Drug Resistance Mutations in Naïve HIV-1 South African Patients, and Construction of Molecular Clones to Phenotype Putative Resistance Mutations

A dissertation in fulfillment for the award of Master of Science degree in Microbiology submitted

By

Mavhandu Lufuno Grace

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School of Mathematics and Natural Sciences
University of Venda, South Africa

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Supervisor: Dr Pascal Obong Bessong
Co-supervisors: Dr David Rekosh
Dr Marie-Louise Hammarskjold

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Declaration

I, Lufuno Grace Mavhandu, hereby declare that the dissertation for the Master of Science degree at the University of Venda, hereby submitted by me, has not been submitted previously for a degree at this or any other university, that it is my own work in design and in execution, and that all reference material contained therein has been duly acknowledged.

Signature ________________ Date 15 March/09

Dedication

I dedicate this work to my heavenly father God who lightens up my way of life. He protected me and gave me strength to do all this work with his almighty power. His blessings made me a fruitful person.

To my dearest mother Janet Sekgokolo Mavhandu, for her unconditional love, support and prayers throughout this work. To all my sisters, Vivian, Vhony, Petty, and my brother Ignatius.

Scientific communications from this work

The investigations described in this dissertation have given rise to the following manuscripts:

1. **Lufuno Mavhandu**, Pascal Bessong, Marie-Louis Hammarskjold, David Rekosh. Design and construction of retroviral vector systems for phenotypic evaluation of putative drug resistant mutations (Submitted to Journal of Virological Methods).

2. **Lufuno Mavhandu**, Marie-Louis Hammarskjöld, David Rekosh, Pascal Bessong. Genetic diversity and drug resistance among chronic HIV infected individuals from Northeastern South Africa (In preparation for Journal of Medical Virology).

Summary

In countries such as South Africa where access to therapy is progressing data is required on patterns of resistance and evolution of resistance. Thirty protease (PR) and 31 reverse transcriptase (RT) amino acid sequences of HIV primary isolates from drug naïve patients from rural settings in South Africa were examined for resistance mutations. Samples were collected between May and August 2007. Phylogenetic analysis showed that all the sequences were HIV-1 subtype C in both the protease and reverse transcriptase genes. The mean genetic distances among the sequences were 0.0170-0.0786 for the protease, and 0.0045-0.0890 for the reverse transcriptase. However, it was noted that 3 pairs of samples 07VGNF5ZA and 07VGNF6ZA, 07VGNF7ZA and 07VGNF8ZA, 07VGNF10ZA and 07VGNF13ZA did not show any genetic variability among their protease sequences. No major resistance mutation was observed among the protease sequences. However, the following minor resistance mutations were noted: L10I/V (3/30), A71T (1/30), and T74S (2/30). Examination of the reverse transcriptase gene for resistance mutations reveal the presence of V118I (1/30), V179D (1/30), K103N (2/30). Most of the RT sequences were wild-type, although V118I (3.3%) and k103N (6.7%) associated with resistance to lamivudine and nevirapine, respectively, were observed. In summary, this study has shown that most of the viruses in Limpopo Province, representing the northeastern part of South Africa are HIV-1 subtype C, and that the prevalence of resistant mutations among the drug naïve patients is still low.

Although combination antiretroviral therapy has resulted in a considerable improvement in the treatment of human immunodeficiency virus type 1 (HIV-1) infection, the emergence of resistant virus is a significant obstacle to the effective management of HIV infection and AIDS. Systems to be used in the testing of phenotypic drug resistance and susceptibility are being developed. These may intimately be used in guiding therapy to improve long term suppression of HIV replication. Two proviral chimeric clones making use of pMJ4 and pNL4-3, and two vector plasmids which deletions of sequences encoding HIV-1 protease or reverse transcriptase were constructed for cloning of HIV-1 PCR products. Growth of constructs was monitored by p24 antigen production. Susceptibility to protease and reverse transcriptase inhibitors was measured by using resistance test vectors that contain a Luciferase indicator gene. Cells were co-transfected with packaging plasmids, pLuc, and pEnv, resulting in the production of virus particles that were used to infect target cells. Luciferase activity was measured following a single round of replication. The chimeric constructs MJ4 carrying the NL4-3 ApaI-HpaI cassette (MJ4/NL4-3) and NL4-3 carrying the MJ4 ApaI-HpaI cassette (NL4-3/MJ4) were successfully developed as shown by restriction digestion analysis. Considering growth of the constructed chimeras NL4-3/MJ4 was better than MJ4/NL4-3 although not robustly. Good p24 production was obtained from all four *gap-pol* plasmids. MJ4/NL4-3 worked better in delivering luciferase to the target cells while NL4-3/ML4 appeared totally devoid of any infectivity. The vectors pCMVGagPol(MJ4)-RREr and pCMVGagPol(NL4.3)-RREr were created and both expressed the viral *gag-pol* protein. Viral inhibition test showed that the vectors can be inhibited by NRTI, NNRTI and PI. Inhibition was seen in all drugs in different concentrations indicating that the system works. The results showed that vector systems constructed can be used to evaluate putative drug resistant mutations, coding for resistance to protease and reverse transcriptase inhibitors, detected in patient viruses. In addition, the system can also be used to evaluate candidate drugs and assist in the development of new drugs that are active against resistant HIV-1 virus.

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List of Abbreviations

HIV	Human Immunodeficiency Virus
UNAIDS	United Nations Joint Programme on AIDS
DoH	Department of Health South Africa
AIDS	Acquired Immune Deficiency Syndrome
RNA	Ribonucleic acid
DNA	Deoxyribonucleic acid
SIV	Simian Immunodeficiency Virus
EIAV	Equine Infectious Anemia Virus
FIV	Feline Immunodeficiency Virus
BIV	Bovine Immunodeficiency Virus
Gp41	glycoprotein 41
Gp120	Glycoprotein 120
p24	Protein
PR	Protease
Pol	Polymerase
Gag	Group Associated Antigen
Env	Envelope
RT	Reverse Transcriptase
RRE	Rev Responsive Element
TAT	Trans-Activator of Transcription
REV	Regulator of Virion protein expression

NEF	Negative Regulatory Factor
VIF	Virion Infectivity Factor
VPU	Viral Protein U
VPR	Viral Protein R
CD4+	Cluster of Differentiation 4
CCR5	C-Chemokine Receptor 5
CXCR4	CX Chemokine Receptor 4
MA	Matrix
CA	Capsid
NC	Nucleocapsid
pr55	Proteins 55
pr160	Protein 160
HAART	Highly Active Antiretroviral Therapy
NRTIs	Nucleoside Analogue Reverse transcriptase Inhibitors
NNRTIs	nonnucleosideanaloguereversetranscriptase inhibitors.
PI	Protease Inhibitor
RTI	Reverse Transcriptase Inhibitor
RT-PCR	Real Time-Polymerase Chain Reaction
ARV	Antiretroviral
IC_{50}	Inhibition Concentration
LTR	Long Terminal Repeat
SV40	Simian virus 40
MMLV	Moloney Murine Leukemia Virus

FI	Fusion inhibitors
EDTA	Ethylene Diamine Tetraacetic Acid
cDNA	Complement
USA	Unites States of America
LB	Luria Bertani
HCl	Hydrochloric Acid
UV	UltraViolet
OD	Optical Density
STBL2	Permissible *E. coli* cells
°C	Degree Celsius
IMDM	Modified Dulbecco's Medium
RPMI	Roswell Park Memorial Institute
CaPo4	Calcium Phosphate
ELISA	Enzyme Linked Immunosorbent Assay
CIP	Calf Intestinal
CMV	Cytomegalovirus
BCS	Bovine Calf Serum
Rpm	Revolution per minute
SRLV	Single-Round Luciferase Virus
AZT	Zidovudine
NVP	Nevarapine
SQV	Saquinavir

Chapter 1:
Introduction and General Review of Literature

1.1 Introduction

Infection with the human immunodeficiency virus (HIV) is a world wide public health concern. The global spread of HIV has been rapid and several countries are dealing with huge health burdens due to HIV. United Nations Joint Programme on AIDS (UNAIDS, 2007) estimated that 33.2 million people were living with HIV, 2.5 were newly infected.

As of January 2007, UNAIDS (2007) estimated that AIDS has killed more than 2.1 million people, making it one of the most destructive pandemic in recorded history. A third of global deaths are occurring in Sub-Saharan Africa, retarding economic growth by destroying human capital (Greener, 2002). Current estimate states that HIV is set to infect 90 million people in Africa resulting in a minimum estimate of 18 million orphans (UNAIDS, 2005). Antiretroviral treatment reduces mortality and morbidity of HIV infection. However routine access to antiretroviral medications is not available in all countries (Palella *et al.*, 1998).

Sub-Saharan Africa remains by far the worst-affected region, with an estimated 22.5 million people infected with HIV at the end of 2007. Prevalence varies between regions. Southern Africa is the hardest hit region, with adult's prevalence rates exceeding 15% in most countries and even 30% in Swaziland and Botswana. Eastern Africa also experience relatively high levels of prevalence with estimates above 10% in some countries, although there are signs that the pandemic is declining in this region, notably in Uganda, Zimbabwe, and Botswana. West Africa on the other hand has been much less affected by the pandemic. Several countries in West Africa reportedly have prevalence rates around 2-3% and no country has yet reached rates above 10%. However, in two of the regions most populous countries, Nigeria and Côte d' Ivoire, between 5 and 7% of their populations respectively are reported to carry the virus (UNAIDS, 2007).

South Africa is the country with the largest number of HIV infections in the world. HIV prevalence data collected from the latest round of antenatal clinic surveillance suggest that HIV infection levels might be leveling off, with prevalence among pregnant women at 30% in 2005 and 29% in 2006 (Department of Health South Africa, 2007). In addition, the decrease in HIV prevalence among young pregnant women (15–24 years) suggests a possible decline in the annual number of new infections. The epidemic varies considerably between provinces, from 15% in the Western Cape and Limpopo 19.3 to 39% in the province of KwaZulu-Natal. (Department of Health South Africa, 2007).

HIV is the etiological agent of acquired immune deficiency syndrome (AIDS), a condition in humans in which the immune system becomes dysfunctional leading to life-threatening opportunistic infections. It ranks as one of the most notorious infectious disease facing human kind, and is thought to have originated in Sub-Saharan Africa during the early twentieth centaury (Gallo *et al.*, 1984).

One characteristic feature of HIV is the high degree of variability of its genome among independent virus isolates. This feature could have impact on many aspects of the biology of HIV, including tissue and target cell specificity, clinical spectrum and pathogenesis, geographic and temporary distribution of virus and prospects of developing an effective-cross reactive vaccine (Wong-stall, 1991; Galloway, 2000). Secondly, infection with HIV sooner or later, creates a window of infections with other microbes which are tuberculosis, pneumonia, and other enteropathogens, notably diarrhea (Gilks, 1997, Harris *et al.*, 2003). And thirdly, there has been a fervent and continuous search for preventive and curative therapeutics in an effort to stem the spread of the pandemic (Pomerantz and Horn, 2003).

1.2 Taxonomy of HIV

Human immunodeficiency virus belongs to the retrovirus family of viruses. This family is distinguishable by the possession of a viral reverse transcriptase which transcribes viral RNA into provirus DNA that is integrated into the host cell genome. The retroviruses are further grouped into three subfamilies; Oncoretroviruses, Spumaviruses and the lentiviruses (Hahn *et al.*, 2000). Lentiviruses have the non-transforming and cytopathic activity, and a relatively long incubation period (Mayo & Pringle, 1998).

The members of the lentivirus family include human immunodeficiency virus (HIV) which infects macrophages and T-lymphocytes. Simian immunodeficiency virus (SIV) which infects primates. SIV infection of 20 different primate species have thus far been identified and confirmed by molecular analyses, and six additional species have been found to harbor SIV-specific antibodies (Hirsch *et al.*, 1999). Other lentiviruses include equine infectious anemia virus (EIAV), which infect macrophages of horses, causing haemolytic anemia, Visna virus which infects sheep's macrophages causing ancelophalopathy, in addition, feline immunodeficiency virus (FIV) which infects the T-lymphocytes of cats causing immunodeficiency, and bovine immunodeficiency virus infects macrophages of the cattle causing lymphadenopathy (Mayo and Pringles, 1998; Hahn *et al.*, 2000). (See Table 1 below).

Table 1: Members of the lentivirus family, hosts, target cells and clinical outcomes (Mayo and Pringle, 1998; Hirsch *et al*., 1999; Hahn *et al*., 2000).

Virus	Host	Primary cell type infected	Clinical disorder
Equine infectious anemia virus (EIAV)	Horse	Macrophages	Haemolytic anemia, encephalopathy
Visna virus	Sheep	Macrophages	Encephalopathy
Caprine arthritis-encephalistis virus (CAEV)	Goat	Macrophages	Immune deficiency, encephalopathy
Bovine immunodeficiency virus	Cow	Macrophages	Lymphadenopathy, Lymphocytosis
Feline immunodeficiency virus	Cat	T-lymphocyte	Immune deficiency
Simian immunodeficiency virus	Primate	T-lymphocytes	Immune deficiency, encephalopathy
Human immunodeficiency virus	Human	T-lymphocytes	Immune deficiency, encephalopathy

1.3 Morphology and Structure of HIV

The mature HIV virion is an icosahedral particle, roughly spherical, with a diameter of approximately 110 nanometers. The outer envelope acquired from the host cell by the virion during budding, is a lipid bilayer, and studded with about 72 spikes. For HIV-1, the projecting portion of each spike is the major surface glycoprotein with a molecular weight of 120 kilodaltons (gp 120), while the arm of the spike is the transmembrane glycoprotein with a molecular weight of 41 kilodaltons (gp 41). Envelope glycoproteins for HIV-2 are gp 105 and gp 36 (Veronese *et al.*, 1985). The central core contains 4 viral proteins, the capsid protein p24, the matrix protein p17, p9 and the nucleocapsid p7 (Robey *et al.*, 1985); two copies of the HIV RNA genome to which p9 and p7, are bound (Theilleux-Delande *et al.,* 2000), and three viral enzymes –reverse transcriptase, intergrase, and protease, essential for viral replication, are also found in the central core (Jetz *et al.*, 2000).

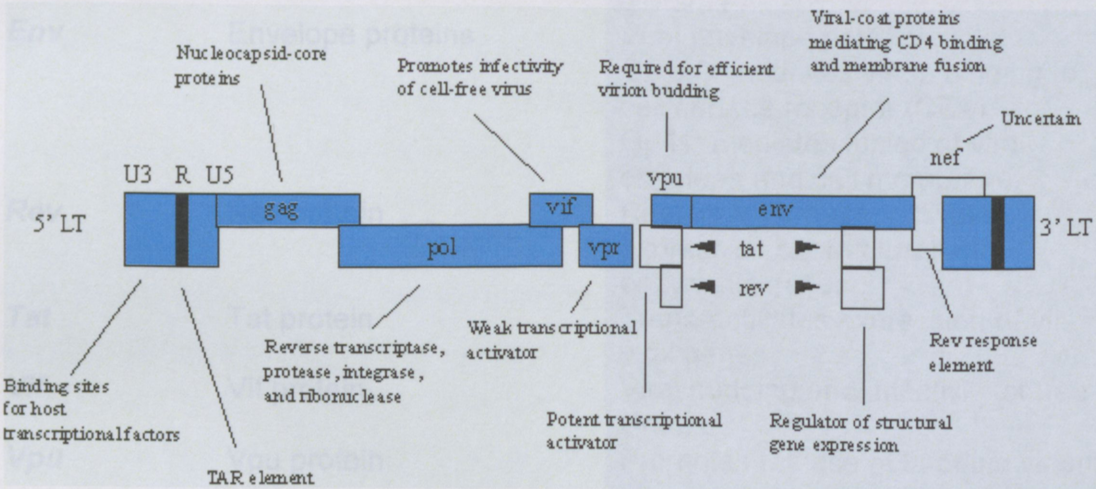

Figure 1.1: A schematic representation of HIV-1 genes (Theilleux-Delande *et al.*, 2000)

Table 1.2: HIV-1 genes, protein products and their functions (Sherman *et al*., 2002)

HIV gene	*Protein product*	*Function*
Gag	Gag/ core protein precursor	Viral core proteins: p24: Major capsid protein p17: Matrix protein. p9: binds to viral RNA. p7: binds to viral RNA.
Pol	Reverse transcriptase	Converts single stranded viral RNA to double stranded DNA.
	Protease	Cleaves the viral polyprotein into structural and functional core proteins.
	Integrase	Integrates viral DNA into host cell genome.
Env	Envelope proteins	Viral envelope proteins: Gp120: mediates virion binding to cell surface receptor (CD4). Gp41: mediates fusion of viral envelope and cell membrane.
Rev	Rev protein	Regulatory protein, activates expression of structural and enzymatic genes.
Tat	Tat protein	Trans-activates expression of all viral genes.
Vif	Vif protein	Viral budding and infectivity of free virions.
Vpu	Vpu protein	Promotes release of budding virions from the host cell.
Nef	Nef protein	Increase viral infectivity; down regulation of CD4.
Vpr	Vpr protein	In nuclear import of the preintegration complex.

1.4 Genetic variability of HIV

HIV-1 strains have diversified extensively through mutation and recombination since their initial transmission to human beings many decades ago. The high error rate of reverse transcriptase combined with the estimated in vivo HIV-1 replication rate of ten billion new virions each day leads to extraordinary genetic diversity of HIV. This means that different genomic combinations may be generated within an individual who is infected by genetically different HIV strains. Recombination results when a cell is simultaneously infected by two different strains of HIV and RNA transcripts from two different viral strains are encapsidated into the same virion particle. This virion then infects a new cell where it undergoes replication. During this phase the RT, by jumping back and forth between the two RNA templates will generate a newly synthesized retroviral DNA sequence that is a recombinant between the two parental genomes. This recombination is most obvious when it occurs between subtypes (Carr *et al.*, 1998).

