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Genotypic characterization of gag-pol cleavage site mutations in HIV-1 infected patients failing HAART

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Genotypic characterization of *gag-pol* cleavage site mutations in HIV-1 infected patients failing HAART

Majoalane Tina Maria Ramatsebe

A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Science in Medicine

Declaration

I, Majoalane Tina Maria Ramatsebe declare that the work presented in this dissertation is my own, unaided work. It is being submitted for the degree of Master of Science in Medicine in the University of Witwatersrand, Johannesburg. This work has not been submitted before for any degree or examination at this or any other University.

& & & & & & . & & & & & & & & & & & & & & & .

& & day of €€€€€€€€€. 2013

Dedication

This dissertation is dedicated to my family, especially my parents Pitso and Qentso, my brothers Thabo and Kamohelo. They have shown me that through patience and understanding, anything is possible.

Publications and Conference Proceedings

Publications

Sigaloff, K. C., T. Ramatsebe, R. Viana, T. F. de Wit, C. L. Wallis and W. S. Stevens (2012). Accumulation of HIV drug resistance mutations in patients failing first-line antiretroviral treatment in South Africa. AIDS research and human retroviruses **28**(2): 171-175.

Tina Ramatsebe, Sergio Carmona, Regina Osih, Willem DF Venter, Wendy S. Stevens and Maria A. Papathanasopoulos. The presence of gag-pol cleavage site mutations in South African HIV-1 subtype C infected patients may account for treatment failure on boosted protease inhibitor containing regimens. AIDS Research and Human Retroviruses (submitted).

Conference Poster Presentations

Ramatsebe, M.T.M., Wallis, C.L., Osih, R., Venter, W.D.F., Stevens, W.S., Papathanasopoulos, M.A. Presence of HIV-1 subtype C gag-pol cleavage site mutations in South African infected patients may account for treatment failure on boosted protease inhibitor containing regimens. Presented at IAS 2011, 17 - 20 July, 2011, Rome, Italy.

Abstract

Sequence analysis from HIV-1 (human immunodeficiency virus type 1) subtype B and more recently subtype C infected patients has revealed that mutations in the HIV-1 protease region that confer drug resistance to boosted protease inhibitor (PI) are selected at the time of virological failure. Mutations in the HIV-1 subtype B gag-pol cleavage sites are thought to be compensatory mutations which arise as a result of PI use. This study investigated the presence of compensatory mutations in the HIV-1 subtype C gag-pol cleavage sites and matched pol genotypes from South African patients failing a boosted PI-based regimen, as compared to antiretroviral drug naïve patients.

A new amplification protocol encompassing the near full-length gag, PR and partial RT was established and used to sequence the HIV-1 gag-pol cleavage sites from 23 proviral DNA samples (p24 antigen cultured peripheral blood mononuclear cells; PBMCs), and 51 patient samples (23 antiretroviral drug-naïve, 26 failing second-line lopinavir/ritonavir containing regimens), all attending the Charlotte Maxeke Johannesburg Hospital. Nucleotide sequences were aligned and codon positions S373Q, A431V, I437T/V, L449P or P453A associated with known gag-pol cleavage site mutations were analysed and recorded. The pol genotypes were established using an in house assay. Antiretroviral drug resistant primary virus isolates were grown from samples from patients enrolled on the CIPRA-SA study, and propagated in co-culture with PHA-activated, IL-2 stimulated PBMCs. HIV-1 gag-pol cleavage sites and pol genotypes for all primary virus isolates were established as described above.

Fifty one of 74 patient samples used to establish the in-house gag-pol cleavage site assay, were successfully amplified and sequenced. Detailed analysis of the five known gag-pol cleavage sites revealed that 5 patient samples (4 PI-exposed, 1 unknown regimen) encoded for the previously described mutations that impact gag-pol cleavage in the absence of any major PR mutations. A further five samples from patients on the failing PI-based regimen had major PR mutations. No known mutations in the gag-pol region were identified in patients failing a first line regimen. The pol mutations described in this study were similar to the findings reported for treatment failures in South African HIV-1 subtype C infected patients. Primary virus was grown from only 25 of the 91 PBMC CIPRA samples. None of the 25 CIPRA-SA primary virus isolates had gag-pol cleavage site mutations, and only 9 harboured known RT antiretroviral drug resistant mutations.

Overall, the presence of HIV-1 gag-pol cleavage site mutations may account for virological treatment failure in 5 of the South African patient samples analysed. Although the gag-pol cleavage site mutations detected in the current study are only present in a small proportion of treatment-experienced South African patients, this may increase due to more patients accessing second line-PI containing regimens. Thus, future genotyping work incorporating the analysis of the gag-pol cleavage sites in addition to the PR and RT regions is warranted. The antiretroviral drug resistant primary viruses obtained provide valuable reagents for future phenotyping studies.

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List of Abbreviations

Units of measurement

%	percent
°C	degree Celsius
µl	microlitre
g	gram
M	molar
mg	milligram
ml	millilit re
nm	nanometres
nM	nanomolar
pM	picomolar
rpm	revolutions per minute
U	units

Amino acids

A	Alanine
R	Arginine
N	Asparagine
D	Aspartic acid
C	Cysteine
E	Glutamic acid
G	Glycine
H	Histidine
I	Isoleucine
L	Leucine
K	Lysine
M	Methionine

F	Phenylalanine
P	Proline
S	Serine
T	Threonine
W	Tryptophan
Y	Tyrosine
V	Valine

Treatment regimens

3TC	lamivudine
ABC	abacavir
APV	amprenavir
APV	fosamprenavir
ATV	atazanavir
AZT	zidovudine
d4T	stavudine
ddC	zalcitabine
ddI	didanosine
DLV	delavirdine
DRV	darunavir
EFV	efavirenz
ETR	etravirine
FTC	emtricitabine
IDV	indinavir
LPV	lopinavir
NFV	nelfinavir
NVP	nevirapine
RPV	rilpivirine
RTV	ritonavir
SQV	saquinavir
TDF	tenofovir

TPV	tipranavir
ENF	enfuvirtide
MVC	maraviroc
RAL	raltegravir

Countries and agencies

IUB	International Union of Biochemistry
NIH	National Institutes of Health
PHRU	Perinatal HIV research unit
SA	South Africa
SANBS	South African National Blood Service
US FDA	United States Food and Drug Administration
USA	United States of America
WHO	World Health Organization

Abbreviations used in the text

AIDS	acquired immunodeficiency syndrome
ART	antiretroviral therapy
ARV	antiretroviral
bp	base pair
CCR5	C-C chemokine receptor type 5
$CD4^+$ cells	CD4 positive cells
cDNA	complimentary deoxyribonucleic acid
CIPRA	Comprehensive International Program of Research in AIDS
CO_2	carbon dioxide
CRF	circulating recombinant forms
CT	Cape Town
CXCR4	CXC chemokine receptor type 4
ddNTPs	dideoxynucleotides
DNA	deoxyribonucleic acid
Dnase	deoxyribonuclease
DTT	dithiothreitol
EDTA-TAE	tris-acetate ethyllenediaminetetraacetic acid

ELISA	enzyme-linked immunosorbent assay
env	envelope
FCS	foetal calf serum
gag	group specific antigen
gp120	glycoprotein 120
gp41	glycoprotein 41
HAART	highly active antiretroviral therapy
HIV-1	human immunodeficiency virus type 1
HIV-2	human immunodeficiency virus type 2
HIVDR	hiv antiretroviral drug resistance
HR1	first heptad repeat region
IC_{50}	concentration required to induce 50% inhibition
IL-2	interleukin-2
INIs	integrase inhibitors
JHB	Johannesburg
kb	kilobase
LTR	long terminal repeat
M	main
MA	matrix
MEGA	Molecular Evolutionary Genetics Analysis
$MgCl_2$	magnesium chloride
MGPs	magnetic glass particles
MT2	melatonin
N	non-M or non-O
NA	no amplification
NC	nucleocapsid
nef	negative factor
NG	no growth
NNRTI	non-nucleoside reverse transcriptase inhibitor
NRTI	nucleoside reverse transcriptase inhibitor
NtRTI	nucleotide reverse transcriptase inhibitor
O	outlier
-OH group	hydroxyl group
PBMCs	peripheral blood mononuclear cells

PBS	phosphate buffered saline
PHA	phytohaemaglutinin
PI	protease inhibitor
PIC	pre-integration complex
pol	polymerase
PR	protease
$PR160^{Gag/Pol}$	Gag-Pol polyproteins
$Pr55^{Gag}$	Gag polyprotein precursor
rev	regulatory viral protein
RNA	ribonucleic acid
RNase H	ribonuclease H
RPMI	Roswell Park Memorial Institute
RTIs	reverse transcriptase inhibitors
RVAs	recombinant vector assays
SU	surface
TAMs	thymidine analog mutations
tat	transcription factor
TB	tuberculosis
TM	transmembrane
TMB	tetramethylbenzide
Tris-HCl	tris-hydrochloride
URFs	unique recombinant form
vif	viral infectivity factor
vpr	viral protein R
vpu	viral protein U

CHAPTER 1

INTRODUCTION

1.1. Introduction to HIV-1

Human immunodeficiency virus type 1 (HIV-1) is a retrovirus that is the causative agent of Acquired Immunodeficiency Syndrome (AIDS), which infects CD4 positive ($CD4^+$) cells, in particular the T-helper lymphocytes in the human immune system (Barre-Sinoussi et al., 1983; Klatzmann et al., 1984; Popovic et al., 1984; Coffin et al., 1986). This leads to decreased $CD4^+$ T cells, and ultimately immunodeficiency, through three mechanisms: i) direct viral killing of infected cells, ii) increase apoptosis rates in infected cells and, iii) killing of infected $CD4^+$ T cells by CD8 lymphocytes that are cytotoxic (Coffin et al., 1986).

Since its identification in the early 1980s, infection with HIV-1 has increased and is now responsible for a global epidemic. There were approximately 34 million individuals living with HIV-1 by the end of 2011, and an estimated 2.5 million new infections in 2011. Furthermore, approximately 1.7 million individuals died from AIDS in 2011. Sub-Saharan Africa remains the most affected region with 23.5 million (69% of the global total) adults and children infected residing on this continent. South Africa is the country in the world with the most HIV-1 infections, with an estimated 5.6 million South Africans living with HIV-1 by the end of 2011 (UNAIDS, 2012).

1.1.1. HIV-1 Diversity

There are two types of HIV, namely HIV-1 and HIV-2 (Coffin et al., 1986). HIV-2 is predominantly found in western Africa and is classified into seven distinct groups (A to G) (Los Alamos HIV Database, 2012). HIV-1 is divided into four groups - M, main; O, outlier and N, non-M or non-O; and more recently, group P of gorilla origin, which was identified in a Cameroonian woman (Peeters et al., 1997; Hahn et al., 2000; Yamaguchi et al., 2006; Plantier et al., 2009). HIV-1 group M is the predominant group circulating worldwide and responsible for the majority of all HIV-1 infections (Wainberg, 2004). This group is subdivided into nine different subtypes (A, B, C, D, F, G, H, J and K), 2 sub-subtypes (A1, A2 and F1 and F2), as well as 58 circulating recombinant forms (CRFs) (Los Alamos HIV Database, 2012) and various unique recombinant forms (URFs) (Rambaut et al., 2001; Brennan et al., 2008; Ragupathy et al., 2011). Subtypes A and D are the most prevalent subtypes in east Africa, with subtype B being mostly found in Europe and the United States of America (USA) (Papathanasopoulos et al., 2003; Vasan et al., 2006; Lutalo et al., 2007). In central and west Africa, subtypes A and G predominate, with subtype C being prevalent in southern

Africa, eastern Africa and India (Papathanasopoulos et al., 2003) (Figure 1.1). Furthermore, HIV-1 subtype C is responsible for over 48% of all new HIV-1 group M infections worldwide, and is the predominant circulating subtype in South Africa (Hemelaar et al., 2011).

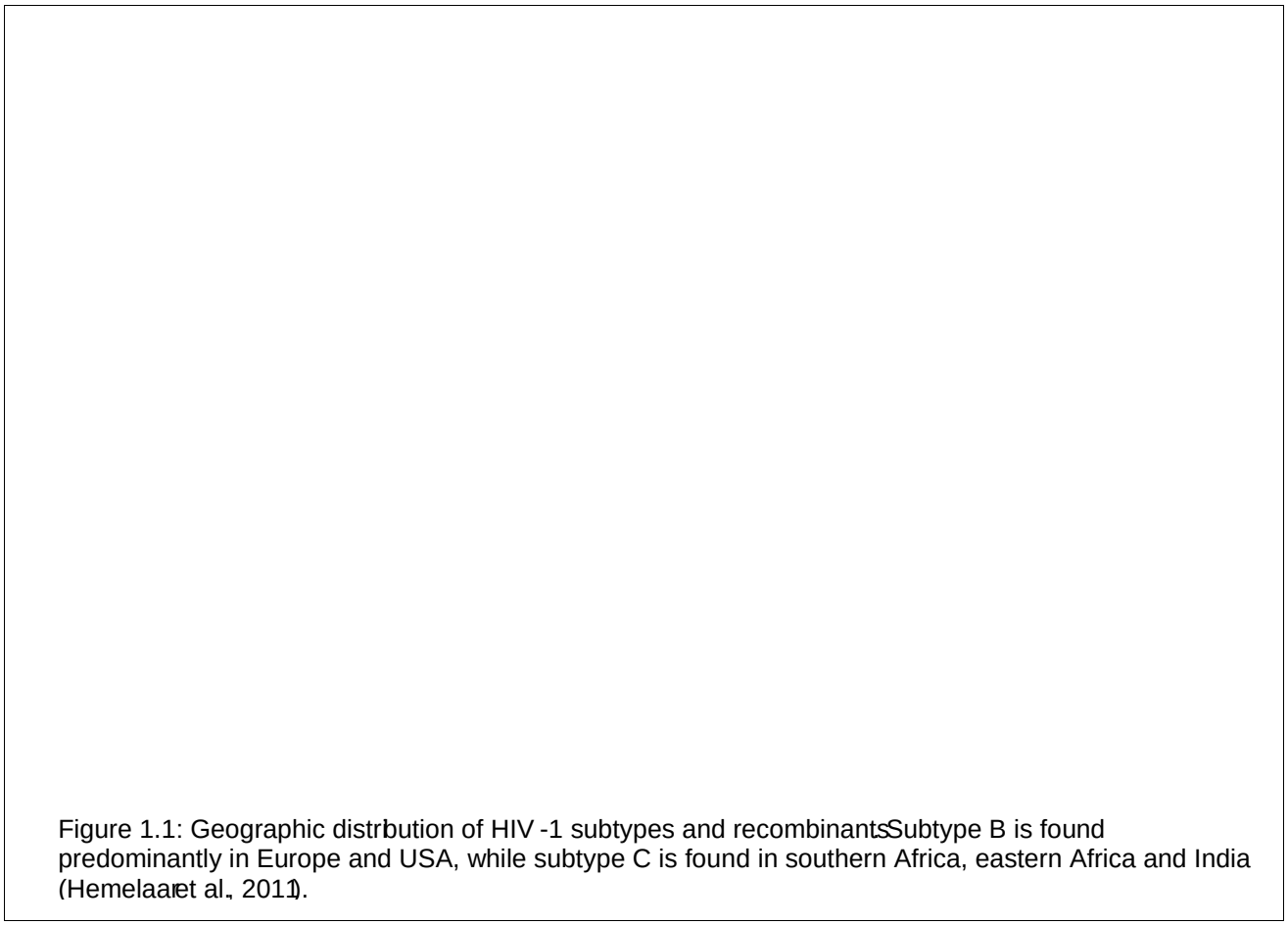


Figure 1.1: Geographic distribution of HIV-1 subtypes and recombinants. Subtype B is found predominantly in Europe and USA, while subtype C is found in southern Africa, eastern Africa and India (Hemelaar et al., 2011).

1.1.2. Morphology of HIV-1

The mature HIV-1 virion, has a spherical outer lipid bilayer that is approximately 110 nanometres (nm) in diameter (Nakai et al., 1989) (Figure 1.2). It is embedded with the trimeric viral transmembrane envelope glycoprotein 41 (gp41), which serves to connect the trimeric viral glycoprotein 120 (gp120) structures to the host derived membrane (Gelderblom et al., 1987; Leis et al., 1988; Gelderblom et al., 1989). A series of structural proteins that organise the viral genome and its associated enzymes for uncoating and replication in a new host cell is contained within the lipid bilayer. The matrix (MA) is the outermost casing that is formed by the protein p17 and is associated with the inner face of the bilayer. Contained in the matrix is the cone-shaped core, whose outer shell is composed of the capsid (CA) protein,

p24 (Gelderblom et al., 1988; Leis et al., 1988; Goto et al., 1990). This capsid surrounds two copies of the positive-sense RNA strands that are covered by the nucleocapsid (NC) protein, p7. Also contained within the capsid are the reverse transcriptase (RT), protease (PR) and integrase enzymes (Figure 1.2) (Gelderblom et al., 1987).

Figure 1.2: Schematic representation of the morphology of HIV-1. The lipid bilayer comprising the gp41 and gp120 trimeric proteins, and the capsid which contains the two single stranded RNA molecules, protease, reverse transcriptase and integrase enzymes are shown. Copied from: https://www.google.co.za/search?hl=en&site=imghp&tbm=isch&source=hp&biw=1311&bih=598&q=structure+of+hiv+virus&oq=structure+of+HIV&gs

1.1.3. HIV-1 Genome

The HIV-1 genome is approximately 9.7 kilobases (kb) long and contains nine open reading frames that encode 3 structural genes (*gag*, *pol* and *env*), 2 regulatory genes (*tat* and *rev*), and 4 accessory genes (*vif*, *vpr*, *vpu* and *nef*) (Figure 1.3), and produce at least 16 proteins. The genome is flanked by two identical LTR (Long Terminal Repeats) which contain transcription initiation sequences (Ratner et al., 1985; Frankel & Young, 1998). The Gag polyprotein precursor ($Pr55^{Gag}$) is proteolytically cleaved by the viral protease to produce MA, CA, NC and p6 protein, as well as two spacer peptides, p2 and p1. The Pol-Gag polyprotein ($PR160^{Gag/Pol}$) is cleaved by the viral protease to produce PR, RT, ribonuclease H (RNAseH) and IN. The *env* gene encodes the surface (SU) gp120 and transmembrane (TM) gp41, which are cleaved by cellular proteases (Watts et al., 2009).

