Characterization of HIV-1 Drug Resistance mutations from plasma and peripheral blood mononuclear cells in patients failing antiretroviral treatment in Bela- Bela, South Africa

A dissertation in fulfilment of the award of Master of Science degree in Microbiology

Submitted By

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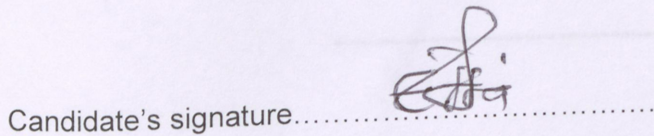
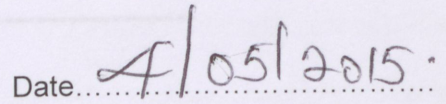
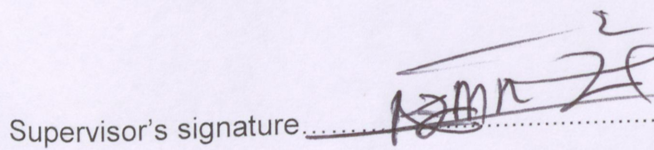
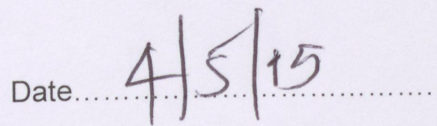
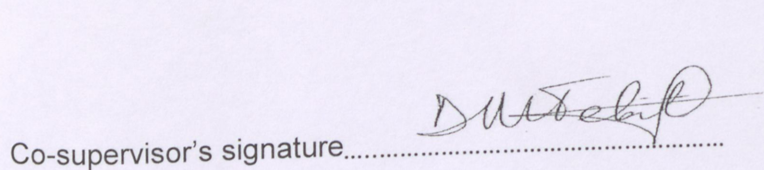
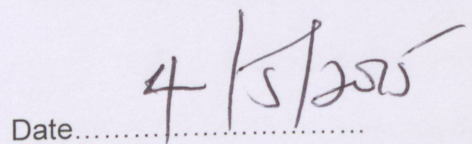
Declaration

I, Elisabeth Mashu Elisabeth, hereby declare that this dissertation for the award of Master of Science in Microbiology at the University of Venda submitted by me has not been submitted before for any degree or examination at this or any other University. It is my own work in design and all the reference materials contained herein have been duly acknowledged.

Candidate's signature..  Date.. 

Supervisor's signature..  Date.. 

Co-supervisor's signature..  Date.. 

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Abstract

BACKGROUND: The current expansion of antiretroviral treatment (ART) in developing countries, such as South Africa, which lacks routine virological monitoring raises concerns on the outcome of the strategy in terms of virological success and the drug resistant burden. With an estimated 3.7% of the patients failing first-line treatment after 2 years and 17.9% after 4 years on treatment, there is qualified need for practical and routine drug resistance testing to provide data to clinicians in order to improve the lives of these patients. Thus, this study was conducted to characterize and compare HIV-1 drug resistance mutations in peripheral blood mononuclear cells (PBMCs) and in the plasma of patients whose therapeutic regimen is failing.

METHOD: The study was nested within the Bela-Bela Wellness clinic, Limpopo Province South Africa. Approval for the study was obtained from the Health, Safety and Research Ethics Committee of the University of Venda. Blood specimens were collected from 23 HIV-infected drug experienced, individuals between July 2013 and October 2014 who met the following criteria: (1) two consecutive viral loads on treatment greater than 1000 copies/ml after previous suppression; (2) one viral load greater than 1000 copies/ml after previous suppression followed by a change in treatment; and (3) one viral load greater than 1000 copies/ml after 180 days on ART without suppression. The protease (PR) and reverse transcriptase (RT) genes of the 23 treatment exposed HIV infected patients failing therapy were PCR amplified, sequenced, subtyped and analysed for the presence of drug resistance mutations.

RESULTS: Twenty one (91%), out of 23 specimens were successfully amplified. Comparison of the amino acid sequence of the PR and RT genes in the cell-associated variants of HIV-1 with that of plasma revealed that 17(81%), out of the 21 specimens tested, exhibited major drug resistance both in PBMCs and plasma. However, the greatest number of mutations were found

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in plasma (D67N, K103N, V106M, Y181C and M184V) occurred most frequently. On the other hand, V106M, K103N and M184V were observed most often in PBMCs. The prevalence of predicted resistance mutations in our study corresponding to respective ART usage were thus: NRTI, (n=11), NNRTI, (n=13) and PI, (n=4). The most common mutations for RT were M184V>K103N>V106M>D67N. It is noteworthy that M230I an NNRTI was found in PBMCs only. Major mutations for PR were M46I> D30N>V82A. Seventeen of the 21 viruses (81%) were HIV-1 subtype C in the polymerase gene; one virus was subtype B (4.8%) and one was a C/B recombinant (4.8%). One virus (4.8%) could not be conclusively typed.

In conclusion, this study firstly, confirmed previous findings that the cellular compartment of blood may contain an archive of drug resistant variants making an interesting compartment for analysing the evolution of drug resistance in a given patient. Secondly, the prevalence of drug resistance mutations observed in Bela-Bela drug experienced individuals is very fairly high; and finally, there appears to be a continual presence of recombinant viruses circulating in the study region.

Key words: Virologic failure; Highly active antiretroviral therapy; Drug resistance; Peripheral blood mononuclear cell; Plasma; Bela-Bela; South Africa.

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List of Abbreviations

AIDS	Acquired Immunodeficiency Syndrome
APOBEC3G	Apolipoprotein B mRNA editing enzyme catalytic polypeptide-like 3G
ART	Antiretroviral treatment
ATP	Adenine triphosphate
AZT	Zidovudine
bp	Base pairs
BLAST	Basic Local Alignment Search Tool
CA	Capsid
CDC	Centre of Disease Control
cDNA	Complementary DNA
Copies/ml	Copies per millitre
CRF	Circulating Recombinant forms
DNA	Deoxyribonucleic acid
ddi	Didanosine
dNTPs	Deoxynucleotide triphosphate
dNTPs A, C,G, T	Adenine Cytosine Guanine, Thymidine
ds-DNA	Double stranded-DNA
EDTA	Ethylene diamine tetra-acetic acid
EFV	Efavirenz
env	Envelope gene
ELISA	Enzyme-Linked Immnunosorbent Assay
Et al	Et alia (and others)
ENV	Envelope protein
e.g.	Example
EQA	External Quality Assessment
FDA	Federal and Drug Administration
FTC	Emtricitabine

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g	Grams
gag	Group antigen gene
Gag	Group antigen protein
GART	Genotypic antiretroviral resistance testing
GDR	Genotypic drug resistance assays
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HIV	Human immunodeficiency virus
HIV-1	Human immunodeficiency virus type-1
HIV-2	Human immunodeficiency virus type-2
HIVNET	Human immunodeficiency virus Network for prevention Trials
i.e	That is
jpHMM	Jumping profile Hidden Markov Model
kg	Kilograms
LAV	Lymphadenopathy-associated virus
LPV/r	Lopinavir/ritonavir
M	Molar
MTCT	Mother- To- Child- Transmission
MA	Matrix
MDR	Multidrug resistance
$MgCl_2$	Magnesium chloride
$MgSO_2$	Magnesium sulphide
MAL-ED	Malnutrition and Enteric Diseases
N	Number
nef	Negative factor gene
Neg/N	Negative control
Nef	Negative factor protein
ng	Nanogram
NC	Nucleocapsid
NAMs	nucleoside analog-resistance mutations

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NICD	National Institute Communicable Diseases
nm	Nanometre
NNRT	Non-nucleoside analogue Reverse Transcriptase Inhibitor
NRTI	Nucleoside analogue Reverse Transcriptase Inhibitor
nt	Nucleotide
NVP	Nevirapine
PBMC	Peripheral blood mononuclear cells
PCR	Polymerase Chain Reaction
PI	Protease inhibitor
PMTCT	Prevention of Mother To Child Transmission
pol	Polymerase enzyme
PCR	Polymerase chain reaction
Pgp	P- glycoprotein
PI	Protease inhibitor
Pol	Polymerase protein
PR	Protease enzyme
QC	Quality Control
QCMD	Quality Control for Molecular Diagnostics
rev	Regulatory of viral expression protein
RNA	Ribonucleic acid
RPM	Revolution per minutes
SA	South Africa
SSA	Statistics South Africa
sd-NVP	Single dose Nevirapine
SIV	Simian immunodeficiency virus
SIVcpz	Simian immunodeficiency virus found in chimpanzee
SIVsm	Simian immunodeficiency virus found in Sooty mangabey
TAC	Treatment Action Campaign
TAMs	Thymidine analog mutations
Taq	an enzyme obtained from Thermus aquaticus

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tat	Transcriptional trans activator protein
TDF	Tenofovir
™	Trade Mark
U	Units
UK	United Kingdom
URF	Unique recombinant forms
USA	United State of America
UV	Ultraviolet
Vpr	Viral protein R protein
vpu	Viral protein U gene
Vpu	Viral protein U protein
vif	Virion infectivity factor gene
Vif	Virion infectivity factor protein
VL	Viral load
vpr	Viral protein R gene
3TC	Lamivudine
d4T	Stavudine
°C	Degree Celsius
>	Greater than
≥	Greater or equal
<	Less than
≤	Less or equal to
µl	Microliter
µM	Micromolar
%	Percentage

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Chapter One: Introduction and Literature Review

1.1 Introduction

The human Immunodeficiency virus (HIV) infects humans and is one of the most serious and devastating pathogens worldwide. Since the beginning of the HIV pandemic, the United Nations Joint Programme on AIDS estimated that, more than 60 million people have been infected with HIV, of which 25 million have died of AIDS related diseases and 2.7 million people were newly infected in 2013 (UNAIDS, 2013).

Even though HIV and AIDS are found in all parts of the world, some areas are more affected than others. The majority of the people infected with HIV/AIDS live in low- and middle income countries. An estimated 70% of all individuals infected with HIV are in Sub-Saharan Africa, which makes it the hardest-hit region in the world: Adolescents and adults are the most infected. Apart from Southern Africa, Eastern Europe and Central Asia have a high HIV prevalence with an estimate of 54.2%. Furthermore, East Africa also experiences a high prevalence with an estimate of about 13% in some of the countries. On the other hand, West Africa has been less affected by the pandemic, with prevalence rates between 3-5%. However, 6-9% in the region's most populated countries of Cote d'Ivoire, Nigeria and the Congo, are reported to be infected with the virus (WHO, AIDS STATS and UNAIDS, 2008-2014). But in the high-income Western countries, HIV infection is mostly common among injecting drug users and men who have sex with men (MSM).These groups are still at a very high risk, but heterosexual transmission has a growing population of new cases in the United States (DCPG, 2013).

South Africa has the largest and the highest HIV profile pandemic worldwide. In 2013, an estimated 6.1 million people were infected with HIV and 240,000 of them dying from AIDS-related illnesses (UNAIDS, 2013). In addition, HIV prevalence remains high in the South African general population, although it varies significantly among the country's regions with Kwazulu-Natal Province having the highest estimated prevalence of 41% and Limpopo Province, 17.9% (DHSA, 2014).

HIV is the etiological agent of AIDS (Acquired Immune Deficiency Syndrome) - a condition which weakens the immune system leading to life threatening opportunistic infections. HIV is a highly polymorphic virus, due principally, to the error-prone reverse transcriptase, the high replication rate and immune pressure to antiretroviral regimens (Arens, 2001; Rosina et al., 2000; Shafer, 2002 and Snoeck et al., 2002). The evolution of HIV has resulted in a complex classification, world

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spread, and intermixing of strains; at least 56 circulating recombinant forms are easily identified (Tebit and Arts, 2011).

Highly Active Antiretroviral Therapy (HAART) has been effective in reducing morbidity and mortality of HIV in both high and low income countries. South Africa, being one of the resource-limited countries, has the largest HAART rollout programme in the world and treatment became available through the national ART rollout in 2004 in the public sector (NDH, 2003, 2004; Mayosi et al., 2013 Palella et al., 1998). This programme initiates HAART to those who have a CD4 count of <350 cells/ul (viral load >1000 HIV RNA copies/ml) or with clinically- defined AIDS condition. Before 2010, the South African treatment guideline recommended the use of stavudine, lamivudine, and efavirenz (with nevirapine replacing efavirenz for pregnant women). Presently, the revised regimen comprises 2 nucleoside reverse transcriptase inhibitors and 1 non-nucleoside reverse transcriptase inhibitor abacavir + lamivudine and efavirenz for children, while stavudine/tenofovir + lamivudine and efavirenz/nevirapine for adolescents and adults for first line options]. For those who are resistant to this option, a second line option is recommended which comprises 2 nucleoside reverse transcriptase inhibitors and 1 boosted protease inhibitor [abacavir + lamivudine + lopinavir/ritonavir for children and stavudine/tenofovir+ lamivudine + lopinavir/ritonavir for adolescents and adults] (S.A Treatment Guide Line, 2013) . Although these regimens are highly effective, there are increasing reports of drug resistance compromising treatment.

In 2012, it was estimated that, about 70% of the HIV population were on ARVs in South Africa, including about 45% of the HIV infected population in the Limpopo Province (UNAIDS, 2013). Overall, 2,5 million HIV-infected patients in South Africa are on antiretroviral therapy (UNAIDS, 2014). Data on drug resistant mutations among drug naïve patients from Waterberg and Capricorn Districts, showed a prevalence of 37% of transmitted drug resistance (Bessong et al., 2005; 2006 and Nwobegahay et al., 2011). Other studies from Sub-Saharan Africa, have reported drug resistant mutation frequencies of 66% to 91% in those failing treatment (Ziad et al., 2010; Nomthandazo et al., 2011 and Aghokeng et al., 2013). Resistance to 3TC and non-nucleoside reverse transcriptase inhibitors (NNRTI), were the most common (Barth et al., 2008; El-Khatib et al., 2010; Hoffmann et al, 2010; Wallis et al.,2010; Marconi et al., 2008; Pillay et al., 2002 and Orrell et al., 2009). In another study by Boulle et al., 2008, they estimated that 3.7% ART patients fail first line treatment after 2 years and 17.9% after 4 years on treatment.

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Unlike in resource-rich countries, HIV treatment in Africa is not accompanied by regular virological monitoring and drug resistance testing. As a result, patients stay on standard first and second line regimens which fail for long periods, with the consequence being the emergence of high-level drug resistance. However, the determination of treatment based on resistance profile of patients' viruses when on treatment and experiencing viral rebound generally relates to a better outcome when compared with the lack of such resistance data. Information on the presence of resistant viruses is important in modifying combination regimens for individuals experiencing virologic failure

The aim of this study was, therefore to evaluate and characterize HIV-1 drug resistance mutations from plasma and peripheral blood mononuclear cells (PBMCs) in Bela-Bela patients failing treatment at point of viral load detection in South Africa.

1.2 Literature Review

1.2.1 Origin, discovery and classification of HIV

It is believed that HIV originated in Central Africa and its likely ancestor the, simian immunodeficiency virus (SIV) in chimpanzees (SIVcpz), is a recombinant virus derived from lentiviruses of the red capped mangabey and greater spot-nosed monkey, or a closely related species (Bailes et al., 2003). The HIV epidemic spread to other African countries and to the homosexual populations of America and Europe. Today the virus has spread worldwide and affects countries at varying degrees (Huet et al., 1990; Gao et al., 1999; Keele et al., 2006; Van et al., 2006; Gilbert at al., 2007; Coffin et al., 1986; Cohen et al., 2008).

HIV belongs to the family of retroviridae and is a member of the Lentivirus sub-group, which has been classified as HIV-1 and HIV-2. The retroviruses are further grouped into 3 subfamilies: Lentiviruses; Oncoretroviruses and the Spumaviruses. HIV is distinguishable by the possession of the viral reverse transcriptase (RT), which transcribes viral RNA into proviral DNA, which is integrated into the host cell (Preston et al., 1988). Most HIVs and SIVs are very similar in genomic structure possessing the structural genes gag, pol and env but with slight shifts in the positioning and the type of accessory genes. The accessory genes vif, rev, vpr and nef are common to all lentiviruses. The role of tat (trans-activating transcriptional factor and rev (regulation of virus gene expression), were initially defined as transcription and RNA regulatory factors in the mid-80s (Haseltine et al., 1984; Sodroski et al., 1984; Rosen et al., 1985). Two other accessory genes, vpu and vpx, are specific to HIV-1 and HIV-2 respectively (Sauter et al., 2009).

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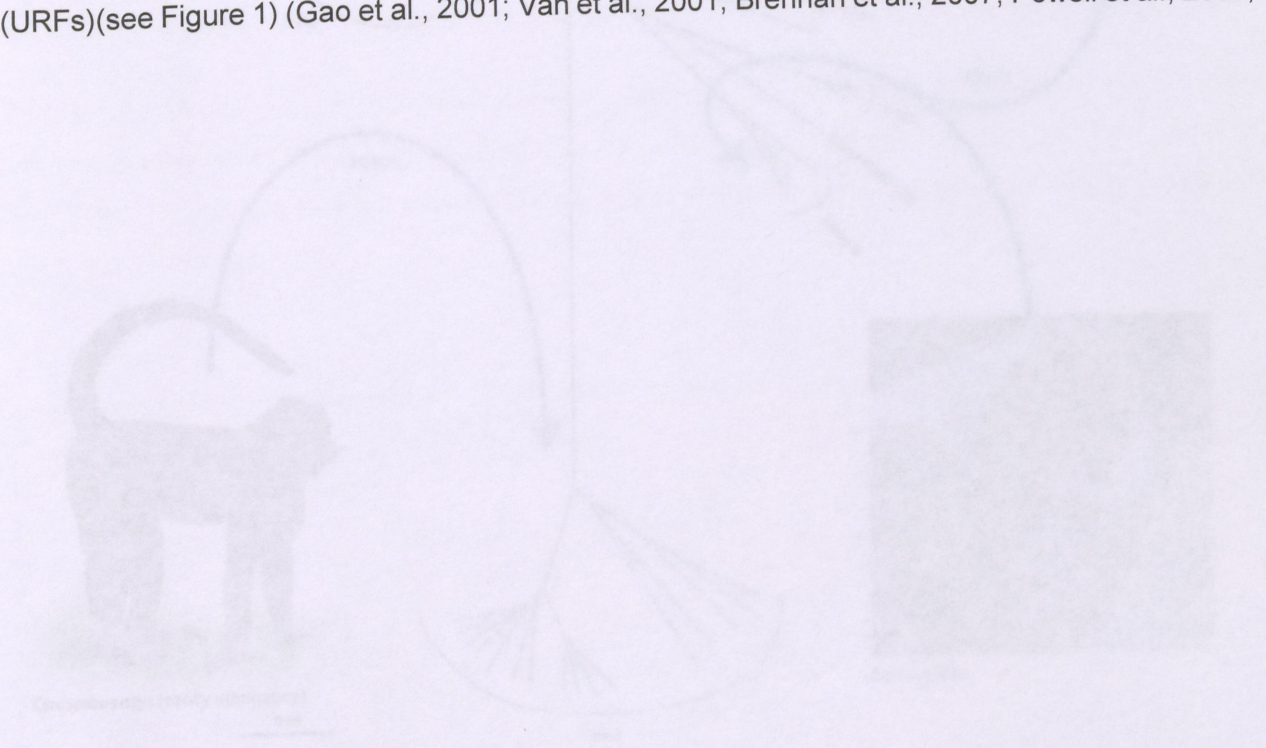
HIV-1 is the virus which was first discovered and termed Lymphadenopathy-associated virus (LAV). It is more virulent, relatively easily transmitted and is the cause of the majority of HIV infections globally (Rambaut et al., 2004). HIV-1 comprises 4 distinct lineages, termed "major (M), "outlier" (O), "new" or non O, non M (N) and the P-groups. HIV-2 is divided into 8 groups, A-H with only groups A and B establishing human transmission chains (see Figure 1.1). HIV-1 group M was the first to be discovered and has been the most successful in establishing the human pandemic. Groups O, P, and N have been limited to west central Africa and all have their epicentre in Cameroon, and are responsible for less than 1-2% of HIV infection in Cameroon (<0.05% of all HIV-1 worldwide) (Simon et al., 1998; Plantier et al., 2009), (Figure 1.1).