Three groups of HIV-1 have been identified on the basis of differences in *env*, *gag*, and *pol* namely major group M consisting of 9 distinct subtypes (A-D, F-H, J and K), group O and group N viruses. Detailed analysis reveals subtypes A and F as comprising sub-subtypes A1, A2, F1 and F2. In addition, infection in certain geographic regions is mostly driven by circulating recombinant forms, comprising intersubtype recombinants and variants with mosaic genomes. HIV-1 group M viruses are responsible for the worldwide epidemic, while over 50% of global infections are attributed to HIV-1 subtype C (Esparza J and Bhamarapravati N, 2000; Kantor R and Katzenstein D, 2004). Group O and N viruses with very low prevalence (Vergne *et al.*, 2003; Roques *et al.*, 2004) are mainly restricted to Cameroon, although group O viruses have been identified in several European countries and North America. On the other hand, HIV-2 viruses clustering into one of seven subtypes (A-G) are prevalent in West Africa. Apart from inter-subtype genetic differences which could attain, for example 35% in the envelope protein, strains of the same subtype can differ by as much as 20%.

South Africa is considered to have experienced two HIV-1 epidemics. The first wave was confined to the homosexual population, with HIV-1 subtype B as the circulating variant (Williamson *et al.*, 1995). Later, HIV-1 subtype C was reported among the heterosexual population (Papathanasopoulos *et al.*, 2003). Subtypes A, D and E (A/E recombinant) have also been identified, although in much smaller proportions (Williamson *et al.*, 1995; Bredel *et al.*, 2000; Van Harmelen *et al.*, 1997). Overall, subtype C is the predorminant variant and drives the epidemic in South Africa and the Southern African region (Van Harmelen *et al.*, 1999; Bredel *et al.*, 2002; Bessong *et al.*, 2006, 2005).

1.5 Phenotypic characteristics of HIV-1

Phenotypic properties of HIV-1 isolates depend on the type of cells infected; the efficiency of infection, and the degree of cytopathic effect upon infection. Earlier characterization of HIV-1 strains was based on tropism for CD4+ cells such as T-cells, macrophages, Langerhans cells, and glial cells. Experiments revealed that certain variants efficiently infect cells of the T-cell lineage and others infect macrophages. As a result, HIV-1 variants were identified as T-cell line tropic and macrophage tropic strains respectively (Miedama *et al.*, 1994).

Certain human cell surface proteins in addition to CD4+ cells have been found to be crucial for HIV-1 entry. At least two co-receptors for HIV-1 believed to mediate fusion between HIV and its target cells have been identified, CCR5 and CXCR4. The chemokine receptor CCR5, expressed by monocytes and lymphocytes mediates entry on non-syncytium inducing (NSI), monocytotropic strain of HIV-1. The chemokine receptor CXCR4 expressed on T-lymphocytes mediates entry of syncytium inducing (SI), T-cell tropic strain of HIV-1 (Rucker & Doms 1998). This explains why monocytotropic strains of HIV can infect both monocytes and primary lymphocytes since both express CCR5, but not T-cell lines (which lack CCR5) and why T-cell tropic HIV-1 cannot infect monocytes (which lack CXCR4). CCR5 and CXCR4 normally function as receptors for chemokines (Berger *et al.*, 1998). The chemokines that bind to the CCR5 are macrophage inflammatory protein 1-alpha (MIP-1α), macrophage inflammatory protein 1-beta (MIP-1β), and regulated upon activation, normal T-cell expressed, and secreted (RANTES); and stromal cell derived factor-1(sdf-1) bind to CXCR4 (De la Luz *et al*., 2004)

1.6 Transmission of HIV

There are three main transmission routes of HIV which have been identified: Sexual route, blood/blood product route, and mother -to- child route (Varghese *et al*., 2002).

The majority of HIV infections are acquired through unprotected sexual relations. Sexual transmission occurs when there is a contact between sexual secretions of one partner with the rectal, genital or oral mucous membrane of another.

The transmission by blood and blood products is particularly important for intravenous drug users, hemophilic and recipients of blood transfusions and blood products (Donegan *et al*., 1994). It is also of concern for person receiving medical care in regions where there is prevalence substandard hygiene in the use of injection equipments. Health care workers (nurses, doctors & lab workers) are also directly concerned, although more rarely. Also concerned by this route are people who give and receive tattoos, piercing, and scarification procedures (Varghese *et al*., 2002).

In mother-to-child route, transmission of the virus from the mother to the child can occur in utero during the last week of pregnancy and at child birth. In the absence of treatment, the transmission rate between the mother and the child is 25%. However, where treatment is available, combined with the availability of caesarian section, this has been reduced to 1%. Breast feeding also presents a risk of infection for the baby (Coovadia, 2004).

1.7 Life cycle of HIV

The main target cells for HIV infection are human CD4+ cells, particularly T-lymphocytes and macrophages. Although mature CD8+ cells are not prime targets for HIV infection, under certain conditions HIV can infect these cells (Paxon *et al.*, 1998). The life cycle of HIV-1 in CD4+ cells can be divided into several stages: Binding – HIV -1 encounters the cell expressing the CD4+ cells receptor and bind to the cell membrane. This process involves the recognition of the CD4+ cells receptor by binding site on the V3 variable domain of gp120, one of the envelope glycoprotein. Membrane fusion and entry of the HIV-1 is done when fusion of the host cell membrane with the viral envelope is facilitated by Gp 41, another *env* glycoprotein of HIV-1, and by the co-receptor CCR5 protein, resulting in the entry of HIV-1 core into the cellular cytoplasm (Moore *et al.*, 1997).

Reverse transcription (RT) is the process where by viral RNA is used as a template by the viral enzyme reverse transcriptase to produce a single complementary strand of DNA using nucleotides in the host cell. HIV-I reverse transcriptase is a heterodimer consisting of a 66 kilodalton (p66) and a 51 kilodalton (p51) subunit. The p51 subunit is created from a p66 subunit. Both p66 and p51 contain the regions necessary for RNA dependent DNA polymerization, but only p66 contains the regions necessary to catalyze DNA dependent DNA polymerization (Dipika Misra and William Knox, 1998). For the structure of RT, refer to the figure1.2. However, this process only proceeds at a substantial rate in activated and proliferating T-lymphocytes. In resting cells, it proceeds inefficiently. The single-stranded virally encoded DNA acts as a template for the production of a complementary DNA strand in Integration process, forming a double stranded “proviral” DNA. This proviral DNA is then incorporated into the host cell genome by viral enzyme integrase (Brown *et al.*, 1989).

In proviral transcription, the proviral DNA is transcribed into viral mRNA by the cell machinery of the host. The transcriptional activity of the HIV-1 provirus is by constitutive host-cell transcriptional factors as well as by the virally encoded *Tat* protein. *Rev* is essential for export and expression of unspliced and singly spliced RNA. The multiple spliced mRNA transcripts encode the viral regulatory proteins *Tat, Rev,* and *Nef*, and are transported into the cytoplasm by host cell factors for translation. The unspliced mRNA also provides new viral genomes and encode *Gag* and *Pol* proteins whereas singly spliced RNA species encodes *Env, Vpr* and *Vpu* for translation (Wong-Stall, 1991).

The last stages of the life cycle of HIV are budding and maturation. In these stages new HIV-1 particles form as the viral core buds through the host-cell membrane, acquiring viral envelope proteins. The viral *Gag* and *Gag-pol* polyproteins are cleaved by the viral protease during or shortly after budding to generate mature infectious HIV-1 particles (Stevenson, 2003).

Figure 1.2: Ribbon model of the two subunits of reverse transcriptase. The p66 subunit is colored teal, and the p51 subunit is colored gold. (Dipika and William, 1998).

1.8 HIV-1 protease

The protease of HIV-1 is a small 99 amino acid aspartic enzyme with a molecular weight of 10779 Da, which mediates the cleavage of *gag, gag-pol,* and *nef* precursor polyproteins. Figure 1.3 below shows the structure of the enzyme protease. Protease cleavage occurs later in the viral life cycle, during virion assembly and maturation at the cell surface. The process is highly specific, temporarily regulated and essential for the production of the infectious viral particles (Jacks *et al*., 1998; Krausslich *et al*., 1989). Each reaction occurs at a unique cleavage that differs in amino acid composition (Billich *et al*., 1988). The structural proteins are formed by cleavage of the pr55 *gag* polyprotein into Matrix (*MA*, p17), Capsid (*CA*, p24), Nucleocapsid (*NC*, p7), p2, p1, p6 *gag*.

The viral enzymes are formed by the cleavage of a second polyprotein, pr160*gag-pol*, a fusion protein derived from ribosomal frame shifting. Although pr160*gag-pol* also contains p17, p24, p7, its C-terminal cleavage sites products are *NC*, a transframe protein (Tfp, p6pol, Protease, RTp66, and integrase (IN) (Ikuta *et al.,* 2000; Tessmer and Krausslich, 1998). Examples of protease cleavage sites are: *MA/CA, CA*/p2, p2/*NC, NC*/p1, p1/*p66gag, NC/Tfp, Tfp/p6pol, p6pol/PR, PR/RTp51, RT/RTp66, and Nef*. Protease activity also initiates virion maturation.

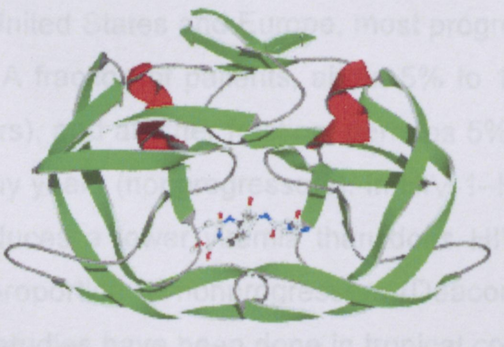

Figure 1.3: The structure of the aspartic enzyme protease (Navia *et al*., 1989).

1.10 Pathogenesis of HIV

After many years of debate on the hypothesis that AIDS is fundamentally an autoimmune disease, it is now clear that autoimmunity is not an essential component of the pathogenesis (Dyer *et al.,* 1997). Instead, disease progression is a direct reflection of virus replication. In the absence of active viral replication, or when viral replication is low (blood viremia levels less than 100 genome copies per milliliter), HIV infection does not progress to clinical immunodeficiency. Conversely, when viral replication is rapid (viremia greater than 100,000 genome copies per milliliter), disease progression is correspondingly rapid. Treatment with antiviral drugs slow disease progression in proportion to their effect in lowering viremia (Moore *et al.*, 2004).

The course of HIV infection can be quite variable. The initial infection is followed by an eclipse phase in which neither viremia nor an antibody response can be found. A virologic "primary infection" with high levels of viremia then appears and persists for several weeks. Up to 30% of HIV-infected persons are reported to experience a mononucleosis-like syndrome of fever, pharyngitis, rash, and depressed CD4 cell counts during this primary phase. Among HIV-infected patients studied in the United States and Europe, most progress from infection to AIDS in 5 to 15 years. A fraction of patients, about 5% to 10%, progress more rapidly (rapid progressors), and another fraction, perhaps 5%, show no evidence of progression over many years (nonprogressors). In HIV-1–HIV-2 studies, HIV-2 infection invariably produces a lower viremia than does HIV-1 and HIV-2 also shows a much higher proportion of nonprogressors (Deacon *et al.*, 1995). Few rigorous natural history studies have been done in tropical countries or in regions where HIV-1 genotypes other than clade B predominate. One study of HIV-1 infections among commercial sex workers in Kenya showed rapid progression to AIDS (within 5 years) in 50% of cases. It is uncertain if the high proportion of rapid progressors in this study in Africa can be attributed to the prevalent viral genotypes (probably clade A and clade D), to the high prevalence of aggressive opportunistic infection (e.g., tuberculosis), to the lack of optimal medical care, or to poor nutrition or other host factors.

There is no evidence that the virus in rapid progressors is more virulent than average. Instead, natural history studies suggest that rapid progressors mount an inadequate immune response. Most patients show low antibodies or cellular responses. Rarely, rapid progressors have been described who appear to be completely but selectively unresponsive to HIV antigens (Biti *et al.*, 1997).

Nonprogressors typically have low plasma viral titers. In some nonprogressors low viral titers are due to infection with grossly replication-defective virus, but in most patients it is uncertain if low viral titers reflect viral factors (poor replication), host genetic factors, or an unusually strong immune response (Mellors, 2001). Nonprogressors do routinely show strong immune responses to HIV proteins, including antibody and cellular responses, but it is unclear if this is cause or effect (Cohen and Walker, 2001).

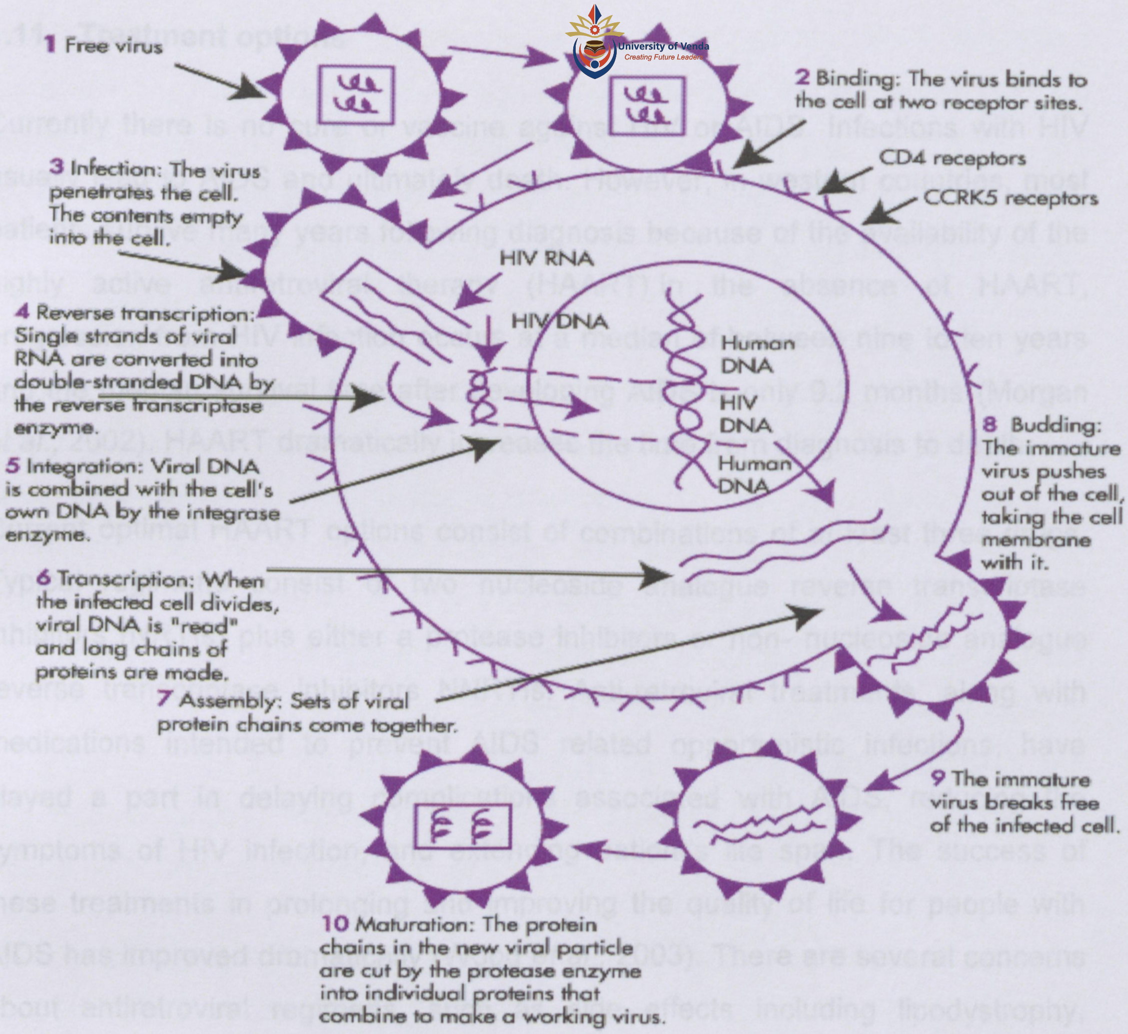

Figure 1.4: Steps involved in the life cycle of HIV-1 (Engenderhealth, 2007)

1.11 Treatment options

Currently there is no cure or vaccine against HIV or AIDS. Infections with HIV usually lead to AIDS and ultimately death. However, in western countries, most patients survive many years following diagnosis because of the availability of the highly active antiretroviral therapy (HAART).In the absence of HAART, progression from HIV infection occurs at a median of between nine to ten years and the median survival time after developing AIDS is only 9.2 months (Morgan *et al*., 2002). HAART dramatically increases the time from diagnosis to death.

Current optimal HAART options consist of combinations of at least three drugs. Typical regimens consist of two nucleoside analogue reverse transcriptase inhibitors (NRTIs) plus either a protease inhibitors or non- nucleoside analogue reverse transcriptase inhibitors NNRTIs. Anti-retroviral treatments, along with medications intended to prevent AIDS related opportunistic infections, have played a part in delaying complications associated with AIDS, reducing the symptoms of HIV infection, and extending patient's life span. The success of these treatments in prolonging and improving the quality of life for people with AIDS has improved dramatically (Wood *et al*., 2003). There are several concerns about antiretroviral regimens, such as side effects including lipodystrophy, dyslipidaemia, insulin resistance, an increase in cardiovascular risks and birth defects (Montensori *et al*., 2004)

1.12 Drug resistance in HIV

A rebound in HIV replication during combination therapy is considered a major cause of treatment failure (Hirsch *et al*., 1996; Vandamme *et al*., 1999). This may be due to the selection or development of drug resistance. Drug resistance arises from mutations in the genes that encode the molecular targets for the drugs. The emergence of amino acid substitutions associated with resistance to *RT* and *PI* has been extensively characterized, and these substitutions can be classified into major and accessory mutations. Major mutations lead to several fold decrease in sensitivity to one or more antiretroviral drugs (Hirsch *et al*., 1996). Accessory mutations may not result in a significant decrease in sensitivity but are associated with an increase in viral fitness (replication capacity). Thus, the appearance of a major mutation in a genome already containing accessory mutations could influence the speed with highly resistant viruses are selected during therapy.

An extremely important aspect of the rate of evolutionary variation for HIV is the selection pressure exerted by the host. This aspect is vividly illustrated by the rapid emergence 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. The process is repeated frequently and cyclically; the dominant clone of the virus elicits a new immune response, becomes controlled, and a new mutational variant takes over as the next dominant viral clone. Many of the mutations and the recombinational events results in the emergence of genomes that are incapable of replication (Bellocchi *et al*., 2005).

The use of protease inhibitors (PIs) has led to a marked reduction in morbidity and mortality in patients with advanced HIV infection. Inevitably, the widespread use of PIs has led to the emergence of drug-resistant HIV variants, many of which display cross-resistance to many inhibitors. The emergence of drug-resistant variants during antiretroviral therapy is a serious obstacle to sustained suppression of the human immunodeficiency Virus type 1 (HIV-1). For that reason, resistance assays are essential to guide clinicians in the selection of optimal treatment regimens.

The introduction of highly active antiretroviral therapy (HAART) and modern regimens, based on triple or quadruple-drug combinations of nucleoside reverse transcriptase inhibitors (NRTIs), nonnucleoside reverse transcriptase inhibitors (NNRTIs) and protease inhibitors (PRIs) has reduced significantly the rate of morbidity and mortality associated with human immunodeficiency virus type 1

(HIV-1) infections (Mocroft *et al*., 2003). Although these combinations can suppress virus replication to undetectable levels for a long period of time, with the consequent immunological reconstitution (Palella *et al*., 1998; Smith *et al*., 2004), many patients experience virological failure as shown by a rebound in the viral load (Ledergerber *et al*., 1999). Besides inadequate drug absorption, poor adherence, low drug potency and persistence of HIV reservoirs, one of the most serious obstacles for HIV suppression is the emergence of drug-resistance variants (Ledergerber *et al*., 1999). The acquisition of viral resistance is accentuated by both, the high-level of in vivo replication and the high error rate of the viral reverse transcriptase (*RT*) (Arts EJ and Wainberg MA (1996). Detection of resistant viruses has become increasingly important to achieve better management of the disease, and therefore, resistance testing has been recommended to facilitate clinicians to choose the optimal drug regimens after the first or multiple treatment failures (Hirsch *et al*., 2003). Furthermore, resistance testing is also recommended in primary HIV infection due to the increased prevalence of HIV resistance in acute infection (Little *et al*., 2002).

1.13 Methods for the evaluation of resistance mutations

The methods for the evaluation of antiviral drug resistance can be classified into genotypic and phenotypic assays. Genotypic testing determines the resistance-related mutation pattern of the virus population of a particular patient by using point hybridization (Stuyver *et al*., 1997) or full sequencing assays (Erali *et al*., 2003). These methods are relatively rapid and easy to perform, but they only provide indirect evidence of drug resistance. Since interpretation of the results depends on the knowledge of the phenotypic resistance and clinical impact of drug resistance mutations, it is difficult to standardize a criteria to analyze the sequence data for sequences with unusual aminoacid substitutions or complex mutation patterns (Hanna and D'Aquila, 2001).

On the other hand, phenotypic assays provide a more direct quantitative resistance measurement, determining the drug concentration fold increase required for achieving the same degree of viral inhibition compared to a wild-type strain (Kellam and Larder, 1995; Boucher *et al*., 1996; Shi and Mellors, 1997; Race *et al*., 1999; Iga *et al*., 2002). Conventional phenotypic assays, based on virus isolation from peripheral blood mononuclear cells, are labor-intensive and time-consuming (Japour *et al*.,1993), and encouraged improvement of the technique. The current available phenotypic assays for testing ARV drug resistance are based on generation of recombinant HIV-1, wherein the protease (*PR)* and *RT* region of HIV-1, amplified from patient's plasma sample by reverse transcription polymerase chain reaction (RT-PCR) is incorporated into recombinant HIV-1 backbone with deleted *PR* and *RT*. The fold change in drug concentration required to inhibit the patient virus sample, in comparison to that required for a wt reference strain, is measured. In addition to measurement of diminution in drug sensitivity, there is a direct quantification of the degree of resistance. The result is expressed as the concentration of the drug inhibiting the virus replication by 50% (IC_{50}). The IC_{50} (or IC_{90}) of test sample is compared with a cut-off value, thereby indicating the factor (fold change) by which this IC_{50} can be increased, with reference to IC_{50} of the wild type strain and still be classified as sensitive.

1.14 Construction of retroviral and other viral vectors.

While other recombinant viral vector systems have been developed, retroviral vectors remain the most popular vector system for gene therapy protocols and drug resistance mutation analysis. This may in part be due to their historical significance as the first vectors developed for efficient gene therapy and the infancy of the field of gene therapy. Vector systems include retroviral, adenoviral, adeno-associated, herpes simplex, and vaccinia vectors. Discussion will focus on the basic biology of these viruses and vector preparation, as well as their applications and limitations in gene transfer and drug resistance analysis.

Human immunodeficiency viruses (HIV) are the most recently discovered members of the retrovirus family and have led to the new classification of lentivirus. Like other members of the retroviral family, the HIV genome contains the *gag, pol* and *env* genes. In addition, several other nonstructural proteins which serve regulatory functions are contained within its genome. Below is the diagram of the HIV genome considered to be one of the most complex among the retroviruses. Figure 1.1 shows the HIV genome and genes.

MMLV vectors, to date, these vectors have been used more than any other gene transfer vehicle. They are produced simply by replacing the viral genes required for replication with the desired genes to be transferred. Thus, the genome in retroviral vectors will contain an LTR at each end with the desired gene or genes in between. The most commonly used system for generating retroviral vectors consists of two parts, the retroviral DNA vector and the packaging cell line.

Retroviral DNA vectors are plasmid DNAs which contain two retroviral LTRs in the region internal to these LTRs for insertion of the desired gene. A portion of a retroviral plasmid DNA vector, LNSX (*Methods in Enzymology*. 1993; 217:584.). The gene of interest is cloned into the multicloning site following the simian virus SV40 promoter (SV). As one can imagine, these plasmid DNAs can be manipulated to meet a variety of needs allowing for multiple applications and the design of very elegant vectors. Packaging cell lines provide all the viral proteins required for capsid production and the virion maturation of the vector. These packaging cell lines have been made so that they contain the *gag, pol* and *env* genes. Early packaging cell lines contained replication competent retroviral genomes and a single recombination event between this genome and the retroviral DNA vector could result in the production of a wild type virus. This led to the term "helper virus contamination" and to this date, with all viral vectors systems, it is important to insure that the vector be free of helper virus. Current packaging systems require that three homologous recombination events occur for any wild type virus production. Since is an extremely rare event, current cell lines are considered to be helper free, although it is still wise to test for contamination of any wild type virus. There have been a wide variety of packaging cell lines produced as well as retroviral DNA vectors, and most are commercially available. For the MMLV vectors it is the packaging cell line which determines if the vector is ecotropic, xenotropic or amphotropic. Dependent upon the target cells it is important to ensure that the correct packaging cell line be chosen.

Table 1.3: Overview of the advantages and disadvantages of retroviral vectors

Advantages	Disadvantages
High transduction efficiency	Requires diving cells for infectivity
Insert size up to 8kb	Low titers (10^6-10^7)
Integrates in host genome resulting in sustained expressing vector	Integration is random
Extremely well studied system	In vivo delivery remains poor, effective only when infecting helper cell lines
Vector proteins not studied in host	

1.15 Lentiviral Vector Systems

The human HIV Lentiviral Vector Systems provide exceptional tools to express any sequence in virtually all mammalian cells. The system consists of three main components: the lentiviral expression plasmids, the lentiviral packaging vector; and a producer cell line for packaging

Lentiviral expression vectors contain the genetic elements responsible for packaging, transduction, stable integration of the viral expression sequence into genomic DNA, and high expression of the inserted effectors sequence. The packaging cell line stably transfected with the packaging plasmids contains the expression elements for the viral coat proteins.

To create functional pseudoviral particles, the lentivector containing the expression construct is co-transfected with the packaging plasmids into the producer cell line. The packaging cells replicate the expression construct and package it into pseudoviral particles. These pseudoviral particles are then purified and used to deliver the expression construct to the cells of interest. Once delivered, the viral expression construct integrates, and the construct is expressed. SBI's lentivectors are self-inactivating. Upon integration into the genome, the 5' LTR promoter is inactivated, which prevents formation of replication-competent viral particles. Thus, the transduced cell cannot produce additional viral particles since the cell does not contain the genes needed to produce the viral capsid, nor is there a functional 5'LTR to enable it to replicate the viral sequence. In addition to yielding very high expression levels, the lentiviral system is very effective at delivering genetic material to almost any mammalian cell including non-dividing cells and whole model organisms. Effective transduction with lentiviral pseudovirus particles does not require cells to be active or growing. The efficiency of lentiviral transduction is close to 100% so this system is ideal as an expression library vector.

1.16 Presentation of this dissertation

The material presented in this study is in three different chapters, preceded by a summary of the entire study. Chapter one presents a review on the biology of HIV comprising epidemiology, prevention, laboratory diagnosis, treatment options, life cycle, and pathogenesis. Chapter two deals with the investigations on drug resistance in naive patients, while chapter three deals with the design and construction of retroviral systems to be used in the phenotypic evaluation of putative drug resistance mutations and the evaluation of candidate drugs. Each of the investigative chapters comprises its own introduction, study rationale, objectives, materials and methods, results and discussion and references.

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Chapter 2:

Drug resistant mutations in naïve chronic HIV-1 infected South Africans

2.1 Introduction

Currently, chemotherapy for HIV infection relies mainly on three main groups of antiretroviral agents, namely reverse transcriptase inhibitors (RTI), protease inhibitors (PI) and fusion inhibitors (FI). However, the most commonly used drugs are the RTI and PI. RTI fall under two groups: nucleoside RTI (NRTI) and non-nucleoside RTI (NNRTI). The nucleoside RTI competes with the natural nucleosides in the synthesis of proviral DNA. Their incorporation causes chain termination. The mode of action of NNRTIs is by binding to an allosteric site on the *RT*, inhibiting viral DNA synthesis. Protease inhibitors block the cleavage of viral polyproteins by protease into structural and enzymatic components. Fusion inhibitors, typified by fuzeon, act on the transmembrane protein gp41and prevents fusion of virus with susceptible cells.

The biggest drawback on the efficacy of PI and RTI is the potential for the development of viral resistance even with the use of highly active antiretroviral therapy (Appay *et al*., 2002; Clevenbergh *et al*., 2002), and this occurs irrespective of subtype. Resistance to RTI and PI is mostly due to genetic changes in the reverse transcriptase (*RT*) or protease (*PR*) gene or selection of a resistant pre-existing viral population in the presence of drug pressure. Alterations in the appropriate gene loci of *RT* or *PR* may lead to the development of mutant enzyme molecules capable of evading inhibitors. The development of resistance to antiretrovirals (ARV) poses a serious challenge to the containment of the course of HIV-1 infection and management of patients. The existence of drug resistant variants in drug-naïve individuals and the transmission of resistant and multi-drug resistant strains further complicate the situation.

2.1.1 Resistance mutations in drug naïve patients

The evolution of resistance as a result of treatment may lead to the transmission of resistant viruses. Previous studies have shown that in countries where most patients are under treatment, the prevalence of resistant viruses in the untreated population could be as high at 20%. On the other hand, in countries were therapy is limited, resistant viruses are rare in the untreated populations (Vergne *et al*., 2000; Pires *et al*., 2004). Prior to 2004 antiretrovirals were available only through private health establishments in South Africa; and due to the scarcity and prohibitive high cost, the majority of patients could not afford them. In early 2004 a national antiretroviral delivery programme was initiated to provide drugs to those who had either a CD4 count less than 200/µl or a clinically defined AIDS condition. The national drug regimen guideline stipulates the use of stavudine, lamivudine and efavirenz as the first-line therapy for those who have never been exposed to antiretrovirals; with nevirapine replacing efavirenz for women of child bearing age in this group. The second-line therapy comprises zidovudine, didansone and lopinavir/ritonavir (DOH, 2004). It has been demonstrated that a determination of a regimen based on the genetic resistance profiles of patients' viruses prior to the initiation of therapy generally correlates with a better outcome, compared to the absence of such resistance data (Shafer, 2002). This information regarding the presence of resistant viruses is important in tailoring combination regimens or to decide whether to prescribe resistance testing prior to initiation of therapy.

Previous studies in South Africa indicate that drug resistance among naïve patients may not be common. No known drug resistance mutations were reported in 2002 against zidovudine, and multiple nucleosides were found in 37 *RT* sequences from drug naive pregnant women from Soweto (Pillay *et al*., 2002). Apart from minor mutations such as L10I, K20R, M36I, M63P and V77I in the protease Gordon *et al*. (2003) did not find any known major protease resistance mutations in 72 patients from KwaZulu Natal reported in 2003. In the same study, no major or minor resistant mutations against NRTI were identified, although

K103N and G190A that causes resistance to NNRTI were detected in two epidemiologically linked patients, and A98G in a third patient.

2.1.2 Study rationale

Countries such as South Africa, where access to therapy is progressing require data on patterns of resistance and evolution of resistance. More and more people are gaining access to anti-retroviral therapy in the Limpopo Province, and this may lead to the selection and development of resistant viruses which could be transmitted to other individuals. Transmission of resistant viruses will decrease the efficiency/ effectiveness of the first line HIV treatment regimen. Therefore it is important to analyze the *gag/ pol* gene from infected, but non treated patients for resistance mutations.

In a previous study done on HIV-1 isolates from Limpopo Province of South Africa a low level of major drug resistance mutations among prevalent viruses in a small sample size from Bela Bela, was observed. One question to answer is whether screening drug inexperienced patients in sites in Limpopo where treatment has been available for a relative longer duration for resistance mutations should be considered before the initiation of therapy? Consequently, this study was undertaken to further contribute to the baseline genetic drug resistance information of HIV in South Africa, by focusing on the Limpopo Province were little data exist on drug naïve patients. The protease and reverse transcriptase genes of primary isolates from newly diagnosed patients were examined for drug resistance mutations, polymorphisms and novel mutations. Such information is relevant in evaluating resistance testing options, interpreting genetic resistance among persons who fail therapy, formulating drug delivery policies, as well as subsequent resistance monitoring, critical in patient management.

2.1.3 Study objectives

2.1.3.1 General objectives

The general objective of the study was to genetically characterize HIV primary isolates from drug naïve patients from the Limpopo Province of South Africa.

2.1.3.2 Specific objectives

The specific objectives were:

1. To determine the genetic subtypes of HIV-1 infected patients from selected sites in the Limpopo Province of South Africa.
2. To determine the intra-genetic diversity of the isolates based on the protease and reverse transcriptase genes.
3. To analyze the protease and reverse transcriptase genes for mutations associated with drug resistance.

2.2 Materials and Methods

2.2.1 Ethical consideration

Ethical clearance for this study was obtained from the Health, Safety and Research Ethics Committee of the University of Venda, South Africa. Permission to recruit patients from clinics and hospitals was obtained from the Limpopo Provincial Department of Health, Polokwane, South Africa

2.2.2 Study area

The specific sites for the sample study were Thohoyandou and Musina in the Vhembe district, and Bela-Bela in the Capricorn district of the Limpopo Province. Thohoyandou is the administrative seat of the Vhembe district with semi-urban developmental infrastructure. Bela Bela is a holiday resort with hotels and camping sites, and Musina, which is a border town with Zimbabwe, is characterized by inter-border commercial activities and stop over for truckers. The prevalence of HIV at these study sites is very high. In addition, people are coming from neighboring countries whose HIV-1 genetic landscapes differ to an appreciable extent from that of South Africa, in terms of known circulating subtypes

2.2.3 Study subjects, sample collection and plasma preparation

HIV infected people were recruited from the different study sites; Thohoyandou, Musina, and Bela Bela, and the informed consent were provided prior participation. Demographic data was collected by means of a questionnaire to capture sex, age, and route of infection and probable place and date of infection, as well as the use of anti-retrovirals.

Five ml of whole blood was collected by venepuncture into EDTA vacutainer tubes from study participants once. Fifty eight blood samples were collected between May and August 2007 from Thohoyandou (01 sample), Musina (40 samples), and Bela-Bela (17 samples). Plasma was prepared by centrifugation of samples at 3000 rpm for 3 minutes. Aliquots of plasma was aspirated aseptically into sterile Nunc tubes and stored at -83°C until subsequent viral RNA extraction.

2.2.4 Viral-RNA extraction

Viral RNA was extracted from 140µl of plasma using the Qiagen Viral RNA Mini Kit (Qiagen, Valencia, USA). Three hundred and ten microliter of buffer AVE was added to lyophilized RNA and aliquoted in 56.0µl aliquots. Fifty microliter of RNA-AVE was then added to 5.6 µl of buffer AVL in a 15 ml tube. Then 560 µl of AVL/Carrier RNA was pipette into 1.5 ml microcentrifuge tube. Two hundred microliter of plasma was centrifuged at 3000 rpm for 1 minute. One hundred and forty of plasma was added to the buffer AVL-carrier RNA and pulse vortex for 15 seconds. This was incubated at room temperature for 10 min. this helps in lysis of the viral particles. Briefly centrifugation was done to remove the drops from the inside of the lid. Five hundred and sixty of ethanol (96-100%) was added to the sample and followed by vortexing for 15 sec. After mixing, brief centrifugation was done. And 630 µl of the ethanol solution was applied carefully to the QIAamp Mini Mini spin column (in a 2 ml collection tube), followed by centrifugation at 8000 rpm for 1 min. The column was placed in a new 2 ml collection tube and discarded the filtrates. The step above was repeated again to apply the remaining lysate to be loaded onto the column. The QIAamp Mini spin column was carefully opened and added 500 µl of buffer AW1, followed by centrifugation at 8000 rpm for 1 min. The column was placed in a clean collection tube and discarded the filtrates. Five hundred microliter of buffer AW2 was added and centrifuged the column at 14000 rpm for 3 min. The column was placed in a clean 1.5 ml microcentrifuge tube, and 60 µl of elution buffer AVE was added and incubated at room temperature for 1 min, and followed by centrifugation at 8000 rpm for 1 min. The extracted RNA was then stored at -20°C for amplification process.

2.2.5 Reverse transcription of HIV-1 *pol* fragment and gel electrophoresis

Viral RNA was transcribed to cDNA using a one-tube reverse transcriptase polymerase chain reaction (RT- PCR) making use of AMV RT (5U) (Roche) and Taq DNA polymerase (0.025U) (Invitrogen), followed by a nested PCR to generate an approximately 1.4-kb product comprising the entire protease gene and at least the first 900 base pairs of the reverse transcriptase gene. The first round primers were Pol1C (2ng/ul), 5'-GAA GGA CAC CAA ATG AAA GAC TGT AC-3' (forward) and RTRv (2ng/ul) 5'-T GAC CCATCA AAA GAC TTG ATAGCTGAAATA-3' (reverse) and the nested primers were (GagP1) (2ng/ul) 5'-CAAGGG GAG GCC AGG GAA TTT-3' (forward) and (Pol2R) (2ng/ul) 5'-CATGGA GTA TAT TAT GAC CCA TCA-3' (reverse).

Amplification was carried out in a BioRAD thermal cycler. The cycling conditions were as follows: one-tube RT-PCR 42ºC for 1 hour followed by 1 cycle of 95°C for 3 minutes then 30 cycles of 95°C for 1min, 58°C for 1 minute, 68°C for 2 minute and a final extension of 72°C for 2 minutes. Cycling conditions for the nested reaction were 95ºC for 3 minutes followed by 30 cycles of 95ºC for 1 minute, 58ºC for 1 minute, 68ºC for 2 minutes, and a final extension of 72ºC for 10 minutes. The nested product was checked for correct size and quantity by 1% agarose gel electrophoresis stained with ethidium bromide and visualized under UV transluminator (Bessong *et al*., 2006).

2.2.6 Purification of PCR products

Amplified PCR products were purified using the Qiaquick PCR purification kit (Qiagen). Five hundred micro liter of buffer PBI was added to 100µl of pcr samples and mixed thoroughly. Twenty four milliliter of 100% ethanol was added to buffer PE before use. The Qiaquick spin columns were placed in a provided 2ml collection tubes, and the samples were applied inside for the DNA to bind to the filter. The columns were centrifuged for 30-60 seconds. The flow-through was discarded and the columns were placed into the same collection tube. To wash the DNa, 750µl of buffer PE was added to the column and centrifuged for 30-60sec. The flow-through was discarded and the columns were placed back in the same tube. Additional centrifugation of one minute was done to remove all the residual ethanol from buffer PE. To elute DNA, Qiaquick columns were then placed in a 1.5 ml micro centrifuge tubes, and 50µl of buffer EB was added in the center of the QiaQuick membrane and centrifuge for 1 min. The purified samples were determined for DNA concentration using the DU 650 Spectrophotometer (A260) and determined the quantity by running 0.8% agarose gel electrophoresis.

2.2.7 Sequencing

The principle of the automated sequencing was to determine the order of the nucleotides of a gene. Automatic population-based sequencing was done on the generated nested PCR product on both strands. Sequencing was done at Geneway Research, California, USA. The primer pairs: Gap1, 5'- CTT CAG AAC AGA CCA GAG C-3' (forward) and Protease 2, 5'-CTC TTC TGG TAA CGG CCA TTG-3' (reverse) was employed to generate the entire protease gene. A partial *RT* gene was sequenced using two sets of primers. PolF, 5'-TTT TCC CAT TAG TCC TAT TGA AAC TGT A-3' (forward) and Pol1R, 5'-CAT GCT ACT CTG GAA TAT TGC TGG TGA TCC-3' (reverse), and Pol2F, 5'-CTG CAT TCA CCA TAC CTA GTA TAA AC-3' (forward) and Pol2R, 5'-TGA TGG GTC ATA ATA TAC TCC ATG-3' (reverse). These primers generate two overlapping fragments which were edited to give approximately 300 amino acids. Sequence editing was done with SeqMan II software.

2.2.8 Phylogenetic analysis

The genetic subtyping was determined by phylogenetic analysis. The test nucleotides sequences and HIV -1 subtype references from Genbank were aligned with ClustalX and phylogenetic trees were generated by the neighbor joining with the PHYLLIP programme, making use of the maximum likelihood model to take care of the differences in the transition and transversion rates.

Tree was rooted with the SIVcpz strain and viewed with the TreeView programme.

2.2.9 Intra-genetic variability among HIV isolates

The intra-genetic variability among the isolates was determined by analysis of the mean genetic distances among the isolates. Nucloetide sequences of the protease and reverse transcriptase genes were aligned with BioEdit followed by mean genetic distance calculations using the Kimura 2-parameter method contained in the BioEdit software.

2.2.10 Drug resistance genotyping

Stanford HIV drug resistance database interpretation programme was used to determine resistant associated mutations found in the sequences under investigation. This interactive programme compares codons of query sequences with resistance coding nucleotides. The resistant sequences are derived from patients failing therapy, and from phenotypic assays. Mutations that on their own result in resistance are referred to as major mutations, while mutations that reduce susceptibility in association with other mutations are referred to as minor mutations. Polymorphisms, which are naturally occurring amino acid changes at sites associated with resistance, are also noted. In addition, resistance related codons were examined and compared to that of subtype B used as the reference in the drug resistance interpretation programme (Bessong *et al.*, 2006).

2.3 Results

2.3.1 Demographics of the study population

Blood samples were collected from 58 patients of whom 43 were females and 13 were males and 2 were unknown. The age ranged from 17-52. None of the patients were on antiretroviral therapy. The numbers of patients for the different AIDS stages were 17 for stage 1, 20 for stage 2, 16 for stage 3 and 3 for stage 4. Only one patient repeal homosexual behavior. Virologic and immunologic data such as viral load and CD4 cell count respectively were not available for any of patients.

Table 2.1: Demographic data of the study group (n=58)

Characteristics	Epidemiologic data
Age (years)	
Range	17-52
Sex	
Females	43 (74%)
Males	13 (22%)
AIDS clinical stage	
I	17
II	20
III	16
IV	3
AIDS	1
Mode of transmission	
Heterosexual	57 (98%)
Homosexual	1 (2.0%)
Antiretroviral therapy	0 (0%)

2.3.2 RT-PCR, nested PCR

Forty-five out of 59 samples (76%) were successfully amplified in the *pol* gene to generate a 1.4 kb product comprising the entire protease and at least the first 300 amino acids of the reverse transcriptase gene. Figure 2.1 shows a representative gel of the amplified products. No product was visible in the negative control showing that there was no contamination.

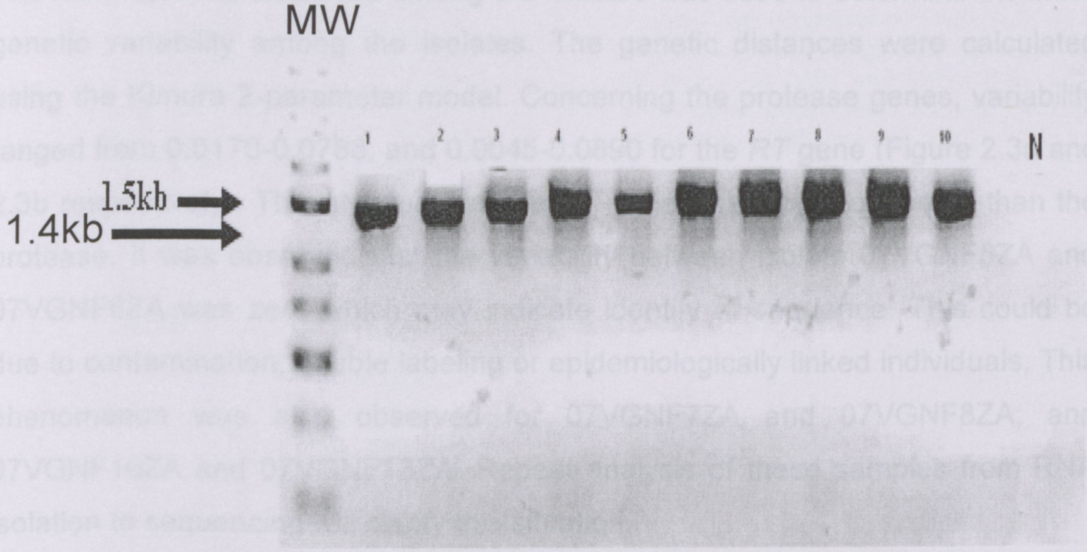

Figure 2.1: Representative 1% agarose gel of a 1.4bp amplified product of HIV-1 pol. Lane M is a 100bp molecular weight marker, lanes 1-10 are test samples, and lane N is a negative control.

2.3.3 HIV subtyping by phylogenetic analysis

All of the 45 PCR products were sequenced. However, 30 were edited reliable in the protease region and 30 in the reverse transcriptase region. The generated nucleotide sequences were subsequently used for genetic analyses including subtyping, inter-subtype variability and drug resistance mutational analyses.

Phylogenetic analysis of the protease and *RT* sequences are shown in Figures 2.2a and 2.2b respectively. The test protease sequences were observed to cluster and intermingle with previously described HIV-1 subtype C protease from the Limpopo Province (AY510031-AY510041), and cluster with references sequences from India (AY515845-515849). A similar profile was observed with the *RT* sequences. This shows that the test viruses were all HIV-1 subtype C at least on the protease and reverse transcriptase gene regions.

2.3.4 Intra-genetic variability among the test sequences

The mean genetic distances 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. Concerning the protease genes, variability ranged from 0.0170-0.0786, and 0.0045-0.0890 for the *RT* gene (Figure 2.3a and 2.3b respectively). This showed that the *RT* gene was more conserved than the protease. It was observed that the variability between isolate 07VGNF5ZA and 07VGNF6ZA was zero which may indicate identify of sequence. This could be due to contamination, double labeling or epidemiologically linked individuals. This phenomenon was also observed for 07VGNF7ZA and 07VGNF8ZA; and 07VGNF10ZA and 07VGNF13ZA. Repeat analysis of these samples from RNA isolation to sequencing will clarify this situation.

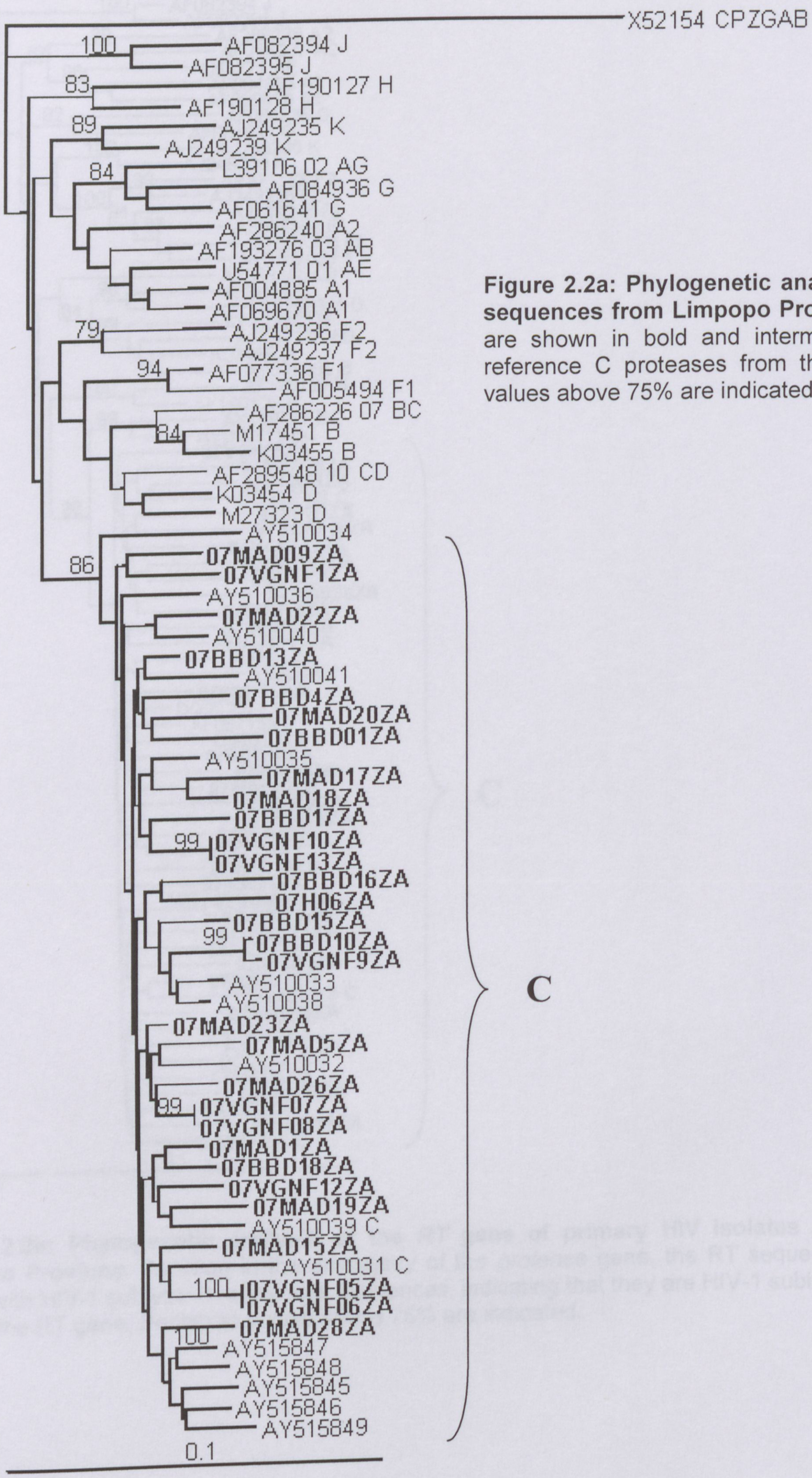

Figure 2.2a: Phylogenetic analysis of HIV *protease* sequences from Limpopo Province. Test sequences are shown in bold and intermingle and cluster with reference C proteases from the Genbank. Bootstrap values above 75% are indicated.

	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	26	27	28	29	30
07MAD1ZA	0.0000																													
07MAD5ZA	0.0524	0.0000																												
07MAD09ZA	0.0452	0.0525	0.0000																											
07MAD15ZA	0.0381	0.0487	0.0525	0.0000																										
07MAD17ZA	0.0596	0.0707	0.0710	0.0634	0.0000																									
07MAD18ZA	0.0452	0.0599	0.0526	0.0526	0.0309	0.0000																								
07MAD19ZA	0.0381	0.0633	0.0673	0.0489	0.0561	0.0526	0.0000																							
07MAD20ZA	0.0634	0.0669	0.0709	0.0630	0.0745	0.0710	0.0671	0.0000																						
07MAD22ZA	0.0346	0.0563	0.0455	0.0564	0.0673	0.0453	0.0636	0.0786	0.0000																					
07MAD23ZA	0.0240	0.0345	0.0382	0.0275	0.0415	0.0311	0.0345	0.0523	0.0346	0.0000																				
07MAD26ZA	0.0415	0.0380	0.0489	0.0451	0.0560	0.0489	0.0523	0.0633	0.0526	0.0239	0.0000																			
07MAD28ZA	0.0381	0.0452	0.0598	0.0345	0.0708	0.0600	0.0561	0.0670	0.0564	0.0345	0.0523	0.0000																		
07BBD01ZA	0.0560	0.0523	0.0634	0.0595	0.0707	0.0636	0.0707	0.0633	0.0673	0.0451	0.0487	0.0668	0.0000																	
07BBD4ZA	0.0528	0.0563	0.0529	0.0490	0.0565	0.0530	0.0528	0.0601	0.0493	0.0347	0.0491	0.0563	0.0563	0.0000																
07BBD10ZA	0.0454	0.0561	0.0673	0.0418	0.0710	0.0675	0.0636	0.0705	0.0602	0.0417	0.0525	0.0418	0.0671	0.0638	0.0000															
07BBD10ZA	0.0346	0.0380	0.0347	0.0310	0.0487	0.0382	0.0453	0.0417	0.0456	0.0171	0.0346	0.0311	0.0488	0.0383	0.0454	0.0000														
07BBD15ZA	0.0308	0.0595	0.0559	0.0451	0.0668	0.0486	0.0523	0.0706	0.0451	0.0309	0.0414	0.0452	0.0704	0.0599	0.0453	0.0416	0.0000													
07BBD16ZA	0.0563	0.0600	0.0639	0.0600	0.0747	0.0639	0.0747	0.0861	0.0603	0.0453	0.0635	0.0564	0.0710	0.0604	0.0602	0.0564	0.0562	0.0000												
07BBD17ZA	0.0451	0.0596	0.0488	0.0486	0.0634	0.0489	0.0671	0.0595	0.0487	0.0381	0.0560	0.0524	0.0560	0.0528	0.0635	0.0417	0.0557	0.0712	0.0000											
07BBD18ZA	0.0242	0.0454	0.0529	0.0419	0.0599	0.0528	0.0383	0.0637	0.0418	0.0276	0.0417	0.0491	0.0563	0.0567	0.0528	0.0384	0.0453	0.0602	0.0489	0.0000										
07VGNF1ZA	0.0559	0.0669	0.0452	0.0632	0.0635	0.0525	0.0708	0.0707	0.0562	0.0488	0.0669	0.0631	0.0669	0.0491	0.0783	0.0453	0.0666	0.0712	0.0522	0.0637	0.0000									
07VGNF5ZA	0.0452	0.0632	0.0599	0.0344	0.0596	0.0526	0.0560	0.0595	0.0488	0.0344	0.0451	0.0561	0.0669	0.0528	0.0599	0.0453	0.0594	0.0673	0.0592	0.0416	0.0634	0.0000								
07VGNF6ZA	0.0452	0.0632	0.0599	0.0344	0.0596	0.0526	0.0560	0.0595	0.0488	0.0344	0.0451	0.0561	0.0669	0.0528	0.0599	0.0453	0.0594	0.0673	0.0592	0.0416	0.0634	**0.0000**	0.0000							
07VGNF7ZA	0.0274	0.0379	0.0488	0.0309	0.0522	0.0416	0.0379	0.0559	0.0453	0.0170	0.0274	0.0451	0.0486	0.0454	0.0524	0.0275	0.0343	0.0561	0.0487	0.0275	0.0595	0.0450	0.0450	0.0000						
07VGNF8ZA	0.0274	0.0379	0.0488	0.0309	0.0522	0.0416	0.0379	0.0559	0.0453	0.0170	0.0274	0.0451	0.0486	0.0454	0.0524	0.0275	0.0343	0.0561	0.0487	0.0275	0.0595	0.0450	0.0450	**0.0000**	0.0000					
07VGNF9ZA	0.0527	0.0634	0.0748	0.0489	0.0710	0.0676	0.0710	0.0705	0.0676	0.0489	0.0598	0.0454	0.0671	0.0639	0.0068	0.0526	0.0526	0.0603	0.0635	0.0602	0.0783	0.0672	0.0672	0.0597	0.0597	0.0000				
07VGNF10ZA	0.0417	0.0489	0.0454	0.0453	0.0600	0.0455	0.0599	0.0563	0.0564	0.0311	0.0454	0.0488	0.0561	0.0457	0.0564	0.0276	0.0488	0.0603	0.0381	0.0529	0.0451	0.0599	0.0599	0.0417	0.0417	0.0565	0.0000			
07VGNF13ZA	0.0417	0.0489	0.0454	0.0453	0.0600	0.0455	0.0599	0.0563	0.0564	0.0311	0.0454	0.0488	0.0561	0.0457	0.0564	0.0276	0.0488	0.0603	0.0381	0.0529	0.0451	0.0599	0.0599	0.0417	0.0417	0.0565	**0.0000**	0.0000		
07VGNF12ZA	0.0311	0.0601	0.0492	0.0456	0.0565	0.0384	0.0456	0.0639	0.0528	0.0312	0.0491	0.0529	0.0638	0.0457	0.0604	0.0420	0.0417	0.0641	0.0491	0.0386	0.0600	0.0528	0.0528	0.0347	0.0347	0.0604	0.0456	0.0456	0.0000	
07H06ZA	0.0598	0.0633	0.0673	0.0561	0.0746	0.0674	0.0746	0.0708	0.0635	0.0488	0.0597	0.0634	0.0670	0.0638	0.0599	0.0525	0.0597	0.0602	0.0631	0.0595	0.0671	0.0704	0.0704	0.0524	0.0524	0.0599	0.0600	0.0600	0.0677	0.0000

Figure 2.3a: Intra-genetic variability analysis of protease nucleotide HIV-1 subtype C isolates. Analysis was done with BioEdit making using of the Kimura 2-parameter method. Sample pairs with genetic distances indicating identity are shown in bold face. The numbers on the horizontal axis represent the corresponding scales at the vertical axis.

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	26	27	28	29	30	
07MAD5ZA	0.0000																													
07MAD8ZA	0.0556	0.0000																												
07MAD13ZA	0.0676	0.0679	0.0000																											
07MAD15ZA	0.0484	0.0618	0.0665	0.0000																										
07MAD16ZA	0.0727	0.0791	0.0863	0.080	0.0000																									
07MAD18ZA	0.0461	0.0570	0.0630	0.0521	0.0792	0.0000																								
07MAD19ZA	0.0588	0.0590	0.0674	0.0650	0.0774	0.0542	0.0000																							
07MAD20ZA	0.0739	0.0828	0.0688	0.0802	0.0965	0.0779	0.0699	0.0000																						
07MAD22ZA	0.0495	0.0460	0.0665	0.0519	0.0728	0.0509	0.0529	0.0813	0.0000																					
07MAD23ZA	0.0650	0.0712	0.0773	0.0676	0.0812	0.0580	0.0660	0.0811	0.0614	0.0000																				
07MAD26ZA	0.0677	0.0727	0.0703	0.0690	0.0867	0.0632	0.0651	0.0800	0.0665	0.0714	0.0000																			
07MAD27ZA	0.0458	0.0590	0.0639	0.0518	0.0689	0.0484	0.0504	0.0713	0.0493	0.0625	0.0591	0.0000																		
07BBD1ZA	0.0448	0.0509	0.0579	0.0508	0.0641	0.0485	0.0505	0.0665	0.0483	0.0542	0.0593	0.0458	0.0000																	
07BBD2ZA	0.0736	0.0702	0.0651	0.0775	0.0824	0.0776	0.0673	0.0812	0.0675	0.0858	0.0787	0.0698	0.0662	0.0000																
07BBD4ZA	0.0543	0.0665	0.0652	0.0544	0.0766	0.0522	0.0577	0.0764	0.0520	0.0459	0.0617	0.0554	0.0508	0.0823	0.0000															
07BBD10ZA	0.0472	0.0462	0.0579	0.0484	0.0753	0.0486	0.0530	0.0714	0.0448	0.0615	0.0568	0.0447	0.0460	0.0639	0.0555	0.0000														
07BBD13ZA	0.0616	0.0679	0.0543	0.0652	0.0828	0.0594	0.0603	0.0617	0.0567	0.0640	0.0704	0.0567	0.0556	0.0701	0.0569	0.0556	0.0000													
07BBD15ZA	0.0591	0.0628	0.0627	0.0627	0.0802	0.0579	0.0662	0.0752	0.0591	0.0687	0.0714	0.0458	0.0544	0.0750	0.0568	0.0568	0.0592	0.0000												
07BBD16ZA	0.0494	0.0677	0.0712	0.0639	0.0827	0.0581	0.0637	0.0701	0.0676	0.0712	0.0665	0.0578	0.0484	0.0810	0.0651	0.0555	0.0641	0.0591	0.0000											
07BBD18ZA	0.0482	0.0627	0.0687	0.0518	0.0702	0.0484	0.0540	0.0774	0.0435	0.0506	0.0556	0.0481	0.0400	0.0686	0.0507	0.0447	0.0506	0.0578	0.0566	0.0000										
07VGNF1ZA	0.0714	0.0789	0.0616	0.0703	0.0891	0.0705	0.0712	0.0738	0.0641	0.0738	0.0558	0.0627	0.0616	0.0725	0.0617	0.0616	0.0641	0.0641	0.0726	0.0628	0.0000									
07VGNF2ZA	0.0542	0.0665	0.0738	0.0641	0.0815	0.0592	0.0674	0.0876	0.0556	0.0639	0.0728	0.0578	0.0616	0.0774	0.0605	0.0532	0.0665	0.0675	0.0651	0.0542	0.0752	0.0000								
07VGNF5ZA	0.0490	0.0591	0.0589	0.0602	0.0776	0.0509	0.0576	0.0687	0.0506	0.0590	0.0591	0.0529	0.0458	0.0662	0.0412	0.0506	0.0543	0.0554	0.0613	0.0446	0.0518	0.0555	0.0000							
07VGNF07ZA	0.0540	0.0630	0.0703	0.0581	0.0742	0.0533	0.0602	0.0766	0.0472	0.0604	0.0619	0.0519	0.0461	0.0665	0.0568	0.0508	0.0606	0.0569	0.0605	0.0448	0.0656	0.0580	0.0580	0.0000						
07VGNF8ZA	0.0544	0.0653	0.0727	0.0556	0.0766	0.0557	0.0626	0.0790	0.0496	0.0627	0.0643	0.0543	0.0485	0.0688	0.0592	0.0532	0.0629	0.0592	0.0629	0.0471	0.0679	0.0628	0.0603	0.0045	0.0000					
07VGNF10ZA	0.0678	0.0765	0.0739	0.0728	0.0890	0.0642	0.0700	0.0789	0.0752	0.0785	0.0728	0.0615	0.0653	0.0687	0.0753	0.0654	0.0704	0.0714	0.0764	0.0689	0.0715	0.0777	0.0664	0.0655	0.0678	0.0000				
07VGNF11ZA	0.0517	0.0640	0.0639	0.0590	0.0727	0.0521	0.0552	0.0701	0.0542	0.0650	0.0604	0.0505	0.0483	0.0772	0.0530	0.0519	0.0603	0.0578	0.0554	0.0505	0.0628	0.0591	0.0505	0.0532	0.0555	0.0738	0.0000			
07VGNF12ZA	0.0603	0.0617	0.0556	0.0689	0.0826	0.0617	0.0602	0.0652	0.0628	0.0699	0.0665	0.0603	0.0603	0.0639	0.0604	0.0569	0.0533	0.0616	0.0639	0.0615	0.0642	0.0689	0.0543	0.0593	0.0617	0.0666	0.0639	0.0000		
07VGNF13ZA	0.0541	0.0579	0.0687	0.0591	0.0787	0.0520	0.0492	0.0725	0.0471	0.0637	0.0651	0.0588	0.0447	0.0649	0.0542	0.0542	0.0520	0.0590	0.0637	0.0458	0.0638	0.0662	0.0517	0.0519	0.0543	0.0640	0.0565	0.0543	0.0000	
07H0601ZA	0.0507	0.0629	0.0628	0.0556	0.0753	0.0414	0.0494	0.0763	0.0495	0.0579	0.0604	0.0506	0.0460	0.0700	0.0496	0.0484	0.0580	0.0543	0.0518	0.0447	0.0593	0.0555	0.0447	0.0461	0.0485	0.0691	0.0482	0.0580	0.0494	0.0000

Figure 2.3b: Intra-genetic variability analysis of reverse transcriptase nucleotide HIV-1 subtype C isolates. Analysis was done with BioEdit making using of the Kimura 2-parameter method. The numbers on the horizontal axis represent the corresponding scales at the vertical axis.

2.3.5 Drug resistance genotyping

The presence of mutations indicating drug resistance was determined by submission of nucleotide sequences to the Stanford HIV drug resistance interpretation algorithm. The interactive programme also provided information on the quality of the sequences in terms of the presence of stop codons, frameshift, and unusual arrangement of coding nucleotides. According to the programme all sequences were of HIV-1 subtype C, reflecting the results obtained from the phylogenetic analysis.

No major resistance mutations were observed among the protease sequences. However, the following minor resistant mutations were noted: L10I/V (3/30), (07MAD20ZA, 07BBD10ZA, 07VGNF9ZA); A71T (1/30) (07BBD1ZA), T74S (2/30) (07MAD22ZA, 07BBD4ZA). Other mutations known to contribute to resistance to one or more protease inhibitors and their frequency of occurrence are shown in Table 2.2.

The nucleoside reverse transcriptase inhibitor (NRTI) resistance mutation V118I was noted in one sample (07VGNF1ZA). This sample also harboured V179D a non-nucleoside reverse transcriptase inhibitor (NNRTI) resistance mutation. K103N a mutation coding for high level resistance to the NNRTI nevirapine, delavirdine, and efavirenz was observed in two samples (07VGNF2ZA and 07MAD5ZA). Overall, 3/31 reverse transcriptase sequences carried major resistant mutations.

No isolate had both protease and reverse transcriptase inhibitor mutations. Figure 2.4a and 2.4b show alignments of the protease and reverse transcriptase amino acid sequences and positions of the resistance mutations observed.

Table 2.2: Secondary mutations and or polymorphisms associated with PI observed among HIV-1 subtype C isolates from Limpopo Province, South Africa (Stanford HIV drug resistance database interpretation programme)

Mutations	Frequency	Resistance potential
T12S	15/30	
I15V	27/30	
G16E	1/30	Highly polymorphic, occurs in 2% to 20% of viruses depending to subtype. May be weakly associated with ATV therapy and response.
L19I/V/T	29/30	
E35D	10/30	
M36I/L	30/30	Weakly associated with PIs. Common in non-subtype B viruses.
H69K	30/30	Highly polymorphic and weakly associated with virologic response to TPV.
L89M/I	24/30	
I93L	29/30	Polymorphism common in persons receiving PIs
N37D	2/30	
R41K	24/30	
R57K	2/30	
K14R/I	2/30	
N37S/T	2/30	
L63S/P/V/F/T/A	19/30	Polymorphism more common in persons receiving PIs.
G68E	1/30	
K20R/M	6/30	Weakly associated with resistance to each of the PIs when present with other mutations. Occurs mainly in subtype B viruses.
I72M	1/30	
D60E	3/30	Slightly more common in viruses from PI-treated compared with untreated persons.
G17D	1/30	
Q61N/E	2/30	
V77I	2/30	Polymorphism associated with NFV therapy.
L97T	2/30	
N98K	1/30	
K45R	2/30	
I64L	1/30	
V82I	1/30	Is common in some non-subtype B; has a little if any effect on PI susceptibility.
P39Q	1/30	

```
             5          15         25         35         45         55         65         75         85         95
Consensus_B  PQITLWQRPL VTIKIGGQLK EALLDTGADD TVLEEMNLPG RWKPKMIGGI GGFIKVRQYD QILIEICGHK AIGTVLVGPT PVNIIGRNLL TQIGCTLNF
Consensus_C  .......... .S..V...I. .......... .....I.... K......... .......... ........K. .......... ........M. ..L......
Consensus    .......... .S..V...I. .......... .....I.... K......... .......... ..?.....K. .......... ........M. ..L......
07MAD1ZA     .......... .S..VE..I. .......... ....DI.... .......... .......... ........K. .......... ........M. ..L......
07MAD5ZA     .......... ....V...I. .......... .....ID... K......... .......... ........K. .......... ........I. ..L......
07MAD9ZA     .......... ....V...I. .......... ....DI.... .......... ......K... ........K. .......... ........M. .........
07MAD15ZA    .......... ....V...I. .......... .....I.... K......... .......... ........K. .......... .......... ..L......
07MAD17ZA    .......... .S.RV...I. .......... .....LS... K......... .......... ..S....EK. .......... ........M. ..L......
07MAD18ZA    .......... .S..V...I. .......... ....DL.... K......... .......... ..P.....K. .......... ........M. ..L......
07MAD19ZA    .......... .S..V...I. .......... .....I.... K......... .......... ........K. .......... ........M. ..L......
07MAD20ZA    .........I ........V. .......... .....I.... K......... .......... ..S.....K. .......... .......... ..L......
07MAD22ZA    .......... .S..V...I. .......... ....DID... .......... .......... ..V.....K. ...S...... ........M. ..L......
07MAD22ZA    .......... .S..V...I. .......... ....DID... .......... .......... ..V.....K. ...S...... ........M. ..L......
07MAD23ZA    .......... .S..V...I. .......... .....I.... K......... .......... ........K. .......... ........M. ..L......
07MAD26ZA    .......... .S.IV...VR .......... .....I.... K......... .......... ........K. ..M....... ........M. ..L......
07MAD28ZA    .......... ........I. .......... .....I.... K......... .........E ........K. .......... ........M. ..L......
07BBD1ZA     .......... ....V.D.V. .......... .....IT... .......... .......... ..P.....K. .T........ ........I. ..L......
07BBD4ZA     .......... ....V..... .......... .....I.... K......... .......... N.P.....K. ...S...... ........M. ..L......
07BBD10ZA    .........V ....V...IR .......... .....I.... K......... .......... ..F.....K. .......I.. ........M. ..L...TK.
07BBD13ZA    .......... ........I. .......... .....L.... K......... .......... ........K. .......... ........M. ..L......
07BBD15ZA    .......... .S..V...IR .......... ....DI.... K......... .......... ........K. .......... ........M. ..L......
07BBD16ZA    .......... .S..V...I. .......... .....I.... K...R..... ......K... ..PL....K. .......... ........M. ..L......
07BBD18ZA    .......... .S..V...I. .......... .....I.... .......... .......... ..V.....K. .......... ........M. ..L......
07VGNF1ZA    .......... ....V...I. .......... ....DI.... .......... .........E ..P.....K. .......... ........M. ..L......
07VGNF9ZA    .........V ....V...IR .......... .....I.... K......... .......... ..S.....K. .......I.. ........M. ..L......
07VGNF12ZA   .......... .S..V...I. .......... ....DI..Q. K......... .......... E.P.....K. .......... ........M. ..L......
07BBD1ZA     .......... ....V.D.V. .......... .....IT... .......... .......... ..P.....K. .T........ ........I. ..L......
07VGNF5ZA    .......... .S..V...V. .......... .....I.... K......... .......... ..V.....K. .......... .......... ..L......
07VGNF6ZA    .......... .S..V...V. .......... .....I.... K......... .......... ..V.....K. .......... .......... ..L......
07VGNF7ZA    .......... .S..V...I. .......... .....I.... K......... .......... ........K. .......... ........M. ..L......
07VGNF8ZA    .......... .S..V...I. .......... .....I.... K......... .......... ........K. .......... ........M. ..L......
07VGNF10ZA   .......... ....V...TM .......... ....DI.... K......... .........E ..P.....K. .......... ........M. ..L......
07VGNF13ZA   .......... ....V...TM .......... ....DI.... K......... .........E ..P.....K. .......... ........M. ..L......
07H061ZA     .......... ....V...I. .......... .....I.... K...R..... .......... ..A.....K. .......... ........M. ..L......
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Figure 2.4a: Amino acid aligment of the protease gene of HIV-1 Subtype C primary isolates from Limpopo province, South Africa. The consensus of test samples (Consesnus) was identical to the that of the global HIV-1 subtype C (Consensus_C), but different from the global subtype B consensus (Consensus_B) at 8 positions viz T12S, I15V, L19I, M36I, R41K, H69K, L89m and I93L. Note that the active site between positions 25-27 is conserved for all the isolates.

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                5          15         25         35         45         55         65         75         85         95         105        115        125        135        145
CONSENSUS_B  PISPIETVPV KLKPGMDGPK VKQWPLTEEK IKALVEICTE MEKEGKISKI GPENPYNTPV FAIKKKDSTK WRKLVDFREL NKRTQDFWEV QLGIPHPAGL KKKKSVTVLD VGDAYFSVPL DKDFRKYTAF TIPSINNETP GIRYQYNVLP
Consensu_C   .......... .......... .......... ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .EG....... .......... ..........
Consensus    .......... .......... .......... ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .E?....... .......... ..........
07MAD5ZA     .......... .......... .......... ....TA..D. .......... .......... .......... .......... .......... .......... ..N....... .......... .EN....... .......... ..........
07MAD8ZA     .......... .......... .......... ....T...A. .......T.. .......... .......... .......... .......... .......... .......... .......... .EG....... .......... ..........
07MAD13ZA    .......... .......... .......... ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .EG....... .......... ..........
07MAD15ZA    .......... .......... .......K.. .E..TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .......... .......... ...LI.....
07MAD16ZA    .......... .......... .......... .....A..E. .......E.. .......... .......... .......... .......... .......... .......... .......... .ES....... .......... ..........
07MAD18ZA    .......... .......... .......... ....T...E. .......T.. .......... .......... .......... .......... .......... .......... .......... .E........ .......... ..........
07MAD19ZA    .......... .......... .......... ....TA..ED .......T.. .......... .......... .......... .......... .......... .......... .......... .EG....... .......... ..........
07MAD20ZA    .......... .......... .......... ....TA..ED .......T.. .........I .......... .......... .......... .......... .......... .......... .E........ ........M. ..........
07MAD22ZA    ......I... .......... .......... ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .EG....... ....V..... ..........
07MAD23ZA    .......... .......... .......... ....T...K. .......T.. .........I .......... .......... .......... .......... .......... .......... HEE....... .......... ..........
07MAD26ZA    .......... .......... .......... ....TA..A. .......T.. .......... .......... .......... ........S. .......... .......... ........I. .EN....... .......A.. ..........
07MAD27ZA    ...S...... .......... .......A.. ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .ES....... .......... ..........
07BBD1ZA     .......... .......... .......... ....KA..E. .......T.. .........I .......... .......... .......... .......... .......... .......... .E........ .......... ..........
07BBD2ZA     ...H...... .......... .......... ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .ES....... .......... ..........
07BBD4ZA     .......... .......... .......S.. ....TA..E. .......T.. .........I .......... .......... .......... .......... .......... .......... HEE....... .......... ..........
07BBD13ZA    .......... .......... .......... ....TA..E. ..*....T.. .......... .......... .......... .......... .......... .......... .......... .E........ .....T.... ..........
07BBD15ZA    .......... .......... .......... ....T...E. .......T.. .......... .......... .......... .R........ .......S.. .......... .......... .EN....... .......... ..........
07BBD16ZA    .......... .......... .......... ....TA..E. .......... .......... .......... .......... .........I .......... .......... .......... .VN....... .......... ..........
07BBD18ZA    .......... .......... .......... ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .E........ .......... ..........
07VGNF1ZA    ....D..... .........R .......... ....TA..E. .......T.. .........I .......... .......... .......... .......... .......... ........I. .EN....... .......A.. ..........
07VGNF2ZA    .......... .......... .......... ....T...E. .......... .......... .......... .......... .........I .......... ..N....... .......... .ES....... .......... ..........
07VGNF5ZA    .......... .......... .......... ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .E........ .......... ..........
07VGNF07ZA   ....A..... .......... .......... ....T...K. .......T.. .......... .......... .......... .......... .......... .......... .......... .EN....... .......... ..........
07VGNF8ZA    ....A..... .......... .......K.. ....T...K. .......T.. .......... .......... .......... .......... .......... .......... .......... .EN....... .......... ..........
07VGNF10ZA   .......... .......... .......... ....T...E. .......T.. .......... .......... .......... .......... .......... ..Q....... .......... .EN....... .......A.. ..........
07VGNF11ZA   .......... .......... .......... ....TA..D. .......T.. .........I .......... .......... .........I .......... .......... .......... .EG....... .......... ..........
07VGNF12ZA   .......... .......... .......... ....T...E. .......T.. .......... .......... .......... .......... .......... .......... .......... .ES....... .......... ..........
07VGNF13ZA   .....D.... .......... .......... ....TA..E. .......T.. ..D....... .......... .......... .R........ .......... .......... .......... .E........ .......A.. ..........
07H0601ZA    .......... .......... .......... ....TA..E. .......T.. .......... .......... .......... .......... .......... .......... .......... .EN....... .......... ..........

                155        165        175        185        195        205        215        225        235        245        255        265        275        285        295
CONSENSUS_B  QGWKGSPAIF QSSMTKILEP FRKQNPDIVI YQYMDDLYVG SDLEIGQHRT KIEELRQHLL RWGFTTPDKK HQKEPPFLWM GYELHPDKWT VQPIVLPEKD SWTVNDIQKL VGKLNWASQI YAGIKVKQLC KLLRGTKALT EVIPLTEEAE
Consensu_C   .......... .......... ..A...E... .......... .........A ......E... K......... .......... .......... ....Q..... .......... .......... .P....R... .....A.... DIV.......
Consensus    .......... .......... ..?...E... .......... .........A ......E... K......... .......... .......... ....Q..... .......... .......... .P....R... .....A.... DIV.......
07MAD5ZA     .......... .....R.... ..E...EM.. .......... .........A ......E... K......... .......... .......... ..A.Q..... .......... .......... .P....R... .....A.... DIV.......
07MAD8ZA     .......... .....R.... ..T...E... .......... .........A ......N... K......... .......... .......... ....Q..T.. .......... .......... .P....R... .......... D.V..N....
07MAD13ZA    .......... .......... ..IK...... .......... .........A ......E... K......... .......... .......... ....Q..D.E D......... .......... .P........ .......... ..V.......
07MAD15ZA    .......... .....R.... ..EK..EV.. .......... .........A ......E... .......... .......... .......... ....Q..... .......... .......... .......... .....A.... DIV.......
07MAD16ZA    .......... .....R.... ..T...E... .......... .........A ......E... .......... .......... .......... ....Q..... .......... .......... .P...GRPVE FPSEQA.... ..V.......
07MAD18ZA    .......... .......... ..E...E... .......... .........A ...K..E... .......... .......... .......... ....Q....E .......... .......... .P....R... ..I..V.... DIV.......
07MAD19ZA    .......... .....R.... ..T...E... .......... .........A ......E... K..L...... .......... .......... ....Q..... .......... .......... .P....R... .....A.... DI........
07MAD20ZA    .......... .......... ..AK...... .......... .......... .V....K... K......... .......... .......... ....Q..N.. .......... .......... .P.....Y.. R....A.... DIV.......
07MAD22ZA    .......... .......... ..T...E... .......... .........A ......N... .......... .......... .......... ....Q..... .......... .......... .K..Q.R... .......... DIV.......
07MAD23ZA    .......... .......... ..A...E... .......... .........A ......E... .......... .......... .......... ....Q..... .......... .......... .P...T.G.. .....A.... DIV.......
07MAD26ZA    .......S.. .......... ..TS..E... .......... T........A ......E... ...L...... .......... .......... ....Q.A... .......... .......... .P..R..H.. .....A.... DIV.......
07MAD27ZA    .......... .......... ..A...E... .......... .........A ......D... .......... .......... .......... ....Q..... .......... .......... .S........ .....A.T.. DIV.......
07BBD1ZA     .......... .....R.... ..A...E... .......... ....T....A ......E... K......... .......... .......... ....Q..... .......... .......... .P....R... .....A.... DIV.......
07BBD2ZA     .......S.. .....R.... ..T....... .......... .........A .V....I... K......... .......... .......... ....L..... .......... .......... .P....R... .......... D.V.......
07BBD4ZA     .......... .......... ..TK..E... .......... .........A ......E... .......... .......... .......... ....K..... .......... .......... .P...T.... .....A.... DI........
07BBD13ZA    .......... .......... ..AK..E... .......... .........A ......E... K......... .......... .......... ....Q..D.E .......... .......... ....Q..... .....A.... DIV.......
07BBD15ZA    .......... .......... ..AK..E.I. .......... .........A ......D... K......... .......... .......... ....Q....E D......... .......... .P........ R......... .IV.......
07BBD16ZA    .......... .......... ..DR...L.. .......... .........A ......E... K......... .......... .......... ....Q..Q.. .......... .......... .P....R... .....A.... DIV.......
07BBD18ZA    .......... .......... ..A...E... .......... .........A ......E... .......... .......... .......... ....Q..... .......... .......... ......R... .....A.... DIV.......
07VGNF1ZA    .......... .......... ..MK....D. .......... .......L.A ..N...D... ...L...... .......... .......... ....Q..D.. V......... .......... .P........ .....A.... DIV.......
07VGNF2ZA    .......... .C........ ..A...EL.. .......... ......EK.A ......E... .......... .......... .......... ....Q....E .......... .......... .P........ ....K.A... DIV.......
07VGNF5ZA    .......... .......... ..TK...M.. .......... .........A ......E... .......... .......... .......... ....Q..D.. .......... .......... .P....R... .......... DIV.......
07VGNF07ZA   .......... .......... ..A...E... .......... .......K.A ......E... K......... .......... .......... .......... .......... .......... ......R... .....A.... DIV.......
07VGNF8ZA    .......... .......... ..A...E... .......... .........A ......E... K......... .......... .......... .......... .......... .......... ......R... .....A.... DIV.......
07VGNF10ZA   .......... .......... ..A....... .......... .........A ......E... K..L...... .......... .......... ....L..... .......... .......... .P..R.RN.. ..I..A.... DIV.......
07VGNF11ZA   .......... .......... ..AK..E... .......... ......LK.A ......E... K......... .......... .........I ....Q..... .......... .......... .S....R... .....A.... DIV.......
07VGNF12ZA   .......S.. .......... ..NK..E... .......... .........A ......G... K......... .......... .......... ....Q..... .......... .......... .P....R... .......... DIV.......
07VGNF13ZA   .......... .......... ..A...E... .......... .........A ......T... K......... .......... .......... ....Q..... .......... .......... .P....R... .....A.... DI........
07H0601ZA    .......... .C........ ..VK..EL.. .......... .........A ......E... K......... .......... .......... ....Q..... .......... .......... .P....R... .....A.... DIV.......
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Figure 2.4b: Alignment of amino acids of the reverse transcriptase of HIV-1 subtype C primary isolates from Limpopo Province, South Africa
The consensus of the test sequences was identical to the global subtype C consensus except at positions 123 and 173 were there was majority residue at a threshold of 50% among the test sequences. However, there were differences between the test consensus and subtype B consensus at 17 positions namely V35T, E36A, T39E, S47T, K122E, D123G, K173A, T200A, Q207E, R211K, V245Q, A272P, K277R, T286A, E291D, V292I, I293V.

2.4 Discussion and conclusion

Treatment with antiretrovirals will inevitably lead to the selection of resistant virus mutants as with most antimicrobials. In addition, since 2004 more and more HIV patients have access to treatment through the public health system. It is therefore expected that the development of resistant variants may lead to their transmission. Information on whether resistant viruses are being transmitted among a population is crucial for drug policy formulation.

This study set out to characterize mutations indicating resistance to antiretrovirals, notably protease and reverse transcriptase inhibitors, in drug naïve chronic HIV infected patients from the Limpopo Province, in the northeastern part of South Africa. Study participants were recruited from Musina, Bela Bela, and Thohoyandou. Viruses have been previously characterized from Bela Bela (Bessong *et al*, 2006,2005) but no study has examined sequences from Musina and Thohoyandou.

Phylogenetic analysis of the protease and reverse transcriptase nucleotide sequences showed that all the viruses examined were HIV-1 subtype C as they clustered with references 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 (Gordon *et al*, 2003; Papathanasopoulos *et al.*, 2003). The phylogeny also revealed that the viruses most probably did not come from a single parental strain, as no sub-clusters were observed.

The genetic variability based on the protease and reverse transcriptase genes of the test isolates showed very little differences among the isolates (figure 2.3a and 2.3b). It was noted that 3 pairs of samples 07VGNF5ZA and 07VGNF6ZA, 07VGNF7ZA and 07VGNF8ZA, 07VGNF10ZA and 07VGNF13ZA did not show any variability among their protease sequences. The observance of sequences with zero mean genetic distance may mean that there was contamination, or that the samples are epidemiologically linked. However, the same samples did not show zero mean genetic distance values in the phylogenetic analysis of reverse transcriptase gene as in the analysis of the protease sequences. Since the same DNA fragment was used to sequence the protease and reverse transcriptase regions, this means that if there was contamination, the pairs of samples that appear to be identical in protease should also have been identical in the reverse transcriptase. But this is not the case. Therefore, it is difficult to explain this observation from the contamination point of view. It is also unlikely that the patients are epidemiologically linked since, as mentioned earlier, the analysis of the reverse transcriptase genes does not show this observation. The most likely explanation is that the sequencing reactions for the protease gene for three of the samples were repeated but with a clerical error during labeling.

The viruses investigated were from HIV infected chronic individuals who have not been receiving antiretrovirals according to information obtained from the questionnaires. Upon analysis of the protease gene for resistance mutations coding for resistance, no primary resistance mutation was noted. However, minor mutation resistance mutations such as L10I/V, A71T, and T74S were observed with a frequency of 3/30, 1/30, 2/30 respectively. A71T polymorphisms occur in 1-2% of untreated persons but become more frequent in persons receiving PIs. T74S is associated with reduced NFV susceptibility. It occasionally occurs in untreated persons. L10I/V occur common in 5-10% of untreated persons and is also associated with each of the PIs when present with other mutations. In addition, several mutations known to be associated with resistance were observed. The presence of several mutations in the protease gene that may contribute to drug resistance has been reported and reviewed by several investigators (Grossman *et al*., 2004; Kantor and Katzenstein, 2004). The phenotypic significance of these mutations and other putative resistance coding mutations in particular when several of them occur in one sequence has also been studied in several systems (Holquin *et al*., 2004, Velazquez-Campoy *et al*., 2002). However, it is becoming evident that a standard approach to evaluate putative resistance mutations especially those found in HIV-1 subtype C viruses, the most common variant worldwide (Siddapa *et al*., 2004; Hemelaar *et al.*, 2006) should be adopted to provide better insights into the significance of these mutations in terms of treatment outcomes.

Examination of the reverse transcriptase gene for resistance mutations reveal the presence of V118I (1/30), V179D (1/30), K103N (2/30). V118I has been shown to code for resistance to NRTI, while V179D and K103N code for resistance to NNRTI. Of particular note is the presence of K103N in 2 patients. K103N confers high level resistance to nevirapine, delavirdine, and efavirenz, and potential low level resistance etravirine. Efavirenz is a component of the first line regimen in South Africa for the treatment of AIDS (D4T/3TC/EFV or NVP). Nevirapine replaces efavirenz in this regimen for pregnant women. The two cases of K103N were found in females. Although at the time of sample collection these women were not pregnant and were not on antiretroviral therapy, it is possible that they received nevirapine during an earlier pregnancy since nevirapine is frequently used as a prophylaxis in the prevention of mother to child transmission of HIV in South Africa. Another possibility is that the K103N mutation arose due to pressure from efavirenz since K103N is selected by efavirenz (Gatanaga *et al.*, 2006; Brenner *et al.*, 2003) and that the patients were subsequally infected with viruses carrying this mutation. It is worthwhile to note that the isolates with the classical resistance mutations were obtained from patients recruited from Musina, a border region with Zimbabwe.

In conclusion, this study has shown that most of the viruses in Limpopo Province, representing the northeastern part of South Africa are HIV-1 subtype C, and that the prevalence of resistance mutations among the drug naïve patients is still low.

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Chapter 3
Construction of molecular clone to phenotype putative resistant mutations

3.1 Introduction

Over the years data has accumulated about the amino acid changes that are related to the development of resistance in HIV-1 subtype B (HIV-1-B), the predominant subtype in North America and Europe. However, regions of the world most hit by the pandemic have non-B subtypes as the common variants. For example, HIV-1-C viruses overwhelmingly predominate in Southern Africa and it is rapidly spreading into Southeast Asia and South America. Overall, subtype C viruses account for more than half of all infections worldwide (Carrion *et al*., 2004). As a result, more understanding is needed on the significance of unknown mutational patterns associated with drug resistance loci in HIV-1-C *PR* and *RT*. Regarding South Africa, earlier studies have indicated the presence of minor, atypical mutations and polymorphisms on positions associated with resistance (Pillay *et al*., 2002; Gordon *et al*., 2003; Bessong *et al*., 2005). The impact of minor resistance mutations and single or multiple candidate drug resistance mutations in HIV subtype C viruses on the propensity to develop resistance once drug pressure is applied is relatively not well described. K20R and M36I in the *PR,* and A98S in the *RT* have been reported to occur significantly more often in subtype C than in HIV-1-B viruses examined from drug inexperienced individuals and most subtype C viruses show significant differences from subtype B sequences in other protease sites (Grossman *et al*., 2001; De Oliveira *et al*., 2003).

Protease inhibitors are generally employed in second line treatment regimens in South Africa. However, due to the fact that some patients may experience virologic failure with the first line regimen comprising RTI, in addition to children who get PI as first line treatment, the use of protease inhibitors is likely to increase. As described in the preliminary results section, K20R, M36I, L63P/A/S/T/V and I93L occurred in high frequencies in drug naïve patients in Limpopo. In a study comparing treatment outcomes in Israel with nelfinavir, a significant accumulation of M36I, M63P and I93L substitutions in patients infected with HIV-1-C than with HIV-1-B was found, and these mutations correlated with reduced replicative capacities. In addition, it was noted HIV-1-C does not select for D30N mutation upon exposure to nelfinavir as does HIV-1-B (Vergne *et al*., 2003), indicating different mutation evolutionary pathways.

3.1.1 Plausible implications of minor and polymorphic substitutions on drug resistance

Analysis of *RT* regions from viruses of different subtypes have revealed silent mutations, polymorphisms and minor mutations that are implicated in the emergence of resistance to nucleoside and non-nucleoside reverse transcriptase inhibitors. Although these changes do not confer resistance in treatment-naïve patients they facilitate the development of resistance. For example the final drug concentration required for the development of mutations that confer resistance to NNRTI has been shown to be significantly lower for subtype C than for subtype B. Also resistant viruses are fully selected more rapidly with subtype C isolates (Wainberg, 2004). The development of novel resistance mutations has also been reported for C viruses. The A98S secondary mutations, associated with resistance to nevirapine, has been reported in Ethiopian subtype C isolates to facilitate the evolution of S98I which confers classical phenotypic resistance to nevirapine (Brenner *et al*., 2003). A baseline polymorphism at codon 106 in subtype C viruses facilitated the development of a novel V106M mutation that confers resistance to efavirenz as well as other members of the NNRTI class of drugs (Wainberg, 2004). In a preliminary analysis done by Bessong *et al* 2006, revealed a prevalence of 11, 5 and 11% for polymorphisms A98S, V118I and V179I in subtype C viruses from Limpopo.

In determining the thermodynamic and relative binding affinities of HIV-1-C proteases relative to HIV-1-B, Velazquez-Campoy and colleagues Velazquez-Campoy *et al*., 2002, reported that natural occurring polymorphisms have an additive effect to resistance mutations on Gibbs energy and lead to significant alterations in the enthalpy and entropy changes associated with inhibitor binding. HIV-1-C protease with the double substitution V82A/I84V, known to confer resistance to all *PR* inhibitors, had an elevated relative vitality in excess of a 1000 fold when they occurred with natural polymorphisms compared to HIV-1-B with the same amino acid changes. Significant increase in protease vitality was observed in the presence of each of indinavir, saquinavir, nelfinavir and ritonavir. The investigated polymorphic changes are found outside of the binding site, indicating that amino acid polymorphisms need not be in the active site to influence binding thermodynamics of inhibitors and amplify the effects even of drug-resistant mutations. This means there are potential significant consequences on the efficacy of current protease inhibitors.

Holguin *et al.*, 2004 investigating the impact of natural polymorphism in HIV-1 non-B subtypes using an *in vitro* recombinant system did not find reduced susceptibilities of HIV-1-C isolates from India to indinavir, ritonavir, saquinavir, nelfinavir, amprenavir and lopinavir, when double and triple amino acids substitutions such as K20R/M36I, M36V/L63P/T, M36I/L63P/S, M36I/L63/S/T and M36I/L63P/V82V/I were taken into account However, chimeric viruses were constructed using a subtype B backbone, and it is not clear if the results would have been different if a subtype C backbone was employed. Polymorphisms may have compensatory roles such as contributing to viral replication efficiency as has been described for L63P under drug pressure (Sune *et al.*, 2004). We have observed this polymorphism in 22% of viruses from Limpopo. Over 80% of HIV-1-C drug naïve isolates harbor the M36I substitution, and Clemente *et al.*, 2003 have shown that M36I and also A71V, when present as natural polymorphisms, could aid the virus in developing active site mutations to escape inhibitor binding while maintaining catalytic efficiency. These initial studies suggest that M36I and the different substitutions at position 63 contribute in enhancing the fitness of HIV-1-C variants. The plausible implication of the effect of multiple polymorphisms on the evolution of resistant viruses is highlighted in the virologic failure in a South African drug naïve patient to a lopinavir/ritonavir containing regimen. Prior to therapy the patient's sequence showed M36I, L63P and I93L. Upon virologic failure other polymorphisms/minor mutations L33F, I54V and V82A were identified. No signature mutations could be associated with the virologic failure, except the accumulation of polymorphisms and minor mutations. The patient had adhered to therapy (Conradie *et al.*, 2004).

3.1.2 Study rationale

In the backdrop of the natural polymorphic nature of HIV-1-C *PR* and *RT* in drug inexperienced patients, the question to answer is whether these substitutions affect viral susceptibility to *PI* and *RTI*, and to what extent does this baseline profile affect the development of resistant viruses. Do these polymorphisms impact evolution of drug resistance and probably lead to a rapid treatment failure? Available systems to evaluate the significance of resistance related mutations are based on the use of an HIV-1 subtype B backbone. However, as has been shown previously, non HIV-1 subtype B isolates such as subtype C, which account for most of the infections worldwide, have appreciable variability in their sequences compared to subtype B. Although it is assumed that the use of a subtype B backbone in the evaluation of patient sequences of other subtypes it would be best to evaluate mutations in the context of the backbone of the DNA fragment under investigation. For example, using a subtype C backbone to evaluation mutation observed in subtype C viruses.

Different systems in which recombinant viruses are generated by either homologous recombination (Hertogs *et al.*, 1998) or direct cloning (Petropoulos *et al.*, 2000; Klimkait, 2002) have demonstrated important advantages for large-scale applications. In addition to be fast and reproducible methods, shorter culture times minimize the outgrowth of minor variants in comparison with conventional phenotypic assays. Some of these phenotypic assays generate infectious viruses (Hertogs *et al.*, 1998, Klimkait, 2002) whereas others are based in single cycle infection assays (Petropoulos *et al.*, 2000).

The present study describes a novel recombinant phenotypic assay that combines two strategies: the generation of recombinant viruses that are able to produce multiple cycles of replication, together with cloning of a reporter gene in the viral vector. Both strategies reduce testing time and represent an advantage in the evaluation of viral isolates with decreased fitness. Therefore, the phenotypic assay described in this study, provides a new tool for assessing drug-susceptibility to *PR* and *RT* inhibitors of clade C patient-derived viruses.

3.1.3 Objectives of the study

3.1.3.1 General objectives

The general objectives of this study were to construct chimeric infectious molecular clones and the vector systems of HIV-1 subtype B and C based on pNL4-3 and pMJ4 respectively. The development of clade C system, in parallel with a clade B one, will allow direct comparisons to be made. MJ4 was chosen since it was one of the few clade C molecular clones that is readily available. A vector system that could be used for phenotyping clade C isolates would be an important reagent for the characterization of known and putative drug resistant mutations in the most common subtype worldwide.

3.1.3.2 Specific objectives

The specific objectives were:

1. To construct and evaluate the infectivity of a chimeric clone based on an HIV-1 subtype C backbone with an insert of the 5' portion of the *pol* gene fragment from the NL4-3 molecular clone.

2. To construct and evaluate the infectivity of a chimeric clone based on HIV-1 subtype B backbone with an insert of the 5' portion of the *pol* gene fragment from MJ4 molecular clone.

3. To construct and evaluate the infectivity of a vector system capable of carrying a *pol* DNA fragment of HIV obtained from clade B or clade C infected patients.

4. To evaluate the susceptibility of vector system to selected protease and reverse transcriptase inhibitors.

3.2 Materials and Methods

3.2.1 HIV infectious molecular clones used in this study

The infectious molecular clones pMJ4 (Ndung'u *et al.* 2001) and pNL4-3 (Adachi *et al.*, 1986) were obtained from the AIDS Reagent Reference programme, National Institutes of Health, Bethesda, USA.

In order to modify pMJ4 so that an HIV *pol* gene from a patient can be inserted and analyzed for susceptibility to protease and reverse transcriptase inhibitors, an ApaI restriction site in the plasmid outside of the HIV sequence had to be destroyed. In this way, the infectious clone would contain unique ApaI and HpaI sites flanking and within the *pol* region of the plasmid in a region on which most mutations coding for resistance to anti-HIV drugs have been described. On the other hand, pNL4-3 has no ApaI site because of a silent single base polymorphism. Therefore, an ApaI site was created to also make the clone to have unique ApaI and HpaI sites in *pol*. It was also necessary to remove any additional HpaI site by substituting a portion of the HXB2 for the region of NL4-3. This engineering allows the cloning of DNA fragments as ApaI-HpaI fragments of protease and reverse transcriptase. The clones used in the experiments described subsequently were therefore pMj4ΔApa1-7 (HR3407) and pNL4-3ApaI^{+} (HR3448) and were obtained from the Thaler Center for AIDS and Human Retrovirus Research, University of Virginia, USA (Pascal Bessong, Marie-Louise Hammarskjold, David Rekosh, unpublished data).

3.2.1. Isolation and Analysis of Recombinant Plasmids

3.2.1.1. <u>Plasmid purification (Large Preparation)</u>

The described plasmids, pNL4-3ApaI^{+} and pMj4ΔApaI-7 were grown in large preparation overnight in *Luria Bertani (*LB) medium containing the appropriate selective antibiotic, ampicilin. Ten grams of LB medium was weighed in 2000 ml flask and added 500ml distilled water, autoclaved for 25 min. The medium was cooled in room temperature and 500ul of ampicilin for resistant plasmids was added in 500 ml LB. Then cells frozen stocks were inoculated into the medium by using the inoculation loop. The starter cultures were vigorously shaken overnight at 30°C. To avoid contamination, the inoculation the cell to the medium was done under the flame. The turbidity of the cell cultures simply means the growth of the cells.

The DNA plasmids were purified using the QIAfilter Plasmid Midi and Maxi Purification Kit (Qiagen). The cell cultures were transferred from 2000 ml flask to 500 ml nalgene container. The bacterial cells were harvested by centrifugation at 12000 rpm for 30 min. Supernatants were discarded and the bacterial pellet was resuspended in 10ml resuspension buffer P1. RNase A solution was added at a final concentration of 100ug/ml to buffer P1 before use, to degrade the RNA. The provided LyseBlue reagent was then added to buffer P1 to prevent the common handling errors that lead to inefficient cell lysis and incomplete precipitation of SDS, genomic DNA, and cell debris. For efficient lysis the vessels used where large enough to allow the complete mixing of the lysis buffer. The bacteria were resuspended completely by vortexing or pipetting up and down until no cell clumps remained. The solution was transferred in 50 ml falcon tube. Ten milliliters of Lysis buffer P2 were added to the solution, and mixed thoroughly by inverting the sealed tubes 4-6 times. Mixing by vortexing was not allowed as this may results in shearing of the genomic DNA. The color of the solution turned blue because of the LyseBlue reagent added in buffer P1. The solution was incubated at room temperature for 5min after mixing. Ten milliliters of chilled Neutralization buffer P3 were added to the lysates, and mixed thoroughly by vigorously inverting the tubes 4-6 times or until the suspension is colorless. After addition of buffer P3, the fluffy white material containing genomic DNA, proteins and cell debris was formed. Precipitation was enhanced by addition of buffer P3 to the lysates. Centrifugation at 12000 rpm for 30 min at 4°C was done. The QIAGEN-tip 500 columns were equilibrated by applying 10 ml Equilibration buffer QBT, and the columns were allowed to empty by gravity flow. The supernatants were filtered into the QIAGEN-tip 500 columns and allowed to enter the resin by gravity flow.

The columns were washed with 2 x 30 ml Washing buffer QC. The first wash was sufficient to remove all contaminants in the majority of plasmid DNA preparations. And the second wash was necessary when large culture volumes of or bacterial strains producing large amount of carbohydrates were used. Plasmid DNA was eluted with 15 ml Elution buffer QF, and the eluates were collected in a 50 ml tubes and the DNA was precipitated by adding 10.5 ml room temperature Isopropanol. The solutions were mixed and centrifuged at 12000 rpm for 30 min at 4°C. The supernatants were carefully decanted, allowing the wash of the pelleted DNA with 5 ml room temperature 70% Ethanol. The supernatants were decanted without disturbing the pellet. Ethanol (70%) removes precipitated salt and replaces isopropanol with the more volatile ethanol; making the DNA easier to redissolve. Then the pellet was air-dried for 5-10 min, and the DNA was redissolved with 200ul TE (10 mM Tris, bring to pH 8.0 with HCl , 1mM Ethylene Diamine Tetraacetic Acid (EDTA)) buffer. The DNA pellet was redissolved by rinsing the walls to recover the entire DNA. Pipetting up and down was not done since it causes shearing of the DNA.

3.2.1.2. <u>Determination of yield (DNA concentration)</u>

To determine the yield, DNA concentration was determined by UV spectrophotometry at 260nm. A UV spectrophotometer was used to measure the fraction of the incident light transmitted through a solution or sample material and, by comparison to the initial intensity of light reaching the sample; it indirectly measured the amount of light absorbed by that sample. For most biological applications however, we measure *absorbance* (Al, also referred to as Optical Density or ODl, where l is the wavelength used for the measurements), the amount of light absorbed by a solution. Absorbance is related logarithmically to transmission thusly. The quantitative DNA was analyzed by running 0.8% agarose gel electrophoresis.

The DNA obtained from the large preparation was used in construction of the infectious chimeric clones. The construction of the proviral clones below starts with the isolation of the parental clones.

3.2.2. Construction of Chimeric Proviral Clones

Several different plasmids were used to construct the two chimeric proviral clones pMj4/NL4-3(HR3874) and pNL4-3/Mj4 (HR3876). Construction of the chimeric clones was carried out from the two HIV-1 clones' pMj4ΔApa1-7(HR3407) and pNL4-3ApaI+ (HR3448). Both these plasmids contain the Apa1-Hpa1 fragments from *gag/pol* gene HIV-1 subtype B and subtype C sequences.

3.2.2.1 Digestion of plasmids

To facilitate subcloning, 0.5ug of pNL4-3ApaI$^+$ and pMj4ΔApaI-7 with the DNA concentration of 0.2334ug/ul and 0.2675ug/ul were completely digested with two restriction enzymes: ApaI and HpaI. Digestion was carried out in 20ul final reation mix of 2ul 10X New England Biolabs (NEB) buffer number 4, 2ul 10x NEB BSA buffer, and 0.5ul 50000 U Apa1 and Hpa1 endonucleases enzymes. These plasmids were digested in 3 hours with ApaI and overnight at 37◦C with HpaI. After 24 hours the digestion mix was run on 0.8% agarose gel electrophoresis to view the expected fragments. Two fragments popped out from both plasmids after cleaving with ApaI/HpaI. MJ4=11kb and 1.6kb, and NL4.3=13kb and 1.6kb respectively.

3.2.2.2 Gel Purification of digestion products

Digestion products were purified using Qiagen Plasmid purification kit (Qiagen, Valentia, USA) and the DNA was excised from the plasmid with appropriate restriction enzymes and purified by agarose gel electrophoresis. The DNA was recovered by using the Freeze-phenol method or the Qiagen gel extraction kit. Freeze Phenol Method- The bands containing DNA fragments were excised from the gel with a clean sharp scalpel and placed in a clean eppendorf tubes. The gel blocks were crushed using the inoculating loop. Add 100ul of TE (10 mM Tris, bring to pH 7.5 with HCl 1 mM Ethylene Diamine Tetraacetic Acid (EDTA)) buffer into the crushed gel. The purpose of TE buffer is to protect DNA or RNA from degradation. Hundred microliters of phenol (pH 8.0) was added and the tubes were vortexed for 10 seconds. Place the solution in -80°C for 5 minutes (min) or until frozen. After 5 min, the tubes were spun down for 10 min at 13000 rpm. The supernatants transfer to a new eppendorf tube. Add 100ul of phenol, vortex for 10 sec and spin at full speed for 2-5 min. The supernatants were transferred to the new tubes and add 100ul chloroform, vortex, spin for 2-5 min and the supernatants were transferred to new tubes. The volume of the supernatants were measured and 1/18 volume of 5Mole of potassium acetate was added to the total volume. And 2 volumes of pure ethanol were added to the supernatant and K-acetate. This mixture was stored at -80°C for 15 min, and spun down at 13000 rpm in 4°C centrifuge for 15 min. The DNA pellet was formed after centrifugation at 4°C. The supernatants were removed from the pellet and saved at -20°C. The DNA pellet was washed with 70% ethanol, spin at 13000 rpm in 4°C centrifuge for 5 min. The supernatants were removed after washing and the pellet was air dried at room temperature. The pellet was then resuspended in 30ul TE buffer or water and kept at 4°C.

Another method used to extract DNA was gel extraction kit from Qiagen. In this method the bands containing DNA fragments were excised and put in sterile eppendorf tubes. The excised bands were weighed in a colorless tube on a table scale. The amount of gel bands weighed was used to get the total volume of solubilization and binding buffer QG. Three volumes of Buffer QG were added to 100mg of the gel containing DNA. Incubate at 50◦C for 10 min or until the gel has completely dissolved. To help dissolve gel, the solution was mixed by vortexing the tube every 2-3 min during the incubation. After the gel has dissolved completely, the color of the mixture should remain yellow, and if not so, if is orange or violet, 10ul of 3M sodium acetate, pH 5.0. Mix the mixture till it changes back to yellow. One hundred microliter of 100% room temperature isopropanol was added to the solution. This step increases the yield of DNA fragments. The QIAquick spin column was placed in a provided 2 ml collection tube provided.

To bind DNA, the samples were applied to the column and centrifuged for 1 min. The supernatants were discarded and the columns were placed in the same collection tube. Five hundred microliter of Buffer QG was added to the columns and centrifuged for 1min. This step helps to remove all the traces of agarose. Discard the supernatants and place the column in the same collection tube. Add 750ul of washing buffer PE, spin down for 1min and spin again for 1min to remove all the excess ethanol. Discard the flow-through place the QIAquick columns into clean 1.5ml microcentrifuge tube. The DNA was eluted by adding 50ul of Elution Buffer EB to the center of the QIAquick membrane and let the columns stand for 1 min and then spin for 1 min. The concentration of the extracted DNA was checked by absorbance spectrophotometer (A260). And to determine the quality of the DNA, 0.8% agarose gel electrophoresis was ran at 126 voltages per centimeter for 30 min.

3.2.2.2 Ligation

DNA fragments generated by digestion of plasmid with two restriction enzymes were incubated with different amounts of ligase for varying periods of time, and then electrophoresed in 1% agarose. Note that even after 5 minutes with ligase, essentially all the fragments have been ligated to one another, and shifted to higher molecular weights. Ligation process was done using the Myles Thaler Center protocol. For a standard reaction, 200ng of a 13kb (NL4.3 with the concentration = 0.0772ug/ul); 11kb (MJ4 = 0.0726ug/ul) plasmid vector were ligated with 100ng of a 1600bp fragment insert (MJ4= 0.0477ug/ul and NL4.3= 0.0566ug/ul) in a volume of 20µl. The big fragment from each plasmid was ligated with the small fragment from the opposite plasmid. The procedure was as follows: Mj411kb/NL4-3 1.6kb and NL4-3 13kb/ Mj4 1.6kb respectively. The mass of the vector was given as 200ng, and to get the mass of the insert this equation was used:

$$Mass\ of\ insert = \frac{3(Length\ of\ Insert\ bp)}{Length\ of\ Vector\ bp} \times Mass\ of\ Vector$$

The following components were assembled in a 1.5 ml tube: 2ul 10x Ligation Buffer (10X = 200mM Tris-HCl pH .6, 100mM MgCl2, 250μg/ml acetylated BSA) (NEB), 1ul 10mM ATP (this is optional, because the buffer already contains ATP), the ratio 3:1 Insert to Vector; and the required volume of nuclease-free water20ul. T4 DNA ligase was added the last and the mixture was gently mixed by stirring with a pipet tip. The T4 DNA ligase joins the blunt and the cohesive end termini. Self ligation was also performed for uncut vector plasmid DNA, with and without DNA ligase. The total volume of 20ul of the ligation mix was obtained and incubated at 16°C 6 hr to overnight. Ligations can be stored at 4°C after heating at 70°C for 10 min to inactivate the enzyme. The optimal incubation temperature for T4 DNA ligase was 14°C and when very high efficiency ligation is desired (e.g. making libraries) this temperature is recommended. However, ligase is active at a broad range of temperatures, and for routine purposes such as subcloning, convenience often dictates incubation time and temperature - ligations performed at 4°C overnight or at room temperature for 30 minutes to a couple of hours usually work well. The ligation mixture was then used directly for transformation.

3.2.2.4 Plasmid Transformation

One hundred microliters of competent cells DH5a were thawed on ice and then transferred to 13 ml pre-chilled tubes. Chilling cells in the presence of divalent cations such as Ca^{2+} (in $CaCl_2$) prepares the cell walls to become permeable to plasmid DNA. Five microliters of ligation mixture were added to the cells and incubated on ice for 30 min, and then briefly heat shocked in 42°C for 90sec. This causes the DNA to enter the cell. The tubes containing the mixture were transferred back to ice and incubated for 2 min. Nine hundred microliters of prewarmed SOC or *Luria bertani* (LB) (10g LB in 500 ml distilled water) media was added to the tubes and shaked at 37°C, 225rpm for 45 min. After that 300ul transformants were spread on LB ampicilin (0.5 ml of ampicilin from -20°C freezer was added into cooled 500 ml LB + 1.5% agar) resistant plates. The tunes were incubated overnight at 37°C. This method works very well for circular plasmid DNAs. An excellent preparation of competent cells will give ~10^8 colonies per microgram of plasmid. A poor preparation will be about 10^4/µg or less. Good non-commercial preps should give 10^5 to 10^6 transformants per microgram of plasmid.

After overnight incubation the colonies were counted on a plate for transformation efficiency, and the following equation was used to determine the transformation efficiency:

$$CFU = \frac{CFU\ in\ Control\ Plate}{Pg\ Ligation\ Mix} \times \frac{10^6}{\mu g} \times Dilution\ Factor$$

To avoid satellite colonies on the plates, the plates should be taken out from an incubator after at least 16hours. Two distinct colonies were picked from the plate, and were grown in LBamp in 5ml tube for Miniprep (Qiagen). This was shaken overnight in 37°C. The Mini preparation was done to obtain small amount of DNA for verification of the ligation if it was successful. At each of the wildtype's plasmid, there is/are unique enzyme sites on the 1.6kb fragment, which are not present in the other plasmid. And in MJ4 plasmid there is Pflm1 site and in NL4.3 there is Age1 site. So, the chimeric plasmids (0.5ug) were cut with Apa1/pflm1 and Apa1/Age1, this is to verify that the chimeric plasmids are correct or the vector really religated to the correct insert and not to itself. The chimeric proviral clones were transformed in Stable2 (STBL2) competent cells, expanded at 30°C and a large preparation done. Digestion was repeated with the DNA extracted from the STBL2 cell line and archived in 60% glycerol stocks in -83°C for future use.

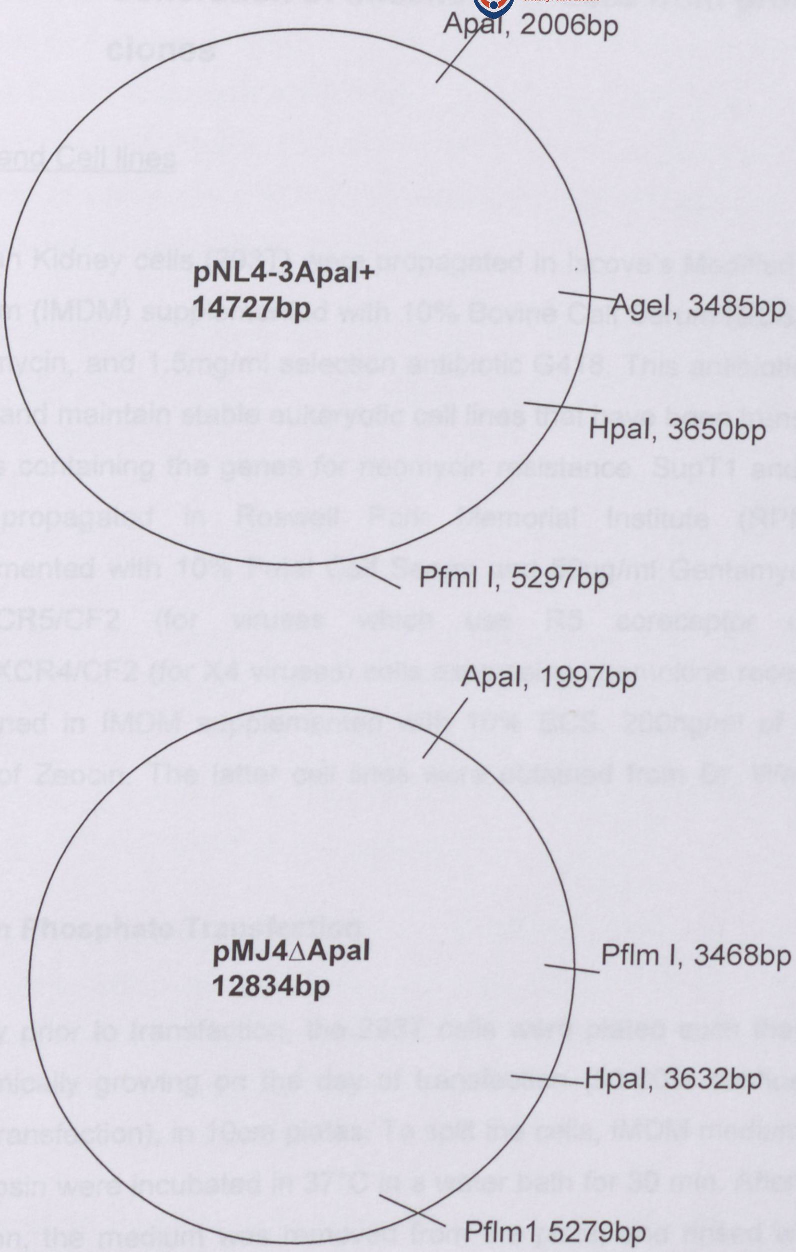

Figure 3.1: The pMJ4ΔApaI and pNL4-3ApaI+

3.2.2.7. Generation of infectious viruses from proviral clones

Cells and Cell lines

Human Kidney cells (293T) were propagated in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% Bovine Calf Serum (BCS), 0, 5 ml of gentamycin, and 1.5mg/ml selection antibiotic G418. This antibiotic is used to select and maintain stable eukaryotic cell lines that have been transferred with vectors containing the genes for neomycin resistance. SupT1 and PM1 cells were propagated in Roswell Park Memorial Institute (RPMI) media supplemented with 10% Fetal Calf Serum and 50ug/ml Gentamycin Sulfate. CD4/CCR5/CF2 (for viruses which use R5 coreceptor usage) or CD4/CXCR4/CF2 (for X4 viruses) cells expressing chemokine receptors were maintained in IMDM supplemented with 10% BCS, 200ng/ml of G418 and 100ng of Zeocin. The latter cell lines were obtained from Dr. Wen Yoan at UVA.

Calcium Phosphate Transfection

One day prior to transfection, the 293T cells were plated such that they are logarithmically growing on the day of transfection (50-60% confluent at the time of transfection), in 10cm plates. To split the cells, IMDM medium together with Trypsin were incubated in 37°C in a water bath for 30 min. After 30min of incubation, the medium was removed from the plate, and rinsed with 10 ml PBS (8g NaCl, 0.2g KCl, 1.44g Na2HPO4, 0.24g KH2PO4, in 800 ml of distilled water. Adjust the pH to 7.4 with HCl. Add H2O to 1 liter. Sterilize by autoclave). The PBS was added on the side of the plate being careful not disturb the monolayer. The monolayer was rinsed by gently rocking the plate back and forth. PBS was removed by drawing out using 9inches Pasteur pipette and discarded. This was done two times. Two milliliters of Trypsin were added and the plate was rocked to ensure that the entire monolayer is covered with the trypsin solution. The plate was incubated until the cells begin to detach, usually 3-5 min. Care should be taken to avoid leaving the cells exposed to the Trypsin longer. Care should also be taken that the cells should not be forced to detach prematurely, as this may results in clumping. The serum containing medium (18 ml) was added in the plate and the cells were pipetted up and down until they are dispersed into a single cell suspension. The cells were transferred to 50 ml tube.

The cells were counted by hemocytometer (Fisher Scientific). Fifty microliter of the cells were mixed with 50ul Trypan blue stain (dye), which is used to measure the viability of the cells. Preparation of 1:1 dilution (50ul of cells and 50ul of the dye) of the cell culture using trypan blue solution was done 20ul of the mixture was then loaded the counting chambers of a with the dilution. The number of stained cells and total number of cells were counted using this device. The calculated percentage of unstained cells represented the percentage of viable cells. After counting, the cells were diluted to 1 x 10^6 ml per plate for seeding. The appropriate volume of the cell suspension to a new plate containing medium, the total volume of the cells and medium was 10 ml. And the plate was incubated overnight at 37°C.

In Calcium Phosphate co-precipitation transfection, there are critical factors that should be taken care of, which are pH and sterility. The pH of the solution should be checked since the solutions are of low ionic strength and care should be taken to ensure that the DNA is not contaminated and that all solutions are autoclaved and filtered.

Two sets of 13 ml tubes were prepared, and in one set 500ul of 2X HEBS (HEPES Buffered Saline) (8.0g NaCl, 0.37g KCl, 0.099g Na2HPO4.2H2O (m.w.141.96), 1.0g Dextrose, 5.0g HEPES, bring to 500 ml with distilled water, pH 7.2). Into the other set 10ug of DNA at the concentration of 0.4273ug/ul (pNL4-3pMj4) and 0.5263ug/ul (pMj4pNL4-2) mixed with 50ul of 2.5M CaCl2 in 10mM 2X HEPES (0.238g HEPES, 27.75g CaCl2, bring to 90 ml with sterile distilled water, pH 7.2). Mixing was done by drawing the solution up and down in a 5 ml pipette. The solution was added drop-wise to the 2XHEPES, blowing bubbles. The mixture stood in room temperature for 30 min and added drop-wise to the surface of the medium containing cells. The dish was swirled gently to mix. The dish was then incubated in 37°C for 6 hours, and the $CaPO_4$ containing medium was removed and replaced with the normal medium. All the transfection processes were done in BL2-Tissue culture lab, and after changing the medium the dish was incubated 72 hours post transfection in BL3 lab (Virus Lab).

After 72 hours post transfection, the cells were harvested. The transfected cells were transferred into 13ml and spun at 2500rpm for 5 min. The supernatant was saved in aliquots in 1.5ml epperndorf tubes and stored at -80°C for p24 analysis and infection assay.

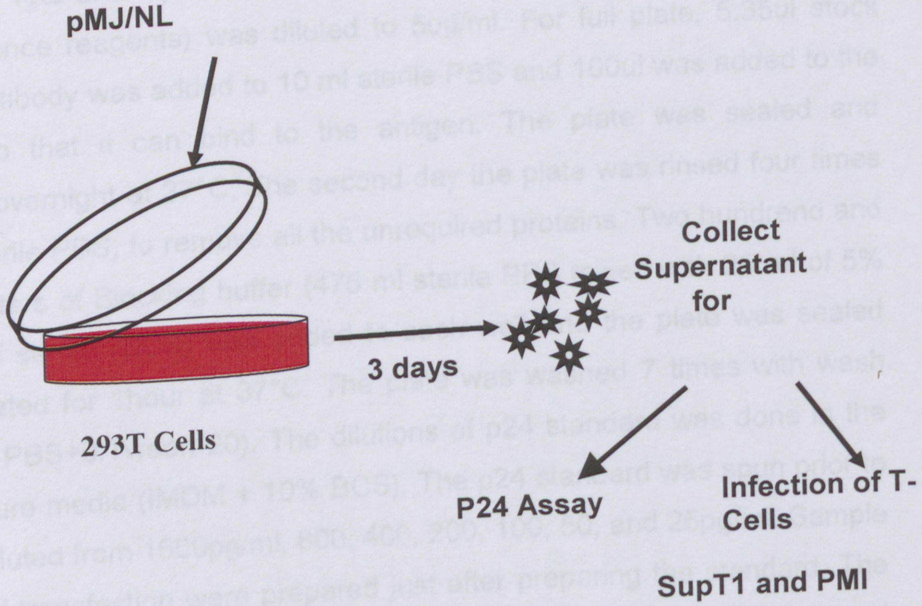

Figure 3.2: Transfection of proviral clones.

3.2.3. Analysis of Viral Proteins

3.2.3.1. P24 Enzyme Linked Immunosorbent Assay (ELISA) (Wehrly and Chesebro, 1997; Coyle *et al.*, 2003)

P24 Assay Procedure

P24 ELISA was performed in 96-well plates which permits high throughput results. The bottom of each well was coated with a protein to which bind to the antibody that needs to be measured. Partially purified, inactivated HIV antigens pre-coated onto an ELISA plate for the first day, Primary antibody: Monoclonal IgG αP24 (HamRek lab in-house supply or NIH AIDS Research and Reference reagents) was diluted to 5ug/ml. For full plate, 5.35ul stock Primary Antibody was added to 10 ml sterile PBS and 100ul was added to the 96-plate so that it can bind to the antigen. The plate was sealed and incubated overnight at 37°C. The second day the plate was rinsed four times with 1X sterile PBS, to remove all the unrequired proteins. Two hundrend and fifty microliters of Blocking buffer (475 ml sterile PBS mixed with 25 ml of 5% bovine calf serum (BCS) was added to each well and the plate was sealed and incubated for 1hour at 37°C. The plate was washed 7 times with wash buffer (1X PBS+0.Tween-20). The dilutions of p24 standard was done in the tissue culture media (IMDM + 10% BCS). The p24 standard was spun prior to use and diluted from 1600pg/ml, 800, 400, 200, 100, 50, and 25pg/ml. Sample dilutions of transfection were prepared just after preparing the standard. The supernatants were thawed prior to use and the tissue culture media was used to dilute the samples. Transfection of 293T cells with gag-pol constructs that express well, generally yield a p24 expression level of 30,000-60,000 pg/ml and the linear range of this assay is roughly 25-800pg/ml (Hamrek lab, University of Virginia, USA). Ten to 1000 dilutions of transfection supernatants were done in both constructs. And 10ul of lysis buffer (9 ml of PBS, 1 ml of 10% Triton X-100, 50ul of Trypan Blue) was added to each well except for the blank, then 100ul of the standard /samples were added to each well appropriate well. The plate was sealed and incubated at 37°C for 2hours or overnight. The plate was washed 7X with wash buffer.

To detect the bound antibodies, a secondary antibody was added to each well except for the blank. The secondary antibody would bind to all human antibodies and is typically produced in a rodent. A dilution of 1/800 was done on the Biotinylated secondary antibody, where in 12.5ul of stock secondary antibody was added to 10ml of Assay buffer (PBS, 10% BCS, 0.5% Triton X-100). And 100ul of this dilution was added to all the wells except for the blank. Seal the plate and incubate for 1hour. The plate was washed with wash buffer 7X. Attached to the secondary antibody is an enzyme Peroxidase Streptavidin. These enzymes can metabolize colorless substrates into colored products. The enzyme was spun prior to use and diluted 1/4000 in 10 ml of assay buffer. Hundred microliter of the dilution mix was added to the to all the plates except for the blank, and the plate was sealed and incubated at 37°C for 30min. Wash the plate 7X with wash buffer. Chromogen or substrate (peroxidase substrate) which changes color when cleaved by the enzyme attached to the secondary antibody. It is very important to protect the substrate from the light with foil and use it within 15 min. Two OPD tablets were dissolved in 10 ml substrate buffer and then 100ul was added in each well including the blank. Incubate at room temperature (in the dark place) for 30 min. To stop the reaction, 50ul of the stop solution (2M H_2SO_4) was added to each well. When the enzyme reaction was complete, the entire plate was placed into a plate reader and the optical density at 492 nm (i.e. the amount of colored product) is determined for each well. The amount of color produced is proportional to the amount of primary antibody bound to the proteins on the bottom of the wells.

3.2.3. T- