	1
V108I	0(0)	1(4)	1
Y181I	2(10)	0(0)	1
Y181V	6(30)	6(25)	0.2
Y181C	1(5)	2(8)	0.7
Y188L	2(10)	0(0)	1
Y188C	4(20)	7(29)	0.2
G190A/S	0(0)	1(4)	0.72
P225H	0(0)	1(4)	1
> 1 Significant mutation (NNRTI)	17(85)	20(83.3)	
Muiltiple (≥ 2) drug resistance (NNRTIs)			
Resistance	16(80)	17(70.8)	
intermidiate	1(5)	3(12.5)	

NOTE. In the table above, unless otherwise stated, No. (%) was calculated from n=19 for first-line therapy and n=24 for second-line therapy.

Table 3.4 (b) frequencies of all mutations observed on the protease gene.

	First-line threapy No. (%)	First -line threapy No. (%)	P
Mutations (PI)			
L10F	0(0)	1(4.2)	1
L10V	0(0)	1(4.2)	1
L10I	0(0)	2(8.3)	1
G16E	0(0)	1(4.2)	1
K20R	1(5.2)	1(4.2)	1
M36I	17(89.4)	16(66.7)	0.1448
M46I	0(0)	5(20.8)	0.0562
I54V	0(0)	5(20.8)	0.0562
Q58E	0(0)	1(4.2)	1
D60E	1(5.2)	1(4.2)	1
I62V	2(10.3)	1(4.2)	0.5751
L63P	7(36.8)	11(45.8)	0.7563
I64M	0(0)	1(4.2)	1
H69K	17(89.4)	19(79.2)	0.437
T74S	0(0)	4(16.7)	0.1175
L76V	0(0)	3(12.5)	0.2425
V77I	0(0)	1(4.2)	1
V82L	0(0)	1(4.2)	1
V82A	0(0)	5(20.8)	0.0562
I93L	19(100)	22(91.7)	0.495
> 1 Significant mutation (NNRTI)	0(0)	5(20.8)	
Muiltiple (≥ 2) drug resistance (PIs)			
Resistance	0(0)	4(16.7)	
intermidiate	0(0)	1(4.2)	

79
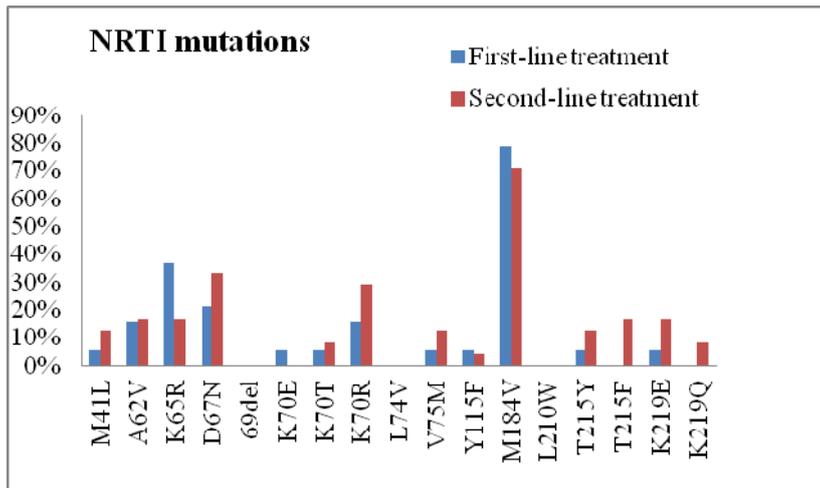
Figure 3.8 HIV-1 Nucleotide reverse transcriptase inhibitor (NRTI) drug resistance mutation frequencies in the reverse transcriptase gene of 19 subjects failing first-line therapy and 24 subjects failing second-line therapy**.**

NRTI Drug resistance
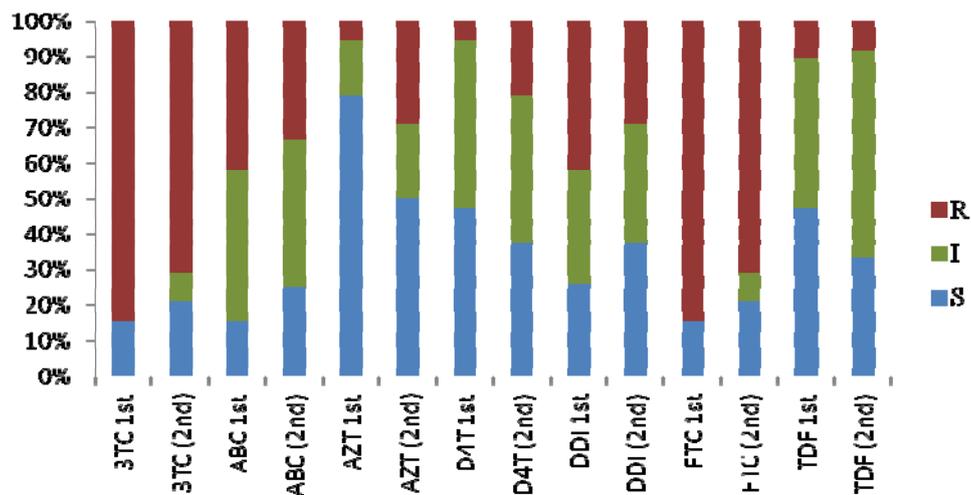
Figure 3.9 shows a Stanford University HIV-1 drug resistance database translation of the NRTI drug resistance pattern found in first-line (n=19) and second-line therapy group (n=24). R= Resistance, I=

80

Intermediate Resistance and S= Susceptible. First-line drugs abbreviated with suffix 1st; Second-line drugs abbreviated with suffix (2nd).
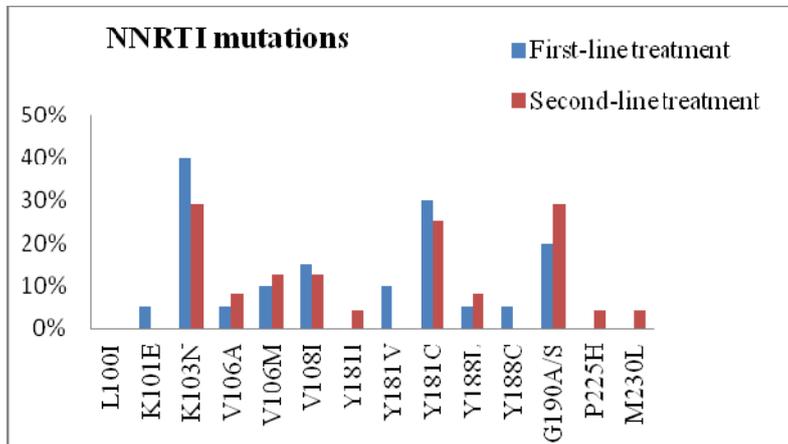
Figure 3.10; HIV-1 NNRTI drug resistance mutation frequencies in the reverse transcriptase gene in 19 subjects failing first-line therapy and 24 subjects failing second-line therapy**.**

NNRTI drug resistance
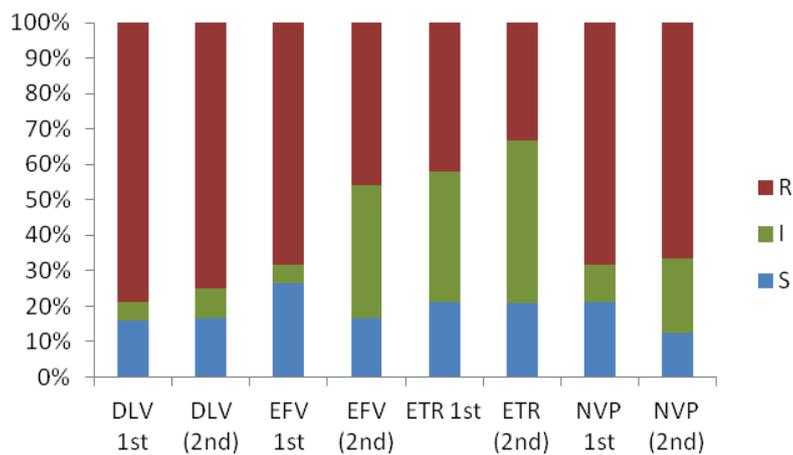
Figure 3.11; shows a Stanford University HIV-1 drug resistance database translation of the NNRTI drug resistance pattern found between the first-line (n=19) and second-line therapy group (n=24). R=

Resistance, I= Intermediate Resistance and S= Susceptible. Second-line drugs were differentiated from first-line with the number (2) at the end of the drug abbreviation.
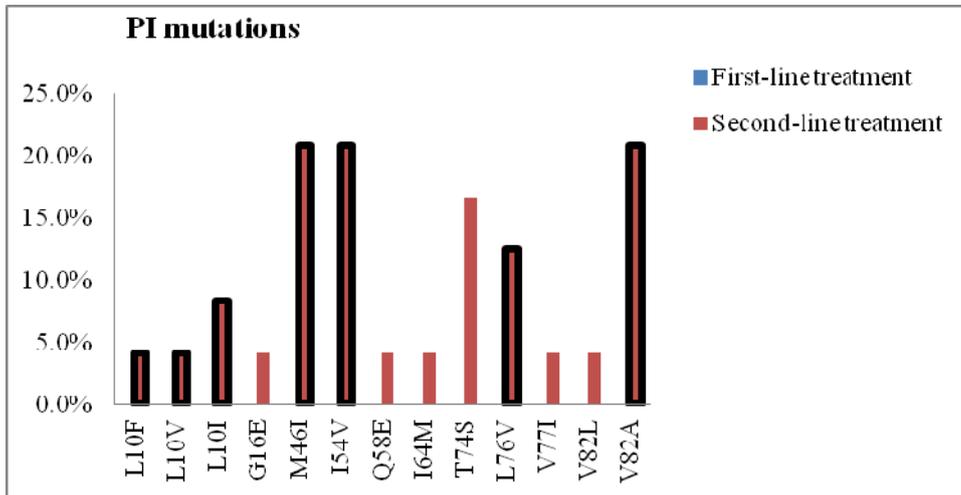
Figure 3.12; HIV-1 protease inhibitor (PI) drug resistance mutation frequencies in n=19 first-line group and n=24 second-line group. All Kaletra drug resistance associated mutations are shown in thick bolder line on the bar graph.

PI Drug resistance
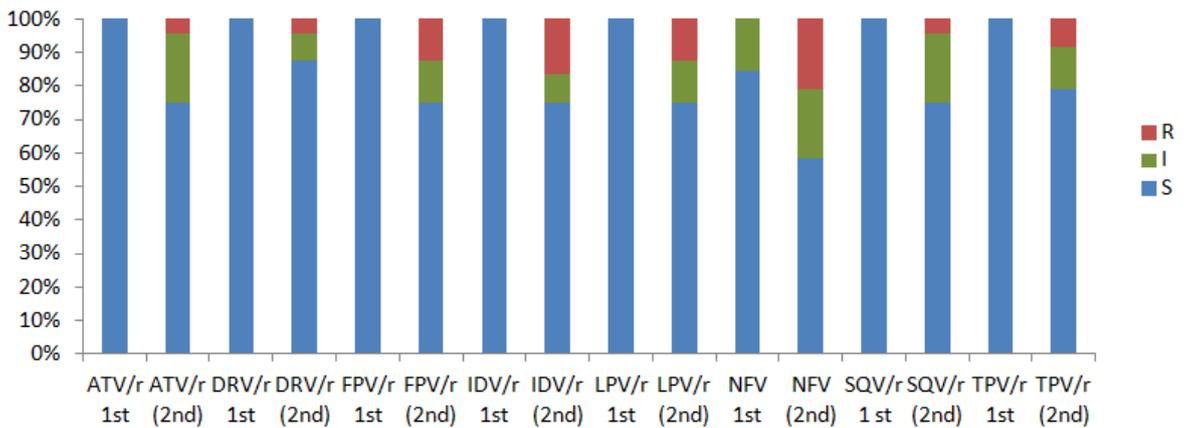
Figure 3.13; shows a Stanford University HIV-1 drug resistance database translation of the PI drug resistance pattern found in first-line group (n=19) and second-line therapy group (n=24). R= Resistance, I= Intermediate Resistance and S= Susceptible. First-line drugs abbreviated with suffix 1st; Second-line drugs abbreviated with suffix (2nd)

CHAPTER 4: DISCUSSION

Assay validation

The in-house genotyping assay we setup was validated in 3 phases to ensure that the quality of the results was not compromised before it could be adopted for routine clinical testing for ART patients.

In the first phase, we compared the assay with an FDA approved ViroSeq HIV-1 Genotyping kit. The University Teaching Hospital (UTH) in Zambia provided us with seven previously "Viroseq" genotyped samples for parallel testing. As is shown in the results section in Tables 3.1 and 3.2, the in-house assay was able to amplify and genotype the *pol* gene of all the seven samples. We compared the degree of similarly for each result between the laboratories using a hamming distance algorithm,[197]. The least comparable sample of the in-house assay sequences had 99.4% homology compared with the corresponding samples of the Viroseq sequence. Some studies have considered a hamming distance of ≥98% as acceptable,[184]. All corresponding sample sequences translated a 99.4% amino acid similarity.

A phylogenetic tree of all the samples was then generated using Bioedit software as described in the materials and method section. The software generated the bootstrap trees using Kimura 2-parameter on the program DNADIST using a transition/transversion ratio of 1.5, which is a model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences[198]. All

corresponding samples had the same topology (comparing > 99%), indicative of their similarity.

After establishing that the in-house assay tested as well as the Viroseq, in the second phase we selected five random samples and repeatedly genotyped them at three different time points to determine the reproducibility of the assay. The least comparable sample had a hamming distance of 99.5% showing the assay was able to reproduce tests. All corresponding sample sequences translated a 100% amino acid similarity. Phylogenetic trees were generated as discussed above and all samples showed the same topology (> 99% similarity).

Lastly, we participated in the National Institute of Health (NIH) Virology Quality Assurance (VQA) program, USA, indentify code; CIDRZ 332, for external quality assurance. We genotyped and submitted consensuses files of 3 five sample panels and our sequences showed a very high homology with other partaking sites in the panel and were scored acceptable (C) for each panel. This assay was adapted and modified from a published assay which performed similarly in the VQA program [184].

HIV-1 mutation pattern

The use of combination antiretroviral therapy has drastically changed the notion that HIV-1 infection is a death sentence by significantly altering the disease course and substantially reducing the morbidity and motility rates [4-6]. The major challenge however as was previously mentioned is the emergence of drug resistant HIV-1 strains and antiretroviral agents being the major contributory cause of treatment failure [199, 200]. A further complication is that HIV-1 drug resistance (HIVDR) mutations have been shown to be transmissible posing the threat of HIVDR in treatment naïve [201-203].

The current cross-sectional study observed a selection of antiretroviral mutations (ARMs) that might result, in HIV-1, during first and second-line HAART virological failure. This study reported a 93% HIV-1C subtype which confirmed previous studies that reported it to be the most prevalent in Zambia [189]. Patients in this study had a wide drug combination variation during both the first-line and second-line drug regimen. The drug combination however followed the recommendation by the WHO for the use of a 2 NRTIs backbone plus one NNRTI during first-line or a 2 NRTIs backbone plus a PI during second-line.

PI Resistance

The main difference between the first-line regimen group and the second-line regimen group was that the NNRTIs drugs were replaced with Lopinavir/Ritonavir. We reported insignificant Lopinavir/Ritonavir drug related mutations in the second-line regimen group and completely none in the first-line regimen experienced group. This confirmed

the hypothesis of this study and of other similar studies that have reported insignificant DRM during HAART which contained Lopinavir/Ritonavir in the regimen [164-166, 204]. Several studies of HIV-1 subtype B in patients experiencing virologic failure after treatment with Lopinavir/Ritonavir have infrequently detected major protease mutations.[164, 205]. However, HIV-1 Subtype C (95%) was most predominant in this study and not many studies have assessed the known drug resistance mutations in patients failing second-line ART or the treatment options that remains. One study by Wallis et al, [204] in which ninety six percent of patients in the study were infected with HIV-1 subtype C, the researcher reported major protease drug resistance mutation following virologic failure with Lopinavir/Ritonavir containing HAART.

According to the latest International antiviral Society (IAS)-USA update of drug resistance mutation in HIV-1, the major drug resistance mutations for Lopinavir/Ritonavir include V32I, I47V/A, L76V and V82A/F/T/S [183, 206]. Genotypic data analysis of the 24 patients on second-line regimen reviewed that, only five patients had Lopinavir/Ritonavir drug resistance associated mutations. These included minor mutations M46I, I54V, L10F/I/V and major mutations L76V and V82A. Mutations M46I, I54V and V82A were present on all the fives sample, *P=0.056* while L76V was present in three *P=0.242*. All the five samples were accompanied with some form of insignificantly present mutations L10F/I/V.

The mutations M46I, I54V and V82A (P=0.0056) presented us with questions for their presence. Some studies have shown that these mutations commonly occur together in previously, PI exposed patients that are failing Lopinavir/Ritonavir containing HAART [206, 207]. It is possible that we observed these mutations in the five patients because

their previous exposures to other PIs were not indicated in their history files. It is not uncommon for patients in Zambia to withhold treatment information as they seek alternative options at different hospitals.

According to the patient's treatment history, patients had been second-line regimen for a median of 52 weeks following regimen switch from first-line regimen. As was described before, WHO recommended NRTI/boosted lopinavir combination therapy have high genetic barrier requiring patients to be on treatment for more than 52 weeks before a significant selection of mutations that confer resistance [164-166]. While the results of patients on the first-line regimen that had virological failure (median CD4, 149 cells/mm^3 and VL 4.3 x 10^4 copies/mL) correlated with the drug resistance pattern in this study (at least 85% had developed greater the 1 drug resistance mutation), PI results for the second-line group did not. Less than 5% of patients on second-line regimen had major or minor PI resistance mutations despite all having virological failure (median CD4 91 cells/mm^3 and VL 4.45 x 10^4 copies/mL). One possible explanation could be that the study might have selected for patients that were not adhering to therapy which resulted in failure to suppress viral replication [10]. We designed the study to include only patients that had virological failure and excluded patients on a similar regimen in which treatment succeeded (VL <1000 copies/mL). One study recently hypothesized that patients were not adherent during second-line therapy due to PI drug severe toxicity [208].

 Another explanation could be that other mutations in the HIV genome could reduce PI susceptibility. Recent studies have indicated that mutations within the *gag* gene may reduce PI susceptibility. This could be another reason we observed virological failure

in the second-line group but insignificant PI drug resistance associated mutations. [209-211].

Reverse Transcriptase inhibitors drug resistance.

During the study, a total of at least eighteen, twelve, six and four patients had a drug history of having taken 3TC, D4T, AZT and FTC respectively in their NRTI backbone during first-line therapy. Nineteen and four out of the 20 patients had at least a history of NVP and EFV respectively. Similarly, twenty-two, twelve, nine, six, three and two patients had a drug history of 3TC, AZT, D4T, TDF, FTC and ABC respectively before initiation of second-line therapy. There was no statistical difference between drug combinations in the first-line therapy group to those taken by patients in the second-line therapy group before they were switched to second-line therapy. Therefore one could hypothesize that patients in second-line therapy had similar genotypic mutation patterns as those we observed in the first-line therapy group. This being the case, patients failing first-line therapy have a limited second-line drug treatment options as only AZT had a drug susceptibility of greater that 50% at the point first-line failure.

NRTI drug resistance

The most prevalent NRTIs antiretroviral mutations (ARMs) observed in this study were M184V (79.5%), D67N (21.1%), K65R (37%), K70R (16.7%), for the first-line therapy group and M184V (71%), D67N (33%), K65R (17%), and K70R (29%) for the second-line group. The results were comparative in a similar study conducted in South Africa in which they reported M184V (64%), D67N (20%), and K70R (16%), as the most prevalent mutations after failure of first-line regimen [212]. The South African study however reported a significantly lower K65R mutation (2.6%) P=0.003 when

compared with our data. This could be attributed the fact that none of the patients had TDF in the drug combination which was used by at least 60.4% of patients in their drug combination for this study. TDF and to a lesser extent ABC have been reported to have a M184/K65R pathway when used in combination with 3TC or FTC [213, 214]. We observed a total of 25% of patients in the study that selected for the K65R mutation in the RT gene. 90% of these patients had a history of TDF while 9% had a history of ABC regime.

The other mutation D67N, K70R, T215F/Y and T219Q are preferably selected by the thymidine analogue. About 79% of patients in this study had a history of either AZT or D4T or both. 27.9% selected for at least $\geq$ 1 thymidine analogue mutation (TAM). 67% of these had a history of AZT and 25% D4T. TAMs have been reported to take two pathways, either TAM1 or TAM2, as was reviewed earlier. When we compared TAMs with one study that was carried out in South Africa by Carole Wallis at el. [190], we noticed a similar ARM pattern for D67N (24.5%), K70R (16.5%), T215Y (15.5%), K219E/Q (14.5%), L210W (7.5%) and T215F (7.5%). In the same study, M184V (72%) were found to be the most common NRTI ARM [190]. This study did not allow us to categorize these pathways as all genotypic results were carried out at one time point and patients had both a known and unknown drug switch history between and across ART classes as we had no control on their ART regimen.

The high prevalence of the M184V mutation in our study could be explained by the presence of 3TC or FTC as a part of the NRTI backbone for triple therapy in all of the patients during first-line therapy. All groups (first and second-line therapy) in this study had up to 95% of 3TC incorporated in the HAART drug combination, while the first-

line group had 21% FTC in their combination therapy and 17% for the second-line during first-line HAART. Similar with Carole Wallis at el, [190] in which all patients had a history of 3TC in all drug regime formation during first-line therapy, our study observed that both groups had a prevalence of 95% 3TC and 21 % FTC.   This mutation has been reported to be selected during 3TC or FTC containing regimes in many other studies and is probably the earliest to emerge during HAART [174-176].

As was reviewed earlier, RT gene mutations M184V, D67N, K70R and K65N have been hypothesized to modify the RT enzyme at the active site in a way that results in a conformational change which selectively rejects thymidine analogues in the pyrophosphorolysis dependant mechanism [215].

AZT had the lowest drug resistance of about 21% and 50% (Resistance plus intermediate resistance) for first and second-line therapy respectively. No significant differences were observed in the second-line therapy group between AZT and 3TC or the other drugs compared.

NNRTI resistance

The most prevalent mutations in the study for NNRTIs were K103N, Y181C and G190A/S. Their prevalence was 40%, 30% and 20% respectively during first-line therapy and 29%, 30%, and 29% respectively during second-line therapy. Mutation YI81C is known to result in drug resistance HIV-1 viruses in all currently approved NNRTIs, while, mutations K103N and G190A/S are known to cause resistance to NVP and EFV. These genotypic mutations are comprised of about 90% of the NNRTI major drug resistance mutations selected during first-line therapy in our study. These findings

confirm previous studies that support the limited use of NNRTIs during second-line therapy if they were used in first-line therapy [216, 217]. A similar study by Sungkanuparph et al. more than 90% patients had drug resistance mutations contributing to NNRTIs resistance following a two year cohort of a fixed-dose combination of stavudine, lamivudine, and nevirapine [218]. Our observations were also similar with another study that reported K103N (38%), Y181C (26%), and G190A (22%) for patients failing first-line therapy [190].

Our study also confirmed other studies that reported a persistence of NNRTI drug resistance mutation during second-line ART despite the drugs not being used [204, 208]. The mechanism by which the K103N, G190A/S and Y181C mutations in the RT gene result in NNRTI inhibition though not certain, have been hypothesized. K103N is suggested to result in NNRTI resistance by blocking drug entry into the RT binding pocket. Studies that have compared the structures of unligated wild type RT with the K103N mutant RT have shown that the mutant structure has hydrogen bonds that are not present in the wild-type; these lead to a conformational change that resembles a closed structural form of the HIV-1 wild-type RT binding pocket [219]. This mutation is known to reduce the susceptibility of EFV and NVP by ~20-fold and ~50-fold respectively but still remains fully susceptible to the second generation NNRTIs, etravirine and Rilpivirin [220, 221].

HIV-1 RT gene mutation Y181C and Y188C in contrast to K103N have been hypothesized to result in drug resistance by causing the loss of an aromatic ring with the inhibitor binding pocket, which is needed for most first generation inhibitors such

as NVP and EFV [222]. This mutation is known to cause drug resistance in all current FDA approved NNRTIs.

Lastly, mutations of glycine to glutamic acid or glutamine at position 190 of the HIV-1 RT has been hypothesized to create a steric bulk within the NNRTI binding site via extensive van der waal interaction residues, leading to a conformational change within the pocket preventing inhibitor binding [223]. The mutation does not cause a reduction in second-generation NNRTIs (ETR and Rilpivirin) susceptibility [183].

Figure 4.2 below shows a Stanford University HIV-1 drug resistance translation of the mutation pattern found between the first and second-line therapy group. AZT had the lowest drug resistance of about 21% and 50% (Resistance plus intermediate resistance) for first and second-line therapy respectively.

Conclusion

This study demonstrated that the previously published affordable "in-house" genotyping assay, effective for genotyping non-subtype B HIV-1[184], could be used as an alternative to the more expensive commercial for kits in Zambia. The assay costs a third of the cost of commercial kits and was designed to effectively genotype HIV-1C which predominate the Zambian population.

The study also gives insight into the selection of the Reverse transcriptase and Protease gene mutations by NRTI/NNRTI or NRTI/PI during routine care in Zambia. Major PI mutations were insignificant in patients exclusively treated with Lopinavir/Ritonavir as the only PI during second-line HAART. The use of most of the FDA approved PI

would therefore be good for subsequent treatment. While RT mutations correlated with virological results and could easily be used to predict treatment failure, PI mutations surprisingly did not correlate with patient virological failure seen in study population. Either other genes that could reduce PI susceptibility such as gag are responsible for drug failure, or patient are not adhering to medication during second-line therapy as a result of enhanced drug toxicity. Including the gag gene as a part of PI drug resistance testing in addition to genotyping the Protease gene could provide additional information about PI resistance. It is possible that the Lopinavir/Ritonavir genetic barrier is lower than most clinical trials have reported considering that most trials excluded gag during genotyping. But again, poor drug adherence could be the cause for the virological failure in this study population.

Lastly, the study reviews that patients have a limited NRTI back bone for use during either second line or third line therapy. We recommend a drug resistance testing to determine NRTIs to be used subsequent to first-line ART failure in Zambia.   AZT, D4T and TDF respectively remain the most effect NRTIs while 3TC and FTC the least effective.

Alternatively, boosted PIs could be used in combination with integrase inhibitors such as Raltegravir where a drug resistance testing is not available although comparative data will be needed.

REFERENCES

1. Kilmarx, P.H., *Global epidemiology of HIV.* Curr Opin HIV AIDS, 2009. **4**(4): p. 240-6.
2. http://www.who.int/hiv/topics/treatment/data/en/index1.html.
3. Aberg, J.A., et al., *Primary care guidelines for the management of persons infected with human immunodeficiency virus: 2009 update by the HIV medicine Association of the Infectious Diseases Society of America.* Clin Infect Dis, 2009. **49**(5): p. 651-81.
4. Perelson, A.S., et al., *Decay characteristics of HIV-1-infected compartments during combination therapy.* Nature, 1997. **387**(6629): p. 188-91.
5. Carpenter, C.C., et al., *Antiretroviral therapy for HIV infection in 1998: updated recommendations of the International AIDS Society-USA Panel.* JAMA, 1998. **280**(1): p. 78-86.
6. Gulick, R.M., et al., *Treatment with indinavir, zidovudine, and lamivudine in adults with human immunodeficiency virus infection and prior antiretroviral therapy.* N Engl J Med, 1997. **337**(11): p. 734-9.
7. UNAIDS, *Progress report summary 2011.* 2011(Accessed 22 December 2011): p. Available   from: <http://www.who.int/hiv/pub/progress_report2011/summary_en.pdf>.
8. Shafer, R.W., et al., *Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated patients.* Ann Intern Med, 1998. **128**(11): p. 906-11.
9. Patick, A.K., et al., *Genotypic and phenotypic characterization of human immunodeficiency virus type 1 variants isolated from patients treated with the protease inhibitor nelfinavir.* Antimicrob Agents Chemother, 1998. **42**(10): p. 2637-44.
10. Fatkenheuer, G., et al., *Virological treatment failure of protease inhibitor therapy in an unselected cohort of HIV-infected patients.* AIDS, 1997. **11**(14): p. F113-6.
11. Deeks, S.G., et al., *HIV RNA and CD4 cell count response to protease inhibitor therapy in an urban AIDS clinic: response to both initial and salvage therapy.* AIDS, 1999. **13**(6): p. F35-43.
12. Kaufmann, G.R., et al., *Treatment response and durability of a double protease inhibitor therapy with saquinavir and ritonavir in an observational cohort of HIV-1-infected individuals.* AIDS, 1998. **12**(13): p. 1625-30.
13. Hirsch, M.S., et al., *Antiretroviral drug resistance testing in adult HIV-1 infection: 2008 recommendations of an International AIDS Society-USA panel.* Top HIV Med, 2008. **16**(3): p. 266-85.
14. Ledesma, E. and V. Soriano, *Spanish consensus conference on drug resistance testing in clinical practice. HIV drug resistance Spanish panel.* AIDS, 1999. **13**(14): p. 1998-2001.
15. Johnson, V.A., et al., *Update of the drug resistance mutations in HIV-1: December 2010.* Top HIV Med. **18**(5): p. 156-63.
16. Gallo, R.C., et al., *Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS.* Science, 1984. **224**(4648): p. 500-3.
17. Chermann, J.C., et al., *Isolation of a new retrovirus in a patient at risk for acquired immunodeficiency syndrome.* Antibiot Chemother, 1983. **32**: p. 48-53.

18.   *Global. Researchers push African approach to prevent HIV spread.* AIDS Policy Law, 2007. **22**(2): p. 2.

19.   Dalgleish, A.G., *Heterosexual spread of HIV.* Dis Markers, 1990. **8**(1): p. 1-3.

20.   Hospedales, J., *Heterosexual spread of HIV infection.* Rev Infect Dis, 1989. **11**(4): p. 663-5.

21.   Sattenspiel, L., et al., *The effects of population structure on the spread of the HIV infection.* Am J Phys Anthropol, 1990. **82**(4): p. 421-9.

22.   Letvin, N.L., *Animal models for AIDS.* Immunol Today, 1990. **11**(9): p. 322-6.

23.   Nermut, M.V., et al., *Further evidence of icosahedral symmetry in human and simian immunodeficiency virus.* AIDS Res Hum Retroviruses, 1993. **9**(10): p. 929-38.

24.   Robertson, D.L., et al., *HIV-1 nomenclature proposal.* Science, 2000. **288**(5463): p. 55-6.

25.   Hemelaar, J., et al., *Global and regional distribution of HIV-1 genetic subtypes and recombinants in 2004.* AIDS, 2006. **20**(16): p. W13-23.

26.   Burke, D.S., *Recombination in HIV: an important viral evolutionary strategy.* Emerg Infect Dis, 1997. **3**(3): p. 253-9.

27.   Arens, M., *Methods for subtyping and molecular comparison of human viral genomes.* Clin Microbiol Rev, 1999. **12**(4): p. 612-26.

28.   Pasquier, C., et al., *HIV-1 subtyping using phylogenetic analysis of pol gene sequences.* J Virol Methods, 2001. **94**(1-2): p. 45-54.

29.   Cardinaud, S., et al., *Identification of cryptic MHC I-restricted epitopes encoded by HIV-1 alternative reading frames.* J Exp Med, 2004. **199**(8): p. 1053-63.

30.   Andreassen, H., et al., *Analysis of the secondary structure of the human immunodeficiency virus (HIV) proteins p17, gp120, and gp41 by computer modeling based on neural network methods.* J Acquir Immune Defic Syndr, 1990. **3**(6): p. 615-22.

31.   Rosen, C.A. and G.N. Pavlakis, *Tat and Rev: positive regulators of HIV gene expression.* AIDS, 1990. **4**(6): p. 499-509.

32.   Henriet, S., et al., *Vif is a RNA chaperone that could temporally regulate RNA dimerization and the early steps of HIV-1 reverse transcription.* Nucleic Acids Res, 2007. **35**(15): p. 5141-53.

33.   Connor, R.I., et al., *Vpr is required for efficient replication of human immunodeficiency virus type-1 in mononuclear phagocytes.* Virology, 1995. **206**(2): p. 935-44.

34.   Miyagi, E., et al., *Vpu enhances HIV-1 virus release in the absence of Bst-2 cell surface down-modulation and intracellular depletion.* Proc Natl Acad Sci U S A, 2009. **106**(8): p. 2868-73.

35.   Frankel, A.D. and J.A. Young, *HIV-1: fifteen proteins and an RNA.* Annu Rev Biochem, 1998. **67**: p. 1-25.

36.   Institute, H., *Human Immunodeficiency Virus and HIV Disease.* 2001(Accessed on 15 April 2010): p. available from http://uhavax.hartford.edu/bugl/hiv.htm.

37.   Luban, J., *The HIV-1 genome.* 2010(accessed on 15 June 2011): p. available from http://www.mimo.unige.ch/luban_lab/research.htm.

38.   Dalgleish, A.G., et al., *The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus.* Nature, 1984. **312**(5996): p. 763-7.

39.   Klatzmann, D., et al., *T-lymphocyte T4 molecule behaves as the receptor for human retrovirus LAV.* Nature, 1984. **312**(5996): p. 767-8.

40.   Clapham, P.R. and R.A. Weiss, *Immunodeficiency viruses. Spoilt for choice of co-receptors.* Nature, 1997. **388**(6639): p. 230-1.

41. Doranz, B.J., et al., *A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors.* Cell, 1996. **85**(7): p. 1149-58.

42. Moore, J.P., *Coreceptors: implications for HIV pathogenesis and therapy.* Science, 1997. **276**(5309): p. 51-2.

43. Chan, D.C. and P.S. Kim, *HIV entry and its inhibition.* Cell, 1998. **93**(5): p. 681-4.

44. Zhu, T., et al., *Genotypic and phenotypic characterization of HIV-1 patients with primary infection.* Science, 1993. **261**(5125): p. 1179-81.

45. Moore, J.P., A. Trkola, and T. Dragic, *Co-receptors for HIV-1 entry.* Curr Opin Immunol, 1997. **9**(4): p. 551-62.

46. Scherer, L., J.J. Rossi, and M.S. Weinberg, *Progress and prospects: RNA-based therapies for treatment of HIV infection.* Gene Ther, 2007. **14**(14): p. 1057-64.

47. Cen, S., et al., *Incorporation of lysyl-tRNA synthetase into human immunodeficiency virus type 1.* J Virol, 2001. **75**(11): p. 5043-8.

48. Cohen, E.A., R.A. Subbramanian, and H.G. Gottlinger, *Role of auxiliary proteins in retroviral morphogenesis.* Curr Top Microbiol Immunol, 1996. **214**: p. 219-35.

49. Fouchier, R.A., et al., *Human immunodeficiency virus type 1 Vif does not influence expression or virion incorporation of gag-, pol-, and env-encoded proteins.* J Virol, 1996. **70**(12): p. 8263-9.

50. Drake, J.W., *Rates of spontaneous mutation among RNA viruses.* Proc Natl Acad Sci U S A, 1993. **90**(9): p. 4171-5.

51. Domingo, E., et al., *Nucleotide sequence heterogeneity of an RNA phage population.* Cell, 1978. **13**(4): p. 735-44.

52. Wain-Hobson, S., *Running the gamut of retroviral variation.* Trends Microbiol, 1996. **4**(4): p. 135-41.

53. Mansky, L.M. and H.M. Temin, *Lower in vivo mutation rate of human immunodeficiency virus type 1 than that predicted from the fidelity of purified reverse transcriptase.* J Virol, 1995. **69**(8): p. 5087-94.

54. Mansky, L.M., *The mutation rate of human immunodeficiency virus type 1 is influenced by the vpr gene.* Virology, 1996. **222**(2): p. 391-400.

55. Zhang, S., et al., *Cytoplasmic retention of HIV-1 regulatory protein Vpr by protein-protein interaction with a novel human cytoplasmic protein VprBP.* Gene, 2001. **263**(1-2): p. 131-40.

56. Miller, R.H. and N. Sarver, *HIV accessory proteins as therapeutic targets.* Nat Med, 1997. **3**(4): p. 389-94.

57. Cushman, M. and P. Sherman, *Inhibition of HIV-1 integration protein by aurintricarboxylic acid monomers, monomer analogs, and polymer fractions.* Biochem Biophys Res Commun, 1992. **185**(1): p. 85-90.

58. Fesen, M.R., et al., *Inhibitors of human immunodeficiency virus integrase.* Proc Natl Acad Sci U S A, 1993. **90**(6): p. 2399-403.

59. Carteau, S., et al., *Inhibitory effect of the polyanionic drug suramin on the in vitro HIV DNA integration reaction.* Arch Biochem Biophys, 1993. **305**(2): p. 606-10.

60. Brainard, D.M., et al., *Clinical Pharmacology Profile of Raltegravir, an HIV-1 Integrase Strand Transfer Inhibitor.* J Clin Pharmacol.

61. Brass, A.L., et al., *Identification of host proteins required for HIV infection through a functional genomic screen.* Science, 2008. **319**(5865): p. 921-6.

62. Parada, C.A. and R.G. Roeder, *Enhanced processivity of RNA polymerase II triggered by Tat-induced phosphorylation of its carboxy-terminal domain.* Nature, 1996. **384**(6607): p. 375-8.

63.   Hope, T.J., et al., *Effector domains of human immunodeficiency virus type 1 Rev and human T-cell leukemia virus type I Rex are functionally interchangeable and share an essential peptide motif.* J Virol, 1991. **65**(11): p. 6001-7.

64.   Chang, D.D. and P.A. Sharp, *Regulation by HIV Rev depends upon recognition of splice sites.* Cell, 1989. **59**(5): p. 789-95.

65.   Emerman, M., R. Vazeux, and K. Peden, *The rev gene product of the human immunodeficiency virus affects envelope-specific RNA localization.* Cell, 1989. **57**(7): p. 1155-65.

66.   Nakielny, S. and G. Dreyfuss, *Nuclear export of proteins and RNAs.* Curr Opin Cell Biol, 1997. **9**(3): p. 420-9.

67.   Nakielny, S., et al., *RNA transport.* Annu Rev Neurosci, 1997. **20**: p. 269-301.

68.   Emerman, M. and M.H. Malim, *HIV-1 regulatory/accessory genes: keys to unraveling viral and host cell biology.* Science, 1998. **280**(5371): p. 1880-4.

69.   Asparuhova, M.B., et al., *Inhibition of HIV-1 multiplication by a modified U7 snRNA inducing Tat and Rev exon skipping.* J Gene Med, 2007. **9**(5): p. 323-34.

70.   Wills, J.W. and R.C. Craven, *Form, function, and use of retroviral gag proteins.* AIDS, 1991. **5**(6): p. 639-54.

71.   Vogt, V.M., *Retroviral Virions and Genomes.* 1997.

72.   Freed, E.O., *HIV-1 gag proteins: diverse functions in the virus life cycle.* Virology, 1998. **251**(1): p. 1-15.

73.   Tritel, M. and M.D. Resh, *The late stage of human immunodeficiency virus type 1 assembly is an energy-dependent process.* J Virol, 2001. **75**(12): p. 5473-81.

74.   Barini, O., *[Antiretroviral drugs].* Rev Assoc Med Bras, 2001. **47**(3): p. 195-6.

75.   Daar, E.S., et al., *Transient high levels of viremia in patients with primary human immunodeficiency virus type 1 infection.* N Engl J Med, 1991. **324**(14): p. 961-4.

76.   Johnston, M.I. and A.S. Fauci, *An HIV vaccine--evolving concepts.* N Engl J Med, 2007. **356**(20): p. 2073-81.

77.   Salazar-Gonzalez, J.F., et al., *Genetic identity, biological phenotype, and evolutionary pathways of transmitted/founder viruses in acute and early HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1273-89.

78.   Veazey, R.S., P.A. Marx, and A.A. Lackner, *Vaginal CD4+ T cells express high levels of CCR5 and are rapidly depleted in simian immunodeficiency virus infection.* J Infect Dis, 2003. **187**(5): p. 769-76.

79.   Kassutto, S. and E.S. Rosenberg, *Primary HIV type 1 infection.* Clin Infect Dis, 2004. **38**(10): p. 1447-53.

80.   Schacker, T., et al., *Clinical and epidemiologic features of primary HIV infection.* Ann Intern Med, 1996. **125**(4): p. 257-64.

81.   Keele, B.F., et al., *Characterization of the follicular dendritic cell reservoir of human immunodeficiency virus type 1.* J Virol, 2008. **82**(11): p. 5548-61.

82.   Geijtenbeek, T.B., et al., *DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells.* Cell, 2000. **100**(5): p. 587-97.

83.   Harris, R.S., et al., *DNA deamination mediates innate immunity to retroviral infection.* Cell, 2003. **113**(6): p. 803-9.

84.   Li, Q., et al., *Peak SIV replication in resting memory CD4+ T cells depletes gut lamina propria CD4+ T cells.* Nature, 2005. **434**(7037): p. 1148-52.

85.   Goonetilleke, N., et al., *The first T cell response to transmitted/founder virus contributes to the control of acute viremia in HIV-1 infection.* J Exp Med, 2009. **206**(6): p. 1253-72.

86.   Lehmann-Grube, F., et al., *Mechanism of recovery from acute virus infection. I. Role of T lymphocytes in the clearance of lymphocytic choriomeningitis virus from spleens of mice.* J Immunol, 1985. **134**(1): p. 608-15.

87.   Wagner, L., et al., *Beta-chemokines are released from HIV-1-specific cytolytic T-cell granules complexed to proteoglycans.* Nature, 1998. **391**(6670): p. 908-11.

88.   Zajac, A.J., et al., *Viral immune evasion due to persistence of activated T cells without effector function.* J Exp Med, 1998. **188**(12): p. 2205-13.

89.   Kalams, S.A. and B.D. Walker, *The critical need for CD4 help in maintaining effective cytotoxic T lymphocyte responses.* J Exp Med, 1998. **188**(12): p. 2199-204.

90.   Phillips, R.E., et al., *Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition.* Nature, 1991. **354**(6353): p. 453-9.

91.   Buchbinder, S.P., et al., *Long-term HIV-1 infection without immunologic progression.* AIDS, 1994. **8**(8): p. 1123-8.

92.   Lifson, A.R., et al., *Long-term human immunodeficiency virus infection in asymptomatic homosexual and bisexual men with normal CD4+ lymphocyte counts: immunologic and virologic characteristics.* J Infect Dis, 1991. **163**(5): p. 959-65.

93.   Pantaleo, G., et al., *HIV infection is active and progressive in lymphoid tissue during the clinically latent stage of disease.* Nature, 1993. **362**(6418): p. 355-8.

94.   Johnson, N. and J.M. Parkin, *Dysregulation of the interleukin-2 receptor alpha- and beta-chain expression in CD4 and CD8 T cells in HIV infection.* Cytometry, 1997. **30**(6): p. 289-95.

95.   Liu, Z., et al., *Elevated CD38 antigen expression on CD8+ T cells is a stronger marker for the risk of chronic HIV disease progression to AIDS and death in the Multicenter AIDS Cohort Study than CD4+ cell count, soluble immune activation markers, or combinations of HLA-DR and CD38 expression.* J Acquir Immune Defic Syndr Hum Retrovirol, 1997. **16**(2): p. 83-92.

96.   Glencross, D.K., et al., *CD8/CD38 activation yields important clinical information of effective antiretroviral therapy: findings from the first year of the CIPRA-SA cohort.* Cytometry B Clin Cytom, 2008. **74 Suppl 1**: p. S131-40.

97.   Silvestri, G., et al., *Nonpathogenic SIV infection of sooty mangabeys is characterized by limited bystander immunopathology despite chronic high-level viremia.* Immunity, 2003. **18**(3): p. 441-52.

98.   Gasper-Smith, N., et al., *Induction of plasma (TRAIL), TNFR-2, Fas ligand, and plasma microparticles after human immunodeficiency virus type 1 (HIV-1) transmission: implications for HIV-1 vaccine design.* J Virol, 2008. **82**(15): p. 7700-10.

99.   Boasso, A. and G.M. Shearer, *Chronic innate immune activation as a cause of HIV-1 immunopathogenesis.* Clin Immunol, 2008. **126**(3): p. 235-42.

100.   Douek, D.C., L.J. Picker, and R.A. Koup, *T cell dynamics in HIV-1 infection.* Annu Rev Immunol, 2003. **21**: p. 265-304.

101.   Hogg, R.S., et al., *Emergence of drug resistance is associated with an increased risk of death among patients first starting HAART.* PLoS Med, 2006. **3**(9): p. e356.

102.   Wu, T.D., et al., *Mutation patterns and structural correlates in human immunodeficiency virus type 1 protease following different protease inhibitor treatments.* J Virol, 2003. **77**(8): p. 4836-47.

103.   Lima, V.D., et al., *Differential impact of adherence on long-term treatment response among naive HIV-infected individuals.* AIDS, 2008. **22**(17): p. 2371-80.

104.   Hirsch, M.S., et al., *Antiretroviral drug resistance testing in adults infected with human immunodeficiency virus type 1: 2003 recommendations of an International AIDS Society-USA Panel.* Clin Infect Dis, 2003. **37**(1): p. 113-28.

105. Bonhoeffer, S., E.C. Holmes, and M.A. Nowak, *Causes of HIV diversity.* Nature, 1995. **376**(6536): p. 125.

106. Biebricher, C.K. and M. Eigen, *What is a quasispecies?* Curr Top Microbiol Immunol, 2006. **299**: p. 1-31.

107. van Rij, R.P., et al., *Persistence of viral HLA-DR- CD4 T-cell reservoir during prolonged treatment of HIV-1 infection with a five-drug regimen.* Antivir Ther, 2002. **7**(1): p. 37-41.

108. Steinhauer, D.A., E. Domingo, and J.J. Holland, *Lack of evidence for proofreading mechanisms associated with an RNA virus polymerase.* Gene, 1992. **122**(2): p. 281-8.

109. Joyce, C.M. and T.A. Steitz, *Function and structure relationships in DNA polymerases.* Annu Rev Biochem, 1994. **63**: p. 777-822.

110. Modrich, P. and R. Lahue, *Mismatch repair in replication fidelity, genetic recombination, and cancer biology.* Annu Rev Biochem, 1996. **65**: p. 101-33.

111. Schaaper, R.M., *Base selection, proofreading, and mismatch repair during DNA replication in Escherichia coli.* J Biol Chem, 1993. **268**(32): p. 23762-5.

112. Loeb, L.A., *Mutator phenotype may be required for multistage carcinogenesis.* Cancer Res, 1991. **51**(12): p. 3075-9.

113. Menendez-Arias, L., *Molecular basis of fidelity of DNA synthesis and nucleotide specificity of retroviral reverse transcriptases.* Prog Nucleic Acid Res Mol Biol, 2002. **71**: p. 91-147.

114. Roberts, J.D., et al., *Fidelity of two retroviral reverse transcriptases during DNA-dependent DNA synthesis in vitro.* Mol Cell Biol, 1989. **9**(2): p. 469-76.

115. Telesnitsky, A. and S.P. Goff, *Reverse Transcriptase and the Generation of Retroviral DNA.* 1997.

116. Coffin, J.M., *HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy.* Science, 1995. **267**(5197): p. 483-9.

117. Eigen, M., *Selforganization of matter and the evolution of biological macromolecules.* Naturwissenschaften, 1971. **58**(10): p. 465-523.

118. Eigen, M. and P. Schuster, *The hypercycle. A principle of natural self-organization. Part A: Emergence of the hypercycle.* Naturwissenschaften, 1977. **64**(11): p. 541-65.

119. Drosopoulos, W.C. and V.R. Prasad, *Increased misincorporation fidelity observed for nucleoside analog resistance mutations M184V and E89G in human immunodeficiency virus type 1 reverse transcriptase does not correlate with the overall error rate measured in vitro.* J Virol, 1998. **72**(5): p. 4224-30.

120. Wain-Hobson, S., *The fastest genome evolution ever described: HIV variation in situ.* Curr Opin Genet Dev, 1993. **3**(6): p. 878-83.

121. Oude Essink, B.B., N.K. Back, and B. Berkhout, *Increased polymerase fidelity of the 3TC-resistant variants of HIV-1 reverse transcriptase.* Nucleic Acids Res, 1997. **25**(16): p. 3212-7.

122. Kohlstaedt, L.A., et al., *Crystal structure at 3.5 A resolution of HIV-1 reverse transcriptase complexed with an inhibitor.* Science, 1992. **256**(5065): p. 1783-90.

123. Polesky, A.H., et al., *Identification of residues critical for the polymerase activity of the Klenow fragment of DNA polymerase I from Escherichia coli.* J Biol Chem, 1990. **265**(24): p. 14579-91.

124. Jacobo-Molina, A., et al., *Crystal structure of human immunodeficiency virus type 1 reverse transcriptase complexed with double-stranded DNA at 3.0 A resolution shows bent DNA.* Proc Natl Acad Sci U S A, 1993. **90**(13): p. 6320-4.

125. Huang, H., et al., *Structure of a covalently trapped catalytic complex of HIV-1 reverse transcriptase: implications for drug resistance.* Science, 1998. **282**(5394): p. 1669-75.

126. Shafer, R.W., *Genotypic testing for human immunodeficiency virus type 1 drug resistance.* Clin Microbiol Rev, 2002. **15**(2): p. 247-77.

127. Tantillo, C., et al., *Locations of anti-AIDS drug binding sites and resistance mutations in the three-dimensional structure of HIV-1 reverse transcriptase. Implications for mechanisms of drug inhibition and resistance.* J Mol Biol, 1994. **243**(3): p. 369-87.

128. Larder, B.A., G. Darby, and D.D. Richman, *HIV with reduced sensitivity to zidovudine (AZT) isolated during prolonged therapy.* Science, 1989. **243**(4899): p. 1731-4.

129. Kellam, P., C.A. Boucher, and B.A. Larder, *Fifth mutation in human immunodeficiency virus type 1 reverse transcriptase contributes to the development of high-level resistance to zidovudine.* Proc Natl Acad Sci U S A, 1992. **89**(5): p. 1934-8.

130. Rhee, S.Y., et al., *Human immunodeficiency virus reverse transcriptase and protease sequence database.* Nucleic Acids Res, 2003. **31**(1): p. 298-303.

131. Domaoal, R.A. and L.M. Demeter, *Structural and biochemical effects of human immunodeficiency virus mutants resistant to non-nucleoside reverse transcriptase inhibitors.* Int J Biochem Cell Biol, 2004. **36**(9): p. 1735-51.

132. Hoggard, P.G., et al., *Effects of drugs on 2',3'-dideoxy-2',3'-didehydrothymidine phosphorylation in vitro.* Antimicrob Agents Chemother, 1997. **41**(6): p. 1231-6.

133. Kewn, S., et al., *Lamivudine (3TC) phosphorylation and drug interactions in vitro.* Biochem Pharmacol, 1997. **54**(5): p. 589-95.

134. De Clercq, E., *New developments in anti-HIV chemotherapy.* Biochim Biophys Acta, 2002. **1587**(2-3): p. 258-75.

135. Himmel, D.M., et al., *Crystal structures for HIV-1 reverse transcriptase in complexes with three pyridinone derivatives: a new class of non-nucleoside inhibitors effective against a broad range of drug-resistant strains.* J Med Chem, 2005. **48**(24): p. 7582-91.

136. El Safadi, Y., V. Vivet-Boudou, and R. Marquet, *HIV-1 reverse transcriptase inhibitors.* Appl Microbiol Biotechnol, 2007. **75**(4): p. 723-37.

137. AIDSInfo, *FDA-Approved Anti-HIV Medications.* 2011(Accessed 22 December 2011): p. http://www.aidsinfo.nih.gov/ContentFiles/ApprovedMedstoTreatHIV_FS_en.pdf.

138. Kaushik, N., et al., *Role of glutamine-151 of human immunodeficiency virus type-1 reverse transcriptase in RNA-directed DNA synthesis.* Biochemistry, 1997. **36**(47): p. 14430-8.

139. Rubinek, T., et al., *The fidelity of 3' misinsertion and mispair extension during DNA synthesis exhibited by two drug-resistant mutants of the reverse transcriptase of human immunodeficiency virus type 1 with Leu74-->Val and Glu89-->Gly.* Eur J Biochem, 1997. **247**(1): p. 238-47.

140. Boyer, P.L., et al., *Sensitivity of wild-type human immunodeficiency virus type 1 reverse transcriptase to dideoxynucleotides depends on template length; the sensitivity of drug-resistant mutants does not.* Proc Natl Acad Sci U S A, 1994. **91**(11): p. 4882-6.

141. Arion, D., et al., *Phenotypic mechanism of HIV-1 resistance to 3'-azido-3'-deoxythymidine (AZT): increased polymerization processivity and enhanced sensitivity to pyrophosphate of the mutant viral reverse transcriptase.* Biochemistry, 1998. **37**(45): p. 15908-17.

142. Canard, B., S.R. Sarfati, and C.C. Richardson, *Enhanced binding of azidothymidine-resistant human immunodeficiency virus 1 reverse transcriptase to the 3'-azido-3'-deoxythymidine 5'-monophosphate-terminated primer.* J Biol Chem, 1998. **273**(23): p. 14596-604.

143. Ren, J., et al., *3'-Azido-3'-deoxythymidine drug resistance mutations in HIV-1 reverse transcriptase can induce long range conformational changes.* Proc Natl Acad Sci U S A, 1998. **95**(16): p. 9518-23.

144. Kohl, N.E., et al., *Active human immunodeficiency virus protease is required for viral infectivity.* Proc Natl Acad Sci U S A, 1988. **85**(13): p. 4686-90.

145. Oroszlan, S. and R.B. Luftig, *Retroviral proteinases.* Curr Top Microbiol Immunol, 1990. **157**: p. 153-85.

146. Kaplan, A.H., M. Manchester, and R. Swanstrom, *The activity of the protease of human immunodeficiency virus type 1 is initiated at the membrane of infected cells before the release of viral proteins and is required for release to occur with maximum efficiency.* J Virol, 1994. **68**(10): p. 6782-6.

147. Miller, M.D., B. Wang, and F.D. Bushman, *Human immunodeficiency virus type 1 preintegration complexes containing discontinuous plus strands are competent to integrate in vitro.* J Virol, 1995. **69**(6): p. 3938-44.

148. Wang, J., et al., *Structural basis of asymmetry in the human immunodeficiency virus type 1 reverse transcriptase heterodimer.* Proc Natl Acad Sci U S A, 1994. **91**(15): p. 7242-6.

149. Spinelli, S., et al., *The three-dimensional structure of the aspartyl protease from the HIV-1 isolate BRU.* Biochimie, 1991. **73**(11): p. 1391-6.

150. Dougherty, W.G. and B.L. Semler, *Expression of virus-encoded proteinases: functional and structural similarities with cellular enzymes.* Microbiol Rev, 1993. **57**(4): p. 781-822.

151. Wlodawer, A. and J.W. Erickson, *Structure-based inhibitors of HIV-1 protease.* Annu Rev Biochem, 1993. **62**: p. 543-85.

152. Gulnik, S.V., et al., *Kinetic characterization and cross-resistance patterns of HIV-1 protease mutants selected under drug pressure.* Biochemistry, 1995. **34**(29): p. 9282-7.

153. Brindeiro, P.A., et al., *Testing genotypic and phenotypic resistance in human immunodeficiency virus type 1 isolates of clade B and other clades from children failing antiretroviral therapy.* J Clin Microbiol, 2002. **40**(12): p. 4512-9.

154. Ceccherini-Silberstein, F., et al., *Identification of the minimal conserved structure of HIV-1 protease in the presence and absence of drug pressure.* AIDS, 2004. **18**(12): p. F11-9.

155. Saskova, K.G., et al., *Enzymatic and structural analysis of the I47A mutation contributing to the reduced susceptibility to HIV protease inhibitor lopinavir.* Protein Sci, 2008. **17**(9): p. 1555-64.

156. Tie, Y., et al., *Atomic resolution crystal structures of HIV-1 protease and mutants V82A and I84V with saquinavir.* Proteins, 2007. **67**(1): p. 232-42.

157. Zambia, M., *Antiretroviral therapy for chronic HIV infection in adults and adolescents. New ART protocols.* 2007 p. 44p.

158. Walmsley, S., et al., *Lopinavir-ritonavir versus nelfinavir for the initial treatment of HIV infection.* N Engl J Med, 2002. **346**(26): p. 2039-46.

159. Delfraissy, J.F., et al., *Lopinavir/ritonavir monotherapy or plus zidovudine and lamivudine in antiretroviral-naive HIV-infected patients.* AIDS, 2008. **22**(3): p. 385-93.

160. Pulido, F., et al., *Lopinavir-ritonavir monotherapy versus lopinavir-ritonavir and two nucleosides for maintenance therapy of HIV.* AIDS, 2008. **22**(2): p. F1-9.

161. de Mendoza, C., et al., *Salvage treatment with lopinavir/ritonavir (Kaletra) in HIV-infected patients failing all current antiretroviral drug families.* HIV Clin Trials, 2002. **3**(4): p. 304-9.

162. Abbott-Laboratories, *Kaletra (lopinavir and ritonavir) Capsule.* 2000. **Accessed 15 October 2011**: p. Available from: <http://dailymed.nlm.nih.gov/dailymed/archives/fdaDrugInfo.cfm?archiveid=1584>.

163. Chandwani, A. and J. Shuter, *Lopinavir/ritonavir in the treatment of HIV-1 infection: a review.* Ther Clin Risk Manag, 2008. **4**(5): p. 1023-33.

164. Murphy, R.L., et al., *Seven-year efficacy of a lopinavir/ritonavir-based regimen in antiretroviral-naive HIV-1-infected patients.* HIV Clin Trials, 2008. **9**(1): p. 1-10.

165. Saez-Llorens, X., et al., *Forty-eight-week evaluation of lopinavir/ritonavir, a new protease inhibitor, in human immunodeficiency virus-infected children.* Pediatr Infect Dis J, 2003. **22**(3): p. 216-24.

166. Kempf, D.J., et al., *Identification of genotypic changes in human immunodeficiency virus protease that correlate with reduced susceptibility to the protease inhibitor lopinavir among viral isolates from protease inhibitor-experienced patients.* J Virol, 2001. **75**(16): p. 7462-9.

167. Friend, J., et al., *Isolated lopinavir resistance after virological rebound of a ritonavir/lopinavir-based regimen.* AIDS, 2004. **18**(14): p. 1965-6.

168. Matloubian, M., R.J. Concepcion, and R. Ahmed, *CD4+ T cells are required to sustain CD8+ cytotoxic T-cell responses during chronic viral infection.* J Virol, 1994. **68**(12): p. 8056-63.

169. Luber, A.D., *Genetic barriers to resistance and impact on clinical response.* MedGenMed, 2005. **7**(3): p. 69.

170. Luber, A.D., *Genetic barriers to resistance and impact on clinical response.* J Int AIDS Soc, 2005. **7**(3): p. 69.

171. Khanlou, H., et al., *Early virologic failure in a pilot study evaluating the efficacy of therapy containing once-daily abacavir, lamivudine, and tenofovir DF in treatment-naive HIV-infected patients.* AIDS Patient Care STDS, 2005. **19**(3): p. 135-40.

172. Jemsek, J., P. Hutcherson, and E. Harper, *Poor Virologic Responses and Early Emergence of Resistance in Treatment Naive, HIV-infected Patients Receiving a Once Daily Triple Nucleoside Regimen of Didanosine, Lamivudine, and Tenofovir DF, 2004.*

. available at: <http://gateway.nlm.nih.gov/MeetingAbstracts/ma?f=102270779.html> Accessed July 14, 2011.

173. Ruane, P. and A.D. Luber, *Possible Causes of Early Treatment Failure With a Novel ARV Regimen.. Expert Column: Medscape HIV/AIDS. 2003(2). .* Available at: <www.medscape.com/viewarticle/460673> Accessed July 14, 2011. .

174. Schuurman, R., et al., *Rapid changes in human immunodeficiency virus type 1 RNA load and appearance of drug-resistant virus populations in persons treated with lamivudine (3TC).* J Infect Dis, 1995. **171**(6): p. 1411-9.

175. Descamps, D., et al., *Mechanisms of virologic failure in previously untreated HIV-infected patients from a trial of induction-maintenance therapy. Trilege (Agence Nationale de Recherches sur le SIDA 072) Study Team).* JAMA, 2000. **283**(2): p. 205-11.

176. Havlir, D.V., et al., *Drug susceptibility in HIV infection after viral rebound in patients receiving indinavir-containing regimens.* JAMA, 2000. **283**(2): p. 229-34.

177. Leon, A., et al., *Early virological failure in treatment-naive HIV-infected adults receiving didanosine and tenofovir plus efavirenz or nevirapine.* AIDS, 2005. **19**(2): p. 213-5.

178. Pozniak, A.L., et al., *Tenofovir disoproxil fumarate, emtricitabine, and efavirenz versus fixed-dose zidovudine/lamivudine and efavirenz in antiretroviral-naive patients:*

virologic, immunologic, and morphologic changes--a 96-week analysis. J Acquir Immune Defic Syndr, 2006. **43**(5): p. 535-40.

179. Siegfried, N.L., et al., *Stavudine, lamivudine and nevirapine combination therapy for treatment of HIV infection and AIDS in adults.* Cochrane Database Syst Rev, 2006(2): p. CD004535.

180. van Leth, F., et al., *Comparison of first-line antiretroviral therapy with regimens including nevirapine, efavirenz, or both drugs, plus stavudine and lamivudine: a randomised open-label trial, the 2NN Study.* Lancet, 2004. **363**(9417): p. 1253-63.

181. Kempf, D.J., et al., *Incidence of resistance in a double-blind study comparing lopinavir/ritonavir plus stavudine and lamivudine to nelfinavir plus stavudine and lamivudine.* J Infect Dis, 2004. **189**(1): p. 51-60.

182. MacManus, S., et al., *GW433908/ritonavir once daily in antiretroviral therapy-naive HIV-infected patients: absence of protease resistance at 48 weeks.* AIDS, 2004. **18**(4): p. 651-5.

183. Johnson, V.A., et al., *2011 update of the drug resistance mutations in HIV-1.* Top Antivir Med, 2011. **19**(4): p. 156-64.

184. Wallis, C.L., et al., *Affordable in-house antiretroviral drug resistance assay with good performance in non-subtype B HIV-1.* J Virol Methods. **163**(2): p. 505-8.

185. Lee, S.Y., et al., *Genotyping of HIV-1 Drug-Resistance: Comparison between in-house Procedure and Visible Genetics TruGene[TM] HIV-1 Assay*

1999 (39: 462).

186. Steegen, K., et al., *A sensitive in-house RT-PCR genotyping system for combined detection of plasma HIV-1 and assessment of drug resistance.* J Virol Methods, 2006. **133**(2): p. 137-45.

187. Sayer, D.C., et al., *Quality assessment program for genotypic antiretroviral testing improves detection of drug resistance mutations.* J Clin Microbiol, 2003. **41**(1): p. 227-36.

188. Schuurman, R., et al., *Worldwide evaluation of DNA sequencing approaches for identification of drug resistance mutations in the human immunodeficiency virus type 1 reverse transcriptase.* J Clin Microbiol, 1999. **37**(7): p. 2291-6.

189. Hamers, R.L., et al., *HIV-1 drug resistance mutations are present in six percent of persons initiating antiretroviral therapy in Lusaka, Zambia.* J Acquir Immune Defic Syndr. **55**(1): p. 95-101.

190. Wallis, C.L., et al., *Varied patterns of HIV-1 drug resistance on failing first-line antiretroviral therapy in South Africa.* J Acquir Immune Defic Syndr. **53**(4): p. 480-4.

191. Jagodzinski, L.L., et al., *Performance characteristics of human immunodeficiency virus type 1 (HIV-1) genotyping systems in sequence-based analysis of subtypes other than HIV-1 subtype B.* J Clin Microbiol, 2003. **41**(3): p. 998-1003.

192. Eshleman, S.H., et al., *Performance of the Celera Diagnostics ViroSeq HIV-1 Genotyping System for sequence-based analysis of diverse human immunodeficiency virus type 1 strains.* J Clin Microbiol, 2004. **42**(6): p. 2711-7.

193. Durant, J., et al., *Drug-resistance genotyping in HIV-1 therapy: the VIRADAPT randomised controlled trial.* Lancet, 1999. **353**(9171): p. 2195-9.

194. Scott, L.E. and D.K. Glencross, *Monitoring reproducibility of single analysis, single platform CD4 cell counts in a high volume, low resource laboratory setting using sequential bead count rates.* Cytometry B Clin Cytom, 2005. **67**(1): p. 31-2.

195. Smerdon, S.J., et al., *Structure of the binding site for nonnucleoside inhibitors of the reverse transcriptase of human immunodeficiency virus type 1.* Proc Natl Acad Sci U S A, 1994. **91**(9): p. 3911-5.

196. Schultz, S.J. and J.J. Champoux, *RNase H activity: structure, specificity, and function in reverse transcription.* Virus Res, 2008. **134**(1-2): p. 86-103.

197. Hamming, R.W., *numerical methods for scientists and engineers* McGraw, 1986. **second edit.**

198. Kimura, M., *A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences.* J Mol Evol, 1980. **16**(2): p. 111-20.

199. Sen, S., S.P. Tripathy, and R.S. Paranjape, *Antiretroviral drug resistance testing.* J Postgrad Med, 2006. **52**(3): p. 187-93.

200. Kuritzkes, D.R., *Preventing and managing antiretroviral drug resistance.* AIDS Patient Care STDS, 2004. **18**(5): p. 259-73.

201. Manosuthi, W., et al., *Patients infected with HIV type 1 subtype CRF01_AE and failing first-line nevirapine- and efavirenz-based regimens demonstrate considerable cross-resistance to etravirine.* AIDS Res Hum Retroviruses, 2010. **26**(6): p. 609-11.

202. Garcia, F., et al., *Transmission of HIV drug resistance and non-B subtype distribution in the Spanish cohort of antiretroviral treatment naive HIV-infected individuals (CoRIS).* Antiviral Res, 2011. **91**(2): p. 150-3.

203. Chilton, D.N., et al., *HIV type-1 drug resistance in antiretroviral treatment-naive adults infected with non-B subtype virus in the United Kingdom.* Antivir Ther, 2010. **15**(7): p. 985-91.

204. Wallis, C.L., et al., *Protease Inhibitor Resistance Is Uncommon in HIV-1 Subtype C Infected Patients on Failing Second-Line Lopinavir/r-Containing Antiretroviral Therapy in South Africa.* AIDS Res Treat, 2011. **2011**: p. 769627.

205. Riddler, S.A., et al., *Class-sparing regimens for initial treatment of HIV-1 infection.* N Engl J Med, 2008. **358**(20): p. 2095-106.

206. Delaugerre, C., et al., *Predictive factors of virologic success in HIV-1-infected children treated with lopinavir/ritonavir.* J Acquir Immune Defic Syndr, 2004. **37**(2): p. 1269-75.

207. Mo, H., et al., *Selection of resistance in protease inhibitor-experienced, human immunodeficiency virus type 1-infected subjects failing lopinavir- and ritonavir-based therapy: mutation patterns and baseline correlates.* J Virol, 2005. **79**(6): p. 3329-38.

208. Levison, J.H., et al., *Virologic failure of protease inhibitor-based second-line antiretroviral therapy without resistance in a large HIV treatment program in South Africa.* PLoS One, 2012. **7**(3): p. e32144.

209. Gupta, R.K., et al., *Full-length HIV-1 Gag determines protease inhibitor susceptibility within in vitro assays.* AIDS, 2010. **24**(11): p. 1651-5.

210. Nijhuis, M., et al., *A novel substrate-based HIV-1 protease inhibitor drug resistance mechanism.* PLoS Med, 2007. **4**(1): p. e36.

211. Young, T.P., et al., *Prevalence, mutation patterns, and effects on protease inhibitor susceptibility of the L76V mutation in HIV-1 protease.* Antimicrob Agents Chemother, 2010. **54**(11): p. 4903-6.

212. Marconi, V.C., et al., *Prevalence of HIV-1 drug resistance after failure of a first highly active antiretroviral therapy regimen in KwaZulu Natal, South Africa.* Clin Infect Dis, 2008. **46**(10): p. 1589-97.

213. Garcia-Lerma, J.G., et al., *A novel genetic pathway of human immunodeficiency virus type 1 resistance to stavudine mediated by the K65R mutation.* J Virol, 2003. **77**(10): p. 5685-93.

214. Winston, A., et al., *The prevalence and determinants of the K65R mutation in HIV-1 reverse transcriptase in tenofovir-naive patients.* AIDS, 2002. **16**(15): p. 2087-9.

215. Gotte, M., et al., *The M184V mutation in the reverse transcriptase of human immunodeficiency virus type 1 impairs rescue of chain-terminated DNA synthesis.* J Virol, 2000. **74**(8): p. 3579-85.

216. Mackie, N., *Resistance to non-nucleoside reverse transcriptase inhibitors.* 2006.

217. Lapadula, G., et al., *Prevalence and risk factors for etravirine resistance among patients failing on non-nucleoside reverse transcriptase inhibitors.* Antivir Ther, 2008. **13**(4): p. 601-5.

218. Sungkanuparph, S., et al., *Options for a second-line antiretroviral regimen for HIV type 1-infected patients whose initial regimen of a fixed-dose combination of stavudine, lamivudine, and nevirapine fails.* Clin Infect Dis, 2007. **44**(3): p. 447-52.

219. Hsiou, Y., et al., *The Lys103Asn mutation of HIV-1 RT: a novel mechanism of drug resistance.* J Mol Biol, 2001. **309**(2): p. 437-45.

220. Azijn, H., et al., *TMC278, a next-generation nonnucleoside reverse transcriptase inhibitor (NNRTI), active against wild-type and NNRTI-resistant HIV-1.* Antimicrob Agents Chemother, 2010. **54**(2): p. 718-27.

221. Rhee, S.Y., et al., *Distribution of human immunodeficiency virus type 1 protease and reverse transcriptase mutation patterns in 4,183 persons undergoing genotypic resistance testing.* Antimicrob Agents Chemother, 2004. **48**(8): p. 3122-6.

222. Ren, J., et al., *Structural mechanisms of drug resistance for mutations at codons 181 and 188 in HIV-1 reverse transcriptase and the improved resilience of second generation non-nucleoside inhibitors.* J Mol Biol, 2001. **312**(4): p. 795-805.

223. Hsiou, Y., et al., *Structures of Tyr188Leu mutant and wild-type HIV-1 reverse transcriptase complexed with the non-nucleoside inhibitor HBY 097: inhibitor flexibility is a useful design feature for reducing drug resistance.* J Mol Biol, 1998. **284**(2): p. 313-23.

APPENDIX A:

Procedure for Qiagen Viral RNA mini kit isolation and purification for In-House Genotyping

VI. PURPOSE/PRINCIPLE: To isolate and purify HIV RNA from Plasma (treated with anticoagulants other than heparin) using the Qiagen Viral RNA mini kit. Samples may be fresh or frozen, but if frozen, should not be thawed more than once

A. Materials

EQUIPMENT: Bench top micro-centrifuge Pipettes (1-10 μl, 10-100 μl, 100-1000 μl), vortex mixer, -20˚C freezer, refrigerator, timer, tube racks.

REAGENTS:

Carrier RNA
WB1 buffer
WB2 buffer
AVE and AVL

MATERIALS:

Matrix tips
Micro centrifuge tubes, 1.5ml
Pipettes
Gloves
Centrifuge

II. SPECIMEN TYPE / STORAGE

Type: Plasma treated with anticoagulants other than heparin

Handling Conditions:
- Sample should be separated from plasma within 2 hours of collection.
- Spin sample at 1000rpm for 15 minute and aliquot into clean RNase free 1.5ml tube

Storage:
- Store sample at -70°C
-

PROCEDURE: (Time required: three hours)

NOTE: Standard (unplugged, non-sterile) pipette tips may be used for this procedure.

Check expiration date of kit and record on Nucleic acid Extraction Record Sheet.

Step	Sample concentration.
1	Record the samples you intend to extract on the Nucleic acid extraction sheet. Include one sample from the previous run that got sequenced as positive control and RNase free deionized water for negative control
2	Put orientation marks on the1.5ml microfuge tube. Mind not to mark the lid tops as these can change position during spin.
3	Add 500ul of sample in each tube and RNase free water in the Negative control
4	Put the tube in the centrifuge with orientation marks facing upwards.
5	Spin in a refrigerated centrifuge at 20,000-22000 x g for one hour at 4°C
6	After centrifugation stops, quickly pipette out 360ul of plasma. This should be done from the position opposite to the orientation marks. Ensure that you do it gently but within two minutes for all samples. Do not run more than 10 samples at the time. If you take longer than 5minutes, repeat the spinning step 5
7	Vortex the remaining 140ul and add 5.6ul of carrier RNA in each tube.
8	Quick spin for 2-4 seconds.
9	Add 560ul of AVL in each tube.
10	Vortex and quick spin
11	Incubate at room temperature (15–25°C) for 10 min.
12	Add 560 µl of ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid.
	Wash and Dry.
13	Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate
14	Carefully open the QIAamp Mini column, and repeat step 13.
15	Carefully open the QIAamp Mini column, and add 500µl of Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.
16	Carefully open the QIAamp Mini column, and add 500µl of Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min. Continue directly with step 18, or to eliminate any chance of possible Buffer AW2 carryover, perform step 10, and then continue with step 18
17	Recommended: Place the QIAamp Mini column in a new 2 ml collection tube (not provided), and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.
	Elution:

18	Place the QIAamp Mini column in a clean 1.5 ml microcentrifuge tube (not provided). Discard the old collection tube containing the filtrate. Carefully open the QIAamp Mini column and add 60 µl of Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min. Store at -20° C for short term > 21day of at -70° C for > 21 day.

APPENDIX B:

Procedure for Reverse Transcription (RT) of RNA for In-house genotyping

PURPOSE/PRINCIPLE: To generate cDNA from RNA using the in-house method HIV-1 cDNA is generated as a template for PCR amplification. HIV-1 RNA is copied into cDNA using the enzyme; expand reverse transcriptase and a specific reverse primer.

VII. MATERIALS AND EQUIPMENT:

A. Materials/Equipment Thermo-cycler UV light enclosure (Dead Air Box, reagent and sample) Timer Area dedicated pipettes -20° C freezer Refrigerator Vortex mixer MicroAmp Tray/Retainer set MicroAMP Caps Biosafety hood Centrifuge Ice tray Waste tray	Microcentrifuge tubes 1.5 ml MicroAmp Reaction tubes 0.2 ml Matrix sterile, plugged-filter tips **B. Reagent** 5x Reverse Transcriptase Buffer DTT (100mM) dNTP (10mM) RNAse Inhibitor Expand Reverse Transcriptase Enzyme Reverse Primer (CWR1 20uM) **C. Specimen** *HIV-1 RNA extracts prepared according to the protocol: RNA Extraction (appendix A).*

Handling Conditions:
- HIV-1 RNA is considered to be infectious. Wear a laboratory coat and gloves whenever handling RNA extracts. These gloves and gowns are not to be removed from the clean room.

- Vials containing RNA extracts should be opened only inside the Dead Air Box in the sample room and never on an open bench.
- Immediately change gloves if visibly contaminated with liquid or damaged.
- Details on waste disposal and decontamination of equipment are provided in the protocol: RNA Extraction.

B. Equipment

Centrifuge, sample

III. STORAGE REQUIREMENTS

- Components of the in-house Genotyping method are stored in the -20^0C freezer in the reagent room. Record the date received and date opened on the reagent lists.

A. Rejection Criteria

1. Do not use haemolysed, contaminated or lipaemic serum or plasma.
2. Hemolyzed samples are unacceptable when the level of hemolysis reaches the point when "printed matter cannot be read through it"

IV. QUALITY CONTROL:

Successful reverse transcription is indicated by amplification of sufficient PCR product for sequencing. A negative control (RNase free deionized water) and positive control are included with each batch of reverse transcription reactions. When results from the subsequent PCR amplification are analyzed (see Agarose Gel Electrophoresis), the negative control detects contamination of reactions with HIV-1 DNA from previous reactions. The positive control indicates that the procedure and reagents for reverse transcription are working properly. Do not process any samples from a run whose negative control indicates amplification after gel electrophoresis.

V. PROCEDURE:

IMPORTANT: Use only sterile, plugged pipette tips for this procedure!

In Sample Dead Air box:

Step	Action
1	Place RNA extracts in the Dead Air Box and thaw at room temperature.
2	Place the Positive Control RNA Extract and Negative RNA Control Extract

109

	in the Dead Air box in the sample room

In the dead air box in the reagent room:

Step	Action
3	Label a 200ul MicroAmp reaction tube for: each sample, negative control, positive control. Label tubes with the 1- or 2-digit numbers indicated on the RT/PCR in-house Record Sheet.
4	Thaw the 5xRT buffer, 100mM DTT, 10mM dNTP and 20u CWR1
5	Add 2.5ul of the reverse primer CWR1 into each microamp tube
6	Transfer to the dead air box in the sample room

In Sample Dead Air box:

Step	Action
7	Place MicroAmp tubes into the retaining rack.
8	Open the MicroAmp tubes. Carefully pipette **8ul of RNA extract** to the bottom of the corresponding tube and mix the sample and primer and cap the tubes,.
9	Add **8 ul of the Positive RNA Control Extract** to the Positive Control tube. This should be the next to last tube.
10	Add **8 ul of the Negative RNA Control Extract** to the Negative Control tube. This should be the last tube.
11	Check that the volume in each tube is similar by lifting the retaining rack and inspecting the tubes. Each tube should contain **10.5 ul.**
12	Place the microAmp tubesonto the thermocycler
13	Select the cycle sequencing program: USER: TMP Method: In-house RT Select "View" and review method for correctness:
14	**Temperature (^{o}C)** **Time** 65 — 10 min 42 — 60 min 4 — ∞
15	Select "Start"
16	Confirm reaction volume is 20 ul, select "Start" The program will start. Note: You will pre-incubate the primers and sample at 65 ^{o}C **for 10 minute. The moment the temperature drops to 42, pulse the**

For step 14, the table reads:

Temperature (^{o}C)	Time
65	10 min
42	60 min
4	∞

In the dead air box in the reagent room-during the 10 minutes make up the following RT master mix:

Step	Action
17	In a 1.5 ml microcentrifuge tube, add the following reagents in order (prepare enough for n samples) : **Reagent** — **Volume for n=1 rxn (ul)**

110

	5x RT Buffer	4
	DTT	2
	dNTP (10mM)	2
	RNAse Inhibitor	0.5
	Expand RT	1
	Final volume	**9.5**
18	Add the RNAse Inhibitor directly into the RT mix. Pipette up and down 3 times to rinse the pipette tip.
19	Add the Expand RT enzyme directly into the brewmix. Pipette up and down 3 times to rinse the pipette tip.
20	IMPORTANT: Vortex the RT brewmix 3-5 seconds (low speed).
21	Return the RT reagents to the freezer.
22	Centrifuge the RT brewmix briefly in the microfuge to bring the contents to the bottom of the tube.
23	Place the RT brewmix into the Eppendorf cold block located in the -20°C freezer in the sample prep area
24	Once the 10 minutes is up, let temperature drop to 42°C and PAUSE the thermocycler
NOTE	When you press PAUSE, the thermal cycler will resume the method after 10 min. You have these 10 minutes to perform the following steps.
25	Remove the microAmp tubes and place on the Eppendorf Cold Block
26	Add **9.5ul** of brew mix to each microamp tube
27	Put the samples back into the Thermal Cycler.
28	Press RESUME to continue at **42°C for 60min**.
29	When finished, hold samples at 4^{0}C for at least 10 minutes or store at -20^{0}C until PCR is performed.

Store the cDNA samples:

If you plan to.....	Then.....
Set-up PCR products within 1 hour	Leave the samples at 4°C on the thermocycler.
Set-up PCR at a later date	Store the cDNA samples in the -20°C freezer in the sample area.

APPENDIX C

Procedure for amplifying cDNA for In-house genotyping

I. **PURPOSE/PRINCIPLE: :** To amplify cDNA The polymerase chain reaction (PCR) is used to amplify a 1.6 kb cDNA from HIV-1 cDNA generated using the protocol: In-house Reverse Transcription (RT) Protocol.   The amplified cDNA includes coding sequences for HIV-1 protease (the entire protein, amino acids 1-99)

111

and HIV-1 reverse transcriptase (amino acids 1-324). This cDNA can be sequenced to identify drug resistance mutations in these HIV-1 proteins

Note: *This procedure is performed in the clean room. Review HIV GENOTYPING LAB PROCEDURAL GUIDELINES before using the clean room.*

II. MATERIALS AND EQUIPMENT:

EQUIPMENT:

Thermal cycler
UV light enclosure (Dead Air Box, reagent and sample)
Timer
Area dedicated pipettes
-20˚C freezer
Refrigerator
Vortex mixer
MicroAmp Tray/Retainer-set
MicroAmp Caps
Biosafety hood
Microfuge
Ice tray
Waste carton

REAGENTS:

5xBuffer
dNTP
CWR-1(5'GCATACTTYCCTGTTTTCAG 3')
CWF-1   (5'GAAGGACACCAAATGAAAGAYTG 3')
Expand reverse transcriptase (Roche) Enzyme
Distilled Water

MATERIALS:

Matrix sterile, plugged-filter tips
MicroAmp Reaction tubes 0.2 ml
Micro centrifuge tubes, 1.5 ml

VI. SPECIMEN TYPE / STORAGE

Type: *HIV-1 cDNA prepared according to the protocol: In-house Reverse Transcription*

Handling Conditions:
- HIV-1 cDNA is considered to be potentially infectious.
- Wear a laboratory coat and gloves whenever handling samples in this protocol. These gloves and gowns are not to be removed from the clean room.
- Vials containing cDNA should be opened only inside the Dead air box in the sample room and never on an open bench.
- Immediately change gloves if visibly contaminated with liquid or damaged.

Details on waste disposal and decontamination of equipment are provided in the protocol: RNA Extraction

Storage:
- Reagents of the PCR are stored in the -20^0C freezer in the reagent room.

  A 50 ml bottle of nuclease-free water is stored at room temperature in the resistance cabinet

VII. PROCEDURE:

IMPORTANT: Use only sterile, plugged pipette tips for this procedure.

In the Dead air box in the reagent room:

Prepare the PCR brewmix: To avoid having to pipette small volumes, prepare enough PCR brewmix for at least 5 reactions.

Step	Action
1	Thaw the PCR buffer, dNTP, Primers: CWR-1 and CWF-1 in the dead air box
2	Determine the volume of PCR brewmix needed as follows: For **n** samples-This will include enough for the samples, the positive and negative control reactions this includes an amount for pipetting error.
4	In a 1.5 ml microcentrifuge tube, add the following in order: | Reagent	Volume for 1 sample
5x Buffer	10ul
dNTP	1ul
CWR-1	1ul
CWF-1	1ul
Enzyme	0.5ul
Water	33.5ul
Total	**47.5ul**	
Note	*Pipetting in the following steps is critical. Follow the protocol exactly as written.*
5	Add the enzyme directly into the PCR mix. Pipette up and down 3 times to rinse the entire enzyme from the pipette tip.
6	Vortex the PCR brew mix 3-5 seconds to mix (low speed).
7	Centrifuge the PCR brew mix briefly in the microfuge to bring the contents to the bottom of the tube.
8	Return the PCR reagents to the freezer.
9	Place the PCR brew mix in the Dead air box in the sample room. IMPORTANT: Proceed immediately to the next step. Do not store the PCR brewmix (see below).

In Dead air box in the sample room:

Step	Action
1	Remove the RT reactions from the thermalcycler and place them in the MicroAmp tray in Dead Air Box. Abort the RT-program on the thermal cycler.
Note	*Pipetting in the following steps is critical. Follow the protocol exactly as written.*
2	Carefully pipette 30ul of the PCR brewmix directly into the sample. Pipette up and down 2-3 times to mix, then cap the tube.
3	Repeat step 2 for each tube. Use a separate pipette tip for each sample.
4	Check that the volume in each tube is similar by lifting the retaining rack and inspecting the tubes. Each tube should contain 50ul.
5	If there are any drops on the side of the tubes, spin the reactions tubes for 5-10 seconds.
6	Discard unused PCR brewmix. Proceed immediately to the next step.

114

Perform the PCR reaction:
The PCR reaction is performed in the in thermo-cyclers the post PCR room.

Step	Action			
1	Place the MicroAmp tubes into the block of the thermal cycler.			
2	Program the thermocycler to the conditions as shown below.			
3	**Number of cycles**	**Temperature (°C)**	**Time**	**Extra**
	-	94	2 min	
	10	94	30 seconds	
		54.5	30 seconds	
		72	2 min	
	35	94	30 seconds	
		55	30 seconds	
		72	2 min*	* Increase of 10 seconds with every cycle
	-	72	10 min	
	-	4	∞	
4	Select "Start"			

Complete the run:
The PCR reaction should take about 4.5 hours. When the program is complete:

Step	Action
1	Remove the 0.2ul tubes from the thermocycler. Turn off the thermalcycler.
2	Place the retaining rack in 2% bleach in the dirty lab.

Store the PCR samples:

If you plan to.....	Then.....
Purify the PCR products within 1 hour	PCR samples may be left in the thermocycler for 1 hour.
Purify the PCR products at a later date	Store the PCR samples in the Sequencing Lab in the freezer inside the box labeled "Unpurified PCR products".

APPENDIX D:

Procedure for GFX Micro-Spin purification of PCR products for In-House Genotyping

I. PURPOSE/PRINCIPLE: To purify PCR products, by removing primers and buffer .Primers and buffers are removed from PCR products using a quick-spin column to purify PCR products for gel analysis and sequencing

B. Materials

EQUIPMENT: Bench top micro-centrifuge Pipettes (1-10 µl, 10-100 µl, 100-1000 µl), vortex mixer, -20˚C freezer, refrigerator, timer, tube racks.

REAGENTS:

RNase/Dnase free deionized water
Illustra GFX PCR DNA purification Band Purification kit.

MATERIALS:

Matrix tips
Microcentrifuge tubes, 1.5ml
Pipettes
Gloves

VIII. SPECIMEN TYPE / STORAGE

Type: PCR products prepared according to the procedure for amplifying cDNA as has been described in, Procedure for amplifying cDNA in-house genotyping, Appendix C

Handling Conditions:
- PCR products are unlikely to be infectious. However, you should wear gloves whenever handling samples in this protocol.
- This procedure may be performed on an open bench.
- Immediately change gloves if visibly contaminated with liquid or damaged.
- All waste should be disposed of in double bagged biohazard waste bins. When the bin is three quarters full, the bag should be twisted closed, sealed with autoclave tape and taken to the autoclave room for autoclaving.

Storage:

- A 1.5 ml microfuge tube of Rnase free water is stored on the bench/fridge in the Post-PCR Lab.
- Illustra GFX PCR DNA purification Band Purification kits are stored in a cabinet in the Post-PCR Lab**.**

IX. PROCEDURE: (Time required: twenty minutes per test or batch of tests)

NOTE: Standard (unplugged, non-sterile) pipette tips may be used for this procedure. Check expiration date of microcons and record on Agarose Gel Record Sheet.

Purify the PCR Products:

Step	Sample capture:
1	Label 2 sets of 1.5 ml Microcon collection vials for each sample. The vials used for final collection should contain the PID#, sample date and date of purification.
2	Assemble the GXF MicroSpin concentrator as described in the package insert.
3	Pipette 500 ul of capture buffer type2 into ≤100ul of sample. If sample volume is greater than 100ul divide the sample and purify using more than one GFX Micro-Spin column. Mix thoroughly
4	Note: if sample contains DNA greater than 5kbp. Do not vortex, as this may cause shearing of the DNA. For each purification that is to be performed, place on GFX, Micro-Spin column into one collection tube.
	Sample Binding:
1	Centrifuge Capture buffer type 2-sample mix briefly to collect the liquid at the bottom of the GFX Micro-Spin column and collection tube.
2	Load the Capture buffer type 2-sample into the assembled GFX Micro-Spin columns and collection tube.
3	Spin the assembled column and the collection tube at 16 000 x g for 30 seconds
4	Discard the flow through by emptying the collection tube. Place the GFX Micro-Spin column back inside the collection tube
	Wash and Dry.
1	Add 500 ml wash buffer type 1 to the GFX Micro-Spin column.
2	Spin the assembled column and collection tube at 16 000 x g for 30 sec **Note:** Wash and Dry steps 1 and 2 can be repeated when purity is paramount, for example if the sample is to be used in a blunt-ended ligation. This extra wash step may reduce yield by 4%
3	Discard the Collection tube and transfer the GFX MicroSpin column into a

	fresh Dnase-free 1.5ml micro centrifuge tube (not supplied in the kit)
	Elution:
1	Add 40ul elution buffer type 6. If the band on electrophoresis gel were weakly bright, as 20ul of elution buffer 6 to the centre of the membrane in the assembled GFX Micro-Spin column and sample Collection tube.
2	Incubate the assembled GFX Micro-Spin column and sample Collection tube at room temperature for 1 minute.
3	Spin the assembled column and sample Collection tube at 16 000 x g for 1 minute to recover the purified DNA.
4	Proceed to downstream application. Store the purified DNA at -20°C

Store the Purified PCR products:

If you plan to.....	Then.....
Analyze the PCR products on an agarose gel or nanodrop within 1 hour	PCR samples may be left on ice for 1 hour or may be put in the refrigerator.
Analyze the PCR products at a later date	Store the PCR products in the -20^{0}C freezer in a box labeled "Purified undiluted PCR Products".

118

APPENDIX E

Procedure for quantification of PCR products for genotyping using the in-house method.

I. PURPOSE/PRINCIPLE:

To purify amplified products, so that they can be quantified and diluted for cycle sequencing. DNA fragments are separated by electrophoresis in agarose gels. DNA bands within the gel are detected by staining with the fluorescent intercalating dye, ethidium bromide, and visualizing with a UV light source. DNA fragments are identified by comparison to fragments from DNA size markers

II. MATERIALS AND EQUIPMENT:

EQUIPMENT: bench-top micro-centrifuge, Pipettes (1-10 μl, 10-100 μl, 100-1000 μl), vortex mixer, -20˚C freezer, refrigerator, timer, tube, Electrophoresis equipment, waste system for ethidium bromide disposal, 1 liter graduated cylinder,

REAGENTS:

Sterile, deionized RNase free water
MassRuler DNA Ladder Gel Loading Solution
Agarose
1 X TAE/TBE buffer
Deionized water

MATERIALS:

Matrix tips
0.2ml strip tubes
gel loading tips
Kimwipes
Activated Charcoal

III. SPECIMEN TYPE / STORAGE

Type: PCR products purified according to the protocol: As stated in the Illustra GFX kit (Appendix D)

Handling Conditions:
- You should wear gloves whenever handling samples in this protocol.
- Ethidium bromide is a carcinogen. It is incorporated into the agarose gels, and also leaches into the buffer chambers during electrophoresis. You must always

119

wear gloves when handling the gels, gel boxes and also when disposing of buffers. Used buffer should be poured into the bottle labeled "Dirty Ethidium Bromide" once the bottle is full the bottle undergoes appropriate disposable (according to national guidelines)
- Gels are photographed using a cannon digital camera.
- Immediately change gloves if visibly contaminated with liquid or damaged.
- All waste (except for gel buffers) should be emptied into the biohazard bin when finished with this procedure.

Use of electrophoresis equipment
- The electrophoresis gel box is equipped with a safety lid. Do not turn the power supply on until the gel box is prepared, the lid is closed, and the electrodes are plugged in.
- Do not open the gel box lid, touch the gel or gel buffer, or unplug the electrodes while the power supply is on. At the end of a run, turn off the power supply first, and then unplug the electrodes.
- You may then open the box and remove the gel.

DNA Mass Ladder and Gel Loading Solution
- The DNA mass ladder and gel loading solution are stored at -20°C in the freezer room while the working dye is stored at 2°C to 8°C fridge in the post PCR room.

IV. PROCEDURE:

A Quantification of DNA using gel electrophoresis

NOTE: Standard (unplugged, non-sterile) pipette tips may be used for this procedure. Before beginning, check the lot numbers of the gels, loading buffer and DNA mass ladder and record on the Agarose Gel Record Sheet.

Prepare 0.5ug/ml ethidium bromide 0.8% gel

Prepare the Samples:

Step	Action
1	Enter sample information onto the Agarose Gel Record Sheet.
2	Place the DNA mass ladder and Gel Loading Solution on the bench.
3	Thaw the purified DNA samples, if necessary.
4	Label a 0.2 ml strip tubes for each sample including positive and negative controls.
5	Add 1 ul of 6x gel Loading Solution to each tube. You may use a single pipette tip for this.
6	Add 5 ul of each purified DNA sample to the appropriate tube. Pipette up and down three times to mix. Use a separate pipette tip for each sample.
7	If needed, micro centrifuge tubes at low speed for 5-10 seconds to collect any drops formed on the sides of the tubes.

Load the Gel: Load samples slowly using the designated gel area pipette. The quantity is critical, if it is used as a reference to determine dilutions prior to sequencing. Carefully place the pipette tip in the well of the gel without piercing the gel bottom. The samples will sink slowly to the bottom of the well. Be careful not to agitate the gel while loading.

Step	Action
1	Vortex the DNA Mass Ladder gently before use
2	Pipette **10 ul** of the DNA Mass Ladder onto the first gel lane.
	Note:If you run a gel with two combs, load both DNA Mass Ladder samples onto the first lanes for each comb
3	Load samples onto the gel, in order listed on the Agarose Gel Record Sheet.
4	Place the samples in the refrigerator while the gel is running so they can be diluted after the gel run (see below).
5	Return reagents to the freezer.

Run the gel:

IMPORTANT: **Agarose gels contain ethidium bromide which is a mutagen**. The ethidium bromide leaches into the running buffer during the gel run. Wear gloves for the following procedures. See other handling precautions above.

Step	Action
1	Place the cover on the gel apparatus.
2	Connect the color coded electrodes to the red and black ports on the power supply.
3	Turn on the power supply. It should be set for constant voltage.
4	Adjust voltage to 75-80 volts (10 volts/cm of gel).
5	Run the gel for about 45-60 minutes. The bromophenol blue dye should migrate approximately 2 cm from the wells.
6	Turn off the power supply.
7	Disconnect the electrodes.
8	Place on the transilluminator and turn on
9	Photograph the gel with the Polaroid camera.
10	Turn off the transilluminator
11	Dispose of the gel in a biohazard waste box.
12	Pour off the running buffer (which contains ethidium bromide) into the funnel of the Dirty Ethidium Bromide Bottle and let the liquid drain (when the buffer becomes cloudy).

A   Quantification of DNA using Nanodrop

The Sample Retention System

Step	Action
1	With the sampling arm open, pipette 1.5 to 2 ul deionized RNase free water onto the lower measurement pedestal (A) and (B)
2	Close the sampling arm and initiate a spectral measurement using the operating software on the PC. The sample column is automatically drawn between the upper and lower measurement pedestals and the spectral measurement made (C)
	NOTE: PC operation software will guide you on to blank if you are starting up the instrument for the first time. For more information, consult the NanoDrop user's manual V3.7 full installed on the operation software.
3	Load the blanking solution (deionized RNase free water) and take the reading. Label it as blank
4	When the measurement is complete, open the sampling arm and wipe the sample from both the upper and lower pedestals using a soft laboratory wipe (kimwipes). Simple wiping prevents sample carryover in successive measurements (D)
5	Repeat the step 1-4 for samples. Remember to label for each sample.
6	Once sample testing is complete, clean the nanodrop as stated in the nanodrop SOP and unplug the instrument.

A   B

122
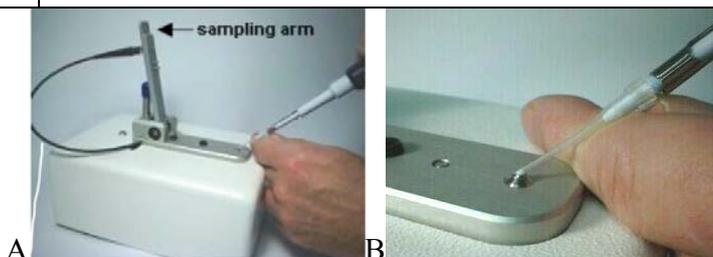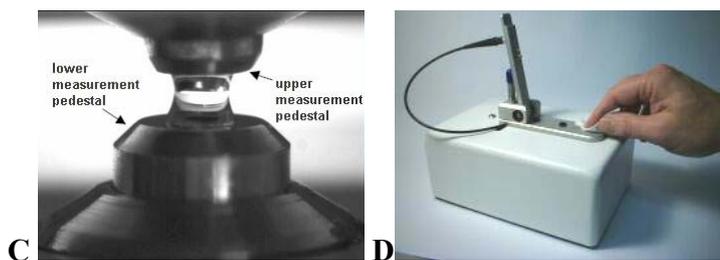
Calculations

The NanoDrop software automatically calculates the absorbance for nucleic acid in ng/ul

If sample concentration is less than 6 ng/ul run a second PCR on the samples.
If concentration is between 6-14 ng/ul, use the neat concentration for sequencing
If concentration is above 14, dilute samples to 10-14 ng/ul for cycle sequencing purposes.

APPENDIX F

Procedure for Cycle Sequencing and purifying using in-house genotyping methodology

I. **PURPOSE/PRINCIPLE:** Dideoxy (chain termination) sequencing is performed using the Big Dye Terminator Kit (Version 3.1) and 5 primers. Standard sequencing includes 2 primers for the sense strand of the PCR products (P41, P42), and 3 primers for the anti-sense strand of the PCR products (P43, P44, P45). Together, these primers provide complete bidirectional sequencing information for the region encoding HIV-1 protease and HIV-1 reverse transcriptase to amino acid 300.

123

II. MATERIALS AND EQUIPMENT:

Absolute Ethanol and Sodium Acetate.
HiDi Formamide 25ml bottle-Aliquotted into 1.5ml Eppendorf tubes
RNAse free deionized water
Big Dye Terminator Kit version 3.1
Primary Primers Primer 21 (5' GGA GGT TTT ATC AAA GTA AGA CAG TAT GA 3') Primer 41 (5' CCTCAAATCACTCTTTGGC 3') Primer 42 (5' AGAACTCAAGACTTTTGGG 3') Primer 43 (5' TGCTGGGTGTGGTATTC 3') Primer 44 (5' TCCCTGTTCTCTGCCAATTC 3') Primer 45 (5' TGGTAAATTTGATATGTCCATTG 3')

III. SPECIMEN TYPE / STORAGE

B. Specimen Type and Storage

- Type: Purified PCR products diluted according to the "Procedure for quantification of PCR products for genotyping using the in-house method" and "Procedure for GFX MicroSpin purification of PCR products for genotyping using the in-house method" SOPs
- Ethanol, stock solution of 3M sodium acetate and deionized RNAse free water are stored in the clean room. Remove one bottle of each for use in the Sequencing lab. When they are empty, get a new bottle from the clean room.
- The Big Dye Terminator Kit and Primers are stored in the –20°C freezer in the clean room.
- The Big Dye Terminator buffer is stored in the 4°C fridge in the clean room.

C. Handing conditions

1) This protocol is performed on the bench in the Sequencing Lab.
2) It is not necessary to use plugged pipette tips for this procedure.
3) Wear gloves whenever handling samples in this protocol.
4) Change gloves if visibly contaminated with liquid or damaged.
5) All waste should be disposed of in Biohazard Waste boxes lined with biohazard waste bags

IV. PROCEDURE:

A. *Preparing the Sequencing Reactions:*

Step	Action
1	Thaw Diluted, purified PCR products, Big Dye terminator Mix and Primers 41, 42, 43, 44, and 45. Take out the Big Dye Terminator Buffer from the fridge
2	Pulse vortex the Big Dye terminator Mix, Primers and Big Dye Terminator Buffer
3	Make up a Mastermix into a 1.5ml RNAse free microfuge tube using each different primer for the number of samples processed: <table><tr><td>Reagent</td><td>Volume for 1 Sample</td></tr><tr><td>Big Dye terminator Mix</td><td>4ul</td></tr><tr><td>Primer (3.2pmol; 41 or 42 or 43or 44 or 45)</td><td>1ul</td></tr></table>

125

	Big Dye Terminator Buffer	2ul
	Water	2ul
	For each pipetting step, lift the plate and directly observe the MasterMix being dispensed into the <u>bottom</u> of the well. Refer to the Sequencing Record Sheet to determine which HIV Seq Mix goes in which well and mix with the PCR product. Use a separate pipette tip for each Master Mix.	
4	Add 1 ul of sample DNA to appropriate well. Make sure you add according to the way you arranged them in the sequencing record sheet. Change tips between samples.	
5	Cover the plate with a rubber full-plate cover or plastic cover seals *Note: Place the A1 position on the cover on the top left corner of the plate*	
6	Check that the volume in each well is similar by lifting the plate and inspecting the well. Each well should contain 20ul.	
7	If any drops are on the sides of the wells, spin the plate, ensure the centrifuge is balanced. Allow the speed to come to approx. 600 rpm (approx. 30 seconds), then stop the spin.	
8	Record the lot # of reagents on the Sequencing Record Sheet.	

Incubating the Sequencing Reactions in the Thermalcycler:

Step	Action		
1	Turn on the ABI 9700 thermalcycler.		
2	Place the retaining rack with the samples in the thermalcycler and close the lid.		
3	Select the cycle sequencing program: USER: TMP Method: Cycle Seq in-house Review the program for correctness. Select "View" and review method for correctness:		
	Number of cycles	Temperature (^{o}C)	Time
	1	96	1min
	25	96	10sec
		55	5 sec
		60	4 min
	-	4	∞
4	Select "Start"		
5	Confirm reaction volume is 20 ul, select "Start" The program will start.		

Complete the run:
The PCR reaction should take about 2.5 hours. When the program is complete:

Step	Action

1	Remove the sequencing plate from the thermalcycler and transfer it to the bench.
2	Turn off the thermalcycler.
3	Proceed to isopropanol precipitation or store reactions in the -20^0C freezer overnight.

B. *To Purify with Ethanol/Sodium Acetate precipitation Method.*

- Prepare the sodium acetate/ethanol solution by combining the following per reaction
  2 uL of 3.0 M sodium acetate, pH 5.2
  50 uL of 100% ethanol
  70% ethanol

Step	Action
1	Add 52 uL of sodium acetate/ethanol solution to each sequencing reaction
2	Seal the tubes with strip caps or the plate with adhesive aluminum foil tape
3	Mix by vortexing. **IMPORTANT!** Mix thoroughly at this step. This is essential for efficient precipitation.
4	Centrifuge at 20,000 X g for 20 minutes.
5	As soon as the centrifuge stops, remove the caps or tape without disturbing the pellets.
6	Immediately place an absorbent paper towel or lint-free tissue on top of the plate and invert it.
7	Centrifuge 150 X g for 1 minute to remove supernatant solution
8	Add 150 uL of 70% ethanol to each well
9	Centrifuge at 2,000 X g for 5 minutes.
10	Immediately place an absorbent paper towel or lint-free tissue on top of the plate and invert it.
11	Centrifuge 150 X g 1 minute to remove supernatant solution
12	When the centrifuge stops and drying is complete, remove the tray/plate and seal with strip caps or aluminum tape, then store at -15 to -25°C in the dark. Analyze the samples within one week. **IMPORTANT!** Use a dark box for storage. The products of sequencing reactions are light sensitive.

Note: Perform the inverted spin immediately after centrifugation to avoid loss of DNA. If the plate sits longer than 1 minute before the inverted spin is performed, spin it for an additional 5 minutes to improve recovery.

C. *Storing the purified sequencing reactions:*

127

If you plan to.....	Then.....
Load the purified sequencing reactions on the 3130xl within 2 hours	Place the reactions in the refrigerator until you are ready to prepare them for analysis on the 3130xl Avoid exposing the samples to light.
Continue another time (samples may be stored for up to six months)	Wrap the plate with the samples with aluminum foil to protect them from light and store them in the -20 freezer.