In addition, the phylogenetic analysis of different HIV type 1 strains from around the world has also revealed that, group M, which is responsible for most of the global HIV pandemic can be further subdivided into 9 subtypes, namely: (A-D, F-H, J and K), 5 sub-sub-types (A1/A2/A3, F1/F2), 56 circulating recombinant forms (CRFs) and several unique recombinant forms (URFs)(see Figure 1) (Gao et al., 2001; Van et al., 2001; Brennan et al., 2007; Powell et al., 2007;



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Castelbranco et al., 2010) (Figure 1.1).

Figure 1.1: Phylogenetic tree of HIV and S IV showing the various linages. The arrows indicate the origin of the virus linage and the years and the approximate dates of transmission (Tebit and Arts: Lancet Infectious Diseases: 2011).

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1.2.2 Structure and Organization of the HIV-1 genome and protein

The mature structure of the HIV-1 genome is an icosahedral particle, roughly spherical with a diameter of approximately 110nm and contains two copies of positive-sense, single-stranded ribonucleic acid (RNA, see Figure 1.2). The HIV genome is approximately 10 kilobases in length. The HIV genome has 3 structural genes: group-specific antigen (gag), which codes for virus core protein, polymerase (pol) gene, which codes for the enzymes, protease (PR), reverse transcriptase (RT), RNase H and integrase (IN) and envelope (env) gene which codes for virus envelope glycoprotein gp120 and this forms the projecting portion of each spike, while on the surface is the transmembrane glycoprotein gp41, which forms the arm of the spike. The central core contains 2 main viral proteins, the capsid protein p24 and the matrix protein p17 (Robey et al., 1985) (see Figure 1.2). The 3 enzymes PR, RT and IN are essential for replication (Jetz et al., 2000). Tat and rev genes are also vital for replication in vitro. Tat is responsible for the regulation of viral transcription and rev controls viral RNA transport and splicing. The other genes: nef, vpu, vpr and vpx are not essential for replication in vitro and are called accessory genes. (see Figure 1. 3). A summary of the HIV-1 viral proteins and their functions are shown in Table 1.1.

HIV-1 differs from HIV-2 in their glycoproteins, transmembrane protein and accessory genes. As mentioned earlier, HIV-1 has gp 120 and gp 41, while HIV-2 has gp105 and gp36 (Veronese et al., 1985). Regarding their accessory genes, HIV-1 possesses the vpu while in HIV-2 the vpu is replaced by the vpx.

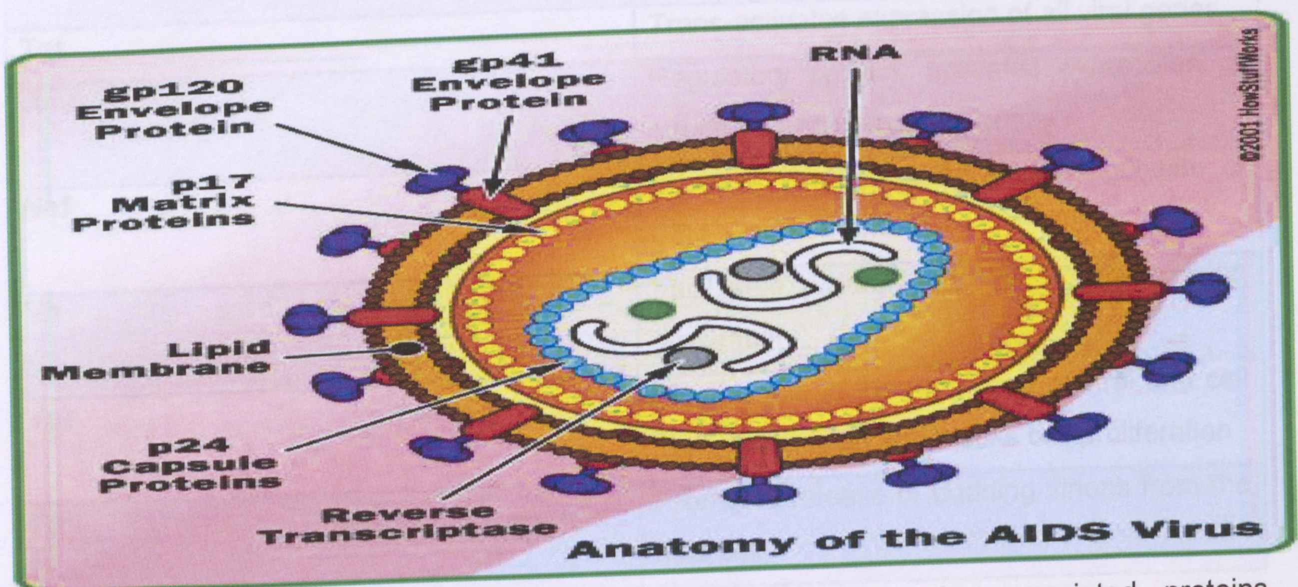

Figure 1.2: A model representation of the HIV virion and its associated proteins (http://science.how stuffwork.com/life/aids 2).

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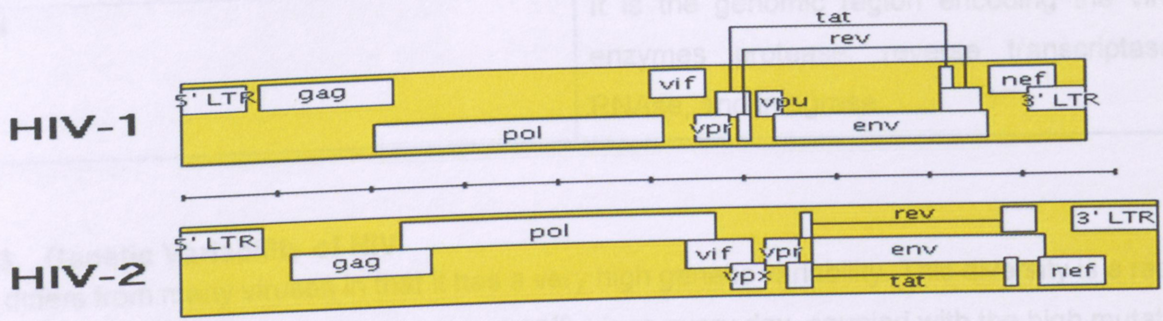

Figure 1.3: Genomic organisation of HIV (http:// www. Nature. Com/ nri / journal: MWCHO).

Table 1.1: HIV-1 viral genes, proteins, protein product and their functions (Frankel et al., 1998; Sherman et al., 2002)

Proteins	Functions
Capsid (CA)	Package the viral RNA
Matrix (MA)	Contains nuclear import signals
Nucleocapsid	Helps with incorporation of Vpr during viral assembly
Gp 120	Surface envelope glycoprotein which enables the viral particle to bind to the host receptor.
Gp 41	Transmembrane glycoprotein, which helps the viral particle to fuse into the host cell.
Tat	Trans-activates expression of all viral genes
Rev	Regulatory protein, activates expression of structural and enzymatic genes
Nef	Increase viral infectivity, down regulation of CD4
Vif	Facilitates viral replication and virion maturation
Vpr	Plays a role in the regulation of viral and cell gene expression and blocks cell proliferation
Vpu	Promotes release of budding virions from the host cell

Pol	It is the genomic region encoding the viral enzymes protease, reverse transcriptase, RNAse, and integrase.

1.2.3 Genetic Variability of HIV

HIV differs from many viruses in that it has a very high genetic variability. This diversity is a result of its high replication rate, which generates 10^{10}virions every day, coupled with the high mutation rate of approximately $3x10^{-5}$ virions per nucleotide base per replication by the reverse transcriptase (RT) which lacks proof reading activity. Also the RT can switch strands during replication leading to recombination (Robert et al., 1995 and Pretson et al., 1988).

During HIV infection, a single cell could be simultaneously infected by two or more different strains of HIV. When simultaneous infections occur, the genome of the progeny virions maybe composed of RNA strands from two different strains. This hybrid virion then infects new cells where it undergoes replication. When this happens, the reverse transcriptase will generate a newly synthesized retroviral DNA sequence, which is a recombinant between the two parental genomes (Hemelaar et al., 2006; Bredell et al., 2002; Wallis et al., 2009 and Tebit and Art, 2011). Inter-subtype recombination occurs when a patient is infected with two different HIV-1 subtypes and during the viral replication, an RNA strand from one virus is co-packaged with an RNA strand from the other, forming new viral particle. When a new replication cycle starts, the two RNA strands of the virus are transcribed to proviral DNA (Ramirez et al., 2008; Taylor et al., 2008).

Four groups of HIV-1(M, N, O and P) have been identified on the basis of differences in the full genome sequences. Group M is the most prevalent and is subdivided into eight subtypes (or clades), sub-sub types (A1/A2A3, F1/F2), circulating recombinant forms (CRFs) and the unique recombinant forms (URFs), which is geographically distinct (Figures 1.1 and 1.2). The most prevalent are subtypes B (found mainly in North America and Europe), A, C and D (found in Africa and Asia). These subtypes form branches on the phylogenetic tree representing the linage of the M group of HIV-1 (see Figure 1.1). Co-infection within distinct sub type's also known as dual infection may give rise to CRFs or URFs.

The highest genetic diversity of HIV is in Africa, especially Central Africa, where HIV is thought to have originated. Currently, HIV is characterised by transmission of numerous genotypes of the virus with subtype C accounting for 52% of infections worldwide. HIV-1 subtype C was first identified in 1980 in Ethiopia, South Africa and Malawi, then it spread to other southern African

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countries (McCormack, 2000; Tebit and Arts 2011), then to Europe, Asia and Brazil. Sub-Saharan Africa has the highest number of patients infected with HIV. At the beginning of the epidemic in South Africa, HIV-1 subtype B viruses were identified among homosexual men who reported contacts in the United States (Williamson et al., 1995). Currently, HIV-1 subtype C viruses are responsible for the epidemic in South Africa (Gorden et al., 2003; Jacobs et al., 2008). HIV-1 subtype diversity can have a major impact on antiretroviral treatment and the development of drug resistance. It may affect the genetic barrier, defined as the amount of viral mutations necessary to result in drug resistant virus. Polymorphisms associated with a particular HIV-1 subtype can affect the genetic barrier towards a particular antiretroviral drug (Beerenwinkel et al., 2005). South Africa is amongst the countries where free HIV treatment was initiated, but constant virological monitoring is limited.

1.2.4 Epidemiology of HIV

In 2011, UNAIDS estimated that approximately a total of 34.2 million people were living with HIV. And from the total, 1.2 million adults and children died of AIDS, accounting for 71% AIDS deaths in South Africa (UNAIDS, 2012). In the same year, an estimated 2.5 million people were newly infected with HIV and an estimated 330.000 of the total were under the age of 15 years. Since the onset of the epidemic, more than 80 million people have contracted HIV and nearly 60 million have died of HIV related diseases. In 2011, 1.7 million people died from AIDS and 230.000 of them were under the age of 15 (UNAIDS, 2012).

Sub-Saharan Africa still remains the most affected region by the global AIDS epidemic and the epidemic continues to be most severe in South Africa, with an estimated 6 million persons more living with HIV, than any other country in the world (UNAIDS, 2013). There was also an estimated number of new infections of 1.4% in 2011, which is a decrease when compared to 1.63% new infections in 2008. In South Africa, of the approximated 6 million HIV infected individuals, more than 600,000 individuals were enrolled into antiretroviral treatment (ART) by mid-2009 (SADH, 2013). Limpopo Province in the north-eastern part of South Africa had an HIV prevalence rate of between 3 and 17% (NAS, 2013).

The nine countries with the highest HIV prevalence in the world are found in Sub-Saharan Africa (UNAIDS, 2010) and the majority (71%) of those living with HIV are women (UNAIDS, 2011). Since 2003, HIV-1 subtype C has been spreading in countries such as Zambia, Lesotho, Zimbabwe and South Africa (Spira et al., 2003). In 2007, National HIV prevalence exceeded 10% in Botswana, Lesotho, Mozambique, Namibia, South Africa, Swaziland, Malawi and Zimbabwe (UNAIDS, 2008).

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1.2.5 Clinical Course of HIV

HIV is often called the silent virus due to its very long incubation period. Infection begins when the virus attaches to CD4 receptors and the chemokine co- receptors (CCR5 and CXCR4). HIV-1 targets all the cells that express the CD4+ molecule on their surface. These cells include; CD4⁺, T-lymphocytes, monocytes, macrophages, follicular dendritic cells and microglial cells in the brain. The primary targets are the CD4⁺, T-lymphocytes, and destruction of these cells is the main cause of AIDS.

The pathogenesis of HIV-1 can be divided into four stages; the first, is the acute viremia phase, also called the primary infection which is asymptomatic and characterized by massive viral replication, resulting in high viral levels (see Figure 1.4). This phase can last for a few weeks. The Second is the chronic phase which is symptomatic. This stage begins after antibodies to the virus are fully developed and the initial immune response is complete. This can last for 28 days to several months. The third is the clinical latent phase which can last up to a year or more. During this stage, the viral load drops to a stable level, termed as a viral set- point. The final is the AIDS stage, which results from long term (chronic) HIV infection, which is defined by an absolute CD4 cell count ≤350 cells/μl and specific opportunistic infections (see Figure 1.4). In the absence of treatment, the average time for the development of AIDS, is about 10 years (Buchbinder et al., 1996 and Lifson et al., 1991). However, there are some HIV-1 infected individuals who remain asymptomatic for over 10-15 years; they are called long-term non-progressors (Buchbinder et al., 1996; Cao et al., 1995 and Panteleo et al., 1991). On the other hand there are some individuals who within 2-3 years or less, progress to the AIDS stage, and are therefore termed rapid progressors (Anzala et al., 1995 and Panteleo et al., 1996). Although HIV-1 and HIV-2 are biologically similar, HIV-2 is less pathogenic than HIV-1 (Jaffar et al., 1997; Marlink et al., 1994

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and Popper et al., 1999).

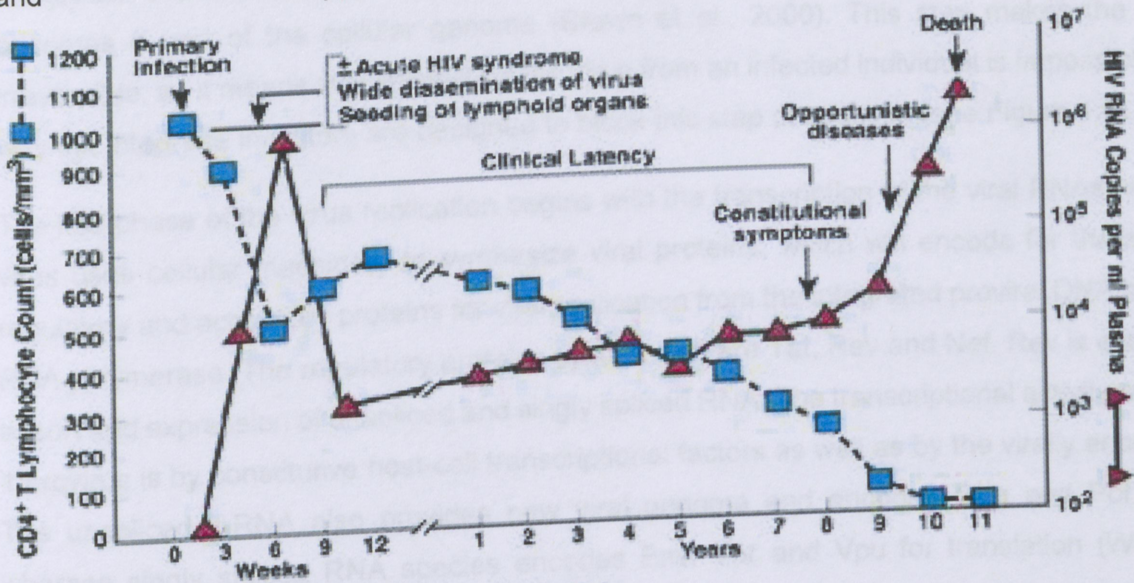

Figure 1.4: Illustration of the clinical progression of HIV (http://www.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/pages/clinicalcourse.aspx).

1.2.6 Replication of HIV and the Mode of Action of ART

The HIV life cycle can be divided into 2 phases. The early phase begins with the recognition of the host cell by the virus and ends when the 2 single strands of RNA are integrated in the host chromosome. The late phase begins when the viral RNA is reverse transcribed into single complementary strand of DNA and ends when the virus matures and leaves the cell.

During the early phase, the virus binds to the surface of the host cell by interaction of the gp120 protein and the CD4 receptor. CCR5 and CXCR4 have been identified as the most essential co-receptors necessary for viral entry and replication of the host cell. Other co-receptors that can be used by HIV include: CCR3, CCR2b, CCR8, CCR9 (Berger et al., 1999). This step can be inhibited by the fusion inhibitors.

Once in the cell, HIV uncoats. The uncoating of the core initiates the reverse transcription process by which RNA is transcribed into complementary DNA by the enzyme reverse transcriptase (RT). The single-stranded virally encoded DNA acts as a template for the production of a complementary DNA, forming a double stranded proviral DNA. This step can be inhibited by reverse transcriptase inhibitors. Reverse transcriptase inhibitors were the first class of HIV inhibitors to be used as drugs (see Figure 1.5).

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Integration of proviral DNA is then incorporated into the host DNA by the integrase enzyme and becomes a part of the cellular genome (Brown et al., 2000). This step makes the infection irreversible, as it means that eliminating the virus from an infected individual is impossible. That's why the integrase inhibitors are designed to block this step of infection (see Figure 1.5).

The late phase of the virus replication begins with the transcription of the viral RNAs, where the virus uses cellular machinery to synthesize viral proteins, which will encode for the structural, regulatory and accessory proteins for viral replication from the integrated proviral DNA by cellular RNA polymerase. The regulatory proteins synthesized are Tat, Rev and Nef. Rev is essential for export and expression of unspliced and singly spliced RNA. The transcriptional activity of the HIV-1 provirus is by constitutive host-cell transcriptional factors as well as by the virally encoded Tat. The unspliced mRNA also provides new viral genome and encodes Gag and Pol proteins, whereas singly spliced RNA species encodes Env, Vpr and Vpu for translation (Wong-Stall, 1991). Several of these are long amino acid chains which must be cleaved by a specific viral protease before new viral particles can be active. Protease inhibitors block viral maturation at this step (Stevenson, 2003) but if this step is not blocked, the virus assembles and becomes mature, then it goes out of the host cell and infects other host cells. Figure 1.5 shows the life cycle of HIV and its mode of action on HAART inhibitors.

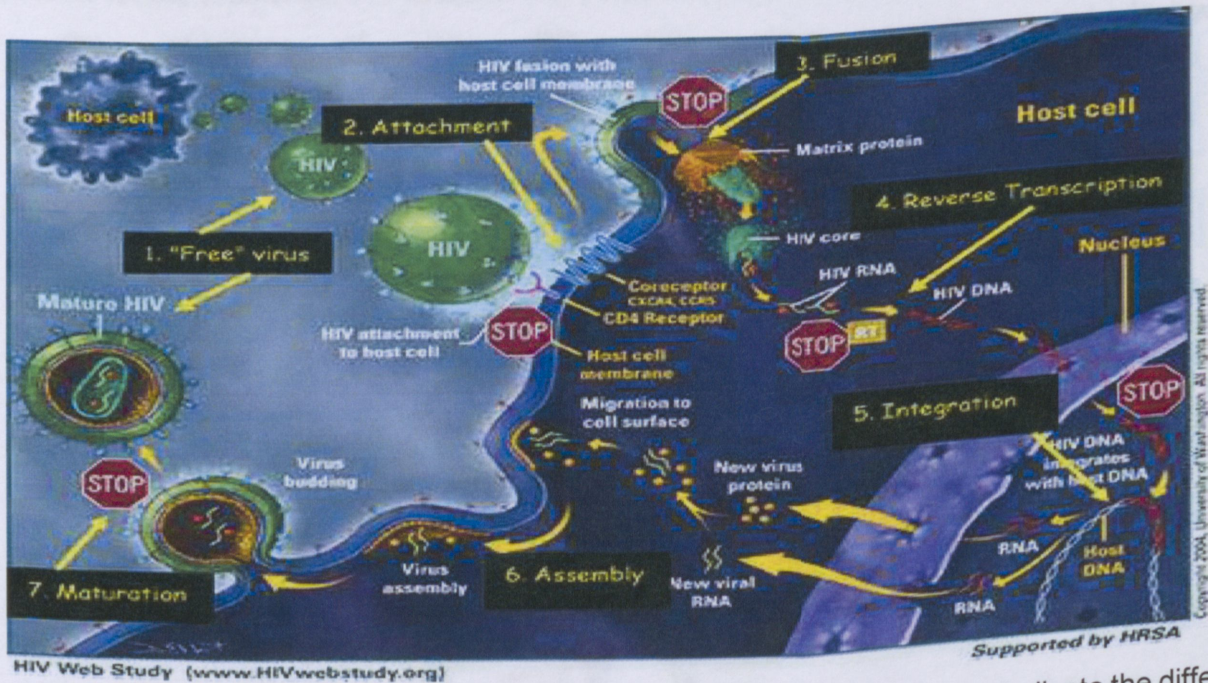

Figure 1.5: Life cycle of HIV and the action of HAART inhibitors. Numbers 1-7 indicate the different stages of the HIV life cycle. The points at which antiviral drugs can act are indicated with a "STOP" sign (UNAIDS, HIV Web Study: www.hivwebstudy.org; 2012).

1.2.7 The HIV-1 enzymes

The HIV-1 pol gene encodes the viral enzymes, protease (PR), reverse transcriptase (RT), RNase H and integrase, which are produced by the cleavage of the Gag-pol polyprotein (Prasad et al., 1990 and Turner et al., 1999).

1.2.7.1 HIV-1 Protease

HIV protease exists as a homodimer consisting of two structural identical subunits made up of 99 amino acids and a molecular weight of 10779 Da, which mediates the cleavage of gag, gag-pol and nef precursor polyproteins. Figure 1.6 shows the ribbon model structure of the protease enzyme. This enzyme targets the cleavage site on the Gag and Gag-pol polyproteins, producing structural and viral enzymes. There are four cleavage sites in the Gag and seven in the Gag-pol polyprotein. Cleavage specificity is determined by four amino acid upstream and three downstream. The active site is positioned in a loop at 25-27 amino acids (Asp, Thr and Gly), which also forms part of the catalytic site. The binding cleft for the substrate is found above the active site, hence giving enough space for the cleft to accommodate a substrate of about seven amino acids in length. The proteolytic cleavage process is highly specific, temporal regulated and essential for the production of infectious viral particles (Jacks et al., 1998). It is worth noting that in the absence of HIV PR, HIV virion remains non-infectious.

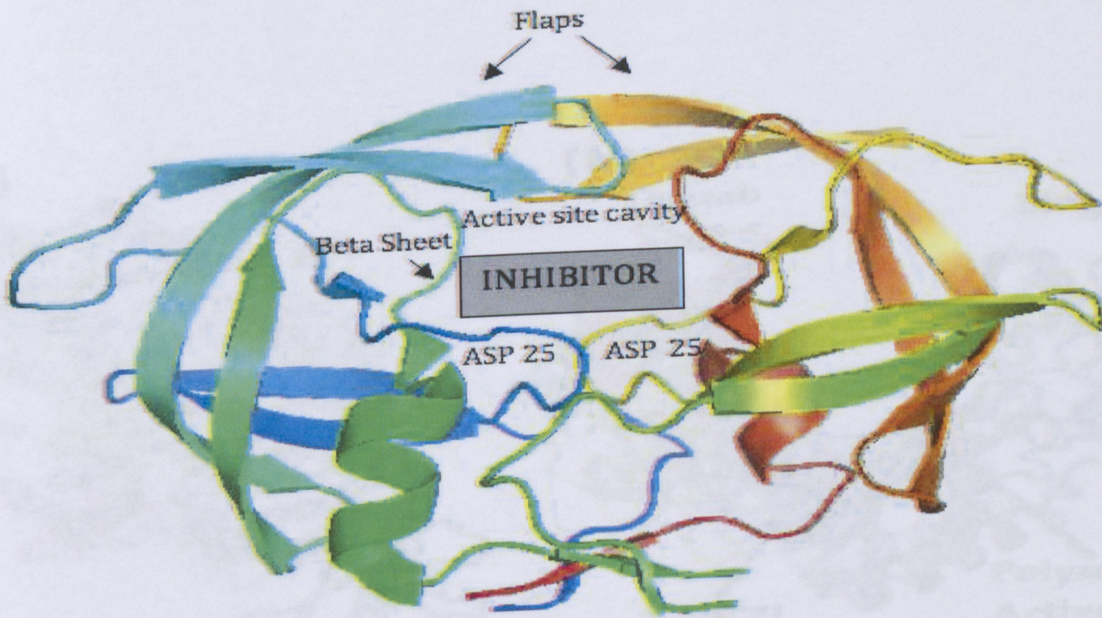

Figure 1.6; Structural model of HIV-1 protease (http:// www. Engender health. Protease.org).

1.2.7.2 HIV-1 reverse transcriptase

The RT has RNA dependant DNA –dependent polymerase activities which enables it to synthesize DNA from RNA and DNA templates. Two subunits p51 and p66 are used for RT cleavage. First, p66 is cleaved from the polyprotein and forms a homodimer which later cleaves closer to the C-terminus to yield a heterodimer containing both p51 and p66. The amino acids for p51 and p66 are 440 and 560 of the RT gene respectively. The structure of the RT domain has 5 subdomains which resembles a hand with fingers, palm and thumb as shown on figure 1.7. The active site of the enzyme lies in the thumb of p66 which contains two alpha helices and the palm acts as a clamp to position the template primer to the active site.

RT has a poor proofreading ability and non-complimentary incorporation of nucleotides occurs which leads to single as well as hyper-mutations, hence responsible for HIV-1 diversity. A frame shift can occur when misincorporation is followed by misalignment of the nucleotides. Slippages of the two DNA strand may occur that can lead to deletions or insertions of one or more nucleotides. Base substitutions can result when slippage occurs with correct insertion and realignment. This can also cross from one template to another resulting in recombination (McCutchan, 2006). All of these factors can lead to variation over a larger portion of the genome and hence contribute to diversity and mutations which can lead to resistance.

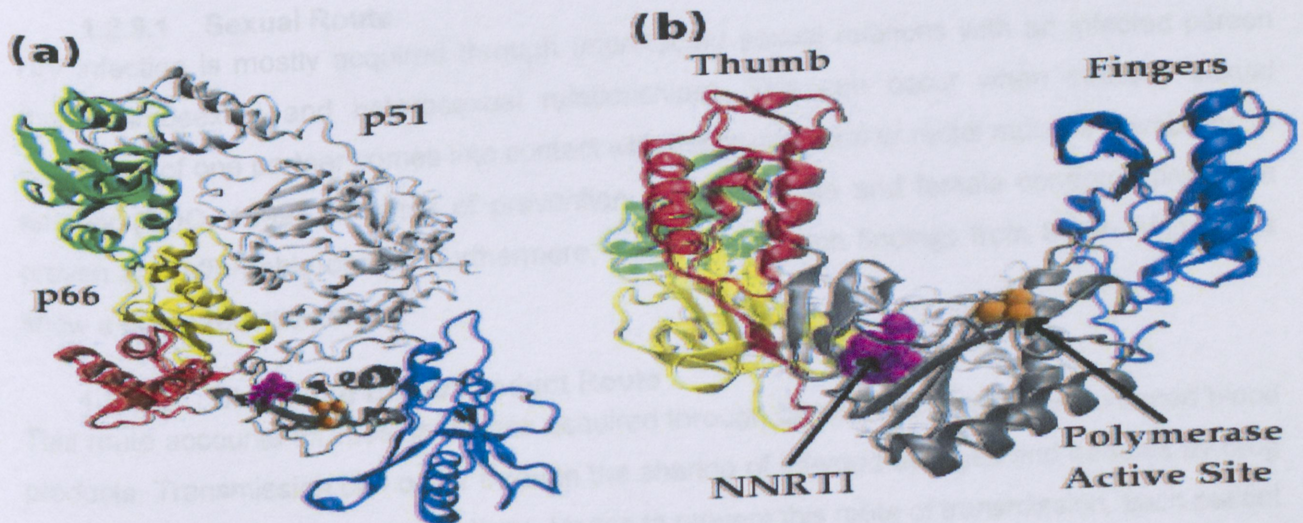

Figure 1.7: Structural model of HIV-1 reverse transcriptase. (http:// www. Engender. Health. reverse transcriptase. org).

1.2.8 Diagnosis of HIV

HIV infection is detected directly through the demonstration of infectious viral antigens as well as indirectly through the demonstration of virus specific antibodies. The most common and simple methods for testing HIV is the rapid test (also called the Enzyme Immuno Assay, EIA), in which human blood is placed on a strip and observed for agglutination. The second, is the Enzyme Linked Immnunosorbent Assay (ELISA), which is an assay, based on the principle of specific antigen-antibody reaction (Connick, 2005). Another common diagnostic method is the p24 assay, which detects the HIV capsid antigen in the serum of an infected individual. P24 antigen assays are more sensitive than the antibody assays.

In addition, polymerase chain reaction (PCR) is a molecular method used in cases where antibody testing may be insufficient to detect if the patient is infected or not. PCR amplifies parts of the virus and can also be used to measure viral load, an important parameter in treatment monitoring. A negative test result means HIV was not detected in the blood of the individual at the time of testing. This does not necessarily mean that the individual is not infected with HIV. The test could have been carried out during the diagnostic window period. The diagnostic window period is defined as the period, the body requires to produce detectable levels of antibodies and this usually takes several weeks (Busch, 1997).

1.2.9 Transmission and Prevention of HIV

In the absence of control, prevention, intervention and treatment, HIV infections can be transmitted through the following three main routes:

1.2.9.1 Sexual Route

HIV infection is mostly acquired through unprotected sexual relations with an infected person (both homosexual and heterosexual relationships). This can occur when infected sexual secretions of one partner comes into contact with the genital, oral or rectal mucous membrane of another (CDC, 1999). In terms of prevention methods, male and female condoms provide a proven and affordable option. Furthermore, male circumcision findings from South Africa trials show a 60% protective effect.

1.2.9.2 Blood and Blood Product Route

This route accounts for most infections acquired through blood transfusion of unscreened blood products. Transmission can occur through the sharing of infected syringes and needles by drug users or even in some hospital settings. Hence to prevent this route of transmission, each patient should be entitled to one needle and syringe and syringes should be used only once and discarded.

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1.2.9.3 Mother-to-Child Transmission (MTCT) Route:

This can occur during pregnancy, labour and delivery or during breast feeding. In the absence of treatment, the transmission rate between the mother and the child is around 25%. However, where the combination HAART and caesarean section are available, this risk can be reduced to as low as 1% (Cooper et al., 2002). The prevention of mother-to-child transmission has become advanced, as the intrapartum transmission has been reduced by the increase in access to interventions, such as single dose of nevirapine to the mother and new born baby. Also, elective delivery by caesarean section can further reduce HIV-1 transmission. The risk of transmission from mother-to-infant has been shown to decrease with the administration of antiretroviral drugs. However, the use of single dose (sd)-NVP regimen leads to the development of HIV-1 resistance mutations to NVP, due to the low genetic barrier of HIV for NVP (Ben et al., 2008). NVP resistance has been shown to be as high as reported in 19-60% of mothers receiving sd-NVP (Eshleman et al., 2001, 2005 and Kurle et al., 2007).

1.2.10 Treatment of HIV-Infection

Currently, there is no vaccine or cure for HIV or AIDS. Nevertheless, HIV-1 positive patients in western countries survive for many more years after diagnosis because of the availability of HAART (Schneider et al., 2004). The success of this treatment has prolonged and improved the quality and life of AIDS patients (Chene et al., 2003 and Wood et al., 2003).

HAART is divided into classes according to their mode of action. Presently, primary HAART options consist of a combination of at least three medications. Usual regimens consist of two NRTIs plus either an NNRTI for first line treatment or PI for second line treatment. Table 1.2 shows the various classes of HAART and their mode of action.

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Table 1.2: Types of HAART, examples and their mode of action (November, 2012).

Class of ARVs	Examples	Mode of action
Nucleoside reverse transcriptase inhibitor	Tenofovir, lamivudine, stavudine, abacavir, didanosine	Prevents the addition of natural nucleoside onto the DNA strand. Hence preventing viral RNA to be transcribed to DNA.
Non-nucleoside reverse transcriptase inhibitors.	Efavirenz, nevirapine ,delavirdine	Binds directly to the RT enzyme, preventing it from transcribing from viral RNA to viral DNA.
Protease inhibitor	Ritonavir/lopinavir,saquinavir, indinavir	Preventing the enzymes from cleaving large viral polypeptides into structural and functional components.
Integrase inhibitor	Raltegravir	Prevents insertion of genetic materials into human cells.
Entry inhibitor	Maraviroc	Binds to human co-receptors preventing viral entry.

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1.2.10.1 South African HIV Treatment Guide Line 2013

In South Africa, three of the five classes of drugs are used. Namely: NRTIs, NNRTIS and PIs. Table 1.3 shows the recommended first-line and second-line treatments for HIV patients.

Table 1.3: Recommended South African Treatment guideline (April, 2013).

Regimens	Children<3 years old	Children>3 years old	Adolescents/ Adults	Pregnant women
First-line	Abacavir, lamivudine + efavirenz	Abacavir, lamivudine + Efavirenz	Tenofovir, lamivudine + efavirenz	Efavirenz is replaced with Nevirapine
Second-line	Abacavir,lamivudine+ Lopinavir/ritonavir	Tenofovir,didanosine+lopinavir/ritonavir	Tenofovir,lamivudine+lopinavir/ritonavir	Lopinavir/ritonavir is replaced with ritonavir (r).

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1.2.11 HIV-1 drug resistance and virologic failure

In 1989, the first HIV-1 antiretroviral drug resistance was reported (Larder et al., 1989). From these findings the authors noted that patients became less likely to respond to AZT therapy after persistent treatment. A rebound in HIV replication during combination therapy is considered a major cause of treatment failure (Hirsch et al., 1996). This may be due to the selection or development of drug resistance. Drug resistance mutations occur within the gene target of the drug which is exposed to sub optimal drug concentrations. For example PR and RT inhibitors will cause mutations in the protease and reverse transcriptase genes respectively which are the molecular targets of the drug (Menéndez-Arias., 2008; Verger et al., 2002; De-Jong et al., 1996 and Daiz et al., 2008).

Some resistance mutations on their own can cause resistance to one or more drugs, and are referred to as primary or major mutations (e.g. M184V). Other mutations can only cause resistance if present in combination with major resistance mutations, and are referred to as secondary or minor mutations (e.g. E138A). Major mutations lead to several fold decrease in sensitivity to one or more antiretroviral drugs (Hirsch et al., 1996 and Shafer., 2004). Minor mutations may not result in a significant decrease in sensitivity but are associated with an increase in viral fitness (replication capacity). Mutations are described by using the first letter of a consensus subtype amino acid, followed by the position of the mutation in the sequence, followed by the amino acid that indicates the mutations, for example M184V. When a mixture of more than one amino acid is reported at one position, the different amino acid letters are written after the position separated by a forward slash (M184M/V), this indicates a mixture of a wild-type amino acid and the mutant amino acid (Clavel et al., 2004 and Shafer, 2004).

An extremely important aspect of the rate of evolutionary changes for HIV is the selection pressure exerted by the host. This aspect is intensely illustrated by the rapid development of drug-resistant variations of HIV. The same type of competition occurs during host-mediated immunoselection pressure, in which viral variants emerge to avoid control by epitope-specific host immune responses (Bellocchi et al., 2005).

1.2.11.1 Protease inhibitor (PI) resistance

Resistance to PI's are due to structural changes that reduce the binding of the protease to the inhibitor. The use of PI's has led to a marked reduction in mortality and morbidity in patients with advanced HIV infection. The unavoidable widespread use of PIs, has led to the emergence of drug-resistant HIV variants, many of which display cross-resistance to the inhibitors. The

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occurrence of drug-resistant variants during antiretroviral therapy is a severe hindrance to sustained suppression of the HIV-1. In this connection, resistant testing is vital in guiding the clinicians in the selection of the best treatment regimens.

1.2.11.2 Nucleoside and Non-Nucleoside reverse transcriptase inhibitors (NRTI and NNRTI) resistance

Resistance to NRTI's develops when the process of blocking the elongation of the proviral DNA during reverse transcription and termination of the DNA chain formation is not achieved by the inhibitor. On the other hand, resistance to NNRTI's arises with changes in the hydrophobic pocket next to the NNRTI binding site (Ren et al., 2008).

Although these combinations of PIs, NRTIs and NNRTIs can suppress virus replication to undetectable levels for a long period of time, with the consequent immunological constitution (Palella et al., 1998 and Smith et al., 2004), many patients experience virologic failure as shown by a rebound in the viral load (Ledergerber et al., 1999). Besides insufficient drug absorption, poor adherence, low drug potency and persistence of HIV reservoirs, one of the gravest problems for HIV suppression is the emergence of drug-resistant variants (Ledergerber et al., 1999). Detection of resistant viruses has become increasingly important to achieve better management of the disease. Hence, resistance testing has been recommended to facilitate the choosing of the ideal drug regimens by clinicians after first or multiple treatment failures (Hirsch et al., 2003).

In addition, mutations in the envelope and integrase genes which confer resistance to the entry and integrase inhibitors have been identified but will not be discussed since these inhibitors are not freely available in South Africa.

1.2.13 Drug resistance testing

Different methods are available for the detection and evaluation of antiviral drug resistance. These methods include; genotypic and phenotypic resistant testing. Genotypic testing is the most common and determines the resistance-related mutation pattern of the virus population by using point hybridization (Stuyver et al., 1997) or population based sequencing and report consensus at each nucleotide (Erali et al., 2001). This method is easy to perform and has a rapid turn-over. This could provide insight to the potential of resistance to emerge and it can also detect transitional mutations that do not cause drug resistance by themselves but indicate the presence of selective drug pressure.

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On the other hand, phenotypic assays measures the direct quantitative resistance and, determines the drug concentration fold increase required for achieving the same degree of viral inhibition as compared to a wild-type (Kellam et al., 1994; Boucher et al., 1992; Hanna et al., 2008 and Shafer, 2004). This method is very expensive and time consuming.

1.2.14 Study Rationale

HIV drug resistance is a major threat to the scaling up of antiretroviral therapy (ARVs) in developing countries, especially in Africa (WHO, 2014). Current South African HIV treatment guideline recommends a combination therapy referred to as Highly Active Antiretroviral Therapy (HAART), which has proven to be extremely useful in reducing morbidity and mortality. Despite the benefits of HAART, a number of patients still experience a major drawback that is associated with the development of major drug resistant mutations. This is usually associated with the use of ARVs (Gatanaga et al., 2007; Maartens et al., 2008; Nachega et al., 2009 and Tang et al., 2004).

In developed countries, HAART has been used with remarkable success, coupled with regular virological monitoring and clinical testing and drug resistance to guide the management of patients (Gupta et al., 2007 and Richmann et al., 2009). Unfortunately, in developing countries, such as South Africa, regular monitoring of patients is a challenge due to the high cost of viral load and drug resistance testing. As a consequence, patients stay on first-line therapy which might be failing for long periods and thus leads to emergence of high-level resistance.

Studies regarding genotypic resistance at viral load rebound among drug experienced individuals have been done worldwide (Tebit et al., 2008; Ziad et al., 2010; Delaugerre et al., 2012; Avelin et al., 2013 and Dolling et al., 2013). A previous study showed that South Africans may harbour non-nucleoside reverse transcriptase(NNRTI) drug resistance mutations (DRM) in patients failing first-line treatment, which is often different from those in other African countries and the rest of the world (Nomthandazo et al., 2011). Furthermore, others have reported subtype distribution and co-receptor usage, prevalence of transmitted drug resistance, clinical determination of anthropometric failure from drug naïve and drug experienced individuals worldwide (Bessong et al., 2005; 2006; Chhagan et al.,2012; Laurent et al., 2006;Oyugi et al., 2007 and Nwobegahay et al., 2011., Avelin et al.,2013).

Given the changing treatment strategies, genotypic and phenotypic resistance patterns continue to evolve. Data on virologic failure at the point of viral rebound detection from drug experienced individuals from an under studied population such as Bela-Bela in Limpopo Province does not

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exist. Therefore, the relation between drug resistance and therapy failures needs further investigation to understand the implications of amino acid changes which lead to major mutations and also to prove the relevance of performing resistance testing in HIV drug experienced individuals. Thus, this study aimed at evaluating and characterizing the genetic drug resistance profile of HIV-individuals experiencing virologic failure at point of viral load rebound detection in Bela-Bela, South Africa.

1.2.14.1 Study Objectives

1.2.14.1 Main Objective

The main goal of this study was to characterized HIV-1 drug resistance mutations from plasma and peripheral blood mononuclear cells in patients failing antiretroviral treatment in Bela-Bela, Limpopo Province, South Africa.

1.2.14.2 Specific objectives

1. To amplify and sequence complete protease and partial reverse transcriptase genes from HAART experienced patients.
2. To determine the subtypes and intra-genetic variability of HIV-resistant viruses in infected individuals failing treatment.
3. To compare the differences in mutations obtained from sequenced amplicons of both viral DNA and viral RNA from each participant.

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CHAPTER TWO: MATERIALS AND METHODS

2.1 Ethical Consideration

The Health, Safety and Research Ethics committee of the University of Venda, South Africa approved the study protocol (SMNS/13/MBY/01/0625). Permission to use public clinics for specimen collection was obtained from the Limpopo Provincial Department of Health, Polokwane and the Bela-Bela HIV/AIDS Wellness clinic permitted the conduct of this study.

2.2 Study population, identification and recruiting of participants

Study participants with prior exposure to ARVs and a rebound in viral load at the Bela-Bela Wellness Clinic of the Limpopo Province, South Africa were recruited sequentially during July 2013- April 2014. The Bela-Bela HIV/AIDS Wellness Clinic was established in 1996 and is located in the township of Bela-Bela which has a population of approximately 63000 inhabitants (Ndejeka and Manhaeve, 2006). This clinic is managed by the HIV/AIDS Prevention Group (HAPG), a non-governmental organization (Ndejeka et al., 2006). Treatment in Bela-Bela began in 2000 that is 4 years before the roll-out of ARVs in South African public health facilities. Bela-Bela is a stop-over point for long distance truck drivers from Botswana, Zambia and Namibia who patronize sex workers during their stay there. Bela-Bela centrum is about 120km from Gauteng Province to the South and Northwest Province to the Southwest. The clinic receives patients from both the urban and rural sectors of the town as it also runs a home based service and a voluntary counselling and testing facility.

For this study, virological failure was defined as:

1. Two consecutive viral loads on treatment greater than 1000 copies/ml after previous suppression.
2. One viral load greater than 1000 copies/ml after previous suppression followed by a change in treatment.
3. One viral load greater than 1000 copies/ml after 180 days on ARVs without suppression.

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2.3 Specimen collection and plasma preparation

Five millitres of venous blood was collected by venipuncture into EDTA vacutainer tubes from each consenting participant. Participants gave written informed consent. Specimens were collected from July 2013 to April 2014. The structured questionnaire which was administered to the participants elicited basic socio-demographic and clinical data which noted information such as age, gender, weight, AIDS stage, presumed mode of infection, place of infection, probable year of infection and type of regimen was obtained from each participant. Explanation of the study was provided in the local languages (Tshivenda, Sesotho and Xitsonga) whenever there was a need. A research code was assigned per blood specimen for confidentiality and easy identification during laboratory procedures.

Plasma was prepared by centrifugation of specimen at 3000 rpm for 3 minutes. Aliquots of plasma was aspirated aseptically into sterile cryovials and stored at -80^{0}C for subsequent viral RNA extraction. PBMCs were isolated from total cells using Ficol- hyplaque method and DNA purification was extracted from the remaining cells using Qiagen Kit as described below.

2.4 Viral RNA extraction, PCR and nested PCR

Five hundred microlitres of plasma was pipetted into a sterile eppendorf tube. The specimen was spun at 4^{0}C for 1 hour at 14,000 rpm. Three hundred and sixty microlitres of the centrifuged plasma was gently pipetted out and stored at -80^{0}C for serological analysis. Viral RNA was extracted from the 140µl plasma left in the tube according to instructions from the Qiagen Viral RNA Mini kit (Qiagen, Valencia, USA). All reagents in the kit were reconstituted as recommended by the manufacturer. The extracted RNA was stored in sterile 1.5 ml microcentrifuge tube at -80^{0}C for RT-PCR. Viral RNA was transcribed to cDNA using the superscript kit (Roche, USA) and Taq DNA polymerase (Invitrogen), followed by first round and nested PCR amplifications. This generated approximately a 1.4-kb product comprising the entire protease gene (PR) and at least the first 900 base pairs of the reverse transcriptase gene (RT). The PCR primers are listed in table 2.1. Polymerase chain reaction for both target gene regions comprised a final reaction of 1µM of forward and reverse primers, 200nm dNTP's (Roche), 4U Taq polymerase (Invitrogen), 5µl DNA template and nuclease free water (Qiagen, Valencia, USA) in a 50µl total volume.

Amplification was carried out in a GSX2 thermal cycler. Thermal cycling conditions for RT-PCR step were as follows: 110^{0}C for the heat lid, an initial denaturation step at 94^{0}C for 2 min, then 30 cycles of 94^{0}C for 30 sec, 52^{0}C for 45 sec, 68^{0}C for 2 min and a final elongation at 68^{0}C for 10

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min and stored at 4⁰C. Cycling conditions for the nested reaction were 95⁰C for 2 min followed by 30 cycles of 95⁰C for 1 min, 58⁰C for 1 min, 68⁰C for 2 min and a final extension of 68⁰C for 7 min and stored at 4⁰C until use.

2.5 DNA isolation from total cells and PCR amplification

Total cells were transferred into sterile 15 ml conical tubes and the PBMCs were separated using the Ficol-hyplaque gradient centrifugation method. DNA was isolated from PBMCs using the Qiagen blood DNA Midi kit (Qiagen, Valencia, USA) according to the manufacturer's specifications. PCR reaction mixture for both target gene region (1.4kb) comprised a final concentration of 2µM of forward and reverse primers, 200mM dNTP's (Roche), 4U FastStart Taq polymerase (Roche), 10x Buffer ($MgCl_2$, 25Mm) (Roche), 2µl of DNA template and nuclease free water (Qiagen, Valencia, USA) in a 50µl total reaction volume.

Amplification was carried out in a GSX2 thermal cycler. Cycling condition for first round PCR were thus: 110⁰C for the heat lid, an initial denaturation step at 94⁰C for 2 min, then 40 cycles of 94⁰C for 30 sec, 52⁰C for 45 sec, 68⁰C for 3 min, and a final elongation at 72⁰C for 10 min and stored at 4⁰C. Cycling conditions for nested reaction were the same as those listed above.

Table 2.1: Primers used for amplification of HIV partial pol region

Primers	Primer direction	Primer Sequence
Gag pol-RRS	Forward	5'-GAG AGG CAG GCT AAT TTT TTA GGG A-3'
Pol RNase H	Reverse	5'-CTT GTA ATT CAG CTT CTG ATT TG-3'
Gag P1	Forward	5'-CAA GGG GAG GCC AGG GAA TTT-3'
Pol 2R	Reverse	5'-CAT GGA GTA TAT TAT GAC CCA TCA-3'

The first pair of primers (Gag-pol-RRS and Pol RNase H) was used for first round amplification using DNA and RNA as templates and the second primer set (GagP1 and Pol2R) was used as nested primers for both amplification methods.

2.6 Visualization of nested PCR amplicons with agarose gel electrophoresis

Using 1% agarose gel prepared in 1 x TAE Buffer (0.04M Tris acetate, 0.001M EDTA), gel electrophoresis was used to resolve all the nested PCR products and verified for expected band

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size. Three µl of 5 mg/ml ethidium bromide (Promega, Madison, USA) was added to the gel to intercalate ds DNA. One microlitre of loading buffer (Promega, Madison, USA) was mixed with 3 µl nested PCR amplicon. Each amplicon was successively loaded in a slot on the gel, including the positive and negative control and 1 kilobasepair marker (Invitrogen). Electric current was set at 300 mAmperes at 80 volts for 35 minutes. After the appropriate migration of the products from the negative to the positive anode as indicated by the changes in the colour dye and the stop of the set-up, the gel was visualized under ultra violet light at a wavelength of 302nm (at this wavelength, ethidium bromide intercalates DNA; PCR amplicons and fluoresces brightly). The gel was visualized and photographed using the UV transillumination gel documentation system (Syngene G, Germany; Version 06-2.d.1).

2.7 Purification of PCR amplicons

The Qiaquick PCR purification kit (Qiagen, Valencia, USA) was used to purify the nested amplicons for sequencing reaction according to the manufacturer's instructions. The nanophotometer (Implen GmbH, Germany; Version 2.2) was used to determine the DNA concentration of the purified samples. The purified products were loaded on a 2% agarose gel and set to run in the same conditions listed above for the confirmation of the expected band sizes. The purified amplicons were stored at -20^{0}C until used.

2.8 Sequence of purified PCR amplicons

Direct population based sequencing reaction was used to sequence both strands of the viral DNA template from nested PCR reaction. The ABI Prism® BigDye™ Terminator v3.1 ready reaction sequencing kit (Applied Biosystems, Warrington, UK) was used for the reaction. This kit is based on the dideoxy method developed by Sanger et al., 1997. The primers for sequencing of HIV partial pol are listed in Table 2.2.

Table 2.2: Primers used for sequencing of HIV partial pol

Primers	Primer Direction	Primer Sequence
Gag P1	Forward	5'-CTT CAG AAC AGA CCA GAG C-3'
Protease 2	Reverse	5'-CTC TTC TGG TAA CGG CCA TTG-3'
Pol 1F	Forward	5'-TTT TCC CAT TAG TCC TAT TGA AAC TGT A-3'

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Pol 1R	Reverse	5'-CAT GCT ACT CTG GAA TAT TGC TGG TGA TCC-3'
Pol 2F	Forward	5'-CTG CAT TCA CCA TAC CTA GTA TAA AC-3'
Pol 2R	Reverse	5'-TGA TGG GTC ATA ATA TAC TCC ATG-3'

The first 2 primers pair (GagP1 and Protease 2) were used to generate the entire protease gene (297 nt) forward and reverse, while the other 2 prime sets (Pol 1F and Pol 1R; Pol 2F and Pol 2R were used to generate a partial RT fragment (~1103nt) forward and reverse.

2.9 Nucleotide Sequence assembles and analysis

2.9.1 Sequence Assembly

The resulting forward and reverse nucleotide sequence electropherograms were assembled, manually edited and translated into predicted amino acid with SeqMan Pro II software, v 8.0 (DNASTAR, Lasergene, USA). BioEdit software Programme was used to align the nucleotide sequences (http://www.mbio.ncsc.edu/BioEdit/bioedit.html).

2.9. 2 HIV genotyping and Recombination analysis

Subtyping of HIV sequences was done by phylogenetic analysis. Polymerase sequences comprising the entire PR and a fragment of RT of the test viruses were aligned using Muscle Clustal X included in Mega 6 software, with representative subtype reference sequences of all the subtypes obtained from GenBank (http://www.ncbi.nlm.nih.gov/genbank,2013). Previously described sequences from Limpopo Province were included in the analysis. Neighbour-joining phylogenetic trees were generated with the PHYLLIP programme, making use of the maximum likelihood model to take care of differences in transition and transversion rates. The reliability of the tree was assessed by bootstrapping of 1000 replicates.

For two isolates (BBRES 10 and 14) which were not clearly distinct using phylogenetic analysis and results obtained from Stanford drug resistance tool, recombination analysing tools; REGA; RIP and jpHMM were used for further analysis. Rega HIV-1 and 2 subtyping tool, v 3.0 was used to determine the HIV-1 subtype (http://dppartners.stanford.edu.Rega.Subtyping) (November, 2013). The jpHMM (jumping profile Hidden Markov Model) (http://jphmm.gobics.de/) (November,

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2010) relates to nucleic acid sequences of numerous alignments to a sequence family for which a classification of sub classes is in existence (Alcantara et al., 2005 and Zhang et al., 2006). jpHMM also detects recombination break points of a test sequence based on HXB2. RIP (Recombination Identification Programme) used to analyse the similarity between and the test isolates and other recombination and pure subtype sequences from gene bank (http://www.ncbi.nih.gov/genbank, 2013).

2.9.3 Intra-genetic variability among HIV isolates

The intra-genetic variability among isolates was determined by the analysis of the mean genetic distance among the isolates, both DNA and RNA. Nucleotide sequences of the protease and reverse transcriptase genes were aligned with BioEdit, followed by mean genetic distance calculation using the Kimura 2-parameter method contained in the Mega 6 software.

2.9.4 Drug resistance genotyping

The test sequences were submitted to the Stanford HIV Drug Resistance database (http://hivdb Stanford.edu) for the examination of drug resistant mutations. For observation of transmitted drug resistance, the same sequences were submitted to the calibrated population resistance program (http://hivdb Stanford. edu, CPR tool). For confirmation of the mutations observed, the International AIDS Society-USA Panel for drug resistance testing guidelines was used (Hirsch et al., 2000). Sequences were deemed high quality if they had no ambiguities, no frame shift, no stop codon, no insertion nor deletion as well as the usual residues.

In order to check for contamination, the edited sequences were blasted against the public dataset using NCBI blast (http://www.ncbi.nlm.nih.gov/blast). Sequences were deemed non contaminant if the identity to previously genotyped samples were lower than 98%.

2.9.5 Avoiding PCR contamination

PCR amplification – based techniques are now used routinely to detect microbial genome in research laboratories. Unfortunately, the superb sensitivity of these techniques make them vulnerable to contamination, hence leading sometimes to false-positive results (Cimino et al., 1990). Steps used for preventing contamination both in pre-amplification and post-amplification, were thus: a PCR cabinet was sterilised with UV light, for each PCR reaction a negative control was included, sterile pipettes and DNA/RNA free PCR tubes were used. Firstly, master mixes were prepared and pipetted into PCR reaction tubes, after which water was added as the negative

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control and finally in a separate sterile working area DNA and RNA template was added. Gloves were often changed to avoid cross contamination. Longer pipette tips were used, preventing the pipette itself from touching the inside of the tubes. Working surfaces and equipment were cleaned regularly with sodium hypochlorite (bleach) and to get rid of the bleach, 70% ethanol was used, DNA and RNA away were also used for sterilization of the PCR cabinet and the pipettes. Finally, phylogenetic analysis was also done to also detect contamination.

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CHAPTER THREE: RESULTS

3.1: Demographic and clinical profile of the study population

Blood specimens were collected from 23 HIV drug experienced patients failing treatment and 13 were females. The mean average age was 34.8 years (range 5-61). The most important risk factor for HIV transmission based on responses to the questions in the questionnaire, was sexual intercourse with a percentage of 65.2%, followed by mother to child transmission 21.7% and blood transfusion 13%. However, the questionnaire did not provide information about method of sexual transmission that is whether it was heterosexual or homosexual. Viral load was available for all 23 patients at the time of sample collection, with values ranging between 342-281380 copies/ml. Fourteen (60.9%) patients were on (D4TorTDF/3TC/EFV); 3 (13.1%) were on AZT/3TC/EFV); 1 (4.3%) was on DDI/3TC/EFV; and 5 children (21.7%) were on ABC/3TC/EFV; with NVP replacing EFV in pregnant women. The questionnaire was also designed to determine the AIDS stage of the patients: 30% (7/23) were stage 4; 26% (6/23) were stage 3; and 22% (5/23) were each stages 1 and 2. Over 70% of the participants had been on treatment for more than 5 years. Table 3.1 shows the detailed demographic and clinical profile of the study population.

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Table 3.1: Demographic and clinical profile of the study participants

Characteristics	Number of study participants
Sex	
Females	13 (56.5%)
Males	10 (43.5%)
Age (Years)	
Mean/ Range	34.8 (5-61 years)
Presumed mode of infection	
Sexual	15 (65.2%)
Mother to child transmission	5 (21.7%)
Blood transfusion	3 (13.0%)
Year of ARV commencement	
2009-2014	5 (21.7%)
2000-2008	18 (78.3%)
Regimen at point of viral rebound	
D4T or TDF/3TC/EFV	14 (56.5%)
ABC/3TC/EFV	5 (21.7%)
AZT/3TC/EFV	3 (13.1%)
DDI/3TC/EFV	1 (4.3%)
CD+4 cell counts (cells/mm³) at viral rebound	
Mean	412.6
Range	6-1196
>350 cells/mm³	10 (43.5%)
<350 cells/mm³	13 (47.8%)
Viral load (copies/ml) at point of rebound	
Mean	695845
Range	342-281380
AIDS stages (WHO)	
Stages 4	30%
Stage 3	26%
Stages 1 and 2	22% each

Note: Blood specimens were collected at the point of viral load rebound detection.

3.2 Amplification of HIV partial pol gene

Approximately 1.4kb encompassing the entire protease (PR) gene (297 nt = 99aa) and partial reverse transcriptase (RT) gene (900 nt = 300aa) was successfully amplified from 21 of 23 specimens on both proviral DNA and viral RNA as templates. Two specimens could not be amplified from either template. In total, 42 PCR products were obtained for subsequent analysis. Representative gel pictures of proviral DNA and viral RNA are shown in Figures 9A and 9B respectively.

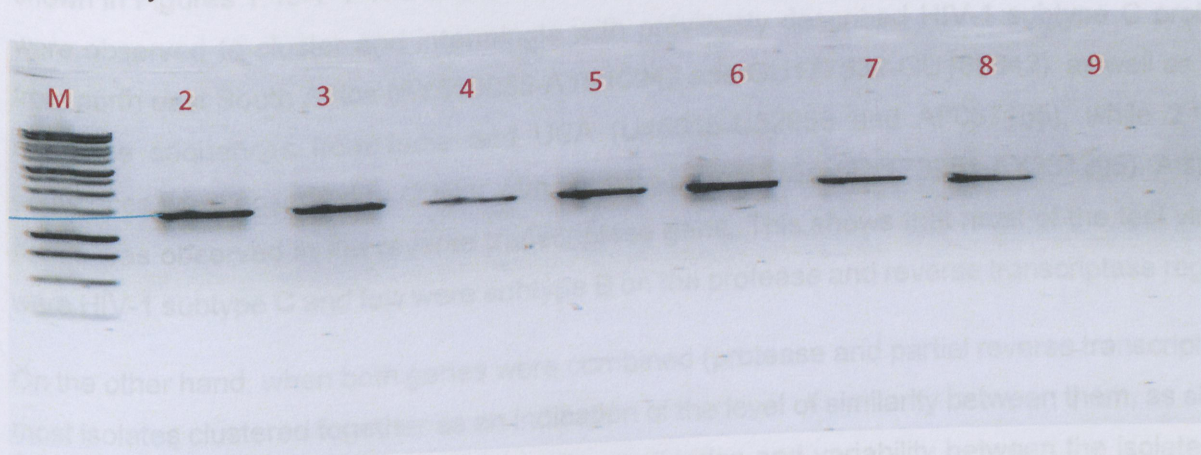

Figure 1.8A: Representative 1% ethidium bromide agarose-stained gel of HIV-1 Pol PCR product, obtained from proviral DNA. Lane M is a 1000bp molecular weight marker, lanes 2-8 are the test specimens and lane 9 is the negative control (nuclease free water), indicating there was no contamination.

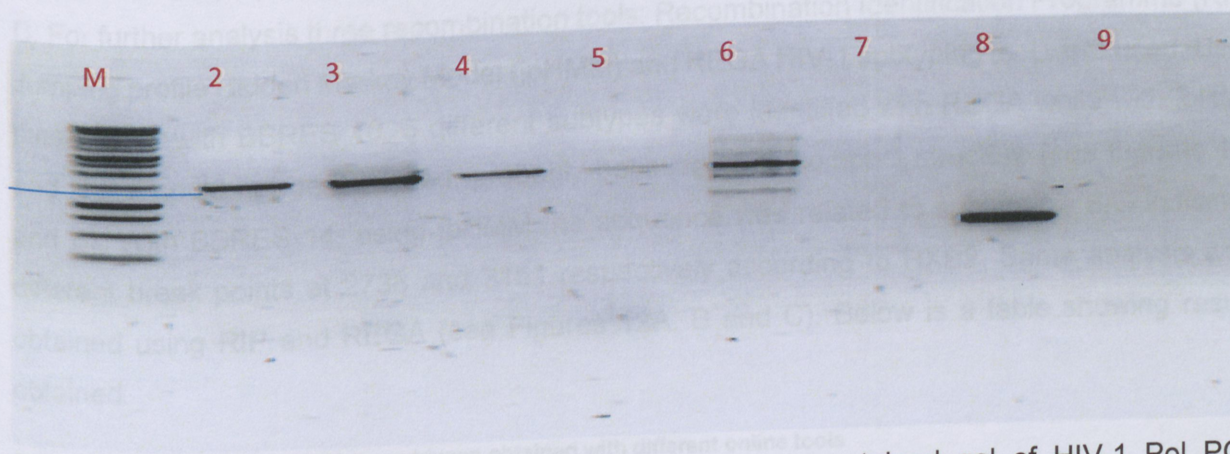

Figure 1.8B: Representative 1% ethidium bromide agarose-stained gel of HIV-1 Pol PCR products, obtained from viral RNA. Lane M is a 1000bp molecular weight marker, lanes 2-6 are test specimens, lane 7 is a negative control (nuclease free water) and lanes 8 and 9 are +ve control (Hela RNA) and –ve control (nuclease free water) contained in the superscript kit.

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3.3 HIV Genotyping and Recombination analyses

Nucleotide sequences were obtained for 38 PR and 42 RT genes. However, 29 sequences were reliable in the protease region, 25 in the reverse transcriptase region. The nucleotides were used for genetic analyses, subtyping and drug resistance mutations interpretation.

Phylogenetic analysis of the protease (for proviral DNA [PD], viral RNA [VD] and PD + VD) are shown in Figures 1.9A, 1.9B and 1.9C respectively, while analysis for reverse transcriptase are shown in Figures 1.10A, 1.10B and 1.10C respectively. The majority of test protease sequences were observed to cluster and intermingle with previously described HIV-1 subtype C protease from north east South Africa (AY510035-AY510042 and GU177532-GU188842), as well as other reference sequences from India and USA (U46016-U52953 and AF067155), while 2 other sequences were observed to cluster with subtype B from USA (AY173951-AY331295). A similar profile was observed in the reverse transcriptase gene. This shows that most of the test viruses were HIV-1 subtype C and few were subtype B on the protease and reverse transcriptase regions.

On the other hand, when both genes were combined (protease and partial reverse transcriptase), most isolates clustered together as an indication of the level of similarity between them, as shown in Figures 1.9C and 1.10C respectively. The similarities and variability between the isolates are outlined in Figures 1.11 and 1.12, which shows the intra-genetic variability and similarities between the isolates.

Furthermore, after phylogenetic analysis, (BBRES 10 and 14) were outliers to pure subtype B and C. For further analysis three recombination tools: Recombination Identification Programme (RIP); Jumping profile Hidden Markov Model (jpHMM) and REGA HIV-1 subtyping tool were used. Using these tools; with BBRES 10, 5 different subtypes were identified with REGA while with jpHMM and RIP it could not be analysed, probably because of its compact structure (see Figures 11A and B). With BBRES 14; using jpHMM the sequence was related to subtype(s) B/C indicating different break points at 2738 and 3151 respectively according to HXB2. Same analysis were obtained using RIP and REGA (see Figures 12A, B and C). Below is a table showing results obtained.

Table 3.2 Table showing the various subtypes obtained with different online tools

Codes	REGA	jpHMM	RIP	Phylogenetic analysis	Stanford
BBRES 10	A/B/C/F/G	Untyped	Untyped	B outlier	B/G
BBRES 14	B/C	B/C	B/C	C outlier	B/C

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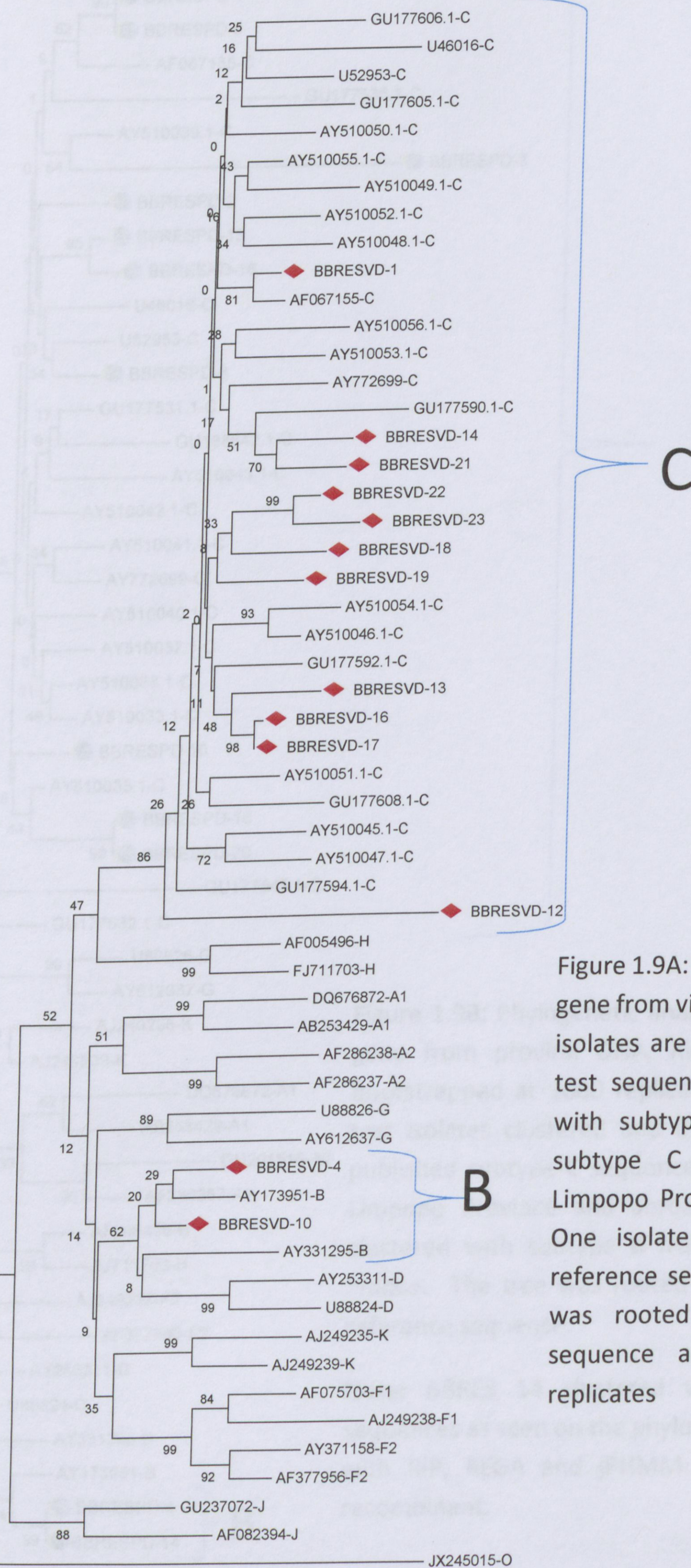

Figure 1.9A: Phylogenetic analysis of protease gene from viral RNA, highlighted red. The test isolates are highlighted in red. Most of the test sequences clustered and intermingled with subtype C references and published subtype C sequences obtained in the Limpopo Province and others from abroad. One isolate clustered with a subtype B reference sequence from the USA. The tree was rooted with a group O reference sequence and boots- trapped at 1000 replicates

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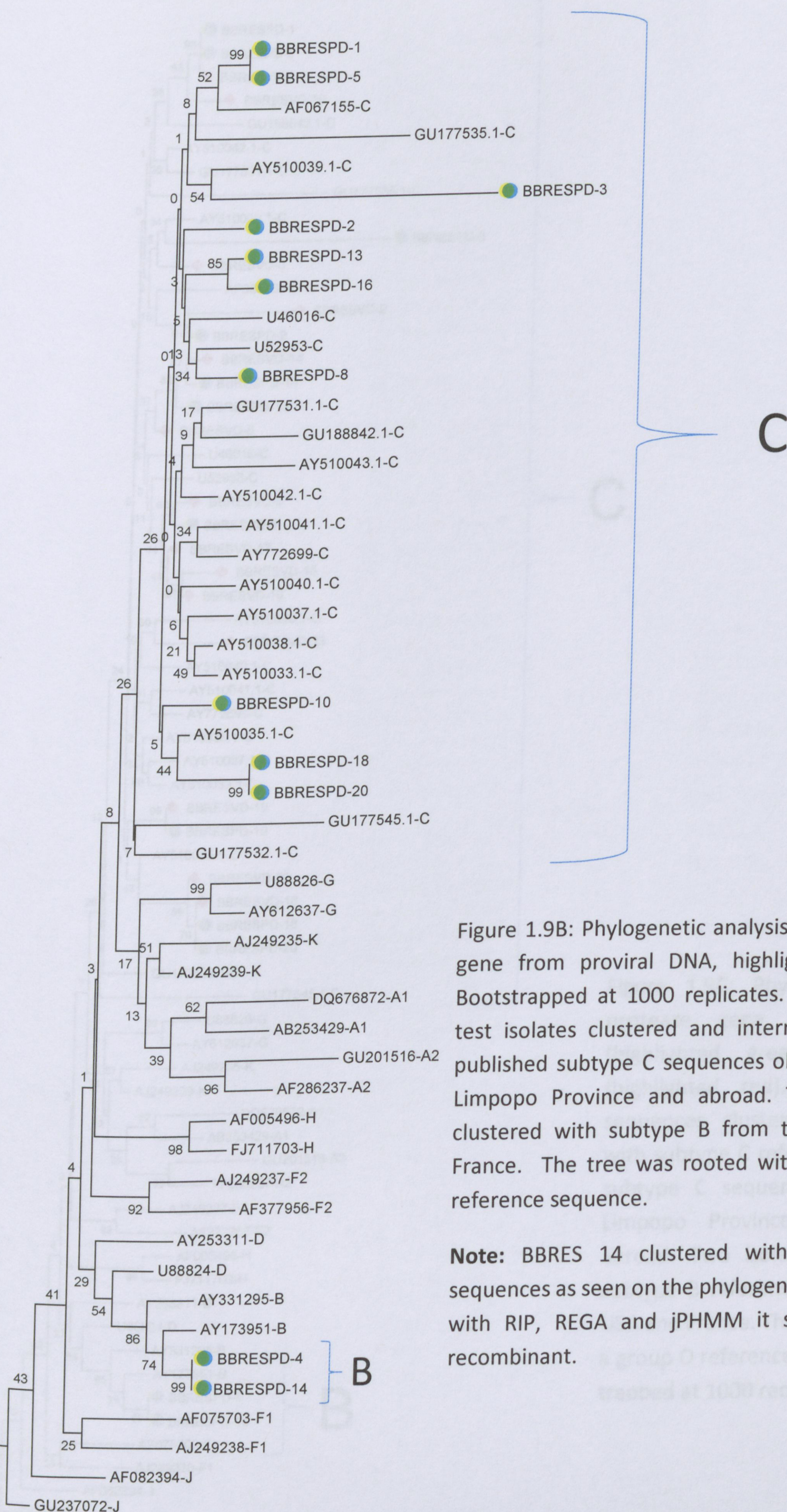

Figure 1.9B: Phylogenetic analysis for protease gene from proviral DNA, highlighted green. Bootstrapped at 1000 replicates. Most of the test isolates clustered and intermingled with published subtype C sequences obtained from Limpopo Province and abroad. Two isolates clustered with subtype B from the USA and France. The tree was rooted with a group O reference sequence.

Note: BBRES 14 clustered with subtype B sequences as seen on the phylogenetic tree and with RIP, REGA and jPHMM it showed B/G recombinant.

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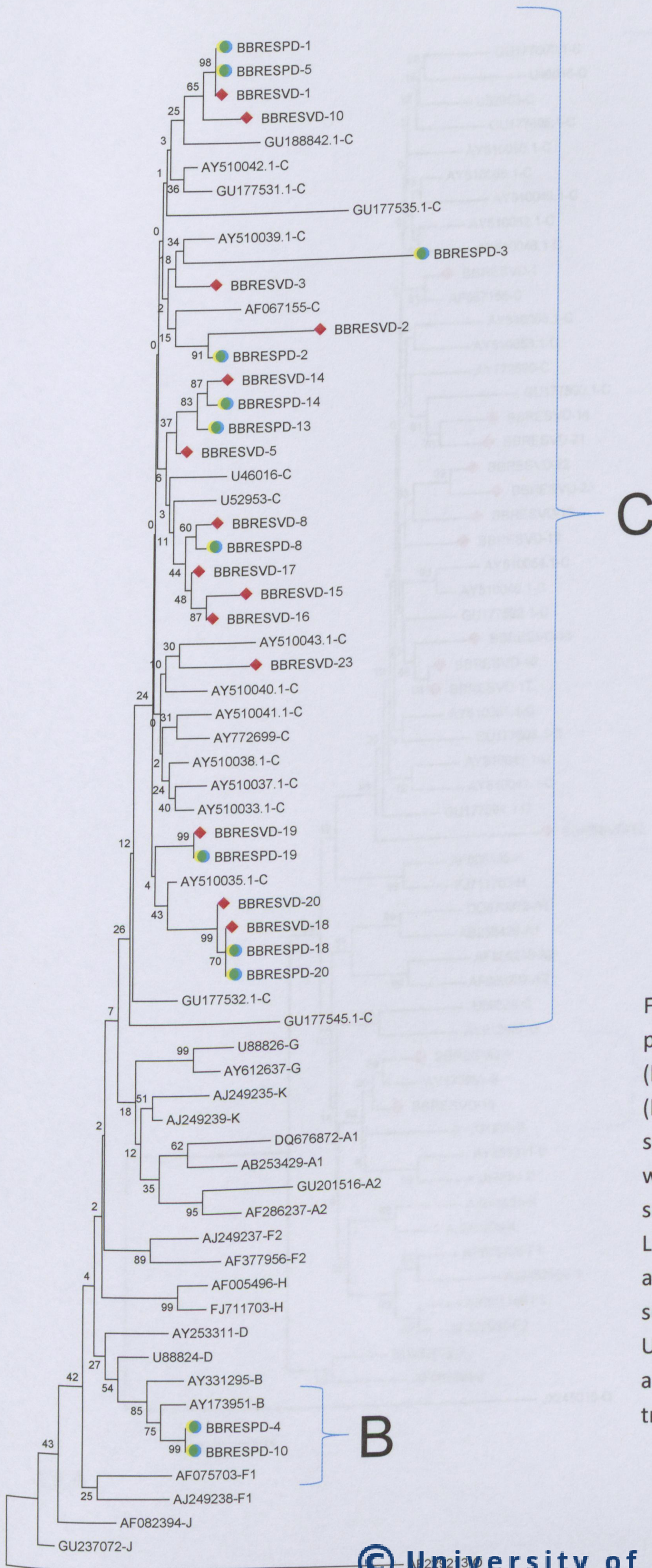

Figure 1.9C: Phylogenetic analysis of protease gene from proviral DNA (highlighted green) and viral RNA (highlighted red). Most of the test sequences clustered and intermingled with subtype C references and published subtype C sequences obtained in the Limpopo Province and others from abroad. Two isolates clustered with a subtype B reference sequence from the USA and France. The tree was rooted with a group O reference sequence and boots-trapped at 1000 replicates.

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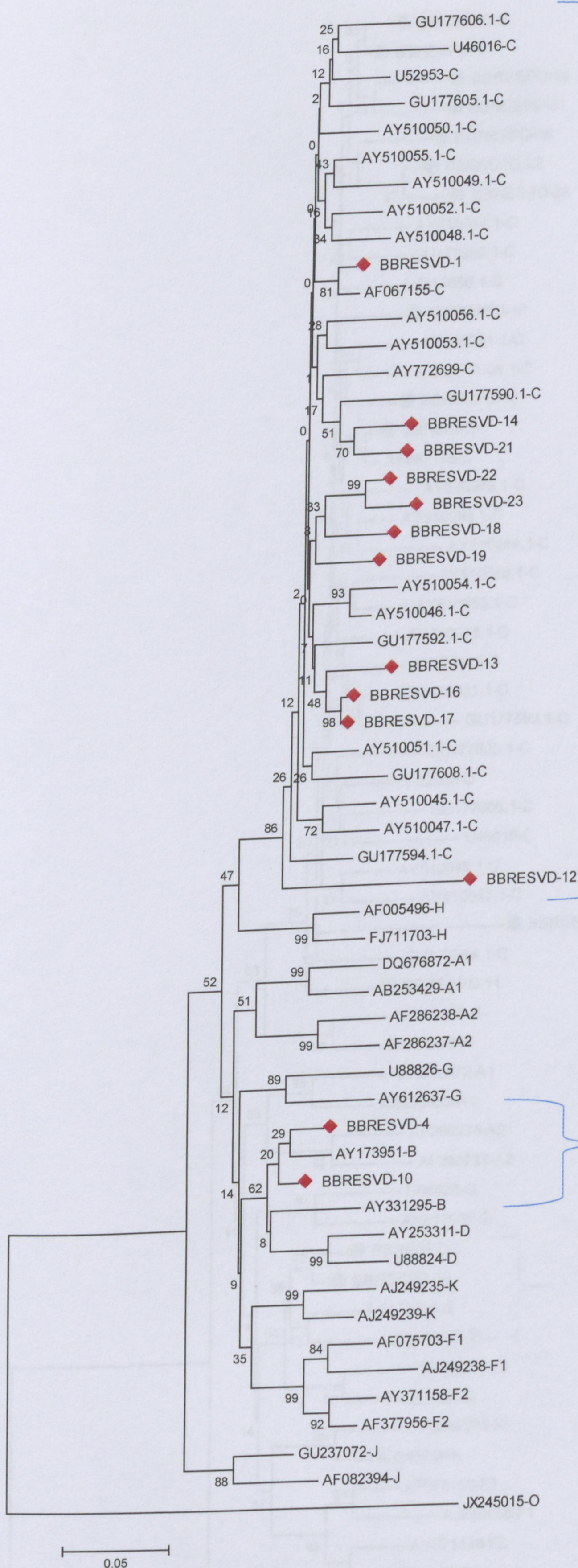

Figure 1.10A: Phylogenetic analysis for reverse transcriptase from viral RNA. Test isolates highlighted red Bootstrapped at 1000 replicates. Most of the test isolates clustered and intermingled with published subtype C sequence obtained from Limpopo Province and abroad. Two isolates clustered with subtype B from the USA and France. The tree was rooted with a group O reference sequence.

3.3.2

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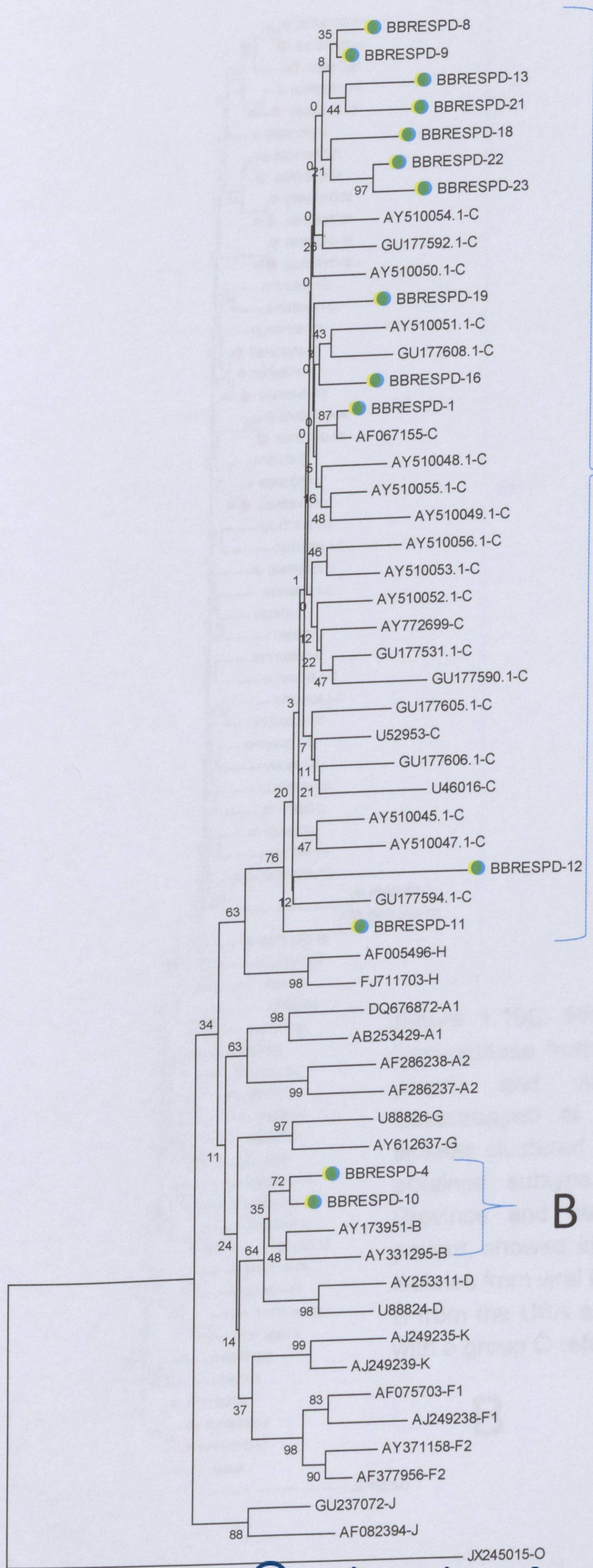

C

Figure 1.10B: Phylogenetic analysis for reverse transcriptase gene from proviral DNA. Test isolates highlighted green. Bootstrapped at 1000 replicates. Most of the test isolates clustered and intermingled with published subtype C sequences obtained from Limpopo Province and abroad. Two isolates clustered with subtype B from the USA and France. The tree was rooted with a group O reference sequence.

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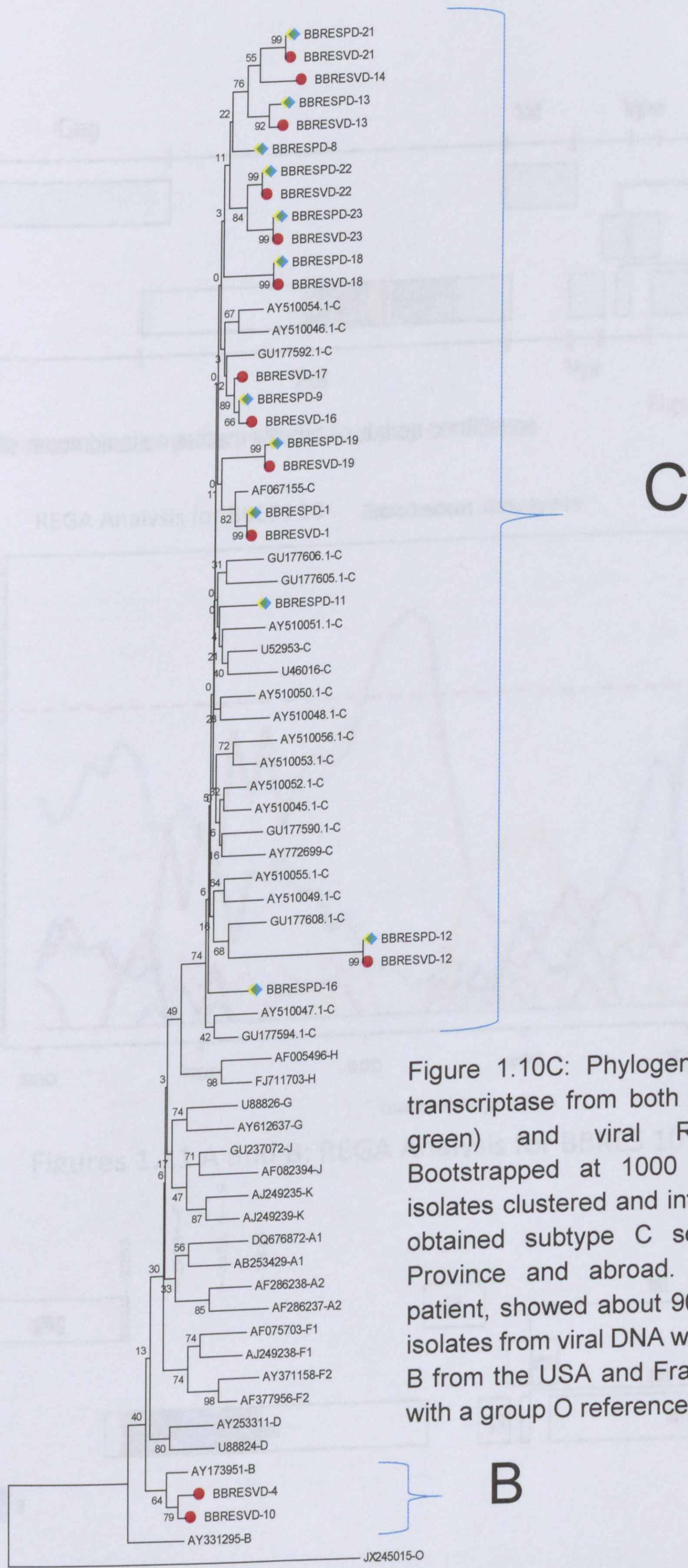

Figure 1.10C: Phylogenetic analysis for reverse transcriptase from both proviral DNA (highlighted green) and viral RNA (highlighted red). Bootstrapped at 1000 replicates. Most of the isolates clustered and intermingled with previously obtained subtype C sequences from Limpopo Province and abroad. Most isolates from the patient, showed about 90% close similarities. Two isolates from viral DNA were clustered with subtype B from the USA and France. The tree was rooted with a group O reference sequence.

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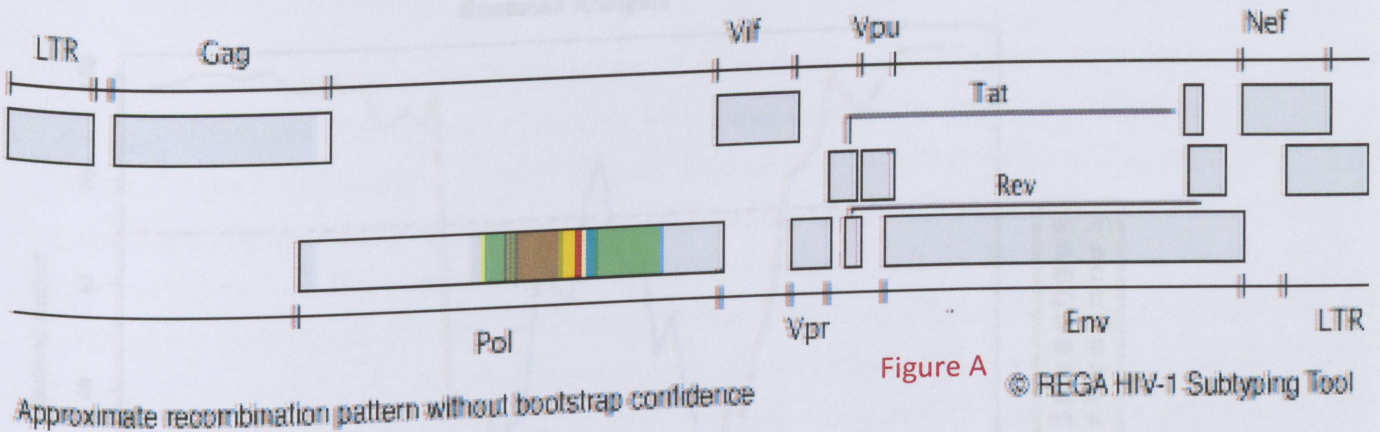
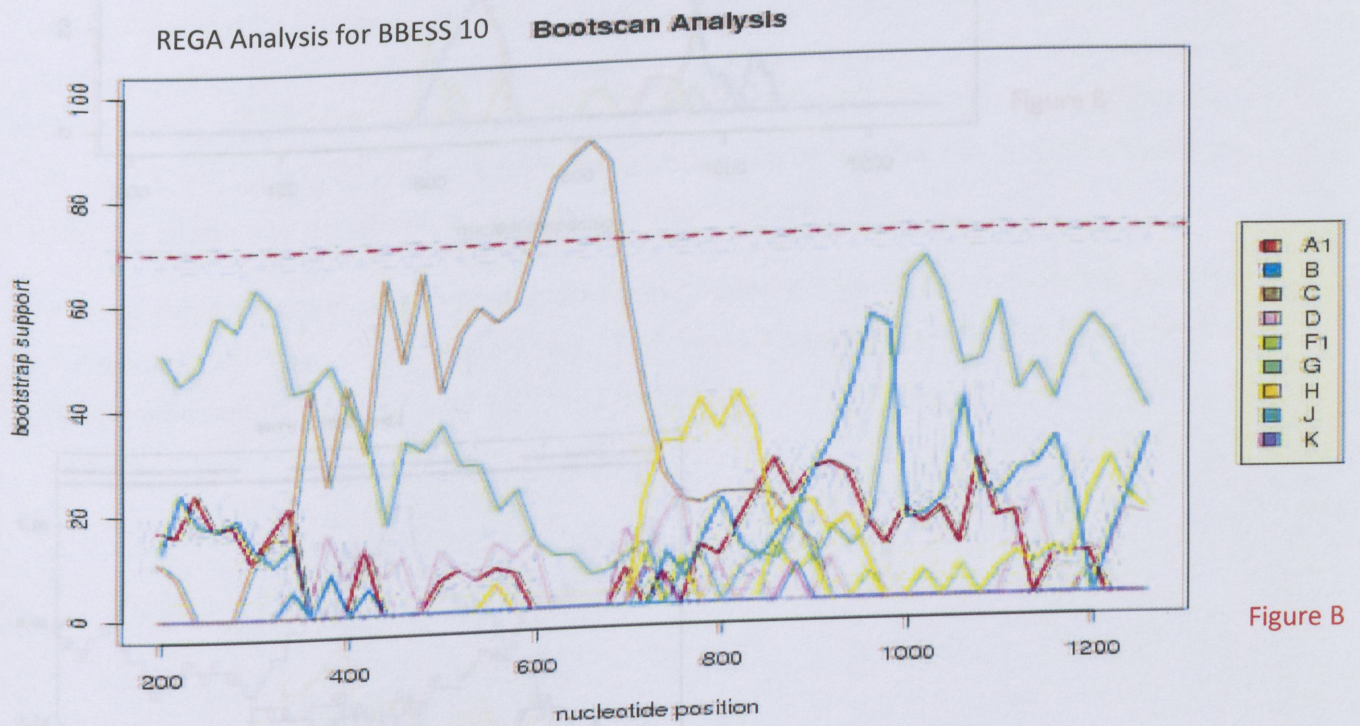
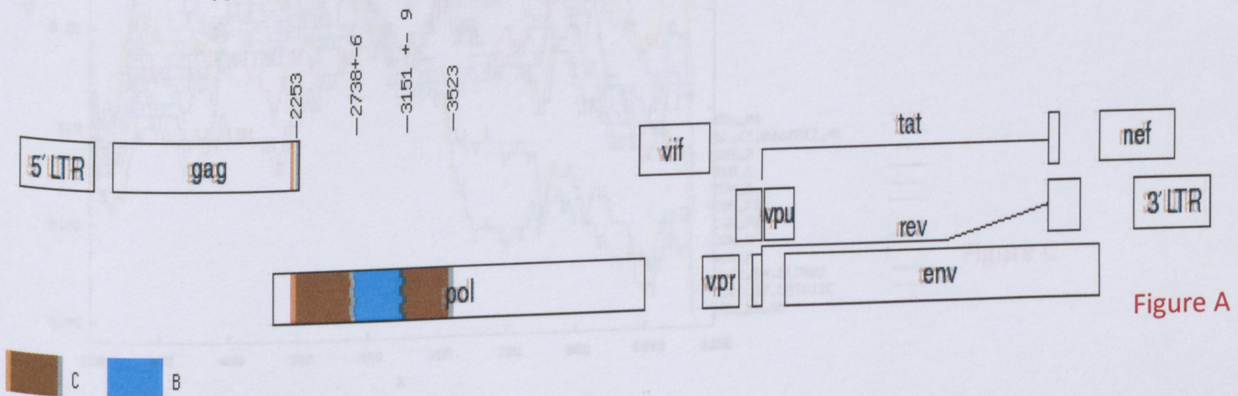
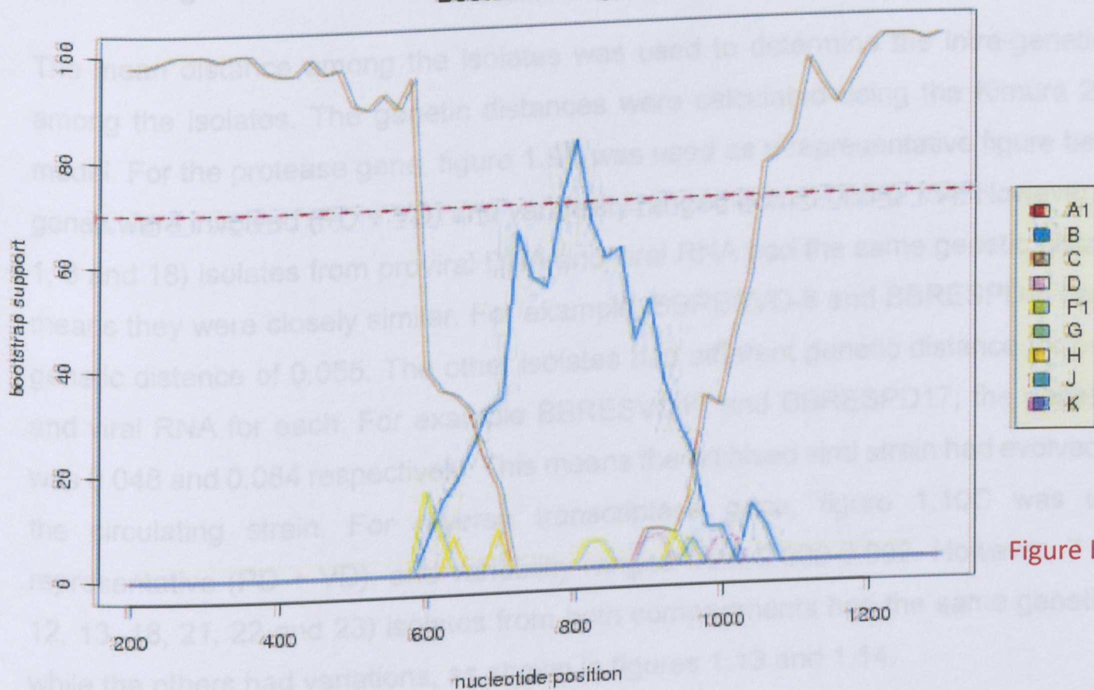
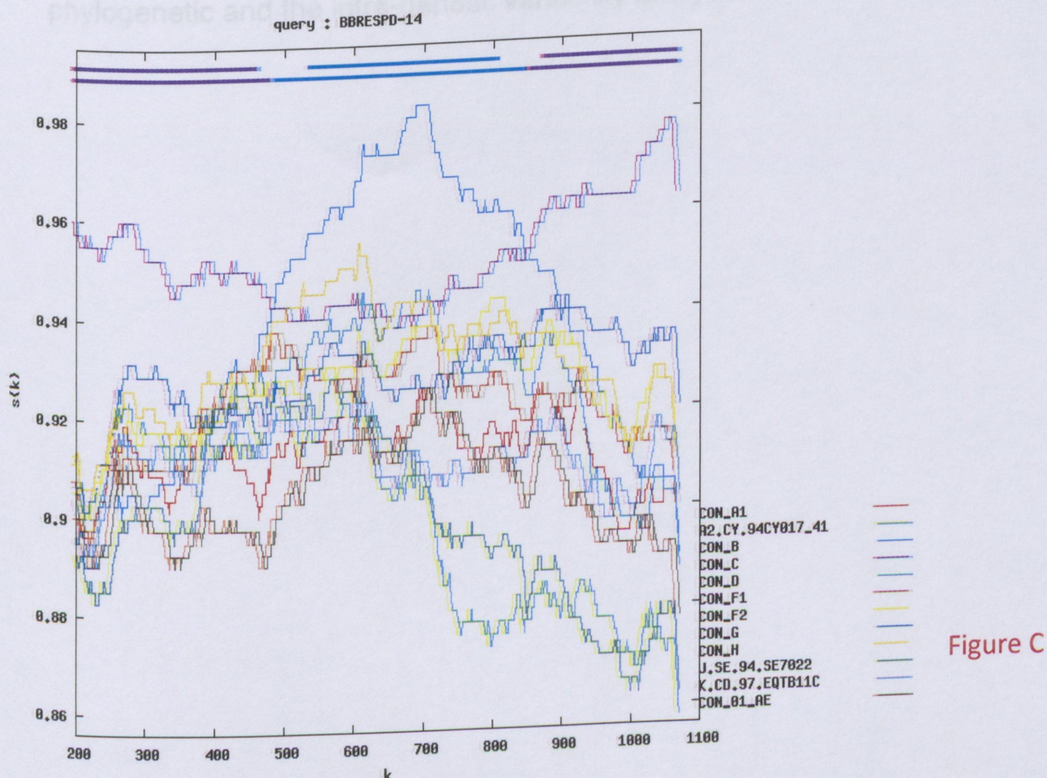




Figures 1.11 A and B: REGA Analysis for BBRES 10







Figures 12 A, B and C represents jpHMM, REGA and RIP analyses, showing B and C recombinant for BBRES 14.

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3.4 Intra-genetic variability among the test sequences

The mean distance among the isolates was used to determine the intra-genetic variability among the isolates. The genetic distances were calculated using the Kimura 2-parameter model. For the protease gene, figure 1.9C was used as a representative figure because both genes were involved (PD + VD) and variability ranged from 0.000-0.134. However, 3 (BBRES 1, 8 and 18) isolates from proviral DNA and viral RNA had the same genetic distance, which means they were closely similar. For example, BBRESVD-8 and BBRESPD-8 had the same genetic distance of 0.055. The other isolates had different genetic distance for proviral DNA and viral RNA for each. For example BBRESVD17 and BBRESPD17, the genetic distance was 0.048 and 0.084 respectively. This means the archived viral strain had evolved more than the circulating strain. For reverse transcriptase gene, figure 1.10C was used as a representative (PD + VD), and variability ranged from 0.000-0.092. However, 7 (BBRES 1, 12, 13, 18, 21, 22 and 23) isolates from both compartments had the same genetic distance, while the others had variations, as shown in figures 1.13 and 1.14.

All these explanations would be best appreciated with reference to the phylogenetic analysis on Figures 1.9C and 1.10C respectively. The same sequences were used for both the phylogenetic and the intra-genetic variability analyses.

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	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
BBRESVD-1																									
BBRESVD-2	0.165																								
BBRESVD-3	0.082	0.152																							
BBRESVD-5	0.076	0.145	0.059																						
BBRESVD-8	0.087	0.158	0.058	0.037																					
BBRESVD-10	0.047	0.184	0.111	0.093	0.129																				
BBRESVD-14	0.075	0.164	0.093	0.042	0.058	0.098																			
BBRESVD-15	0.117	0.170	0.087	0.053	0.037	0.148	0.075																		
BBRESVD-16	0.093	0.152	0.064	0.031	0.015	0.123	0.053	0.021																	
BBRESVD-17	0.088	0.145	0.064	0.026	0.021	0.117	0.047	0.026	0.005																
BBRESVD-18	0.087	0.191	0.070	0.064	0.093	0.116	0.110	0.122	0.099	0.093															
BBRESVD-19	0.087	0.164	0.064	0.058	0.070	0.117	0.081	0.086	0.064	0.058	0.081														
BBRESVD-20	0.081	0.184	0.065	0.058	0.087	0.110	0.105	0.117	0.093	0.087	0.005	0.075													
BBRESVD-23	0.070	0.172	0.087	0.069	0.092	0.110	0.075	0.110	0.086	0.081	0.092	0.086	0.086												
BBRESPD-1	0.000	0.165	0.082	0.076	0.087	0.047	0.075	0.117	0.093	0.088	0.087	0.087	0.081	0.070											
BBRESPD-2	0.076	0.080	0.070	0.064	0.075	0.105	0.081	0.092	0.069	0.064	0.098	0.081	0.093	0.081	0.076										
BBRESPD-3	0.232	0.283	0.197	0.211	0.240	0.261	0.239	0.254	0.232	0.232	0.239	0.246	0.232	0.246	0.232	0.232									
BBRESPD-4	0.167	0.268	0.154	0.153	0.173	0.110	0.185	0.193	0.166	0.166	0.128	0.153	0.122	0.186	0.167	0.179	0.330								
BBRESPD-5	0.000	0.165	0.082	0.076	0.087	0.047	0.075	0.117	0.093	0.088	0.087	0.087	0.081	0.070	0.000	0.076	0.232	0.167							
BBRESPD-8	0.070	0.158	0.064	0.042	0.015	0.110	0.064	0.042	0.021	0.026	0.087	0.075	0.081	0.086	0.070	0.069	0.232	0.166	0.070						
BBRESPD-13	0.082	0.165	0.082	0.031	0.048	0.105	0.021	0.064	0.042	0.037	0.099	0.076	0.093	0.064	0.082	0.081	0.239	0.166	0.082	0.053					
BBRESPD-14	0.081	0.171	0.099	0.037	0.064	0.092	0.021	0.081	0.059	0.053	0.105	0.082	0.099	0.081	0.081	0.087	0.239	0.179	0.081	0.070	0.026				
BBRESPD-16	0.167	0.268	0.154	0.153	0.173	0.110	0.185	0.193	0.166	0.166	0.128	0.153	0.122	0.186	0.167	0.179	0.330	0.000	0.167	0.166	0.166	0.179			
BBRESPD-18	0.087	0.191	0.070	0.064	0.093	0.116	0.110	0.122	0.099	0.093	0.000	0.081	0.005	0.092	0.087	0.098	0.239	0.128	0.087	0.087	0.099	0.105	0.128		
BBRESPD-19	0.087	0.164	0.064	0.058	0.070	0.117	0.081	0.086	0.064	0.058	0.081	0.000	0.075	0.086	0.087	0.081	0.246	0.153	0.087	0.075	0.076	0.082	0.153	0.081	
BBRESPD-20	0.087	0.191	0.070	0.064	0.093	0.116	0.110	0.122	0.099	0.093	0.000	0.081	0.005	0.092	0.087	0.098	0.239	0.128	0.087	0.087	0.099	0.105	0.128	0.000	0.081

Figure 1.13: Intra-genetic variability analysis of protease nucleotide HIV-1 isolates for both proviral DNA and viral RNA. Analysis was done with MEGA 6 making use of the Kimura 2-parameter method. Specimen pairs with genetic distance are shown in bold face. The number on the horizontal axis represents the corresponding specimens on the vertical axis.

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3.5 Drug resistance interpretation

Using Stanford Drug Resistance Interpretation Algorithms (SDRI) and Calibrated Population Resistance tool (CPR), drug and transmitted resistance mutations were detected and confirmed with IAS. These interactive programmes also provided information on the sequence quality in terms of stop codons, frame shift, unusual residues, insertions and deletions. Table 3.3 shows the types and distribution of both major and minor drug resistance mutations in the HIV-1 pol gene amplified from proviral DNA and viral RNA.

Table 3.3: Antiretroviral drug resistance in 21 patients receiving multiple ART at the Bela- Bela Wellness clinic in North East South Africa.

Patient Codes	Antiretroviral drugs received at VL rebound	Duration on ART years	Drug resistance at VL rebound
BBRES 1	D4T, 3TC, EFV	8	NFV, 3TC, DDI, FTC, ABC, EFV, NVP, RPV
BBRES 2	D4T, 3TC, EFV	8	3TC, FTC, AZT, DDI, ABC, TDF, EFV, NVP, RPV
BBRES 3	D4T, 3TC, EFV	5	EFV, NVP, RPV
BBRES 4	D4T, 3TC, NVP	7	Susceptible
BBRES 5	D4T, 3TC, EFV	8	Susceptible
BBRES 8	D4T, 3TC, NVP	6	3TC, FTC, EFV, NVP
BBRES 9	ABC, 3TC, EFV	6	Susceptible
BBRES 10	TDF, 3TC, EFV	5	Susceptible
BBRES 11	AZT, DDI, EFV	5	DDI, ABC, TDF, EFV, NVP
BBRES 12	TDF, 3TC, EFV	3	Susceptible
BBRES 13	AZT, DDI, EFV	5	3TC, FTC, AZT, EFV, NVP, RPV
BBRES 14	ABC, 3TC, EFV	6	3TC, FTC, EFV, NVP
BBRES 15	ABC, 3TC, EFV	3	3TC, FTC, EFV, NVP
BBRES 16	DDI, 3TC, EFV	2	3TC, AZT, D4T, FTC, NVP
BBRES 17	TDF, 3TC, EFV	6	3TC, FTC, EFV, NVP
BBRES 18	ABC, 3TC, EFV	2	3TC, FTC, EFV, NVP
BBRES 19	TDF, 3TC, EFV	6	EFV, NVP
BBRES 20	TDF, 3TC, EFV	10	DDI, TDF, EFV, NVP
BBRES 21	AZT, 3TC, EFV	2	3TC, EFV
BBRES 22	TDF, 3TC, EFV	8	EFV, NVP
BBRES 23	TDF, 3TC, EFV	1	3TC, DDI, FTC, ABC, EFV, NVP

ART, antiretroviral therapy; VL, viral load; 3TC, lamivudine; AZT, zidovudine; EFV, efavirenz; FTC, Emtricitabine; NVP, nevirapine; D4T, stavudine; DDI, didanosine; TDF, tenofovir; RPV, rilpivirine; NFV, nelfinavir; ABC, abacavir.

The mutation patterns associated with drug resistance and viral load detected in proviral DNA and viral RNA revealed that 4 of the 23 patients' specimen with rebound in viral load did not demonstrate any resistant mutations in both compartments table 3.4. The viral load varied from 342-281380copies/ml, with *BBRES 17* having the lowest value and *BBRES 23* having

the highest value. Careful examination of the patients' records by the clinicians revealed that these participants were non-adherent to treatment. Furthermore, the greatest number of mutations was found in specimens amplified from viral RNA.

3.5.1 Drug resistant mutations detected in the PR gene

Of great significance, 4 (17%) of the 23 patients' (BBRES 1, 2, 14 and 19) showed major PI resistance mutations; D30N, M46I, V32I and V82A in viral RNA but not in proviral DNA (Table 3.4). The viral load ranged between 916 for *BBRES 19* to 3090 for *BBRES 2*. Likewise, 4 (17%) minor mutations; A71V, L33I, L33F and I47V were observed in the viral RNA of *BBRES 4, 14 and 19* but not proviral DNA. The viral load for these specimens ranged between 670 *for BBRES 4* to 2800 copies/ml for *BBRES 14*. Common polymorphisms; T74S and L89V were detected for both proviral DNA and viral RNA. Table 3.4 shows types and distribution of drug resistance mutations in HIV-1 pol gene amplified from proviral DNA and viral RNA.

3.5.2 Drug resistant mutations detected in the RT gene

It is noteworthy that, 17 (74%) of the 23 patients' specimens showed major RT resistance mutations in both proviral DNA and viral RNA and of these 17, only 6 (35%); *BBRES 11,14,17,18,19,23* showed the same number and types of mutations in both proviral DNA and viral RNA with viral load between 342 for *BBRES 17* to 281380 copies/ml for *BBRES 23,* while 8 (47%); *BBRES 1,2,4,8,13,16,20 and 21* revealed more resistance mutations in viral RNA compared to proviral DNA, with viral load ranging from 670 for *BBRES 4* to 127219 copies/ml for *BBRES 21* and 3 (17%); *BBRES 3,15 and 22* showed resistant mutations in viral RNA only; with viral load ranging between 25748 for *BBRES 3* to 226674 copies/ml for *BBRES 22*. NRTI and NNRTI mutations detected were; (M41L, A62V, K65R, D67N, K70R, L74I, V75I/S, A98G, L100F/V, K103N, V106M, G109A/E, Q151L, E138Q V179D, M184V, Y181C, Y188L/H T215Y, K219Q, H221Y and H225Y) shown in table 3.4.

It is also worth noting that 3 (13%) isolates; *BBRES 1,2 and 14*; with viral load between 916 for *BBRES 19* to 3090 copies/ml for *BBRES 2,* harboured both major protease and reverse transcriptase drug resistance mutations. PR and RT mutations were; D30N, M46I, V32I and V82A for PR and A62V, K65R, D67N, K103N, V106M and M184V for RT. In addition, 4 isolates; *BBRES 5,9,10 and 12,* with viral load between 859 for *BBRES 12* to 10163 copies/ml for *BBRES 9* did not show any drug resistance mutations. Furthermore, 4 isolates; *BBRES 1, 2, 16, 17* showed hypermutations, with viral load ranging between 342 for *BBRES 17* to 3090 copies/ml for *BBRES 2*, shown in table 3.4.

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3.5.3 Analysis of discordant mutations by class of inhibitors

Examination and analysis of the number of mutations detected in the proviral DNA and viral RNA revealed that 71% (n=15) of the 21 drug resistant patients' specimen had at least 2 mutations associated with NRTIs. As shown in table 3.4, 10 specimens had ≥2 NRTI mutations in proviral DNA and 13 specimens had ≥2 NRTI mutations in viral RNA. Three proviral DNA specimens and 1 specimen from viral RNA did not contain NRTI mutations.

On the other hand, mutations associated with NNRTI were detected in 15 viral RNA specimens, compared to 13 detected in proviral DNA's. Two viral RNA and 3 proviral DNA specimens did not contain NNRTI mutations as, shown in table 3.4.

3.5.4 Analysis of the occurrence of specific drug resistance mutations

Analysis of the occurrence of mutations related to NRTI resistance showed that the mutations repeatedly detected in the viral RNA and proviral DNA compartments in this study population was M184V (76% and 41%); followed by D67N (29% and 18%); K70R (24% and 18%); A62V (18% and 12%); K219Q (18% and 12%); T215Y (18% and 6%); K65R (12% each); L74I (6% each) and V75I/S (6% and 0%) for viral RNA and proviral DNA respectively, as shown on Figure 1.15.

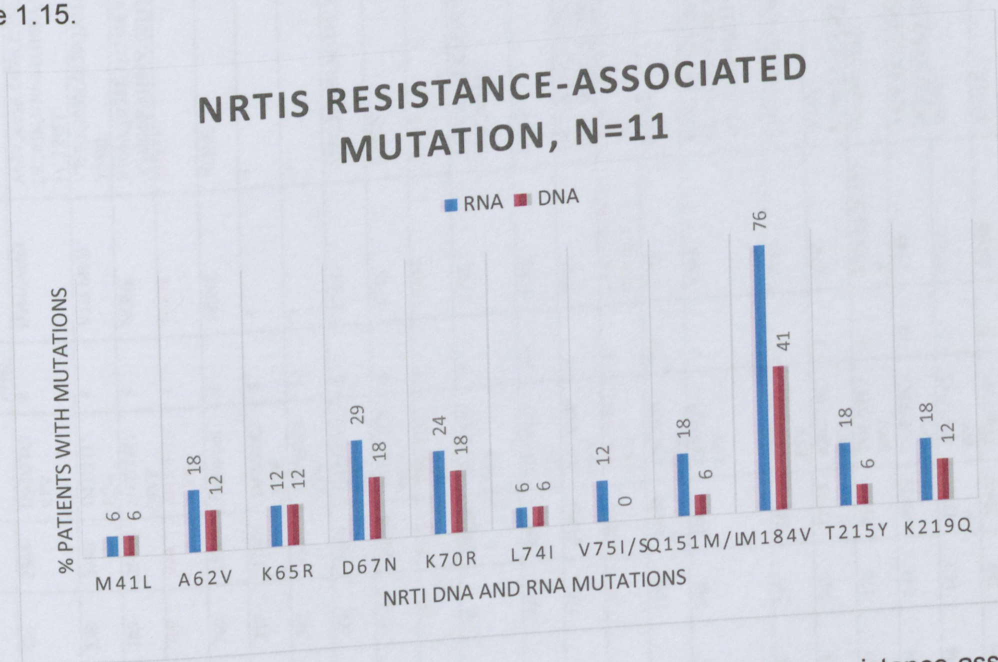

Figure 1.15: Nucleoside reverse transcriptase inhibitor (NRTIs), resistance-associated mutations in circulating HIV-1 RNA and archived HIV-1 DNA genotypes from long term treated individuals experiencing a rebound in viral load from Bela-Bela Wellness clinic. Major codons associated with resistance to NRTIs are indicated on the x-axis. The percentage of patients harbouring mutations in HIV-1 DNA (red) and in HIV-1 RNA (blue) are indicated on the y-axis.

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Patient codes	Gender	Age	CD4	VL	ART Regimen	ART Duration (Yrs)	PR Mutations (RNA)	RT Mutations (RNA)	PR Mutations (DNA)	RT Mutations (DNA)	Hypermutations	Resistance interpretation	Sub-type (RNA) PR/RT	Sub-type (DNA) PR/RT
BBRES 1	F	40	414	2364	D4T/3TC/ EFV	8	D30N,M46I	A62V,K65R,D67N,K70R,V75 I,K103N,V106M,G109A,M18 4V,T215Y	NONE	V106M,M184V	Present	High-level resistance	C/C	C/C
BBRES 2	M	61	338	3090	D4T/3TC/ EFV	8	V32I,M46I	V75S,K103N,E138Q,M184V, Y188L	NONE	K103N,M184V	Present	High-level resistance	C/C	C/C
BBRES 3	F	36	186	25748	D4T/3TC/ NVP	5	NONE	V106A,E138K,Q151M,V179D ,Y188H/E,M184V,T215Y	NONE	NONE	Absent	High-level resistance	C/C	C/C
BBRES 4	F	37	209	670	D4T/3TC/ NVP	4	NONE	M184V	NONE	M184V,L100F	Absent	High-level resistance	B/B	B/B
BBRES 5	NA	36	209	1830	Unknown	8	NONE	NONE	NONE	NONE	Absent	No Resistance	C/C	C/C
BBRES 6	M	39	387	11661	D4T/3TC/ NVP	8	*	*	*	*	*	*	*	*
BBRES 7	F	42	562	652	TDF/3TC/ EFV	11	*	*	*	*	*	*	*	*
BBRES 8	F	39	600	1200	D4T/3TC/ EFV	6	T74S	A98G,K103N,M184V,P225H	T74S	A98G,K103N,M184V	Absent	High-level resistance	C/C	C/C
BBRES 9	M	10	783	10163	ABC/3TC/ EFV	6	T74S	K103N	T74S	NONE	Absent	Resistance	C/C	C/C
BBRES 10	F	33	788	1773	TDF/3TC/ EFV	5	NONE	NONE	NONE	NONE	Absent	No Resistance	B/G	B/G
BBRES 11	F	38	42	27424 1	AZT/DDI/ EFV	5	T74S	A62V,K65R,K103N,V106M,Y 181C,M184V	NONE	A62V, K65R,K103N,V106M Y181C	Absent	High-level resistance	C/C	C/C
BBRES 12	F	49	701	859	TDF/3TC/ EFV	NA	T74S	M184V	T74S	NONE	Absent	Resistance	C/C	C/C
BBRES 13	M	43	716	61354	AZT/DDI/ EFV	5	T74S	D67N,K70R,K101H,K103N,G 109A,M184V,K219Q	T74S	D67N,K70R,K101H,K 103N	Absent	High-level resistance	C/C	C/C
BBRES 14	M	11	720	2800	ABC/3TC/ EFV	6	L33I/F,T74S,V 82A184V	D67N,K70R,K103NQ151L,M 184V	T74S	D67N,K70R,K103NQ1 51L,M184V	Absent	High-level resistance	B/C	B/C
BBRES 15	M	5	1196	10408 7	ABC/3TC/ EFV	NA	T74S	M184V	NONE	NONE	Absent	High-level resistance	C/C	C/C
BBRES 16	M	14	540	1000	DDI/3TC/ EFV	2	T74S	M41L,D67N,K70RA98G,V17 9D,Y181C,Y188L,M184V,T2 15Y, K219Q,H221Y	T74S	M41L,D67N,K70R,A9 8G,V179D,Y181C,Y18 8L,T215Y,K219Q,H22 1Y,M230I	Present	High-level resistance	C/C	C/C
BBRES 17	M	56	247	**342**	TDF/3TC/ EFV	6	T74S	L100V,G109A,K103N,M184V	NONE	L100V,G109A,K103N, M184V	Present	High-level resistance	C/C	C/C
BBRES 18	F	13	245	27503 3	ABC/3TC/ EFV	2	T74S	K103N, M184V,K219QP225H	NONE	K103N,K219Q,K103N, P225H	Absent	High-level resistance	C/C	C/C
BBRES 19	F	40	170	916	TDF/3TC/ EFV	6	V32I,L33F,I47 V	V106M,Q151L	T74S	V106M	Absent	High-level resistance	C/C	C/C
BBRES 20	M	44	244	48423	TDF/3TC/ EFV	10	T74S	A62V,K65R,A98G,L100F,V1 06M,G109A,Y188L,H221Y	NONE	A62V,K65R,L100F,V1 06M	Absent	High-level resistance	C/C	C/C
BBRES 21	F	36	14 2	12721 9	AZT/3TC/ EFV	2	T74S	M184V	T74S,L33F	A98G,G109A,Y188L, M184V,H221Y	Absent	High-level resistance	C/C	C/C
BBRES 22	F	29	360	22667 4	TDF/3TC/ EFV	8	NONE	K103N	NONE	NONE	Absent	High-level resistance	C/C	C/C
BBRES 23	M	49	6	**28138 0**	ABC/3TC/ EFV	1	NONE	L74I,K103N,V106M,M184V	NONE	L74I,K103N,V106M, M184V	Absent	High-level resistance	C/C	C/C

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Table 3.4: Comparison and distribution of drug resistance mutations in HIV-1 pol gene amplified from proviral DNA and viral RNA from drug experienced individuals in Bela-Bela, Limpopo Province, South Africa. F = Female, M = male,* = specimens which could not be amplified, [legend swatch] = specimen isolates with hypermutations [legend swatch] = specimen isolates with discordance in subtypes, VL = viral load (cell/ml), CD4 = cell differentiation counts (cells/mm³), ART = antiretroviral treatment, PR = protease, RT = reverse transcriptase, yrs. = years, DNA = deoxyribose nucleic acid and RNA = ribose nucleic acid. M230I was found only in an achieved viral isolate and not in the circulating viral isolate (BBRES 16).

Blood specimens were collected at viral load rebound. Also, drug resistant mutations were detected using Stanford drug resistance tool and the Calibrated population resistant tool. All mutations were confirmed using the International AIDS Society Programme.

On the other hand, mutations associated with NNRTI resistance in DNA and RNA, showed higher incidence for K103N (59% and 47%); followed by V106M (29% each); G109A and Y188H/L (24% each); A98G and L100F (17% each); Y188L/H/E (24% and 12%); H221Y (12% each); P225H, V179D and Y181C (12% each) and E138Q (12% and 0%) and P236L (6% and 0%) for RNA and DNA respectively. M230I was observed in proviral DNA (6% and 0%), as illustrated in Figure 1.16.

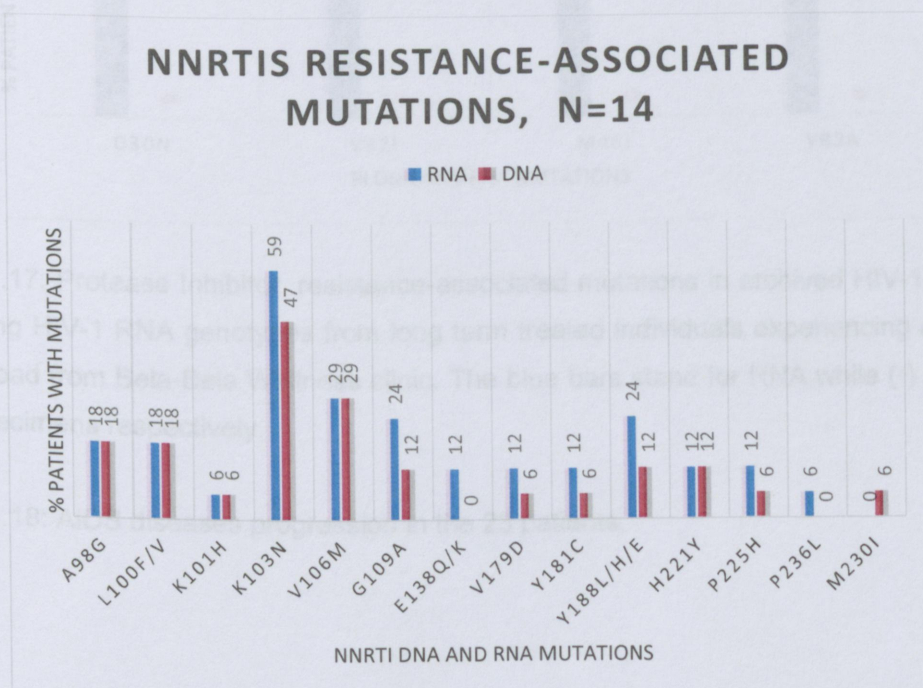

Figure 1.16: Non-nucleoside reverse transcriptase inhibitor, resistance-associated mutations in archived HIV-1 DNA and circulating HIV-1 RNA genotypes from long term treated individuals experiencing a rebound in viral load from Bela-Bela Wellness clinic. Major codons

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associated with resistance to NRTIs are indicated on the x-axis. The percentage of patients harbouring mutations in HIV-1 DNA (red) and in HIV-1 RNA (blue) are indicated on the y-axis.

Analysis and interpretation of PI mutations showed that T74S and L33F/I, which are polymorphisms, occurred most frequently in the study population. Specifically, T74S was observed in 70% of both viral RNA and proviral DNA specimens. The frequency of major protease associated mutations in DNA and RNA were; D30N (6% and 0%); M46I (12% and 0%); V32I (12% and 0%) and V82A (6% and 0%) respectively (Figure 1.17).

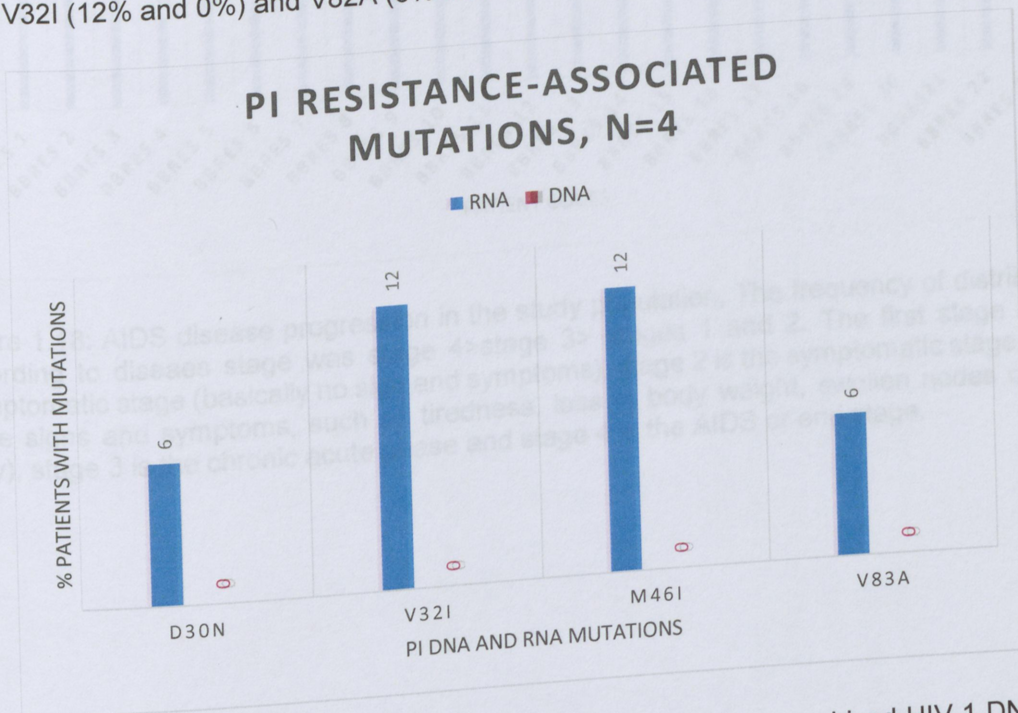

Figure 1.17: Protease Inhibitor, resistance-associated mutations in archived HIV-1 DNA and circulating HIV-1 RNA genotypes from long term treated individuals experiencing a rebound in viral load from Bela-Bela Wellness clinic. The blue bars stand for RNA while (◦) stands for DNA specimens respectively.

Figure 1.18: AIDS diseases progression in the 23 patients.

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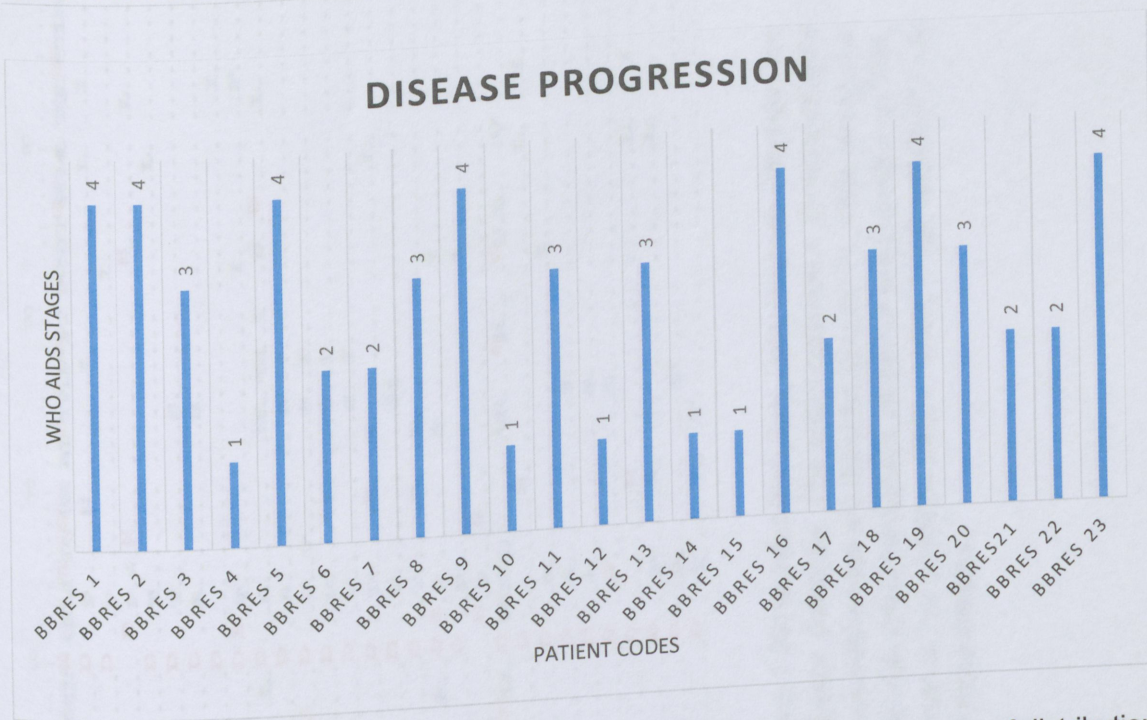

Figure 1.18: AIDS disease progression in the study population. The frequency of distribution according to diseaes stage was stage 4>stage 3> stages 1 and 2. The first stage is the asynptomatic stage (basically no sign and symptoms), stage 2 is the symptomatic stage (with some signs and symptoms, such as tiredness, loss in body weight, swollen nodes on the body), stage 3 is the chronic acute phase and stage 4 is the AIDS or end stage.

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Figure 1.19: Amino acid alignment of the protease gene of HIV-1 subtype B and C (codons 1-99) isolates from Bela-Bela, Limpopo Province. The alignment was generated for 21 isolates. The global subtypes B and C from HIV sequence database. The entire consensus sequences were aligned using BioEdit. All positions that agree with the global subtype B and C consensus sequences are denoted by a dot (.), while ambiguous amino acid are denoted by an asterisk (*). The test consensus was identical to the global subtype C consensus, except at seven positions (T19L, R20K, D35E, E37N, E60D, L63P and T74S). It differs from the global subtype B consensus at four positions (M36V, V69R, I77M, and I93F). Note that the active site between positions 25-27 (Asp, Thr and Gly) is conserved except for three isolates.

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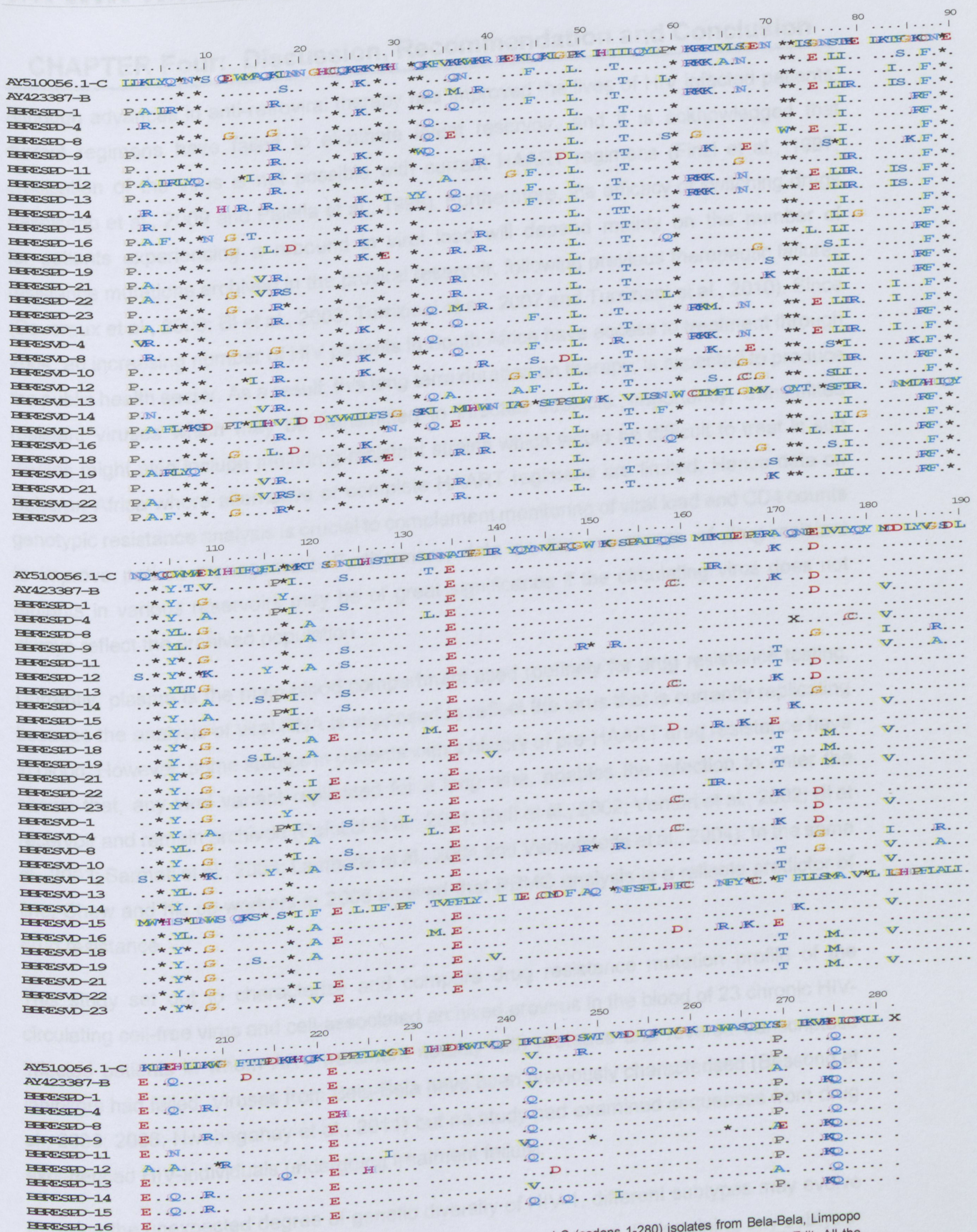

Figure 1.20 : Amino acid alignment of reverse transcriptase gene of HIV-1 subtype B and C (codons 1-280) isolates from Bela-Bela, Limpopo province. The alignment was generated for 30 isolates DNA and RNAQ. The entire consensus sequences was aligned using BioEdit. All the positions that agree with the consensus are denoted (.), while ambiguous positions are denoted *.

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CHAPTER Four: Discussion, Recommendation and Conclusion

Although advances in anti-retroviral therapy has improved the lives of HIV infected patients, current regimens have failed to eliminate latent reservoir, and it is acknowledged that eradication of the virus is not possible with current HAART regimens (Finzi et al., 1997; Palmisano et al., 2009 and Palella et al., 1998). Furthermore, the efficacy of switching drugs for patients experiencing a rebound in viral load will depend mainly on the number of resistance mutations archived in the proviral reservoir, following previous therapeutic failures (Devereux et al., 2000; Bl et al., 2003; Turriziani et al., 2007 and Turriziani et al., 2010). Since 2004, an increasing number of HIV patients in South Africa have access to treatment through the public health sector. As a result, this long term duration on therapy, is expected to produce resistant viruses which may be transmitted to infected subjects. Importantly, transmitted viruses might also include multidrug-resistant strains which would be difficult to treat in sub Saharan Africa where alternative or complete HAART regimens are limited. Hence data on genotypic resistance analysis is crucial to complement monitoring of viral load and CD4 counts to improve patient management. Furthermore, data on the occurrence of drug-resistant mutants in various reservoirs may be of great significance if the circulating virus does not always reflect the archived population.

Currently, plasma is the main blood compartment used routinely for drug resistance testing, because the analysis of viral RNA is supposed to reflect the virus that is currently replicating in blood. However, some studies in patients with a history of pre-HAART drug resistance have shown that, any viral variant replicated for a long time, enables the infection to enter the reservoir and remain archived (Palucci et al., 2001; Ruff et al., 2002; Venturi et al., 2002; Bi et al., 2003; Sarmati et al., 2003; Lambotte et al., 2004 and Verhofstede et al., 2004). In the same line, Chew and his co-workers in 2005 showed that PBMC analysis is a reliable predictor of drug resistance.

This study set out to characterize and compare drug resistance mutation profile of the circulating cell-free virus and cell-associated archived provirus in the blood of 23 chronic HIV-infected patients in whom ARV treatment notably with protease and reverse transcriptase inhibitors had failed. Viruses from Bela-Bela have been previously characterised (Bessong et al., 2005; 2006; Nwobegahay et al., 2011) but no study had examined sequences from drug experienced HIV-individuals undergoing treatment failure.

Knowing the unexpected degree of genetic diversity of HIV-1, different subtypes may evolve in different ways when under host immune and selective pressure. Determining any subtype-specific difference in the pattern of resistance mutation would clearly be of great significance for treatment management and also for a better understanding for resistance mechanism.

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Phylogenetic analysis of the protease and reverse transcriptase nucleotide sequences showed that, 18 (85.6%) viruses examined were HIV-1 subtype C as they clustered with the reference subtype C sequences from South Africa and abroad. This finding is in agreement with previous genetic characterization of HIV from other parts of South Africa which have shown that HIV-1 subtype C is the predominant variant in South Africa (Bessong et al., 2005; Gordon et al., 2003; Papathanasopoulos et al., 2003). Further, one virus was subtype B; 1(4.8%) was a C/B recombinant and 1 (4.8%) virus could not be conclusively typed. Since these CRFs have not been reported previously in the region, and only the partial pol gene were analysed, a full-length genomic analysis of the virus would be required to reveal its complete mosaic nature.

The genetic variability of the protease and reverse transcriptase and a combination of both genes of the test isolates showed differences among the isolates (Figures 1.13 and 1.14). It was noted that 3 pairs of specimens *BBRESVD-1* and *BBRESPD-1, BBRESVD-8* and *BBRESPD-8* and *BBRESVD-18; BBRESPD-18* did not show variability among their protease sequences.

DNA genotyping detected significantly fewer resistance mutations in the RT gene and none in the PR gene as compared to RNA genotyping which showed mutations both in the PR and RT genes. Actually, mutations conferring resistance to at least one antiretroviral drug were detected exclusively by RNA genotyping or exclusively by DNA genotyping in (56%) and (35%) of patients for NRTIs, (48%) and (30%) of patients for NNRTIs and (23%) and (0%) of patients for PIs respectively. Of great interest, about 10% of the population carried more drug resistance in DNA than in RNA and one NNRTI mutation M230I was detected only in DNA, which suggested that analysis of plasma virus might not be sufficient to understand the drug-resistant evolutionary status of patients experiencing virologic failure. In addition, these additional mutations found in the cellular compartment probably arose from previous therapy. These patients had at one point, been treated with drugs able to select for the mutations found in DNA.

It is critical to point out that, this analysis is based on the use of a routine assay, such as population-based sequencing, which does not allow the detection of minor viral variants. Cloning or single genome sequencing would probably have detected more archived mutations (Paolucci et al., 2001; Verhofstede et al., 2004 and Palmer et al., 2005), as would sequenced DNA from selected latent T cells, the main cellular HIV reservoir (Ruff et al., 2002). Furthermore, several reports have indicated that, undetected drug resistant minority variants are known to persist when antiretroviral therapy is discontinued or changed (Delaugerre et al., 2001; Devereux et al., 2000; Hance et al., 2001 and Izopet et al., 2002).

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In the population studied, many NRTI, NNRTI and PI mutations were found in RNA than DNA, which reflects the therapeutic history of the patients. It is worth noting that these patients had received treatment for at least 5 years and above. This difference in resistant mutations in both compartments, suggests that plasma viruses at the time of regimen failure are enriched for resistant viruses, leading to a better capture of the resistant genotypes than in the mononuclear cells which may have a huge library of archived variants, the majority of which may be missing resistance mutations. An alternative suggestion is that, in patients on effective HAART, cells infected by archived resistant provirus could be diluted by more recent uninfected cells and hence less readily detected.

NRTIs and NNRTIs constitute the first line of therapy in sub-Saharan Africa (Laurent et al., 2005 and Tee et al., 2006). The combination TDF/d4T+3TC+EFV is the recommended first-line therapy in South Africa. Upon analysis of the PR and RT genes for resistance mutations, 11 (64%) mutations were detected in NRTIs, 13 (76%) mutations for NNRTIs and 4 (23%) for PIs. The most common mutations detected in increasing number of frequency were: M184V>K103N>V106M>D67N for RT and M46I>D30N>V82A>for PR, corresponding to the regimen administered.

Analysis of PI mutations showed that T74S and L33F/I occurred most frequently in our study population. T74S is associated with reduced NFV susceptibility. Most specifically, T74S was observed in 70% of both viral RNA and proviral DNA specimens. Further analysis of the major PR mutations such as D30N, M46I and V82A, confirmed these mutations in viral RNA only and non in proviral DNA. It is worth noting that these major mutations were detected in 4 individuals even though the PIs were not part of their current regimen at the time of resistance testing.

The high frequency of M184V reveals the extensive use of 3TC. Specimens analysed in this project came from patients who were 3TC experienced. The selection of thymidine analogue mutations such as D67N, M41L and T215Y was attributed to treatment with AZT and D4T. Of note, is the 3 viral RNA and 2 proviral DNA specimens containing a K65R mutation which arose from patients who had never been treated with regimens like DDI, ABC, or TFV, which are known to be the main cause of this mutation. The high occurrence of K103N and V106M reflects the wide use of EFV and NVP.

Overall these results clearly show that, patients receiving extensive antiretroviral regimen but experiencing therapeutic failure, harbour diverse drug-resistant variants which could be archived as proviral DNA found in the cellular compartment of blood. This viral population (proviral DNA) does not necessarily resemble the population found in the plasma compartment (viral RNA). This may suggest, ultimately that, proviral DNA may constitute a reservoir for

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drug-resistant variants and might replenish plasma with drug resistant HIV variants in certain circumstances. Also with the framework of this study, we had the prospect to address and gain new understanding into the issue of archived and resistant variant from HAART experienced individuals in Bela-Bela. Of great ncte, M230I as mentioned earlier, was observed in the DNA. This mutation was selected after in vitro exposure of HIV to rilpivirine (RPV) a novel NNRTI. Furthermore drug resistant variants might also display a different ability to persist in the reservoir according to the mutation involved (Martinez et al 1999). This might explain why mutation M230I was found only in DNA. Moreover, the apolipoprotein B mRNA-editing, enzyme-catalytic (APOBEC)-induced resistance mutation mechanism could explain the mutation in archived proviral DNA. APOBEC is a cellular antiviral factor that is responsible for numerous guanosine (G) to adenosine (A) changes in the HIV provirus (Mulder, 2008).

Future studies will fully reveal the appearance of the complete structure of the viruses in BBRES 10 and 14. Secondly a larger sample size will reveal clearly the scope and types of drug resistance mutations and finally because population based sequencing was used for looking at the mutations, further studies using deep sequencing or single genome sequencing will be applied to detect the minority population of drug resistance.

In conclusion, this study suggests that PBMCs (the cellular compartment of blood) may act as an archive of drug resistant variants, thus making an interesting reservoir for studying the resistant potential and evolution of resistant viruses in a given patient. Furthermore, owing to the fact that, mutations may revert over time in the absence of drug pressure, this might also lead to transmitted resistant viruses. Thirdly, the prevalence of drug resistance mutations observed in a small cohort of drug experienced individuals in Bela-Bela is very high. Finally, there appears to be a fairly high prevalence of non HIV-1 subtype C viruses in the study region.

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