Figure 1.3: Schematic representation of the HIV-1 genome. HIV-1 genome comprised of 9 genes and encodes 15 proteins. (http://www.hiv.lanl.gov/content/sequence/HIV/IMAGES/hxb2genome.gif)

1.1.4. Lifecycle of HIV-1

The life cycle of HIV-1 is regulated by both viral and host cellular proteins, and takes approximately 2.6 days (Figure 1.4) (Perelson et al., 1996). The beginning of the infection process occurs when the HIV particle recognizes the host's T-lymphocytes that express the CD4 surface receptors on their surface (Klatzmann et al., 1984; Popovic et al., 1984). The viral surface envelope glycoprotein, gp120, sequentially binds to CD4 and subsequently to either the CXCR4 or CCR5 co-receptor. Binding of the co-receptor induces conformational changes in the gp41 to form a six helix bundle, which ultimately leads to fusion of the viral and host cellular membranes, thus allowing entry of the p24 CA into the host cytoplasm (Alkhatib et al., 1996; Deng et al., 1996; Dragic et al., 1996; Feng et al., 1996). Once the virus is in the cytoplasm, a series of uncoating processes occur. In these processes, the CA is lost, while some MA and NC and essential enzymes; namely IN, RT and PR; are retained to form part of the viral complex (Miller et al., 1997).

Reverse transcription of viral RNA into complementary DNA (cDNA) by reverse transcriptase is initiated. The viral cDNA, IN and several other viral and host proteins form the preintegration complex, which localizes across the nuclear membrane into the nucleus where irreversible integration of the viral DNA into genomic DNA occurs. This process is catalysed by the enzyme integrase. Once viral and host genomes are integrated, the virus then uses the host's transcription and translation machinery to synthesize its own proteins (Miller et al., 1997; Schroder et al., 2002).

The viral proteins assemble at the cell membrane. A membrane curve is created by the assembled Gag proteins, leading to the formation of a bud, in which the envelope

glycoproteins are embedded. The Gag and Gag-Pol polyproteins are partially cleaved by the viral protease, and the immature virion is released. Post budding, complete cleavage of the Gag and Gag-Pol polyproteins results in mature virions that are capable of infecting a new round of infection (Figure 1.4) (Goto et al., 1990; Nguyen & Hildreth, 2000).

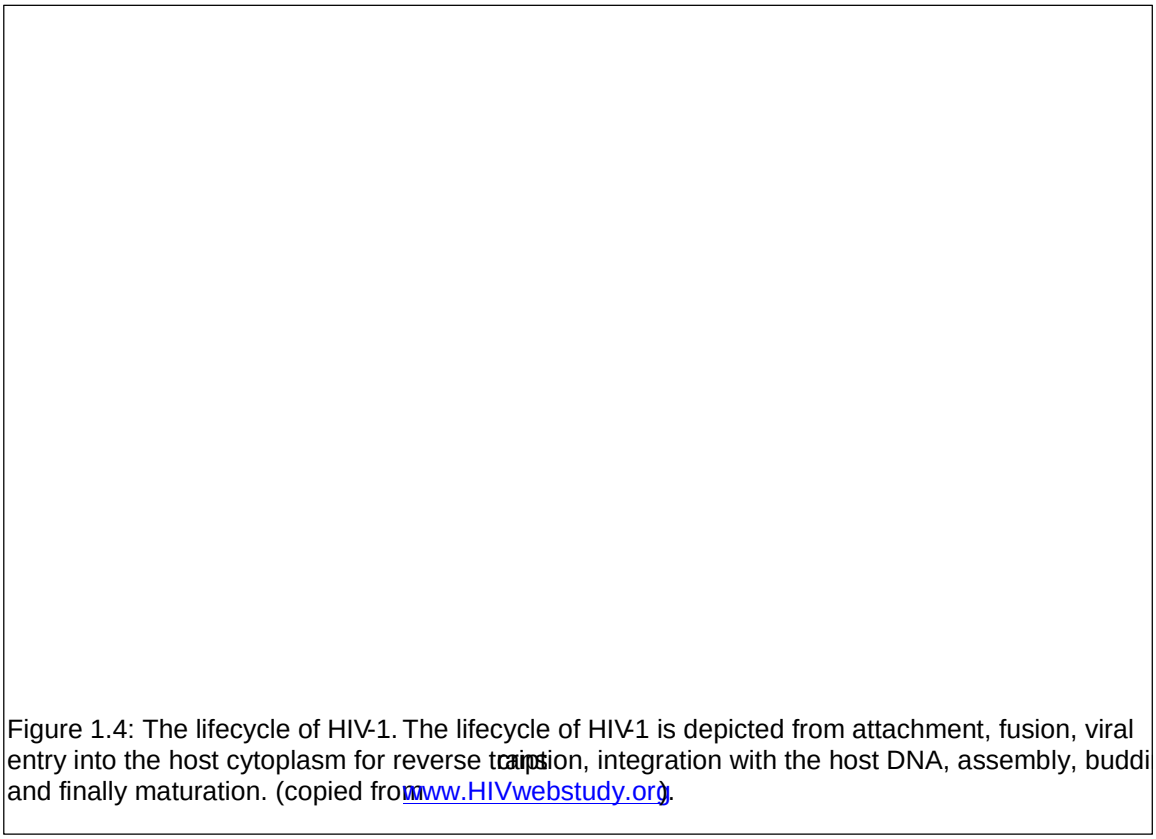


Figure 1.4: The lifecycle of HIV-1. The lifecycle of HIV-1 is depicted from attachment, fusion, viral entry into the host cytoplasm for reverse transcription, integration with the host DNA, assembly, budding and finally maturation. (copied from www.HIVwebstudy.org).

1.1.5. HIV-1 Pathogenesis and disease progression to AIDS

The natural history of HIV-1 infection and disease progression to AIDS is shown in Figure 1.5a. After transmission occurs, primary HIV-1 infection, HIV-1 is initially allowed to replicate uncontrollably, until finally the host manages to control viral replication and the viral load (measured in RNA copies/ml) reaches a set point. The host maintains the viral set point and stable $CD4^+$ T cell counts for approximately 10 years (clinically asymptomatic phase, in a typical progressor). At some critical point, $CD4^+$ T cells are severely depleted and the viral load increases dramatically. At this stage, the HIV-1 infected individual becomes susceptible to a whole spectrum of life threatening opportunistic infections or cancers, and is diagnosed with AIDS (Fauci et al., 1996).

In the absence of antiretroviral therapy (ART), a person with AIDS will die within 2 years. However, the introduction of successful ART leads to the rapid decrease in viral load and

undetectable levels and an increase in CD4+ T cell counts (Figure 1.5b) (Fauci et al., 1996; Guadalupe et al., 2003).

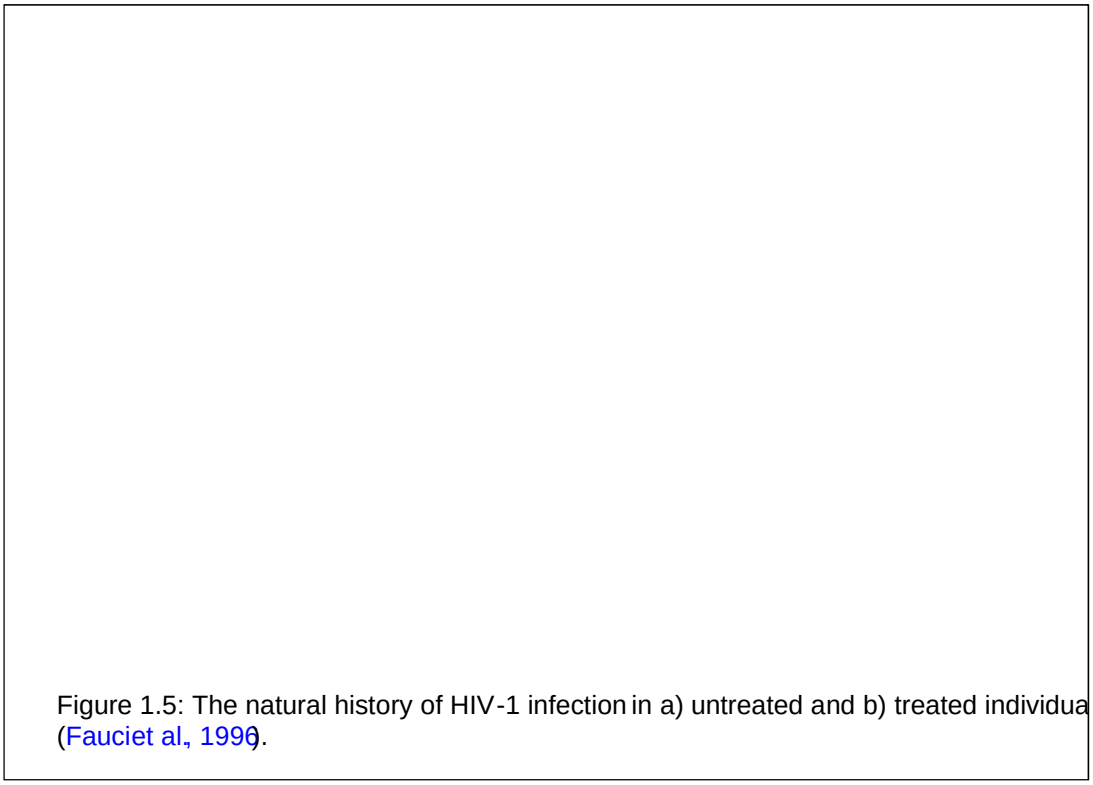


Figure 1.5: The natural history of HIV-1 infection in a) untreated and b) treated individuals (Fauci et al., 1996).

1.2 Antiretroviral drug treatment of HIV/AIDS

Highly active antiretroviral therapy (HAART) was introduced in 1996 and is the current standard for treatment of HIV-1 infected patients. HAART involves triple therapy consisting of a nucleoside analogue reverse transcriptase inhibitor (NRTI) backbone (consisting of two drugs) and either a non-nucleoside analogue reverse transcriptase inhibitor (NNRTI) or protease inhibitor (PI) (Hammer et al., 1996; Hammer et al., 1997; Carpenter et al., 2000). This combinatorial therapy has been shown to greatly reduce the morbidity and increase the life-span of HIV-1 infected patients by preventing progression to AIDS (Fauci et al., 1996; Palella et al., 1998; Guadalupe et al., 2003). HAART is measured as the patient achieves an undetectable viral load (<50 RNA copies/ml) within 12 weeks of treatment initiation.

1.2.1. FDA approved Antiretroviral Agents

There are currently 26 antiretroviral drugs that have been approved by the United States Food and Drug Administration (USFDA), excluding fixed-dose drug combinations

(http://www.fda.gov/ForConsumers/byAudience/ForPatientAdvocates/HIVandAIDSActivities/default.htm). These include:

1.2.1.1. Reverse Transcriptase Inhibitors (RTIs)

Since the RT plays a major role in the life cycle of HIV-1, it has become a fundamental target of antiretroviral (ARV) drugs. RTIs include both nucleoside/tide RTIs (NRTI) and non-NRTIs (NNRTIs) and have become integral components of ARV regimens (Xu et al., 2010).

NRTIs are structural analogues of the natural substrates used to synthesize viral cDNA, and compete with them for incorporation into the growing viral DNA chain. Because RT inhibitors lack a 3'-hydroxyl group on the deoxyribose moiety, subsequent incorporation of nucleotides into the nascent DNA is blocked, thereby terminating the synthesis of proviral DNA (Mitsuya et al., 1985). In order for their antiviral activity to be exerted, NRTIs must be phosphorylated intracellularly by host kinases to their active 5'-triphosphate (Balzarini et al., 1994). Examples of NRTIs are zidovudine (AZT), didanosine (ddI), stavudine (d4T), lamivudine (3TC), abacavir (ABC), zalcitabine (ddC), tenofovir (TDF) and emtracitabine (FTC).

NNRTIs are hydrophobic compounds with diverse structures that bind specifically and non-competitively to the hydrophobic pocket of RT that is located close to the enzyme active site (Balzarini, 1999; De Clercq, 1998; Sluis-Cremer et al., 2006). This action impairs its polymerase activity, therefore causing a conformational change in which the catalytic activities of the enzyme are affected. (Spence et al., 1995; Sluis-Cremer & Tachedjian, 2008). These agents do not need to be phosphorylated to induce their activity and are not incorporated into the viral DNA. They are generally only effective against HIV-1, not other retroviruses, including HIV-2 (De Clercq, 1998). Examples of NNRTIs include nevirapine (NVP), efavirenz (EFV), delavirdine (DLV), rilpivirine (RPV) and etravirine (ETR).

1.2.1.2 Protease Inhibitors (PIs)

PIs work by specifically binding to the active site of the aspartic-1 HIV PR, thus inhibiting the processing of protein products needed for the core proteins and enzymes. As a result, viral particles are incompletely formed and non-infectious are released (Matsuoka & Aizawa et al., 2003; Nijhuis et al., 2007). Examples of PIs include amprenavir (APV), atazanavir (ATV), darunavir (DRV), fosamprenavir (APV), indinavir (IDV), lopinavir (LPV), nelfinavir (NFV), ritonavir (RTV), saquinavir (SQV) and tipranavir (TPV).

In addition, some mutations in gag can adapt the gag/gag-pol cleavage site or improve the incorporation of PR in the virion in order to optimize the cleavage activity of a mutated PR enzyme (Nijhuis et al., 2007).

1.2.1.3. Entry inhibitors

Entry inhibitors prevent the virus from infecting the CD4+ T cell by either inhibiting fusion between the viral and the host cell membrane or by blocking the receptors (Kilby et al., 1998; Kilby et al., 2002; Pohlmann & Doms, 2002). The fusion inhibitor, enfuvirtide (ENF), inhibits fusion by binding to the gp41 first heptad repeat region (HR1) and preventing formation of the six helix bundle (Wild et al., 1993; Chan et al., 1997; Chan & Kim, 1998). The co-receptor antagonist maraviroc (MVC) binds to the CCR5 co-receptor, thereby disrupting the sequential gp120 binding. MVC only prevents viral entry of CCR5-utilizing viruses (Dorr et al., 2005; Fatkenheuer et al., 2005).

1.2.1.4. Integrase Inhibitors (INIs)

The INI Raltegravir (RAL) is a *β*-diketo acid derivative approved by the FDA as an IN strand transfer inhibitor administered twice daily. RAL acts to inhibit HIV-1 integrase by sequestering the divalent metal cofactors bound in the active site of the enzyme (Hazuda et al., 2000; Grobler et al., 2002; Markowitz et al., 2006; Kassahun et al., 2007).

1.2.2. Current ART options in South Africa

The South African comprehensive public health prevention program, initiated in April 2004, has enrolled approximately 1.8 million patients onto their ART roll-out program to date (UNAIDS, 2012). The 2010 South African ARV treatment guidelines describe two standardised regimens for all patients accessing care (The South African Antiretrioviral Treatment Guidelines 2013). The updated first-line regimen for adults and adolescents consists of the following drugs: TDF, 3TC/FTC and EFV/NVP. In the case of tuberculosis (TB) co-infected patients, EFV is preferred and NVP is administered to women of a child bearing age who are not on a reliable contraception. Patients on a d4T-containing first line regimen (d4T, 3TC, EFV/NVP) that exhibit no side effects will remain on this regimen, and patients with contraindications for TDF will be placed on AZT, 3TC and EFV/NVP. The second line is now comprised of TDF, 3TC/FTC and LPV/r if the patient failed a d4T or AZT-based first line regimen, and AZT, 3TC and LPV/r if they failed on a TDF-based first line regimen. Patients failing the second line regimen are asked to „consult an expert….

1.2.3. Antiretroviral drug treatment failure

A major downfall of HAART is treatment failure, which can be attributed to patient non-adherence, poor response to antiretroviral drug regimens, toxicity to ART or the evolution/emergence of HIV-1 drug resistance as a result of the error-prone RT enzyme during replication or drug pressure (Richman et al., 2004).

The emergence of HIV antiretroviral drug resistance (HIVDR) in resource limited settings is compounded by inadequate resources and insufficient health care infrastructures, resulting in an increasing cumulative number of patients who harbour resistance mutations (acquired), and have the potential to also transmit antiretroviral drug resistant virus (Little et al., 2002, Zaccarelli et al., 2005). Currently, as a result of high costs, no provisions to monitor for the emergence of HIV-1 drug resistance have been made in the South African public sector. However, with the recent publication of HIV drug resistance testing, validated in house assays, which are about 50% cheaper than the FDA approved commercial assays, will provide a way to monitor this HIV-1 drug resistance (Conradie et al., 2012). Several reports have highlighted antiretroviral drug treatment failures in South African HIV-1 subtype C infected HAART patients attributed to the emergence of drug resistant virus (Wallis, Mellors, et al., 2010, Singh et al., 2011, van Zyl et al., 2011, Hamers et al., 2012).

1.3 Antiretroviral drug resistance (HIVDR)

The development of drug resistance depends on the heterogeneity and size of the HIV-1 population in an infected individual, the ability of the virus to replicate under antiretroviral drug pressure, the ease of mutations developing and the effect of drug resistance mutations on drug susceptibility and viral fitness (Shafer, 2002). A possible single point mutation occurs between 10^{-4} and 10^{-5} times a day in an HIV-1 infected antiretroviral drug naïve individual (Coffin, 1995). It is unknown whether the emergence of HIVDR, in response to therapy, is due to resistant strains that may already exist in untreated patients or that pre-existing strains are sensitive, but HAART is not 100% effective and the resistant mutant is created de novo during treatment (Ribeiro & Bonhoeffer, 2000).

As mentioned above, there is emerging data available on ARV drug resistance in South African HIV-1 subtype C infected individuals accessing public sector regimens (Wallis, Mellors, et al., 2010, Singh et al., 2011, van Zyl et al., 2011, Hamers et al., 2012). ARV drug

resistance studies conducted in subtype C infected individuals failing therapy revealed that HIV-1 subtype C developed similar ARV mutation profiles to subtype B, with the exception of V106M (Brenner et al., 2003; Morris et al., 2003; Lai et al., 2010) and the higher prevalence of K65R (Brenner et al., 2006; Doualla-Bell et al., 2006; Invernizzi et al., 2009). However, there is no evidence that there are subtype specific naturally occurring polymorphisms that contribute to differences in antiviral drug treatment outcome. For both K65R and V106M the changes are silent nucleotide (AA) changes (Turner et al., 2004; Coutsinos et al., 2009; Invernizzi et al., 2009). In the case of K65R, resistance mutation results from an AAA-to-AGA transition in HIV-1 subtype B, but from an AAG-to-AGG transition in subtype C (Invernizzi et al., 2009). For V106M, the selection of this mutation in subtype C viruses results from a single nucleotide change from wild-type (GTG to ATG), whereas in subtype B it is a transition from GTG to GTA (Turner et al., 2004). The protease polymorphisms in subtype C have been shown to have no impact on HIVDR and do not increase in frequency when exposed to PI (Wallis et al., 2011).

1.3.1. Resistance to NRTIs

Resistance to NRTIs occurs via two mechanisms. In the first mechanism, the RT enzyme is capable of discriminating between the NRTI and the natural deoxynucleotide triphosphate (dNTP) substrate. Mutations such as M184V, K65R, L74V and Q151M occur by this process (Deval et al., 2002; Deval et al., 2004). In the second mechanism, the removal of the chain-terminating NRTI from the 3, terminus of the primer after it has been inserted into the viral DNA is greatly enhanced. Thymidine analog mutations (TAMs) are known mutations that occur via this process and examples of these are D67N, L210W, T215Y and K219Q. (Boyer et al., 2001). The K65R mutation may emerge at a higher frequency in HIV-1 subtype C infected patients on certain NRTI containing regimens and its rapid emergence has been shown to confer resistance to TDF in cell culture (Brenner et al., 2006; Doualla-Bell et al., 2006; Invernizzi et al., 2009). Lamivudine rapidly selects for M184V and it has been previously shown that in subtype B infected patients the mutation delays the emergence of TAMs, which are involved in NRTI cross-resistance. This mutation also impairs the enzymatic function of HIV RT, reduces viral replicative capacity and improves drug susceptibility to zidovudine, stavudine and tenofovir (Grossman et al., 2001; Ait-Khaled et al., 2002; Zaccarelli et al., 2003; Petrella et al., 2004).

1.3.2. Resistance to NNRTIs

After NNRTI treatment failure, selected mutations arise in the enzyme pocket targeted by these compounds and as a result decrease the affinity of the inhibitors for the enzyme (Shafer & Schapiro, 2008). NNRTI resistance mutations include Y181C, V106M and K103N. Interestingly, the V106M mutation has been previously shown to be a subtype-C specific mutation in patients failing EFV and is a result of a naturally occurring polymorphism which occurs at this codon (Brenner et al., 2003; Morris et al., 2003). The K103N mutation, is selected for by NVP, and found to occur at a greater frequency and in higher levels in women infected with subtypes C and D as compared to subtype A (Eshleman et al., 2001; Flys et al., 2006).

1.3.3. Resistance to PIs

Under PI pressure, the PR region can develop mutations through approximately 20 different amino acid changes which confer antiretroviral drug resistance (Condra et al., 1995; Condra et al., 1996; Schmit et al., 1996). Resistance to PIs usually occurs slowly from the accumulation of multiple primary and secondary mutations. Primary mutations can cause resistance to one or more PIs by themselves; however the level of resistance is usually not enough to interfere with the antiviral activity of these drugs. Therefore, the accumulation of additional primary and secondary mutations results in higher levels of resistance required for clinically significant reductions in drug susceptibility (Condra et al., 1995; Molla et al., 1996; Kempf et al., 2001).

Some of the examples of resistance to PI are: D30N, I54V, V82A/F/T, I84A, M46I/L and L76V (Zhang et al., 1997; Bally et al., 2000; Malet et al., 2007; Lambert-Niclot et al., 2008). These arise from inside the substrate binding domain of the enzyme or at distant sites that enables full recovery of viral replication ability (Walmsley et al., 2002). Mutations that develop generally occur in the PR active site resulting in a decrease of drug binding, but with impaired enzymatic activity (Bally et al., 2000; Bleiber et al., 2001). Studies conducted by Grossman et al., (2001) and Douella-Bell et al., (2006) revealed that HIV-1 subtype C infected patient on NFV containing regimens developed resistance to this PI through distinct mutational pathways, different from subtype B.

1.3.4. Resistance to Entry inhibitors

Mutations in the gp41 amino acids 36-45 have been related to ENF resistance and therapy failure. However, mutations in other regions of the envelope as well as coreceptor usage may affect susceptibility to ENF (Reeves et al., 2002, Sista et al., 2004, Xu et al., 2005). By contrast, HIV-1 develops resistance to MVC by adapting to binding to CCR5 in the presence of the drug (Trkola et al., 2002, Dorr et al., 2005, Westby et al., 2007).

1.3.5. Resistance to INIs

Resistance to RAL has been associated with three major pathways, which involve amino acid substitutions at three key positions in the IN protein: Y143R/C, Q148H/R/K, or N155H, alone, or accompanied by other mutations (Cooper et al., 2008, Malet et al., 2008, Fransen et al., 2009). The Q148H/R/K and N155H mutations are thought to play a role in eliciting conformational changes within the binding pocket and thus resulting in an increase in the binding energy of INIs. In contrast, the Y143 residue is thought to connect to RAL by pi stacking between the tyrosine phenol ring the RAL 1,3,4 oxidiazole group. (Hare et al., 2010).

1.3.6. Mutations in the gag-pol cleavage sites

Mutations in the gag-pol cleavage sites are thought to be compensatory mutations which arise as a result of PI use, and have generally been described at two cleavage sites, p7/p1 and p1/p6 and a few within gag (Table 1.1)(Zhang et al, 1997, Bally et al., 2000, Kaufmann et al., 2001, Fehér et al., 2002, Gatanaga et al., 2002, Parkin et al., 2005, Malet et al., 2007).

Table 1.1: Known mutations in the HIV-1 gag-pol cleavage sites affecting protease inhibitor susceptibility (Zhang et al., 1997, Bally et al., 2000, Kaufmann et al., 2001, Fehér et al., 2002, Gatanaga et al., 2002, Parkin et al., 2005, Malet et al., 2007).

CODON	NUCLEOTIDE POSITION (HXB2)	GAG CLEAVAGE SITE	WILD TYPE	MUTATION
S373	1115	p2/p7	TCA	CAA, CAG
A431	1289	p7/p1	GCT	GTT, GTC, GTA, GTG
I437	1307	p7/p1	ATC	GTT, GTC,GTA, GTG, ACT, ACC, ACA, ACG
L449	1343	p1/p6	CTT	CCT, CCC, CCA, CCG
P453	1355	p1/p6	CCA	TTA, TTG, CTT, CTC, CTA, CTG

Several researchers have investigated the impact of gag-pol cleavage site mutations on viral fitness (Nijhuis et al., 2007). When HIV-1 is under extensive PI pressure, it develops mutations in PR which results in a decrease in viral replication, but when compensatory mutations develop in the p7/p1 or p1/p6 cleavage sites, the viral replicative fitness is restored back to 92% of that of the wild type virus (Zhang et al., 1997, Tamiya et al., 2004). Moreover, these gag-pol mutations contribute to a positive viral fitness with or without drug pressure (Condra et al., 1995, Doyon et al., 1996, Zhang et al., 1997).

1.3.7. Association of gag-pol cleavage site mutations with PR mutations

The gag cleavage sites have been associated with some PR mutations. The A431V mutation in the p7/p1 cleavage site has been shown to be associated with M46I/L, I54V, V82A/T/F and L76V PR mutations. In vitro, the A431V mutation is mostly associated with enhancing the gag processing by the mutated PR in viruses that are highly resistant to PIs.

The P453L mutation, in the p1/p6 cleavage site, is associated with I84A and L90M protease mutations and is involved in influencing the development of the PR resistance mutations. (Zhang et al., 1997, Bally et al., 2000, Cote et al., 2001, Matsuoka-Aizawa et al., 2003, Matsuoka-Aizawa et al., 2006, Malet et al., 2007, Lambert-Niclot et al., 2008).

The S373Q and L449P polymorphisms have been associated with K20I/R/M and L89M/I PR mutations (Zhang et al., 1997, Bally et al., 2000, Malet et al., 2007, Nijhuis et al., 2007). The S373Q mutation contributes negatively on the impact of HIV-1 replication (Malet et al., 2007). L449P mutation substitution might act as compensatory mutations which allow an increase of the cleavage activity of the mutant PR (Parrouy et al., 2010).

The I437T/V gag cleavage mutation, in the p7/p1 cleavage site, is associated with the L76V PR mutation (Lambert-Niclot et al., 2008). However, it has been also identified to increase PR resistance without major PR mutations (Nijhuis et al., 2007).

Interestingly, sequence analysis from HIV-1 subtype B infected patients failing a PI containing regimen (lopinavir and ritonavir) revealed an infrequent accumulation of known PR mutations (Riddler et al., 2008). More recently, these findings have been mirrored in South African study which revealed that only 7% of HIV-1 subtype C infected patients failing a PI containing ARV drug regimen, had major PR mutations (Wallis et al., 2011). We

speculated that in these patients, compensatory mutations in gag may be responsible for PI treatment failure in the absence of known PR mutations.

1.4. Laboratory monitoring of antiretroviral drug resistance

Currently, there are two antiretroviral drug resistance testing methods available, namely phenotyping and genotyping.

1.4.1. Phenotyping for antiretroviral drug resistance

Phenotyping measures the ability of HIV-1 to replicate in the presence of an antiretroviral drug/s, and determine the inhibitory concentration of antiretroviral agents that decrease HIV-1 replication by 50% (IC_{50}) in tissue culture. This assay provides a direct quantitative measure of drug resistance or susceptibility of the isolate relative to a reference strain. Phenotyping precisely reflects the contribution of each mutation and potential mutation interactions to ARV drug resistance. However, it is relatively difficult to perform, expensive, and requires dedicated facilities (Hanna & D'Aquila, 2001).

1.4.1.1. Phenotyping using peripheral blood mononuclear cells (PBMCs)

The use of PBMCs in phenotypic studies is the standardized antiviral drug susceptibility assay for clinical HIV-1 isolates. This protocol involves the isolation and amplification of HIV-1 from PBMCs of infected patients by cocultivation with phytohemagglutinin (PHA)-stimulated PBMCs from seronegative donors. A virus stock is generated in this manner and then titrated to determine the virus infectivity. This is subsequently used in a drug susceptibility inhibition assay and the IC_{50} can be calculated on the basis of infection by measuring HIV-1 p24 antigen levels (Japour et al., 1993).

1.4.1.2. Phenotyping using recombinant vectors

Recombinant vector assays (RVAs) includes the PCR amplification of complete RT and PR sequences from either cultured or uncultured patients PBMCs. The patient-derived sequences are then introduced into a reference backbone plasmid containing the full length HIV genome with the deleted corresponding sequences. This yields a recombinant virus that is then cultured in cell lines. Subsequently, the virus is used in a drug susceptibility inhibition assay (Harada et al., 1985; Kellam & Larder, 1994).

1.4.2. Genotyping for antiretroviral drug resistance determination

Genotyping assays are the most commonly used sequencing based method for detecting resistant HIV-1 isolates. It is a qualitative assay that is based on the detection of specific mutations in the HIV genes that target enzymes (protease, reverse transcriptase or integrase) or entry (gp41) and its interpretation involves the use of algorithms which depict drug susceptibility or resistance. Genotypic tests are able to detect mutations that are present as mixtures and provide insight into the potential for resistance to emerge. However, the evidence of drug susceptibility that is obtained from this assay is indirect. This is because they cannot always differentiate between one homogenous virus population with multiple mutations and multiple populations, in which each have a few different mutations. A genotypic assay is less labour intensive, requires less skill and is more affordable than phenotyping. Therefore, this assay is widely used in clinical practice to assist the selection of active antiretroviral drug regimens. Its major limitation concerns the complexity of results interpretation which still awaits a general consensus with regards to non-B HIV-1 subtype variations (Hanna & D'Aquila, 2001, Vandamme et al., 2001, Shafer, 2002).

The commercially available ViroSeq HIV-1 Genotyping System, which is FDA approved, is regarded as the gold standard tool for the determination of ARV drug resistance mutations in HIV-1 RT and PR (Celera Diagnostics, USA) (Eshleman et al., 2004). Due to subtype-specific sequence differences of primer binding, the genotyping of non-B subtypes has proven to be difficult with this assay. This was evident in a study conducted by Maes and colleagues whereby the performance of the primer set provided by the kit was lower for all non-B subtypes (Maes et al., 2004).

More affordable in house HIV-1 genotyping assays have been developed in various laboratories, including the Genotyping laboratory at the Charlotte Maxeke Johannesburg Academic Hospital in South Africa, which overcome the subtype issue (Wallis, Papathanasopoulos, et al., 2010).

To date, most of HIV-1 genotypic and phenotypic antiretroviral drug resistance data has been generated from studies with HIV-1 subtype B viruses. Subsequently, results from these two assays have formed the basis of generating algorithms used in predicting antiretroviral drug resistance in non-B subtypes (Richman et al., 2004).

1.5. Aim of the study

The abovementioned data highlights a need for continued evaluation of antiretroviral drug resistance patterns in South African HIV-1 subtype C infected patients failing the first and second line regimens. There is also a paucity of data on the role of the gag-pol cleavage sites in association with PR/RT mutations in HIV-1 drug resistant viruses. Moreover, there is no published data on phenotypic susceptibility evaluation of antiretroviral drug resistant HIV-1 subtype C primary virus isolates against the various antiretroviral drugs.

Therefore, the overall aim of this study was to identify patterns of gag-pol cleavage site and RT/PR mutations that emerge in HIV-1 subtype C infected South Africans receiving either first-or-second line ART, and to generate HIV-1 drug resistant viruses for future use in in vitro phenotypic inhibition assays.

This was achieved by the following objectives:

- To establish an HIV-1 subtype C specific RT-PCR protocol to amplify and sequence the gag-pol cleavage sites
- To evaluate the gag-pol genotyping assay on patients failing the first- and second-line regimens.
- To isolate antiretroviral drug resistant HIV-1 subtype C primary virus isolates from selected South African patients failing the first- and second-line regimens.
- To determine the gag-pol cleavage site and PR/RT antiretroviral drug resistance profiles from the resulting antiretroviral drug resistant HIV-1 subtype C primary virus isolates.

CHAPTER 2

MATERIALS AND METHODS

2.1. Participant samples used in this study

A total of 165 participant samples were used for various analyses throughout the course of this study and included samples from ARV drug-naive patients, as well as patients failing first and second line ARV drug regimens. Ethical clearance for the use of patient samples for this project was obtained from the University of the Witwatersrand Committee for Research on Human Subjects (ethics number M10743, Appendix A).

2.1.1. Proviral DNA from primary virus isolates from antiretroviral drug-naive patients

Twenty three proviral DNA samples were sourced from p24 antigen PBMCs. These included primary viral isolates (99LT) obtained from seven ARV drug-naive vertically infected children attending a clinic at Chris Hani Baragwanath Hospital, Johannesburg (Tiemessen et al., 2009) in 1999, and 16 well characterised primary HIV-1 isolates (05FV) from ARV drug naïve AIDS patients (isolated during 2005) attending the Charlotte Maxeke Hospital, Johannesburg (previously Johannesburg General Hospital) (Connell et al., 2009). Selected samples were initially used to set up the PCR component of the in house gag-pol RT-PCR assay and the remainder to subsequently obtain data.

2.1.2. Plasma samples from patients failing first or second line ARV regimens at the Charlotte Maxeke Johannesburg Academic Hospital

Fifty one patient samples that were previously sent for routine HIV-1 drug resistance genotyping from the Charlotte Maxeke Johannesburg Academic Hospital during 2009 were selected for the purposes of this study. Of the 51 samples, 26 were from ART-exposed patients (labelled 09TR) and 25 from patients with unknown ARV drug exposure history (labelled 09ZAP). Selected samples were initially used to set up the RT component of the in house gag-pol RT-PCR assay, and the remainder to subsequently obtain data.

2.1.3. PBMC samples from patients prior to treatment initiation, or failing first or second line ARV regimens, enrolled on the CIPRA-SA cohort

A total of 91 PBMC samples used for the evaluation of ARV drug resistance profiles were retrieved from the CIPRA-SA cohort. CIPRA (Comprehensive International Program of Research in AIDS) was a National Institutes of Health (NIH) funded programme that involved two clinic sites, namely; the perinatal HIV research unit (PHRU), Chris Hani Baragwanath in Soweto, Johannesburg (JHB) and Masiphumelele in Cape Town (CT). The

programme started in September 2002 with the first patients being enrolled in these projects by the first quarter of 2005 and funding continued until August 2011 (Wallis et al., 2012).

PBMCs were obtained from a total of 91 samples from HIV-1 infected patients of the CIPRA-SA cohort, at baseline (n=21), and at virological failure (n=70). Of the baseline samples, none were from JHB and 21 were from Cape Town., while for the virological failures, 49 were from JHB and 21 were from Cape Town. Virological failure was defined by either as less than 1.5 log drop in viral load measurements from baseline to 12 weeks or two consecutive viral load measurements greater than a 1000 RNA copies/millilitre (ml) after week 24. A summary of the demographic data and clinical characteristics of the CIPRA participants used in this study are shown in Table 2.1.

Table 2.1: Demographics and clinical characteristics at the time of treatment initiation of the 91 patient samples at the two CIPRA-SA sites.

CHARACTERISTICS	JOHANNESBURG SITE (n = 49)	MASIPHUMELELE SITE (n = 21 ART naïve; n = 21 ART exposed)
Age, median years	32.6	32.9
Female gender (%)	39 (80%)	15 (71%)
CD4 cell count, median cell/mm^3	170	129
Plasma HIV-1 viral load, median log_{10} copies/ml	171 060	854 486
Drug regimen (%)		
d4T, 3TC, EFV	24 (49%)	16 (76%)
d4T, 3TC, NVP	17 (35%)	2 (10%)
d4T, 3TC, LPV/r	6 (12%)	2 (10%)
d4T, 3TC, NFV	2 (4%)	1 (4%)

d4T: Stavudine, 3TC: Lamuvudine, EFV: Efavirenz, NVP: Nevirapine, LPV/r: Lopinavir/ritonavir, NFV: Nelfinavir.

The PBMC samples were used to co-culture potential drug resistant primary HIV-1 isolates, and the p24 antigen positive culture supernatant was used to extract viral RNA for use in the newly established in-house gag-pol RT-PCR assay to determine the presence of gag-pol cleavage site mutations

2.2. Development of an in-house gag-pol genotyping assay to characterize the HIV-1 gag-pol cleavage sites

An RT-PCR and sequencing protocol to amplify the HIV-1 subtype C gag and partial pol to evaluate the presence of mutations in gag-pol in the cohorts described in section 2.1 was designed and optimised.

2.2.1. Nucleic acid Extraction

2.2.1.1. Extraction of proviral DNA

Proviral DNA was extracted from the p24 antigen positive cultures from the passage 1 PBMCs described in section 2.1.1, using the High Pure Template Preparation Kit (Roche, Mannheim, Germany) as per manufactures, instructions. Briefly, the p24 antigen positive PBMCs were pelleted by centrifugation at 200 g for 10 minutes and re-suspended in 200µl of phosphate buffered saline (PBS) (Sigma, Buchs SG, Switzerland). Two hundred microlitres of Binding buffer and 40µl of proteinase K were added and incubated for 10 minutes at 70°C. Proteinase K was used for sample lysis and the inactivation of endogenous DNases. To this mixture, 100µl of isopropanol (MERCK, Wadeville, Gauteng, South Africa) was added, mixed well, and added into the upper reservoir of the filter tube. Samples were centrifuged in a 5810 Centrifuge (Eppendorf AG, Hamburg, Germany) at 8 000 X g for 1 minute. The flowthrough liquid was discarded, and 500µl of inhibitor removal was added to the upper reservoir and centrifuged for 1 minute at 8000 X g. The flowthrough liquid was discarded and 500µl of wash buffer was added to the upper reservoir, and centrifuged for 1 minute at 8 000 X g. This step was repeated, followed by final 10 seconds spin at 8 000 X g to remove residual wash buffer. The collection tube was then discarded and the filter tube inserted into a clean 1.5ml reaction tube and DNA was eluted by the addition of pre-warmed (70°C) elution buffer (200µl) and centrifugation for 1 minute at 8 000 X g. The extracted proviral DNA samples were stored at -20°C until further use.

2.2.1.2. Extraction of viral RNA

HIV-1 RNA was isolated from 200µl plasma (section 2.1.2; or culture supernatant, section 2.1.3) by using the MagNa Pure LC total nucleic acid isolation kit (Roche, Mannheim, Germany) on the automated Roche MagNa Pure LC analyser. The kit uses patient plasma/culture supernatant to isolate total nucleic acids using magnetic bead technology.

Briefly, the samples were lysed during an incubation process which used a buffer that contained a chaotropic salt and Proteinase K. The chaotropic salt disrupted the dimensional structure in macromolecules such as protein, DNA and RNA, and denatured them, whilst the proteinase K digested the cellular proteins that are present. Magnetic Glass Particles (MGPs) were subsequently added, which the nucleic acids bound to. Any unbound substances were removed by several washing steps. The purified total nucleic acid was eluted with a low salt buffer in a total volume of 100µl. Eluted viral RNA was stored at -70°C until used.

2.2.2. Establishment of an in-house gag-pol genotyping assay

Extracted proviral DNA from samples 05ZAFV3 and 05ZAFV6 were initially used to set up the PCR component of the RT-PCR reaction. Once this was optimized, viral RNA from samples 09ZAP16 and 09ZAP25 were used to set up the step of RT of the RT-PCR amplification reaction. The optimized RT-PCR protocol is described below.

2.2.2.1 RT-PCR and Sequencing Primers used in the in-house assay

Primers for the RT-PCR amplification and sequencing of an approximately 3002 base pair (bp) fragment encompassing the near full length gag, protease and up to codon 67 in RT were selected from a panel of available primers in our laboratory (Table 2). These primers were designed from the 2002 consensus HIV-1 subtype C sequence from the Los Alamos database (http://www.hiv.lanl.gov). The two PCR primers covered nucleotides from positions 798 to 2772 according to the HXB2 sequence. The five sequencing primers ensure sequencing primer coverage according to HXB2 sequence from nucleotide 1400 to 2271.

Table 2.2: Primers to RT-PCR amplify and sequence the *pol* region of HIV-1

PRIMER	POSITION (HXB2)	DIRECTION	PRIMER SEQUENCE	USE
CWCS2	2753 - 2772	Reverse	5,• CCCAAAAGTCTTGAGTTCT-3,	RT and PCR
CWGAG1	798 • 804	Forward	5,• GCGAGAGCGTCAYTATTAA-3,	PCR
CWGAG2	1400 • 1418	Forward	5,-CATCAATGAGGARGCTGC-3,	SEQUENCING
CWGAG3	2002 • 2020	Forward	5,-GCAGGGCCCCTAGGAAAA-3,	SEQUENCING
CWGAG4	2252 • 2271	Reverse	5,-GCAAAAGAGTGATTTGAGG-3,	SEQUENCING
CWGAG5	1481 • 1500	Reverse	5,-AGTTCCTGCTATGTCACTTC-3,	SEQUENCING
CWGAG6	890 • 908	Reverse	5,-TCYCTGCTTGCCCATACT-3,	SEQUENCING

2.2.2.2. Reverse Transcriptase-Polymerase Chain Reaction Amplification

For the cDNA synthesis (RT step), the Transcriptor High Fidelity cDNA synthesis kit (Roche, Mannheim, Germany) was used, as per manufacturer,s instructions. The reverse primer CWCS2 (2.5 µM) was added to 8 µl of the extracted RNA, and denatured at 65°C for 10 minutes. This was followed by addition of 1 x Expand buffer containing magnesium chloride ($MgCl_2$), 10mM dithiothreitol (DTT), 1mM dNTPs, 1.0U RNase Inhibitor and 2.5U Expand Reverse Transcriptase to a total volume of 20µl, and heated at 42°C for 60 minutes. Reverse transcription was carried out using a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Foster City, USA).

For PCR amplification, the Expand High Fidelity PLUS PCR System (Roche, Mannheim, Germany) was used, as per manufacturer,s instructions, to generate a 2000bp amplicon. Briefly, 5 µl of the extracted proviral DNA or 20 µl of the reverse transcription product, 0.4mM of the forward CWGAG1 and reverse CWSC2 primers, 1x PCR buffer (containing $MgCl_2$), 0.2mM dNTPs, 0.05U Taq enzyme and X µl RNase free water (Fermentas, Burlington, Canada) were added to a total volume of 50 µl. Amplification was carried out using a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Foster City, USA). The thermocycling conditions consisted of an initial denaturation step of 94°C for 2 minutes. This was followed by 10 cycles of denaturation at 94°C for 30 seconds, annealing at 50°C for 30 seconds; and elongation at 72°C for 2 min; and a further 30 cycles of denaturation at 94°C for 30 seconds, annealing at 50.5°C for 30 seconds and elongation at 72°C for 2 minutes with

an additional 5 seconds extension per cycle, and a final extension at 72°C for 10 minutes followed by a 4°C hold. The amplicons were resolved by agarose (1%) gel electrophoresis for 60 minutes at 110 volts, and viewed under a UV transilluminator (Appendix B1 and B2).

2.2.2.3. Column Purification of PCR amplicons

Column purification was performed using the GeneJet™ PCR Purification Kit (Fermentas, Burlington, Canada) to purify PCR products by removing the primers, dNTPs, buffers, etc. from solution. In this purification system, a reaction mixture containing DNA was combined with the binding buffer and added to a purification column. A chaotropic agent in the binding buffer denatured proteins and promoted DNA binding to the silica membrane in the column. Briefly, 40µl of binding buffer was added to 40µl of PCR mixture and mixed thoroughly. This solution was then transferred to the GeneJet™ purification column, centrifuged for 60 seconds at 12000 x g (Centrifuge 5415 D) (Eppendorf, AG, Hamburg, Germany) and the flow-through discarded. Seven hundred microliters of wash buffer was added to the column, centrifuged for 60 seconds at 12000 x g and the filtrate was discarded. The empty purification column was placed back into the collection tube and centrifuged for an additional 1 minute at 12000 x g to completely remove any residual wash buffer. The column was then transferred to a clean 1.5 ml microcentrifuge tube and 50µl of the elution buffer was added to the membrane and centrifuged for 1 minute at 12000 x g. The purified samples resolved by agarose (1%) gel electrophoresis containing 30pM ethidium bromide (Sigma, USA) in a 1xTAE buffer (Fermentas Life Sciences, Lithuania) for 60 minutes at 110 volts, viewed under a UV transilluminator. The size and concentration of the amplicons was established by comparing it to a DNA Mass Ladder (MassRuler™ DNA ladder, Mix, ready-to-use Fermentas Life Sciences, Lithuania). gel electrophoresis was performed before and after PCR purification to check the purity of the PCR amplicons in order for a sequencing reaction to be successful.

2.2.2.4. Automated dideoxy Sequencing

The amplicons obtained in section 2.2.2.3 were diluted according to their concentration, and sequenced using a cycle sequencing reaction with the ABI PRISM® BigDye® Terminator version 3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA). A set of 5 primers were used for complete coverage of the region: 2 forward primers, CWGAG2 and CWGAG3; and 3 reverse primers CWGAG4, CWGAG5 and CWGAG6 (Table 2.2). Cycle sequencing was performed by adding 3.2 pM of each primer to 2µl of amplicon, 0.5 x of the

Big Dye Terminator Kit version 3.1 sequencing mix, 0.5 x of sequencing buffer (containing Tris-HCl and $MgCl_2$) and 11µl of RNase free water to a total volume of 20µl. The cycle sequencing mix includes fluorescently labelled ddNTPs, known as dideoxynucleotides, which lack a 3'-OH group that is required for the formation of a phosphodiester bond between two nucleotides, therefore terminating DNA strand extension and resulting in DNA fragments of varying lengths. Each base has a differently coloured fluorescent dye that can be excited by a laser. (Sanger et al., 1977; Smith et al., 1985).

The cycling conditions consisted of an initial denaturation step of 96°C for 1 minute, followed by 25 cycles of denaturation at 96°C for 10 seconds, annealing at 50°C for 5 seconds, elongation at 60°C for 4 minutes and a final 4°C hold. Cycle sequencing was performed on a GeneAmp PCR system 2700 thermocycler (Applied Biosystems, Foster City, USA).

The removal of primers, unbound, ddNTPs buffer, etc. after cycle sequencing was achieved by an isopropanol precipitation. Briefly, 75% isopropanol was freshly prepared by transferring 7.5 ml of 100% isopropanol and 2.5 ml of RNase free water into a 15 ml Nunc tube (Nunc, AEC Amersham). Eighty microlitres of isopropanol was added to each 20 µl sequencing reaction. The plate was mixed by pipetting up and down and covered with a plate cover. The plate was centrifuged at room at temperature in the 5810 Centrifuge (Eppendorf, AG, Hamburg, Germany) at 2000 x g for 45 minutes. After centrifugation, the plate cover was removed and the plate was inverted and centrifuged at 700 x g for 1 minute. The plate was left to air dry for 10 minutes and the samples resuspended in 20 µl of Hi-Di™ Formamide (Applied Biosystems, Foster City, USA) and covered with septa.

Sequence chromatograms were generated by using an automated ABI PRISM® 3730 Genetic Analyzer (Applied Biosystems, HITACHI, Foster City, USA). The sequencer carries out capillary electrophoresis for size separation, detection and recording of dye fluorescence. The data output is shown as fluorescent peak trace chromatographs. Sequence information was processed by the ABI PRISM ® 3730 Data Collection Software version 1.1, and all generated sequence data was edited using the Sequencing analysis 3.3 program (Applied Biosystems, Foster City, USA).

2.2.2.5. Genotypic Analysis

All generated sequences were assembled and manually edited using Sequencher v4.8 software (Genecodes Corporation, Ann Arbor, USA). This software allows for sequence assembly, contig editing and mutation editing. Polymorphisms were detected by direct inspection of sequence chromatograms as displayed in Sequencher. The sequences were imported into the software and were trimmed to remove any ambiguous data. Contigs were assembled using the Dirty Data algorithm, as this is best suited for data that may include occasional ambiguities or mismatches that are generated by automated sequences. The minimum match percentage and minimum overlap were 85% and 20 bases, respectively. Fragments were viewed by using the „view contig assemble… application according to position, 5, to 3,, within the contig. This was done to make sure that all sequencing primers were orientated in the correct orientation. Sequences were also checked for complete, bi directional coverage (sense and antisense sequence) from at least one primer in each direction over the entire region analysed. Sequences were checked and edited by using the „edit assembled chromatogram… application. Nucleotide mixtures are common in clinical HIV samples. Whenever there were more than one nucleotide (green adenine, A; blue cytosine, C; red thymine, T and black guanine, G) at a given position, the sequence was evaluated to check whether it was a sequencing artefact or a true mixture. Once it was determined that a true mixture was present, Editing Palette was used to select the correct Code for the International Union of Biochemistry (IUB code, http://www.cortec.ca/iubcodes.html) (Appendix B, Table B1) and the letter was entered into the consensus sequence.

Once manual editing was completed the sequences were saved in fasta format and were submitted to the Stanford HIV Drug Resistance database (http://hivbd.standford.edu) to determine if there were any known mutations in the PR region. Reports were generated and analysed. To validate the mutations determined by the HIV Drug Resistance Database, sequences were submitted to the Viroscore software (TherapyEdge 3.8.4, https://196.36.218.99).

A multiple alignment of the gag-pol with reference sequences from HIV-1 subtypes A to K, and CRF01_AE and CRF02_AG (http://hiv-web.lanl.gov), were generated using ClustalX

(version 2.0.11) http://www.ebi.ac.uk/Tools/clustalx). CLUSTAL X is a tool used for analysing multiple sequences, with the most closely related groups of sequences aligned first. This programme provides a single environment in which the user can perfom multiple alignments, view results, process and improve the alignment simultaneously (Thompson et al., 1997). The generated Clustal X alignment was used to construct a Neighbour-joining phylogenetic tree in MEGA 5.05 (http://www.megasoftware.net) with the Kimura two-parameter model. The stability of the nodes was assessed by bootstrap analysis (1000 replicates), and bootstrap values greater than 70% were considered significant. MEGA (Molecular Evolutionary Genetics Analysis) is a programme used to perform automatic and manual sequence alignments, analysing sequence alignments to estimate evolutionary distances, inferring phylogenetic trees and testing evolutionary hypotheses (Tamura et al., 2011). The Kimura two-parameter method infers evolutionary distances in which transitions and transversions are treated as separate entities.

In addition, the generated Clustal X alignment was manually edited with GeneDoc version 2.6.002 (www.psc.edu/biomed/genedoc), and the codon positions associated with mutations in the gag-pol cleavage sites, specifically p7/p1 and p1/p6 (codon positions 373, 431, 437, 449 and 453) were extensively analysed and compared amongst all samples.

2.3. Determination of gag-pol cleavage site mutations in antiretroviral drug naive and treatment experienced cohorts

The newly established RT-PCR gag-pol genotyping protocol described in section 2.2 was used to establish the presence/absence of HIV-1 gag-pol cleavage site mutations in all 165 plasma and PBMC samples described in section 2.1.

2.4. HIV-1 drug resistance genotyping using an in-house pol genotyping assay

The validated in-house pol genotyping assay used in our Genotyping laboratory for the routine patient monitoring for the presence of HIV-1 antiretroviral drug resistance (Wallis, Papathanasopoulos et al., 2010) to currently available ARV drugs was used to amplify, sequence and genotype the pol region (approximately 1550 bp) of all 165 plasma and PBMC samples described in section 2.1. This in-house RT-PCR assay includes all of PR and up to position 355 in RT.

The sequence data generated from the overlapping regions in PR and RT for the newly developed gag-pol genotyping assay and the validated pol genotyping assay were assembled and compared to ensure nucleotide sequence homology between the two assays, as well as assay accuracy by comparison of the Stanford HIV-1 ARV drug resistance mutation profiles generated by the two methods.

2.5. Antiretroviral drug resistant primary HIV-1 viral isolation

PBMC samples from the Comprehensive International Program of Research on AIDS in South Africa (CIPRA-SA) cohort were selected based on the gag-pol cleavage site mutation and pol genotyping results, and used to isolate ARV drug resistant primary HIV-1 virus isolates.

2.5.1. PBMC isolation from HIV-1 seronegative blood donors

Two leukocyte-enriched whole HIV-1 negative blood (buffy coats) were received weekly from the South African National Blood Service (SANBS). The buffy coat pack was cut at the bottom and the contents were emptied into a 50 ml tube. The two donors were kept separate. The approximately 50ml buffy coat sample was divided between two 50ml tubes and diluted up to 30ml with fetal calf serum-phosphate buffer saline (FCS-PBS) (Appendix B3) in each tube. Fifteen millilitres of Ficoll-Hypaque (Amersham Pharmacia Biotech, Uppsala, Sweden) was placed in a 50ml tube, one for each donor. The diluted blood was then layered over the Ficoll and centrifuged at 850 x g for 30 minutes without a brake (Centrifuge 5810 R, Eppendorf, North America). Most of the upper layer was removed and discarded and the PBMCs at the interface were carefully removed, without disturbing the Ficoll, and transferred into a sterile 50ml centrifuge tube. These cells were washed 3 times in 40ml 1-PBS by resuspension and were centrifuged at 210 x g for 10 minutes. The cell pellets were resuspended in 20ml of Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich Company, Steinheim, Germany) (Appendix B4). Ten microliters of the cell suspension was taken and added to 90µl of Trypan Blue solution (0.4%) (Sigma-Aldrich Company, Steinheim, Germany) in a 1.5ml Eppendorf tube (Eppendorf AG, Hamburg, Germany). Ten microliters of the Trypan Blue-cell mixture was taken and placed on a Haemocytometer slide and cells were counted. The volume required to give a final concentration of 1.5 x 10^6 cells/ml in RPMI was calculated. The cell suspension was adjusted accordingly in sterile tissue flasks, with no more than 100ml/flask. PHA (Sigma-Aldrich Company, Steinheim, Germany) (Appendix B5) was added to cells to give a final concentration of 1µg/ml and incubated in an $37^{\circ}C$, 5% CO_2

upright position at 37ºC, 5% CO_2 for 3 days. Cells from different donors were not mixed at this stage.

2.5.2. Isolation of ARV drug resistant primary HIV-1

Before use, the 30ml of donor cells from section 2.5.1 were placed in 50ml centrifuge tubes and centrifuged at 290 x g for 10 minutes. The supernatant was carefully discarded and cells were resuspended in 30ml of fresh RPMI, recounted and set back up to 2×10^6 cells/ml by diluting accordingly. Two millilitres of donor one, 2ml of donor two and 50µl of interleukin 2 (IL-2) (Roche, Mannheim, Germany) per millilitre were placed in a 25cm^3 sterile tissue flask. This was set aside. The infected PBMCs were thawed rapidly. Nine millilitres of fresh RPMI and 1ml of the infected PBMCs were added into a 15ml centrifuge tube, and centrifuged at 290 X g for 10 minutes. The supernatant was carefully discarded and the pellet was resuspended in 1ml of RPMI. This was finally added to the 25cm^3 tissue flask containing the donor mixture and incubated at 37ºC with 5% CO_2 for up to 28 days.

Cultures were maintained by replacing 50% of the total volume of culture medium with fresh medium containing IL-2 (Roche Diagnostics, Mannheim, Germany) on days 4, 10, 17 and 24 and with PHA-stimulated donor PBMCs on days 7, 14, 21 and 28. The primary viral cultures were monitored weekly (days 7, 14, 21 and 28) for viral growth indicated by the rise in p24 antigen. Cultures with negative p24 readings by day 28 were discarded. Cultures that reached high levels of p24 were expanded into 10ml. Once the expanded cultures yielded high viral titer, they were centrifuged at 290 X g for 10 minutes. The infected primary PBMCs and infected supernatant was stored at -80ºC until required.

2.5.3. p24 Antigen enzyme-linked immunosorbent assay (ELISA)

The Vironostika HIV Uni-Form II Ag/Ab (bioMérieux, Marcy l'Etoile, France) was used to monitor p24 levels as a measure of viral growth in the PBMC culture experiments, as per manufacturers, instructions. All preparation of reagents used in this assay are listed in Appendix B6. Briefly, 100µl of the specimen diluent was pipetted into all wells and to this 50µl of the supernatant from the infected PBMCs was added. Both the negative and positive (HIV-1 antigen positive) controls were included. The strips were covered with an adhesive plate sealer and incubated for 60 minutes at 37ºC. After the incubation period, the wells were washed 5 times with a phosphate buffer. One hundred microliters of the tetramethylbenzide (TMB) substrate solution was added to each well and incubated for 30 minutes at room

temperature in the dark. The reaction was stopped by adding 100µl (1 molar) of sulphuric acid to well and the strip holder was tapped to ensure thorough mixing. The absorbance of the solutions in the well was read at 450 nm with a microplate reader and the 620 nm was dual wavelength reference.

2.5.4. RNA extraction from p24 antigen culture supernatants

Viral RNA was extracted from p24 antigen positive culture supernatants of primary virus isolates from section 2.5.2 using the NucliSENS easyMag Extractor (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's recommendations, with a few exceptions. Briefly, 200µl of culture supernatants or plasma was placed in a disposable sample vessel and loaded onto the extractor, and the „Generic Extraction Protocol… was used. After the viral lysis incubation, 50µl of magnetic silica, which captured all the target nucleic acids, was added to each sample, and the extractor was restarted. The NucliSENS easyMag magnetic device attracted all the magnetic silica, enabling the system to purify the nucleic acids through several washing steps. The heating step released the nucleic acids from the silica. At the final step, the samples were eluted in 25µl of eluate, in which the magnetic particles were separated from the eluate by the magnetic device. The eluted viral RNA was stored at -80ºC until further use.

2.5.5. Analysis of gag-pol cleavage site mutations and pol genotypes of ARV drug resistant primary virus isolates

Extracted viral RNA isolated from culture supernatant of primary virus isolates (section 2.5.2) was used to genotype the gag-pol cleavage sites and pol genotypes

The ARV drug resistance profiles obtained directly from patient plasma were compared to that obtained from the primary virus isolate supernatant. This was necessary since HIV-1 exists as quasispecies and culturing may select for outgrowth of isolates with different mutation profiles than that seen in the patient. In addition, the profiles were linked to the ARV drug regimen of each patient. For subtyping, all sequences, together with reference sequences from HIV-1 subtypes A to K, and CRF01_AE and CRF02_AG (http://hiv-web.lanl.gov), were aligned in Clustal X, and used to construct Neighbour-Joining phylogenetic tree in MEGA 5.05 (http://www.megasoftware.net) with the Kimura two-parameter model.

CHAPTER 3

RESULTS

3.1. Demographic and clinical data of samples used in this study

A summary of the demographic data and clinical characteristics of the 165 patient samples used in this study are shown in Table 3.1, with details of each patient in Appendix C. Of the 165 samples available for the purposes of this study, 44 were ARV drug naive, 25 had unknown ARV drug history and 96 were ARV drug exposed (70 on a first line regimen and 26 on a second line regimen).

Table 3.1: Demographic data and clinical characteristics of the 165 patients used in this study.

Enrolment characteristics	
Female	109 (66.1%); n= 165
Viral Load (RNA copies/ml) average; range	245510 (140 to 7 500001); n= 165
Regimen Prescribed	n=96
Stavudine-Lamivudine-Efavirenz	40
Stavudine-Lamivudine-Nevirapine	19
Stavudine-Lamivudine-Lopinavir/r	8
Stavudine-Lamivudine-Nelfinavir	3
Zidovudine-Didanosine-Lopinavir/r	18
Efavirenz-Lamivudine-Lopinavir/r	4
Zidovudine-Lamivudine-Lopinavir/r	3
Zidovudine-Stavudine-Lopinavir/r	1

3.2. Development of an in-house assay to sequence the HIV-1 gag-pol cleavage sites

An optimised RT-PCR protocol to evaluate the presence of mutations in gag-pol in HIV-1 subtype C infected populations was established. Overall, this protocol used an automated viral RNA extraction method, amplification of the viral RNA using subtype C specific primers; column based PCR amplicon purification and automated sequencing using of subtype C specific primers.

The PCR component of the RT-PCR protocol was optimised using the extracted proviral DNA from samples 05ZAFV3 and 05ZAFV9 and amplified an approximately 2kb fragment

encompassing the near full length gag, protease and up to codon 67 of the reverse transcriptase (Figure 3.1). Four of the five sequencing primers consistently generated sequences for all samples (Figure 3.2). CWGag6 did not generate sequence for any samples. The RT-PCR protocol was optimised using the extracted viral RNA from samples 09ZAP16 and 09ZAP25 (results not shown). Similarly to the proviral DNA amplicons, CWGAG6 did not generate sequence for any samples.

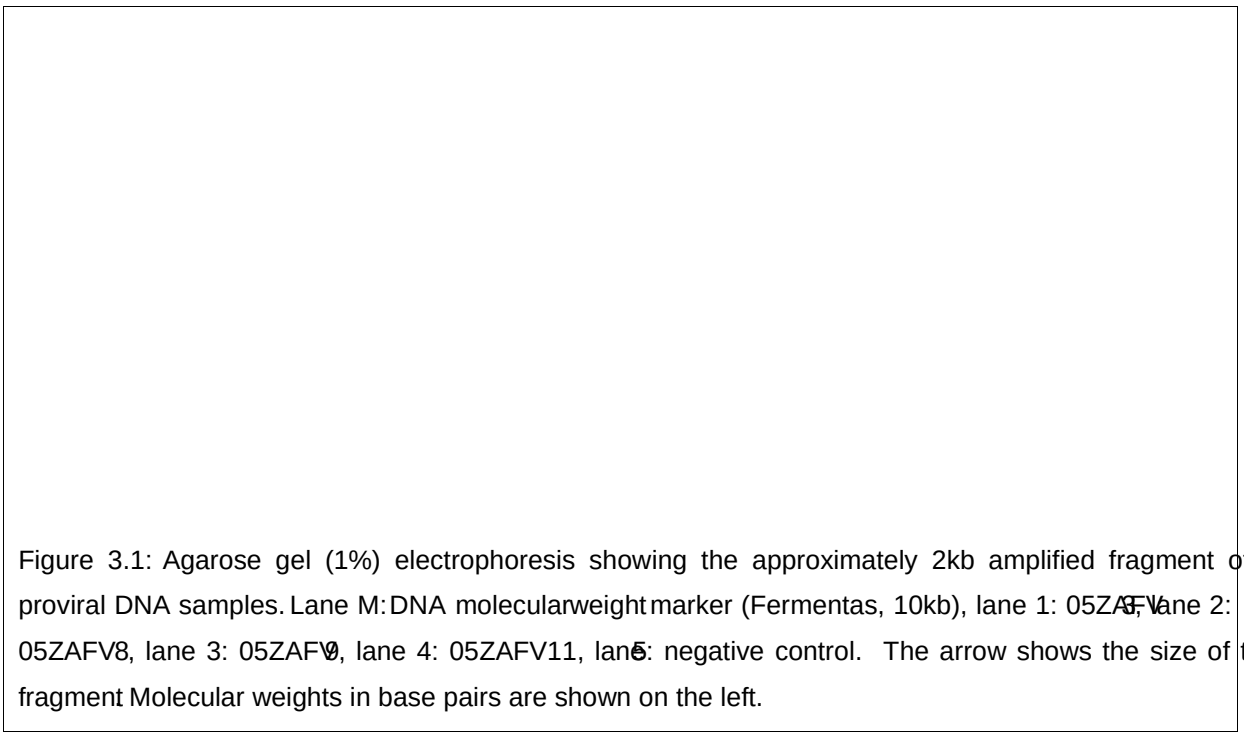


Figure 3.1: Agarose gel (1%) electrophoresis showing the approximately 2kb amplified fragment of proviral DNA samples. Lane M: DNA molecular weight marker (Fermentas, 10kb), lane 1: 05ZAFV7, lane 2: 05ZAFV8, lane 3: 05ZAFV9, lane 4: 05ZAFV11, lane 5: negative control. The arrow shows the size of the fragment. Molecular weights in base pairs are shown on the left.

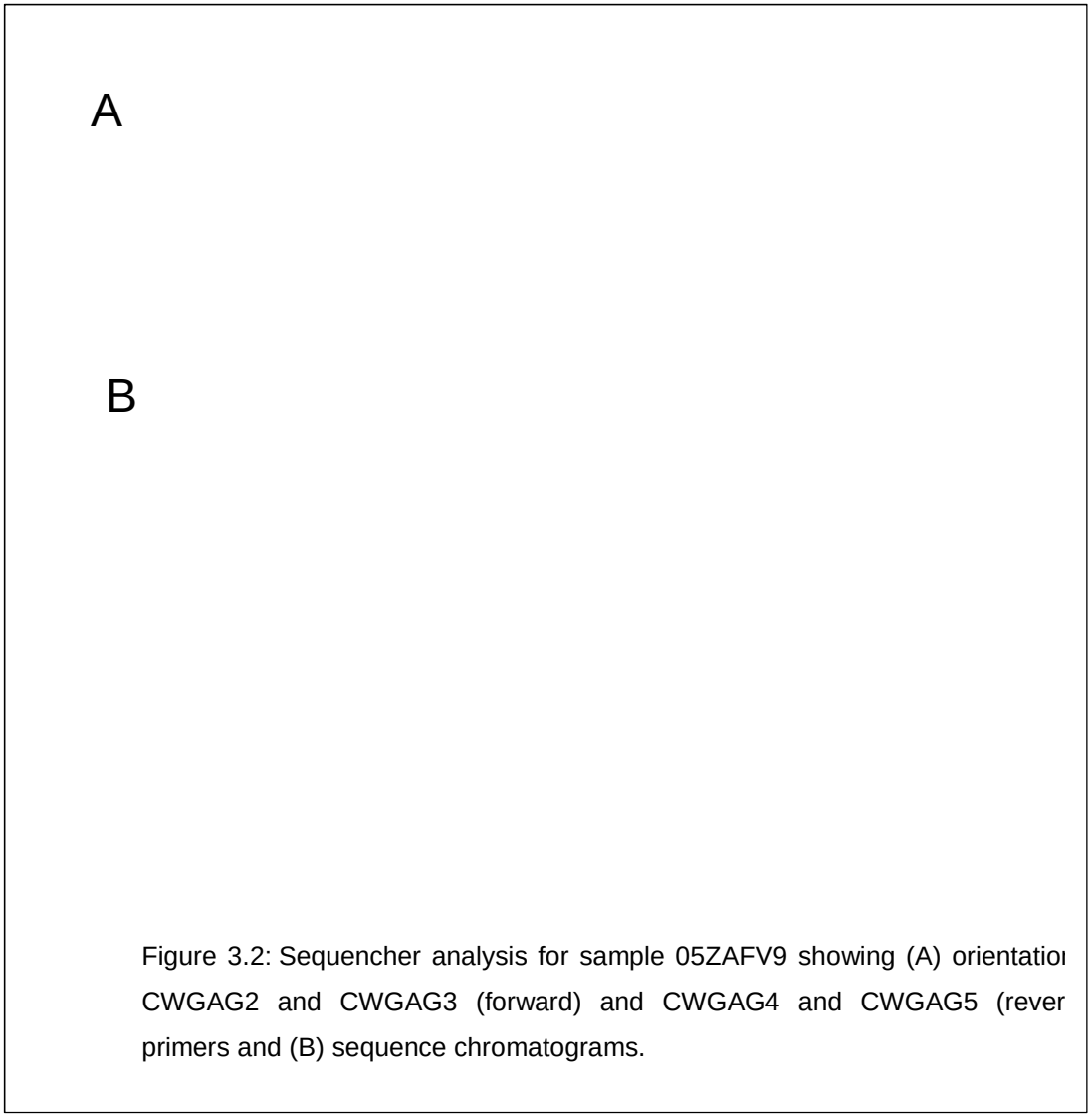


Figure 3.2: Sequencher analysis for sample 05ZAFV9 showing (A) orientatio CWGAG2 and CWGAG3 (forward) and CWGAG4 and CWGAG5 (rever primers and (B) sequence chromatograms.

3.3 Genetic characterization of the gag-pol cleavage sites and pol genotypes

Extracted viral RNA isolated from plasma or culture supernatant as well as proviral DNA was used to genotype the gag-pol cleavage sites and pol genotypes. The five gag-pol codons associated with changes in PI susceptibility were extensively analysed and compared amongst all the ARV drug naïve and ARV drug exposed samples, as well as compared to the HIV-1 subtype B HXB2 sequence. All the HIV-1 antiretroviral drug resistance pol genotypes were submitted to the Stanford University HIV drug resistance database.

3.3.1. Primary virus isolates from antiretroviral drug-naive patients (proviral DNA)

Overall, 18 of the 23 (78.26%) and all 18 (100%) proviral DNA samples were successfully amplified and sequenced using the newly developed gag-pol cleavage site mutation and in house genotyping assays, respectively. None of the samples contained any of the known gag-pol cleavage site mutations, nor any major PR and RT mutations, with only 3 having minor PR mutations (Appendix C).

3.3.2. Plasma samples from patients failing first or second line ARV regimens at the Charlotte Maxeke Johannesburg Academic Hospital

Of the 51 plasma samples, 33 (64.7%) were successfully HIV RT-PCR amplified and sequenced using the newly developed gag-pol cleavage site mutation protocol and in house genotyping assay, respectively. This included 16 of the 25 (64%) with unknown drug history and 17 of the 26 (65.4%) failing a second line regimen.

Only 5 patients (15.2%) had gag-pol cleavage site mutations (Table 3.1). These included viruses from patients 09ZAP25 and 09TR05 that contained the mutation P453L; patient 09TR06 that contained the L449P and P453L mutations; patient 09TR09 that contained the S373Q and I437T mutations; while patient 09TR25 contained the I437T gag-pol cleavage site mutation. It was also noted that the nucleotide sequences of the S373, I437, L449 and P453 sites in the gag-pol cleavage sites were highly polymorphic, and generally coded for an amino acid sequence change (Appendix D). 10 patients contained the A431V mutation

A further five 5 patients (15.2%) had major PR mutations (Table 3.1; Figure 3.3). These included viruses from patient 09ZAP16 that contained the M46L mutation; patient 09ZAP17 that contained the L90M mutation; 09TR03 that contained the V32I, M46L, I47V and I54V mutations; 09TR21 that contained the M46I, I47V and I50V mutations; and 09TR24 that contained the M46L and L76V mutations. Eight (24.2%) patients had one or two minor PR mutations (Appendix C).

Table 3.2 Summary of the gag-pol cleavage sites and pol genotypes from 33 plasma samples from patients failing first or second line ARV regimens at the Charlotte Maxeke Johannesburg Academic Hospital. Samples with mutations are highlighted in yellow. NRTI- Nucleoside reverse transcriptase inhibitors; NNRTI- nonNRTI. Mutations are highlighted in yellow.

PID	PR mutations		RT mutations		gag-pol cleavage sites (excluding A431V mutation)			
	Major	Minor	NRTI	NNRTI	S373	I437	L449	P453
VIRAL RNA -UNKNOWN								
09ZAP01	None	None	None	None	None	None	None	None
09ZAP02	None	None	None	None	None	None	None	None
09ZAP03	None	None	None	None	None	None	None	None

Table 3.2 Summary of the gag-pol cleavage sites and pol genotypes from 33 plasma samples from patients failing first or second line ARV regimens at the Charlotte Maxeke Johannesburg Academic Hospital. Samples with mutations are highlighted in yellow. NRTI- Nucleoside reverse transcriptase inhibitors; NNRTI- Non NRTI. Mutations are bolded and highlighted in yellow.

09ZAP04	None	L10V	None	None	None	None	None	
09ZAP06	None	None	None	None	None	None	None	None
09ZAP07	None	None	None	None	None	None	None	None
09ZAP10	None	None	None	None	None	None	None	None
09ZAP13	None	None	None	None	None	None	None	None
09ZAP14	None	None	None	None	None	None	None	None
09ZAP16	M46L	None	None	None	None	None	None	None
09ZAP17	L90M	T74S	None	None	None	None	None	None
09ZAP18	None	None	None	None	None	None	None	None
09ZAP19	None	None	None	None	None	None	None	None
09ZAP21	None	None	None	None	None	None	None	None
09ZAP22	None	T74S	None	None	None	None	None	None
09ZAP25	None	None	None	None	None	None	None	P453L
VIRAL RNA -DRUG EXPOSED								
09TR02	None	L10I, T74S	None	K103N	None	None	None	None
09TR03	V32I, M46L, I47V, I54V	L33V, T74S	M184V	K103N	None	None	None	None
09TR05	None	None	M184V	None	None	None	None	P453L
09TR06	None	None	M184V	K103N, V106M, F227L	None	None	L449P	P453L
09TR07	None	None	M184V	K103N	None	None	None	None
09TR08	None	None	M184V	K103N	None	None	None	None
09TR09	None	T74S	None	T215D, A98Q	S373Q	I437T	None	None
09TR10	None	None	None	K103N	None	None	None	None
09TR11	None	L10I	M184V	Y188L	None	None	None	None
09TR12	None	None	None	G190A	None	None	None	None
09TR14	None	T74S	M184V	None	None	None	None	None
09TR20	None	None	M184V	K103N	None	None	None	None
09TR21	M46I, I47V, I50V	None	None	None	None	None	None	None
09TR22	None	None	M184V, K65R	V106M, A62V, V179D	None	None	None	None
09TR24	M46L,L76V	None	M41L	None	None	None	None	None
09TR25	None	None	M184V, M41L	F227L	None	I437T	None	None
09TR26	None	None	M184V	T215Y, Y188L	None	None	None	None

Figure 3.3: The frequency of the major and minor protease mutations in all 33 ARV naïve and ARV exposed patients. (Major mutations, Minor mutations).

Sixteen (48.5%) patients had RT mutations, while 9 had both NRTI and NNRTI mutations (Table 3.1,Figure 3.4).The major NRTI mutations were M184V (n=11, 33.3%), M41L (n=2, 6.06%) and K65R (n=1, 3.03%). The K103N NNRTI mutation was the most prevalent (n=9, 27.3%),followed by V106M (n=2, 6.06%).

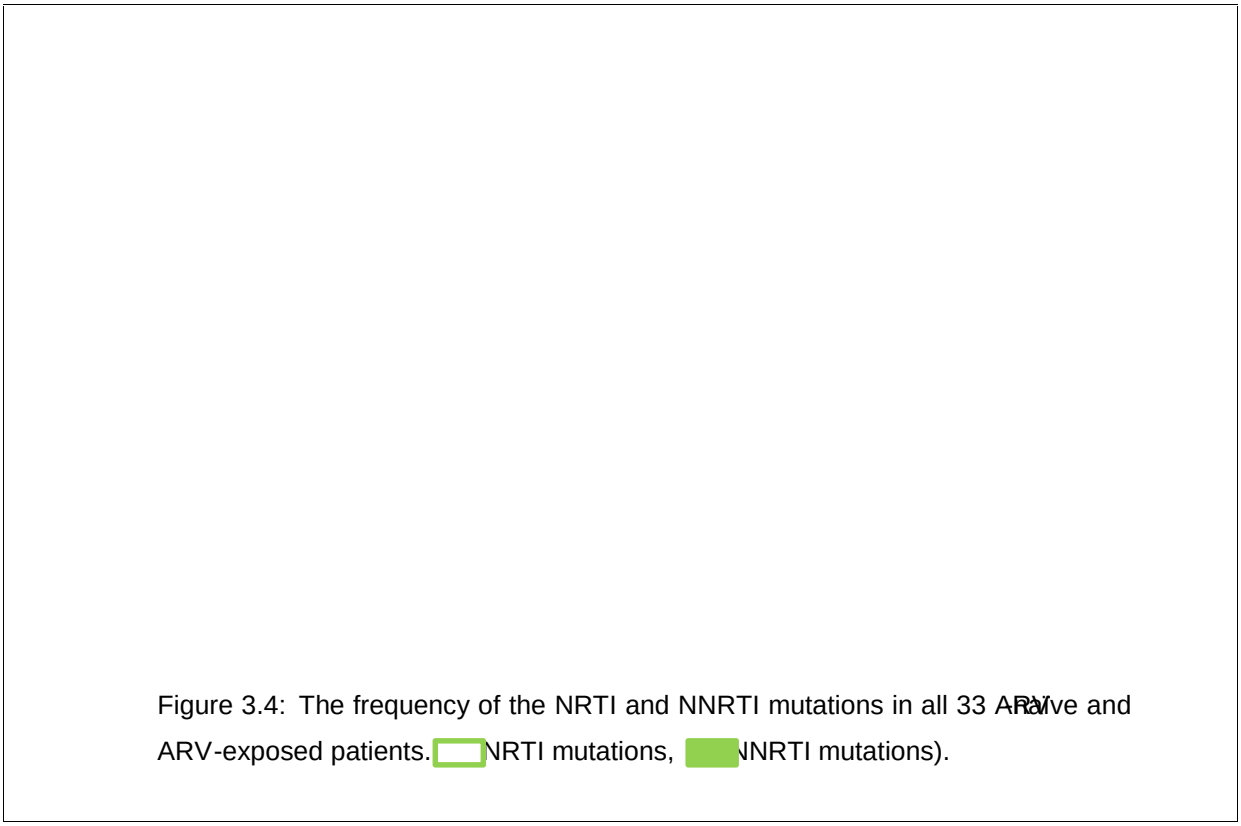


Figure 3.4: The frequency of the NRTI and NNRTI mutations in all 33 ARV-naïve and ARV-exposed patients. (NRTI mutations, NNRTI mutations).

Overall, it was observed that none of the gag-pol cleavage site mutations were observed in combination with any major PR mutations (Table 3.2). Four of the patients with gag-pol mutations had mutations present which would have reduced the susceptibility to NRTIs and/or NNRTIs.

3.4. Subtyping

Phylogenetic tree analysis was performed on the gag-pol nucleotide sequences from the 33 successfully sequenced samples, and revealed that all samples clustered within HIV-1 subtype C with significant bootstrap values, and confirmed that there was no cross contamination of samples (Figure 3.5).

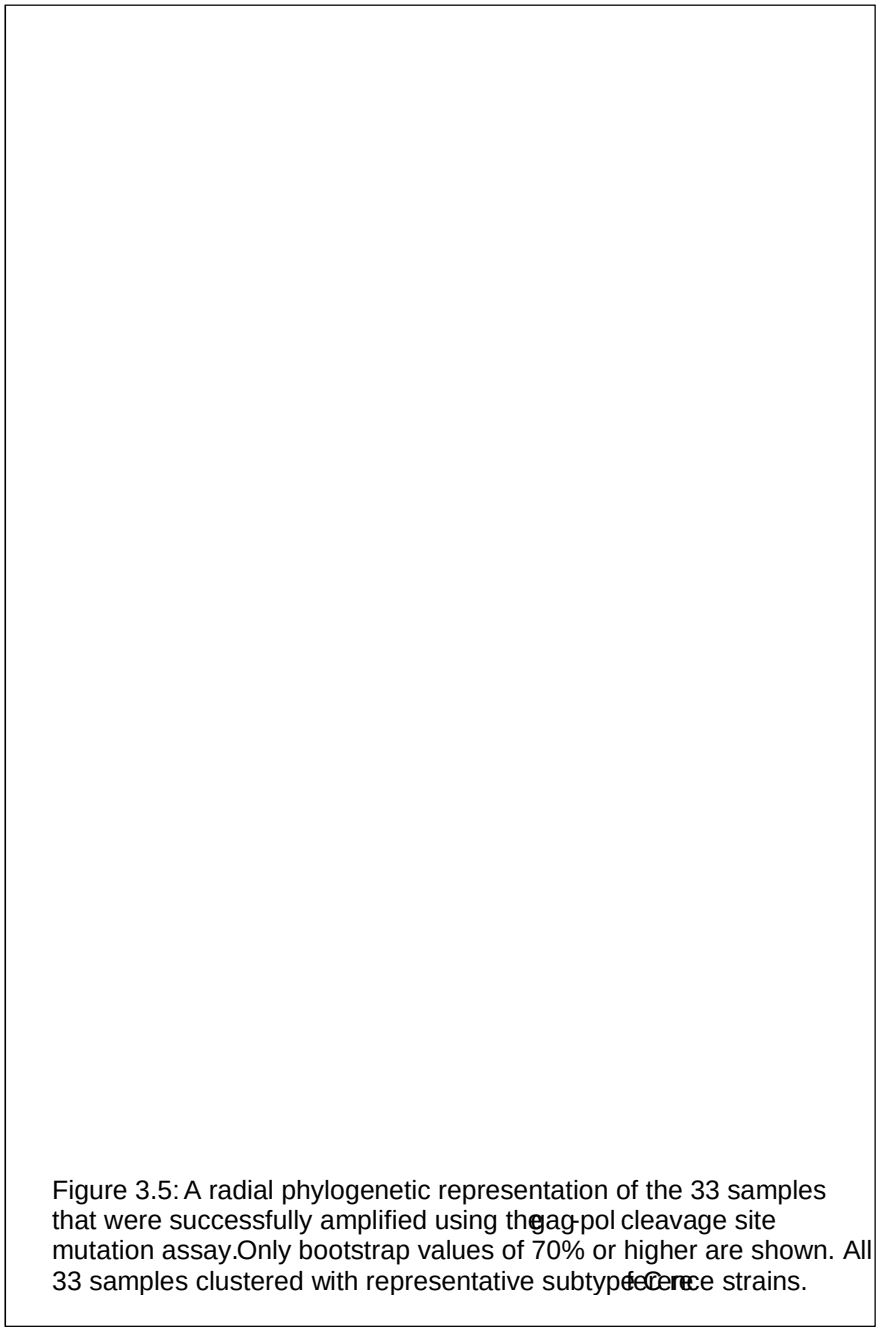


Figure 3.5: A radial phylogenetic representation of the 33 samples that were successfully amplified using the gag-pol cleavage site mutation assay. Only bootstrap values of 70% or higher are shown. All 33 samples clustered with representative subtype C reference strains.

3.5. Isolation of antiretroviral drug resistant primary virus isolates

3.5.1. Co-cultivation experiments

All 91 CIPRA-SA PBMC samples were grown in co-culture experiments, of which only 31 (34.1%) primary virus isolates were obtained. These included, 5 of 21 (23%) virus isolates from CT baseline PBMC samples and 26/49 (53 %) virus isolates from JHB failure PBMC

sample grew. None of the 21 primary virus isolates from CT failure PBMC samples were obtained from co-culture experiments.

The 5 primary virus isolates from the CT baseline PBMC samples included 11MR29, 11MR32, 11MR36, 11MR37 and 11MR39. All patients were subsequently exposed to d4T-3TC-EFV first line regimen. The 26 primary virus isolates, and the antiretroviral drug regimens the patients were exposed to from the JHB failure PBMC samples are listed in Table 3.3.

Table 3.3: Primary virus isolates obtained from PBMCs of patients failing antiretroviral therapy, enrolled in the Johannesburg arm of the CIPRSA cohort. d4T: Stavudine, 3TC: Lamuvidine, EFV: Efavirenz, LPV/r: Lopinavir/rtv, NVP: Nevirapine, NFV: Nelfinavir

PID AT FAILURE	REGIMEN
11MR44	d4T-3TC-EFV
11MR45	d4T-3TC-EFV
11MR47	d4T-3TC-LPV/r
11MR52	d4T-3TC-LPV/r
11MR53	d4T-3TC-EFV
11MR55	d4T-3TC-EFV
11MR56	d4T-3TC-NVP
11MR58	d4T-3TC-EFV
11MR62	d4T-3TC-EFV
11MR63	d4T-3TC-NVP
11MR64	d4T-3TC-NVP
11MR65	d4T-3TC-EFV
11MR66	d4T-3TC-NVP
11MR69	d4T-3TC-EFV
11MR70	d4T-3TC-EFV
11MR75	d4T-3TC-NVP
11MR77	d4T-3TC-NFV
11MR78	d4T-3TC-NVP
11MR79	d4T-3TC-EFV
11MR80	d4T-3TC-LPV/r
11MR81	d4T-3TC-NVP
11MR82	d4T-3TC-EFV
11MR83	d4T-3TC-LPV/r
11MR84	d4T-3TC-EFV
11MR86	d4T-3TC-NVP
11MR89	d4T-3TC-EFV

3.5.2 Sequence analysis of gag-pol cleavage site mutations and pol genotypes from primary virus isolate culture supernatant.

Viral RNA was extracted from the p24 antigen positive culture supernatant from the 31 successfully grown primary virus isolates. Twenty five of the 31 (81%) samples were

successfully amplified and sequenced using the gag-pol cleavage site protocol (Figure 3.6A). Subsequently, only the 25 samples were used to obtain the pol genotype (Figure 3.6B). The non-specific bands seen on the agarose gels did not interfere with the sequencing reaction.

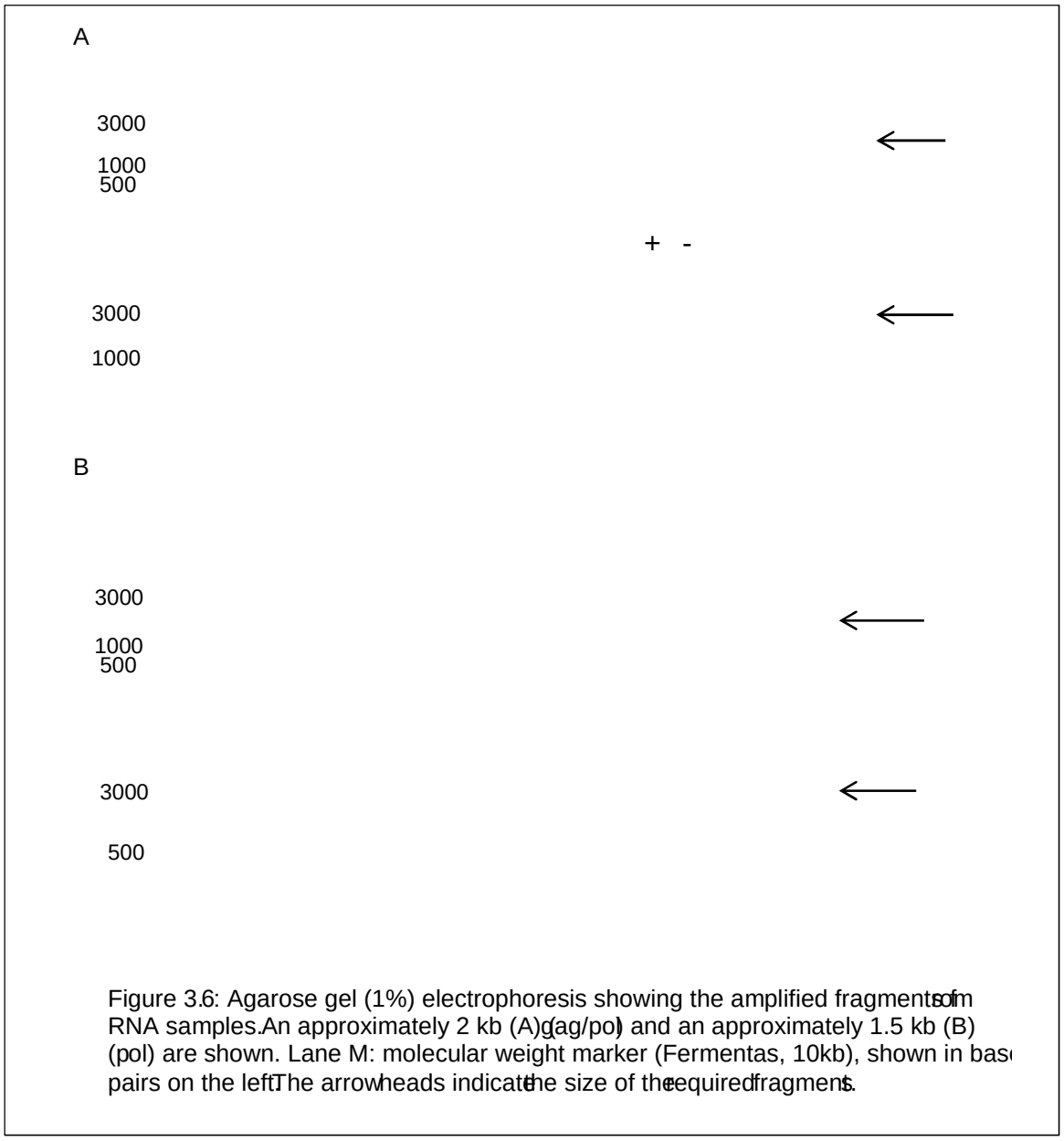


Figure 3.6: Agarose gel (1%) electrophoresis showing the amplified fragments from RNA samples. An approximately 2 kb (A) (gag/pol) and an approximately 1.5 kb (B) (pol) are shown. Lane M: molecular weight marker (Fermentas, 10kb), shown in base pairs on the left. The arrowheads indicate the size of the required fragments.

The five gag-pol codons associated with changes in PI susceptibility were also extensively analysed and compared amongst the ARV drug naïve and ARV exposed samples, as well as compared to the HIV-1 subtype B HXB2 sequence. None of the patients had any of the 5 known gag-pol associated cleavage site mutations

Only 9 (36%) patients had mutations in the RT region. The M184V NRTI mutation was observed in 5 patients (20%), while 8 (32%) had the K103N NNRTI mutation. None of the patients had major PR mutations (Table 3.3).

Table 3.4: Mutation profiles found in p24+ culture supernatant. Mutations are bolded and highlighted in yellow.

			MUTATIONS FOUND IN p24 + CULTURE SUP		
PID	GENDER	REGIMEN	PI	NRTI	NNRTI
11MR29	F	d4T-3TC-EFV	None	None	None
11MR32	F	d4T-3TC-EFV	None	None	None
11MR36	M	d4T-3TC-EFV	None	None	None
11MR37	M	d4T-3TC-EFV	None	None	None
11MR38	M	d4T-3TC-EFV	None	None	None
11MR44	F	d4T-3TC-EFV	None	None	K103N
11MR45	M	d4T-3TC-EFV	A71T (minor)	None	None
11MR52	F	d4T-3TC-LPV/r	None	None	None
11MR55	M	d4T-3TC-EFV	None	None	None
11MR58	M	d4T-3TC-EFV	None	None	None
11MR62	F	d4T-3TC-EFV	None	None	K103N
11MR64	F	d4T-3TC-NVP	None	None	None
11MR65	F	d4T-3TC-EFV	None	None	None
11MR69	F	d4T-3TC-EFV	None	None	K103N
11MR70	F	d4T-3TC-EFV	None	None	None
11MR75	F	d4T-3TC-NVP	None	V179D	None
11MR78	F	d4T-3TC-NVP	None	M184V	K103N, V108I, Y181C
11MR79	M	d4T-3TC-EFV	None	M184V	K103N

Table 3.4: Mutation profiles found in p24+ culture supernatant. Mutations are bolded and highlighted in yellow.

11MR80	F	d4T-3TC-LPV/r	None	None	None
11MR81	F	d4T-3TC-NVP	None	M184V	K103N, K238N
11MR82	F	d4T-3TC-EFV	None	M184V	K103N
11MR83	F	d4T-3TC-LPV/r	None	None	None
11MR84	F	d4T-3TC-EFV	None	None	None
11MR86	F	d4T-3TC-NVP	None	None	None
11MR89	F	d4T-3TC-EFV	None	M184V	K103N

CHAPTER 4
DISCUSSION AND CONCLUSIONS

HAART, which is the current standard for treatment of HIV-1 infected patients in South Africa, has greatly reduced the morbidity and mortality associated with HIV-1 infection. However, the greatest challenge of successful HAART is the development of antiretroviral drug resistance. This study aimed to identify patterns of gag-pol cleavage site and RT/PR mutations that emerge in HIV-1 subtype C infected South Africans receiving either first- or second line ART, and to isolate HIV-1 drug resistant primary viruses for future use in in vitro phenotypic inhibition assays.

An optimised gag-pol assay for the amplification and sequencing of an approximately 2 kb fragment encompassing the near full length gag and partial pol for the analysis of mutations associated with reduced susceptibility to PI was established. The RT-PCR and PCR amplification was successful for 72.4% of all samples, and included 33 of the 51 patient plasma samples (16 of 25 with unknown drug history and 17 of the 26 failing a second line regimen), 25 of the 31 primary virus isolate supernatants, and 18 of the 23 proviral DNA samples, respectively. All of the amplified samples were successfully sequenced. To the best of our knowledge, this is the first report describing the establishment of such an assay for HIV-1 subtype C. Assays targeting gag-pol in non-B subtypes, such as HIV-1 subtype G have been described (Knops et al., 2010). The gag-pol and pol genotyping results for each sample were analysed in parallel. Phylogenetic tree analysis of all samples confirmed they were HIV-1 subtype C, which is as expected since this is the major subtype that occurs in South Africa (Hemelaar et al., 2011).

Since the 18 successfully amplified and sequenced proviral DNA samples were antiretroviral drug naïve, it was expected that none of the samples contained any of the known gag-pol cleavage site mutations, nor any major PR and RT mutations, with only 3 of them having A71V and T74S minor PR mutations. These naturally occurring polymorphisms, which occur in antiretroviral drug naïve patients infected with subtype C virus, have been documented in other studies and are known to be associated with a decreased susceptibility to NFV (Vergne et al., 2006; Toor et al., 2011).

Polymorphisms that occur naturally in the PR of HIV-1 subtype C would be expected to lead to changes in the gag-pol cleavage sites. Furthermore, HIV-1 cleavage sites are highly more diverse in subtype C than in subtype B (de Oliveira et al., 2003). Similar findings were observed in this study. An analysis of the gag-pol cleavage sites in the viral RNA of the 33

successfully amplified and sequenced patient plasma samples revealed that the gag-pol cleavage sites were variable with respect to the subtype B consensus sequence HXB2. The nucleotide sequences of the S373, I437, L449 and P453 sites in the gag-pol cleavage sites were variable in contrast to the A431 site that was highly conserved (100%). These sites were highly polymorphic and coded for an amino acid change (Appendix D).

The natural variation at subtype C gag-pol cleavage sites may play a role in disease progression and the response to therapy (de Oliveira et al., 2003; Verheyen et al., 2009; Larrouy et al., 2010). This was evident in our study as 4 of the 5 of the PI exposed patients who had mutations at these sites had reduced susceptibility to PI therapy as a result of these compensatory mutations. Interestingly, the mutations in the gag-pol cleavage sites did not occur with associated mutations in the PR. Therefore, it can be suggested that these compensatory mutations might confer drug resistance directly (Borman et al., 1996; Mammano et al., 1998; Dam et al., 2009). However, this needs to be further investigated phenotypically since these 4 samples also harboured RT mutations. The mutations seen in the 5 patients included viruses from patient 09ZAP25 and 09TR05 that contained the mutation P453L; patient 09TR06 that contained the L449P and P453L mutations; patient 09TR09 that contained the S373Q and I437T mutations; while patient 09TR25 contained the I437T gag-pol cleavage site mutation. No patients contained the A431V mutation. It is possible that patient 09ZAP25 (unknown drug history) was on a failing PI regimen.

The P453L mutation is a polymorphism found in some drug naive viruses (Dam et al., 2009). However, this mutation is seen with a higher frequency in resistant viruses carrying the I50V, V82V and I84V mutations in PR (Bally et al., 2000; Maguire et al., 2002). This was not observed in our subtype C patient samples because this mutation was absent in drug naive patients and it was not associated with any PR mutations in the 3 patients that harboured viruses with this gag-pol cleavage site mutation.

The L449P and S373Q gag-pol cleavage site mutations have been controversially discussed as natural polymorphisms or as compensatory cleavage site mutations selecting certain PR mutations. The L449P and S373Q as natural polymorphisms is further reinforced by the fact that viruses that had these mutations showed fewer primary PR mutations (Bally et al., 2000). However, these cleavage site mutations were seen in two patients in our study, and occurred

without any of the PR mutations K20I, I50V and L89M, which the S373Q and L449P gag-pol cleavage site mutations are associated (Maguire et al., 2002, Malet et al., 2007).

Nijhuis et al (2007) suggested that in patients who lacked primary PR mutations, the I437T mutation in the gag-pol cleavage site is associated with a 5-fold increase in resistance to PI (Nijhuis et al., 2007). This may be the case with the I437T mutation seen in two of the PI exposed patients. The association of the gag-pol cleavage site mutation with the L76V PR mutation was not observed in any of the exposed patients, which is in contrast with previous studies that have reported the association of these two mutations (Lambert-Niclot et al., 2008).

Studies in HIV-1 subtype B have shown that the gag-pol cleavage site mutation A431V is associated with the M46I/L, I54V and L76V PR mutations (Lambert-Niclot et al., 2008, Verheyen et al., 2009). Furthermore, the association of this gag-pol cleavage site mutation with PR mutations has also been observed in patients infected with HIV-1 subtype B and were LPV-naive; neither of these two findings were observed in this study and may be attributed to a difference in association of these mutations in HIV-1 subtype C. The resistance produced by PR mutation L76V was more pronounced by the presence of M46I/L and A431V mutations in gag-pol cleavage sites (Nijhuis et al., 2007). Results from this study are not in agreement with these finding, since there was an absence of the A431V gag-pol mutations in LVP-experienced patients (Lambert-Niclot et al., 2008).

Interestingly, four of the five patients harbouring gag-pol mutations had mutations in the RT region. This is most probably directly attributed to the first- and second-line regimen failures observed in these patients. Further studies need to be elucidated to explore the relationship between these two regions.

Only 5 of the 33 (15.2%) patients had major PR mutations, which would have resulted in reduced susceptibility to LVP. The PR mutations observed were in patient samples 09ZAP16 that contained the M46L mutation; 09ZAP17 that contained the L90M mutation; 09TR03 that contained the V32I, M46L, I47V and I54V mutations; 09TR21 that contained the M46I, I47V and I50V mutations; and 09TR24 that contained the M46L and L76V mutations (Figure 3.3). The infrequency of the PR mutations observed in this cohort is similar to that observed in HIV-1 subtype B and other HIV-1 subtype C patients (Riddler et al.,

2008; Wallis et al., 2011). Furthermore, for LVP/r resistance to develop, more than 6 of the possible 17 mutations associated with resistance need to develop, although rare single mutations (V32I, I47V, or L76V) observed in 3 of the 5 patients can reduce LPV susceptibility.

Minor PR mutations were observed in 8 of the 33 patient plasma samples in this study. These naturally occurring HIV-1 subtype C polymorphisms (L10I/V, L33V, T74S) have also been observed by other groups (Kantor & Katzenstein, 2003). These minor mutations would not be expected to impact on the efficacy of boosted LPV and therefore unlikely to be the reason these patients were accessing a failing PI regimen at time of failure (Champenois et al., 2008). The L10V/I mutation occurs in 5-10% of antiretroviral drug naïve patients and reduces the activity of the PIs ATV/r, FPV/r, IDV/r, LPV/r, NFV and SQV/r. This polymorphism has been shown to be weakly associated with a reduced response of the virus to some of the PIs (Mintsa-Ndong et al., 2009). According to the Stanford HIV Drug Resistance Database, the L33V is a polymorphism that does not appear to be related to PI therapy or drug resistance. T74S is shown to be associated with reduced NFV susceptibility and occurs in about 5% of naïve patients with subtype C viruses (Deforche et al., 2006).

The observed phenotype of PI resistance not mapping to the PR coding region suggests that there might be mutations alternative regions. When comparing HIV-1 subtype C PR to that of subtype B, it is observed that subtype C PR is highly conserved at the amino acid level (Velazquez-Campoy et al., 2001, Gordon et al., 2003). Polymorphic mutations in subtype C PR have been linked to the high catalytic activity of the PR (Velazquez-Campoy et al., 2001). This is in contrast to the subtype B PR which has a much slower catalytic activity (Kräusslich et al., 1989). Furthermore, polymorphisms in subtype C are located outside the catalytic site of the protease in regions that would be expected to impede the activity of the enzyme toward its natural substrates (e.g. gag-pol cleavage sites), thus leading to the evolution of the cleavage site itself (de Oliveira et al., 2003). This, therefore, needs to be further investigated by conducting phenotyping work on gag/pol cleavage sites.

Overall, 16 of the 33 ARV-exposed patients had RT mutations, with 9 having both NRTI and NNRTI mutations. The high percentages of NNRTI mutations K103N (27.3%), V106M and Y188L (both 6.1%) are likely the result of first-line failure of a NNRTI-containing regimen. The high prevalence of the K103N mutation has been mirrored by other studies which

suggest that this mutation is found to occur at a greater frequency and in higher levels in women infected with subtypes C as compared to subtypes A (De Aleman et al., 2001, Flys et al., 2006). The presence of the M184V, K65R and TAMs present are likely selected by NRTI present in either the first- or second-line regimens.

Ten of the 33 exposed patients did not possess gag-pol cleavage site mutations, nor did they have PR mutations. This finding suggests that non-adherence to the regimens may have contributed to the viral failure observed. A study by Pulido et al (2009) supports this finding, as it indicated that loss of viral suppression on a LPV/r regimen was linked to a low baseline CD4 count or haemoglobin levels and medication non-adherence (Pulido et al., 2009). Non-adherence may be linked to side effects/toxicity from the ARVs the patient were taking (ddI, AZT or LPV/r) (Venhoff et al., 2007). Other possible reasons for viral failure may include host genetic factors linked to metabolism of PIs (Rempf et al., 1997).

The co-cultivation of PBMCs from infected patients with uninfected donor PBMC is one of the method that has been used to isolate HIV for phenotypic assays (Japour et al., 1993). Only 31 of the 91 of primary virus isolates were obtained. This low isolation success rate (34%) is expected since antiretroviral drug resistant viruses often are less fit than wild type viruses (Kusumi et al., 1992, Robinson et al., 2000). Of these 31 primary virus isolates, 25 were successfully RT-PCR amplified and sequenced using the gag-pol cleavage site and pol genotyping assays.

None of the 25 patients had any of the known gag-pol associated cleavage site mutations. This is in contrast to a study conducted by Verheyen et al, whereby 10% of the therapy-naïve viruses exhibited compensatory cleavage site mutations (Verheyen et al., 2006). The meaning of compensatory cleavage site mutations in naïve viruses still remains undetermined. However, our results are in agreement with the study of Bally et al which found that cleavage site mutations are exclusively in treatment-exposed viruses (Bally et al., 2000). Interestingly, despite all patient samples showing NRTI and/or NNRTI mutations at treatment failure (Wallis et al., 2012), only nine (36%) of the primary virus isolates had mutations in the RT region. The most observed NRTI and NNRTI mutations were M184V and K103N, respectively. This was a low outcome of results. Some studies suggest the use of PBMCs in co-cultivation studies is not ideal application since it involves the isolation of fresh PBMC

and the long culture times have been shown to select for minority or less-resistant variants (Kusumi et al., 1992; Robinson et al., 2000).

Since the matching plasma samples were unavailable, future studies will have to be conducted to find out more about the association of mutations found in viral RNA from plasma and PBMCs. However, studies have shown a strong relation in drug resistance mutations between primary virus isolates cultured from PBMCs and HIV-1 RNA from plasma (Young et al., 1998; Diallo et al., 2012).

In conclusion, this study describes the matched gag-pol and pol genotypes associated with patients failing a first and second line regimen. No known mutations in the gag/pol region were identified in patients failing a first line regimen. By contrast, results revealed that major PR mutations were present, albeit rare in patients failing a second line regimen and not found in combination with mutations in the gag/pol region that would impact on LPV/r susceptibility. Furthermore, the pol antiretroviral drug resistance mutations described in this study were similar to the findings reported for treatment failures in South African HIV-1 subtype C infected HAART patients. The absence of resistance in other patients suggests that non-adherence could be the cause of virological failure. Further studies on the gag-pol cleavage sites in association with PR and RT regions need to be conducted. Furthermore, the high polymorphic regions of gag-pol in HIV-1 subtype C compared to subtype B should be further evaluated to determine if there are additional mutations in subtype C that impact on PI susceptibility. The antiretroviral drug resistant primary viruses obtained during the course of this study provide valuable tools that will allow us to perform subtype C specific phenotyping in future.

CHAPTER 5

APPENDICES

Appendix A: Human Ethics approval certificate

Appendix B: The preparation of reagents used for the development and evaluation of antiretroviral drug resistance mutations.

B1: Gel Preparation

A 1% agarose gel was prepared by weighing out 1 g of agarose (Bioline, London, United Kingdom) into a 250 ml conical flask and 100 ml of 1 x Tris acetate ethyllenediaminetetraacetic acid (EDTA) (TAE) buffer (Fermentas, Burlington, Canada) was added. The flask was swirled to allow for mixing. The agarose was dissolved in a microwave for about 1 minute. After the agarose was dissolved, it was left for 5 minutes on the bench to cool down to about 60°C. Three microlitres of ethidium bromide (30 µl) (Sigma-Aldrich Company, Steinheim, Germany) was added and the mixture swirled. The gel was slowly poured into the tray containing one set of combs, and any bubbles formed were pushed to the side using a disposable tip. The gel was left to set and then submerged into the gel tank containing 1 x TAE running buffer.

B2: Sample Preparation

Samples were prepared by transferring 5 µl of 6x Mass Ruler Loading Solution (Fermentas, Burlington, Canada) into fresh microfuge tubes. Five microliters of DNA product were added into each tube and the solution was mixed by pipetting up and down. The well of the gel was loaded with 10 µl (103ng/ µl) of O'GeneRuler™ DNA Ladder Mix (Fermentas, Burlington, Canada). The DNA Ladder Mix is a solution of DNA molecules of varying lengths used in agarose gel electrophoresis. It is used as a reference to estimate sizes of unknown DNA molecules. The samples were loaded after the well containing the ladder. The samples were resolved for 60 minutes at 110 volts.



Figure B1: A 10kb DNA ladder (Fermentas, Burlington, Canada) (Accessed on 23 January 2012) http://www.fermentas.com/templates/files/tiny_mce/family_images/s33_fam.jpg)

Table B1: IUB genetic code

IUB code	Mixture of bases
R	A, G
S	G, C
M	C, A
W	A, T
K	T, G
Y	T, C
D	A,T, G
B	G, T, C
H	C, T, A

B3: Preparation of 2% fetal calf serum in phosphate buffer saline (FCS-PBS)

Two per cent of FCS-PBS was prepared by adding 10ml of FCS (Invitrogen GmbH, Darmstadt, Germany) in 500ml of 1x PBS (Sigma-Aldrich Company, Steinheim, Germany).

B4: Preparation of Roswell Park Memorial Institute (RPMI) medium

The Roswell Park Memorial Institute (RPMI) medium (Sigma-Aldrich Company, Steinheim, Germany) was completed by adding 10% FCS, 100x L-Glutamine (Invitrogen GmbH, Darmstadt, Germany.) and 100x penicillin/streptomycin (Invitrogen GmbH, Darmstadt, Germany) in 500ml of RPMI and stored at room temperature.

B5: Preparation of phytohemaglutinin (PHA)

Five millilitres of distilled water was added to 50mg PHA (Sigma-Aldrich Company, Steinheim, Germany). Fifteen millilitres of fresh supplemented RPMI was added to give a final concentration of 2.5mg/ml. Five millilitre aliquots were made and stored at -20ºC.

B6: Preparation of reagents used in the p24 assay

Negative Control

This control contained human serum that was nonreactive for HBV, HCV and HIV antigen.

Positive Control (HIV-1 antigen positive control)

The positive control contained inactivated HIV-1 p24.

Phosphate Fluid

The buffer concentrate was diluted 1:25 with distilled water. Therefore, 100 ml of the buffer concentrate was poured in a flask and filled up to 2500 ml with distilled water. At least 25 ml of phosphate buffer was prepared for each microelisa strip.

TMB Substrate Solution

This solution was prepared by adding equal amounts of both the tetramethylbenzide (TMB) solution with urea peroxidase solution according to the number of the wells being run. For example, for a full 96 well plate, 6ml of TMB solution was added to 6ml of urea peroxidase solution.

Sulphuric Acid

To make up a 1 molar solution of sulphuric acid, 5.36 ml of acid was added to 94.64 distilled water to a final volume of 100 ml. This was stored at room temperature.

Appendix C

Table C1: Demographic data and clinical characteristics of the 165 patient samples used in this study. NA: not amplification; NG: no growth. Mutations are bolded and highlighted in yellow.

PID	PR mutations		RT mutations		gag-pol cleavage sites					Drug history	Gender	VL
	Major	Minor	NRTI	NNRTI	S373	A431	I437	L449	P453			
PBMC € DRUG NAIVE												
99ZALT4	None	None	None	None	None	None	None	None	None	naive	unknown	unknown
99ZALT5	None	None	None	None	None	None	None	None	None	naive	F	1074
99ZALT21	None	None	None	None	None	None	None	None	None	naive	F	6588
99ZALT39	None	A71V	None	None	None	None	None	None	None	naive	F	12180
99ZALT42	None	None	None	None	None	None	None	None	None	naive	M	23564
99ZALT46	None	None	None	None	None	None	None	None	None	naive	M	6024
99ZALT49	None	None	None	None	None	None	None	None	None	naive	unknown	unknown
05ZAFV2	NA	NA	NA	NA	NA	NA	NA	NA	NA	naïve	M	22900
05ZAFV3	None	None	None	None	None	None	None	None	None	naïve	F	unknown
05ZAFV5	NA	NA	NA	NA	NA	NA	NA	NA	NA	naïve	M	>750000
05ZAFV7	None	None	None	None	None	None	None	None	None	naïve	F	598000
05ZAFV8	None	T74S	None	None	None	None	None	None	None	naïve	F	660000
05ZAFV9	None	None	None	None	None	None	None	None	None	naïve	M	736000
05ZAFV10	None	T74S	None	None	None	None	None	None	None	naïve	M	>750000
05ZAFV11	None	None	None	None	None	None	None	None	None	naïve	M	>750000
05ZAFV12	NA	NA	NA	NA	NA	NA	NA	NA	NA	naïve	M	924000
05ZAFV13	None	None	None	None	None	None	None	None	None	naïve	F	378000
05ZAFV14	None	None	None	None	None	None	None	None	None	naïve	F	unknown
05ZAFV15	None	None	None	None	None	None	None	None	None	naïve	F	343000
05ZAFV23	NA	NA	NA	NA	NA	NA	NA	NA	NA	naïve	F	37000
05ZAFV25	None	None	None	None	None	None	None	None	None	naïve	F	>750000
05ZAFV26	None	None	None	None	None	None	None	None	None	naïve	F	unknown
05ZAFV28	NA	NA	NA	NA	NA	NA	NA	NA	NA	naïve	M	>750000

Table C1: Demographic data and clinical characteristics of the 165 patient samples used in this study NA: not amplification; NG: no growth. Mutations are bolded and highlighted in yellow.

VIRAL RNA € UNKNOWN DRUG EXPOSURE												
09ZAP01	None	None	None	None	None	None	None	None	None	unknown	F	18000
09ZAP02	None	None	None	None	None	None	None	None	None	unknown	F	270000
09ZAP03	None	None	None	None	None	None	None	None	None	unknown	F	12000
09ZAP04	None	L10V	None	None	None	None	None	None	None	unknown	F	18000
09ZAP05	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	F	6400
09ZAP06	None	None	None	None	None	None	None	None	None	unknown	M	9100
09ZAP07	None	None	None	None	None	None	None	None	None	unknown	M	32000
09ZAP08	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	F	1500
09ZAP09	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	F	11000
09ZAP10	None	None	None	None	None	None	None	None	None	unknown	M	6100
09ZAP11	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	F	25000
09ZAP12	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	M	4300
09ZAP13	None	None	None	None	None	None	None	None	None	unknown	M	3900
09ZAP14	None	None	None	None	None	None	None	None	None	unknown	F	4800
09ZAP15	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	M	43000
09ZAP16	M46L	None	None	None	None	None	None	None	None	unknown	M	31000
09ZAP17	L90M	T74S	None	None	None	None	None	None	None	unknown	M	21000
09ZAP18	None	None	None	None	None	None	None	None	None	unknown	F	5000
09ZAP19	None	None	None	None	None	None	None	None	None	unknown	F	29000
09ZAP20	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	F	34000
09ZAP21	None	None	None	None	None	None	None	None	None	unknown	F	19000
09ZAP22	None	T74S	None	None	None	None	None	None	None	unknown	M	47000
09ZAP23	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	M	220000
09ZAP24	NA	NA	NA	NA	NA	NA	NA	NA	NA	unknown	F	160000
09ZAP25	None	None	None	None	None	None	None	None	P453L	unknown	F	260000

Table C1: Demographic data and clinical characteristics of the 165 patient samples used in this study. NA: not amplification; NG: no growth. Mutations are bolded and highlighted in yellow.

						VIRAL RNA € DRUG EXPOSED						
09TR01	NA	NA	NA	NA	NA	NA	NA	NA	NA	AZT, ddI, LPVr	M	<LDL
09TR02	None	L10I, T74S	None	K103N	None	None	None	None	None	AZT, ddI, LPVr	F	490000
09TR03	V32I, M46L, I47V, I54V	L33V, T74S	M184V	K103N	None	None	None	None	None	AZT, ddI, LPVr	F	310000
09TR04	NA	NA	NA	NA	NA	NA	NA	NA	NA	AZT ,ddI, LPVr	F	420
09TR05	None	None	M184V	None	None	None	None	None	P453L	AZT, 3TC, LPVr	F	74000
09TR06	None	None	M184V	K103N, V106M, F227L	None	None	None	L449P	P453L	AZT ,ddI, LPVr	F	19000
09TR07	None	None	M184V	K103N	None	None	None	None	None	AZT, ddI ,LPVr	F	8680
09TR08	None	None	M184V	K103N	None	None	None	None	None	AZT, ddI, LPVr	M	4100
09TR09	None	T74S	None	T215D,A98Q	S373Q	None	I437T	None	None	AZT ,ddI ,LPVr	F	220000
09TR10	None	None	None	K103N	None	None	None	None	None	AZT, d4T ,LPVr	M	39000
09TR11	None	L10I	M184V	Y188L	None	None	None	None	None	AZT, ddI ,LPVr	F	1200
09TR12	None	None	None	G190A	None	None	None	None	None	EFV, 3TC ,LPVr	F	15000
09TR13	NA	NA	NA	NA	NA	NA	NA	NA	NA	AZT, ddI, LPVr	M	400
09TR14	None	T74S	M184V	None	None	None	None	None	None	AZT, 3TC ,LPVr	M	150000
09TR15	NA	NA	NA	NA	NA	NA	NA	NA	NA	AZT, ddI ,LPVr	M	300
09TR16	NA	NA	NA	NA	NA	NA	NA	NA	NA	EFV, 3TC ,LPVr	F	<LDL
09TR17	NA	NA	NA	NA	NA	NA	NA	NA	NA	AZT ,ddI ,LPVr	F	870
09TR18	NA	NA	NA	NA	NA	NA	NA	NA	NA	EFV, 3TC ,LPVr	F	140
09TR19	NA	NA	NA	NA	NA	NA	NA	NA	NA	AZT, 3TC ,LPVr	M	630
09TR20	None	None	M184V	K103N	None	None	None	None	None	AZT, ddI ,LPVr	M	850
09TR21	M46I, I47V, I50V	None	None	None	None	None	None	None	None	AZT, ddI, LPVr	F	3000000
09TR22	None	None	M184V, K65R	V106M, A62V, V179D	None	None	None	None	None	AZT ,ddI, LPVr	M	1700
09TR23	NA	NA	NA	NA	NA	NA	NA	NA	NA	EFV, 3TC ,LPVr	M	<LDL
09TR24	M46L, L76V	None	M41L	None	None	None	None	None	None	AZT, ddI, LPVr	F	3700
09TR25	None	None	M184V, M41L	F227L	None	None	I437T	None	None	AZT ,ddI, LPVr	F	1080
09TR26	None	None	M184V	T215Y,Y188L	None	None	None	None	None	AZT, ddI ,LPVr	M	81900

Table C1: Demographic data and clinical characteristics of the 165 patient samples used in this study. NA: no amplification; NG: no growth. Mutations are bolded and highlighted in yellow.

PBMC € DRUG NAIVE												
11MR01	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	7 020
11MR02	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	100 001
11MR03	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	M	1 700
11MR04	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	6 060
11MR05	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	51 700
11MR06	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	10 700
11MR07	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	100 001
11MR08	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	1 790
11MR09	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	M	100 001
11MR10	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	60 500
11MR11	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	M	11 400
11MR12	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	2 290
11MR13	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	M	1 680
11MR14	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	1 910
11MR15	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	M	100 001
11MR16	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	M	55 100
11MR17	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	6 100
11MR18	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	M	5 590
11MR19	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	2 260
11MR20	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	29 400
11MR21	NG	NG	NG	NG	NG	NG	NG	NG	NG	naive	F	63 600

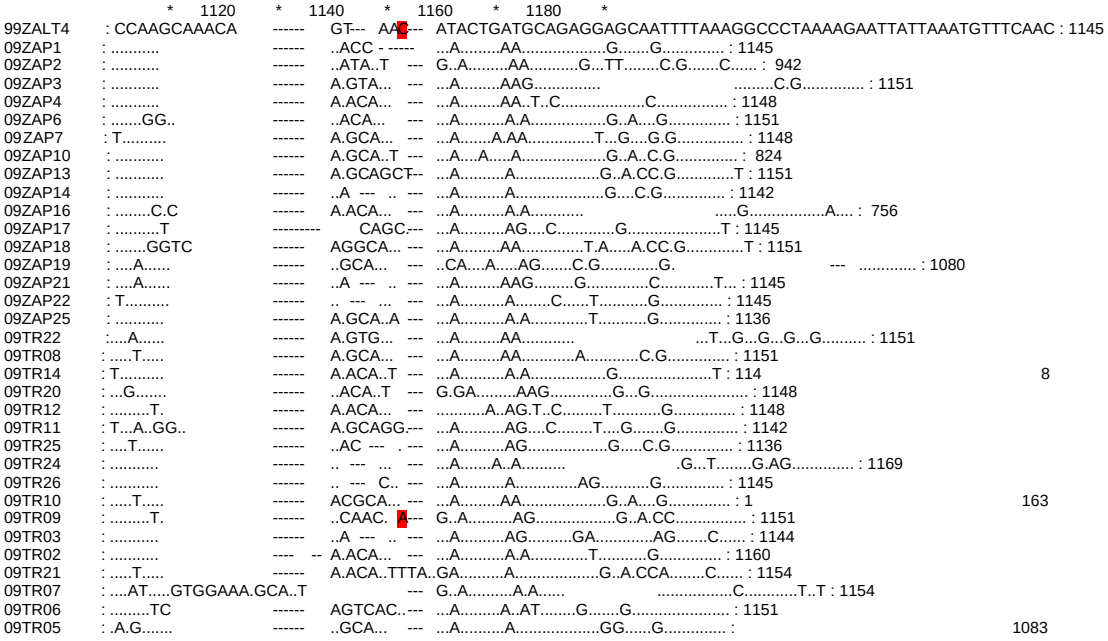
Table C1: Demographic data and clinical characteristics of the 165 patient samples used in this study. NA: no amplification; NG: no growth. Mutations are bolded and highlighted in yellow.

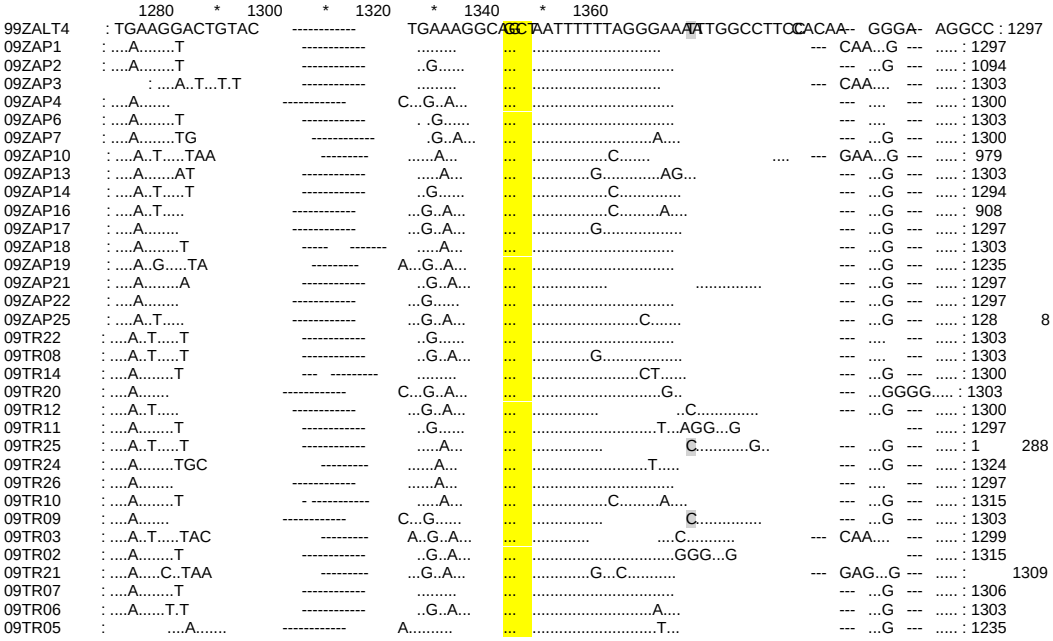
PBMC € DRUG EXPOSED												
11MR22	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NFV	F	24 800
11MR23	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	750 001
11MR24	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	210 000
11MR25	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	261 000
11MR26	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	854 000
11MR27	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	623 000
11MR28	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	221 000
11MR29	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	F	287 000
11MR30	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-LPVr	F	107 000
11MR31	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	78 700
11MR32	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	F	945 000
11MR33	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	94 800
11MR34	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-LPVr	F	139 000
11MR35	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	99 900
11MR36	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	M	460 000
11MR37	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	M	1 680 000
11MR38	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	M	7 500 001
11MR39	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	2 260 000
11MR40	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	751 000
11MR41	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	306 000
11MR42	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	292 000
11MR43	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	609 000
11MR44	None	None	None	**K103N**	None	None	None	None	None	d4T-3TC-EFV	F	ND
11MR45	None	**A71T**	None	None	None	None	None	None	None	d4T-3TC-NFV	F	58 200
11MR46	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	2 890
11MR47	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	152 000
11MR48	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NFV	F	8 990
11MR49	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	204 000
11MR50	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	122 000
11MR51	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	111 000
11MR52	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	F	5 180
11MR53	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	249 000
11MR54	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-LPVr	F	308 000
11MR55	None	None	None	None	None	None	None	None	None	d4T-3TC-NVP	F	16 600
11MR56	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	750 001
11MR57	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	155 000
11MR58	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	M	218 000
11MR59	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-LPVr	F	74 400
11MR60	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	30 500
11MR61	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	28 500
11MR62	None	None	None	**K103N**	None	None	None	None	None	d4T-3TC-NVP	F	455 000
11MR63	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	33 100

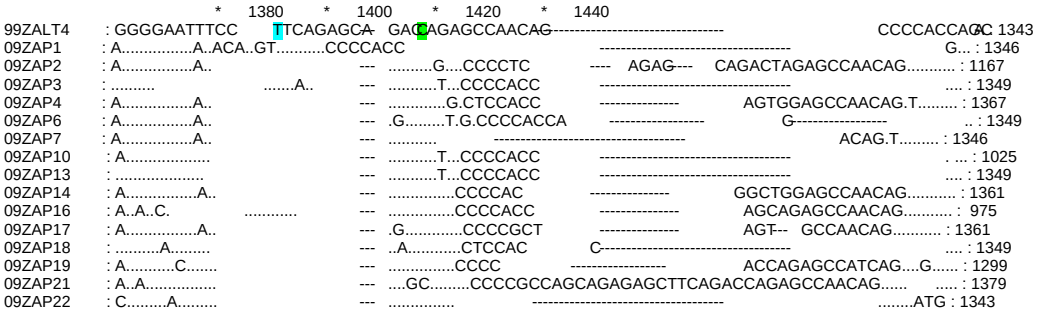
Table C1: Demographic data and clinical characteristics of the 165 patient samples used in this study. NA: no amplification; NG: no growth. Mutations are bolded and highlighted in yellow.

11MR64	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	M	58 700
11MR65	None	None	None	None	None	None	None	None	None	d4T-3TC-NVP	F	298 000
11MR66	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	468 000
11MR67	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	37 000
11MR68	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	39 400
11MR69	None	None	None	K103N	None	None	None	None	None	d4T-3TC-NVP	F	138 000
11MR70	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	F	448 000
11MR71	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-LPVr	F	168 000
11MR72	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	12 500
11MR73	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	15 100
11MR74	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	M	71 400
11MR75	None	None	None	V179D	None	None	None	None	None	d4T-3TC-NVP	F	25 200
11MR76	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-LPVr	F	28 700
11MR77	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-LPVr	F	110 000
11MR78	None	None	M184V	K103N, V108I, Y181C	None	None	None	None	None	d4T-3TC-EFV	M	70 300
11MR79	None	None	M184V	K103N	None	None	None	None	None	d4T-3TC-LPVr	F	215 000
11MR80	None	None	None	None	None	None	None	None	None	d4T-3TC-NVP	F	293 000
11MR81	None	None	M184V	K103N, K238N	None	None	None	None	None	d4T-3TC-NVP	F	637 000
11MR82	None	None	M184V	K103N	None	None	None	None	None	d4T-3TC-EFV	M	293 000
11MR83	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	F	24 500
11MR84	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	F	399
11MR85	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	266 000
11MR86	None	None	None	None	None	None	None	None	None	d4T-3TC-EFV	M	347 000
11MR87	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	93 000
11MR88	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	18 900
11MR89	None	None	M184V	K103N	None	None	None	None	None	d4T-3TC-NVP	F	201 000
11MR90	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-NVP	F	162 000
11MR91	NG	NG	NG	NG	NG	NG	NG	NG	NG	d4T-3TC-EFV	F	80 400

Appendix D







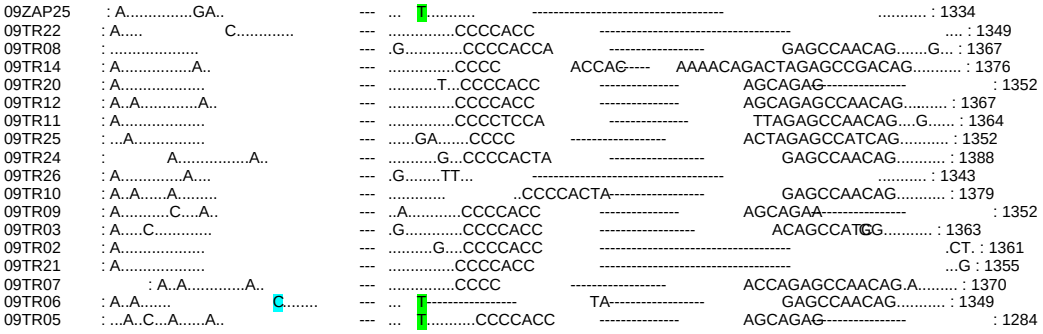


Figure D1: An analysis of the gag-pol cleavage sites in the 33 viral RNA samples. The highlighted amino acids represent the gag-pol cleavage site mutations. (Red: S373Q; Grey: I437T; Turquoise: L449P; and Green: P453L. Also represented is the highly conserved A431V cleavage site. This is s in yellow.

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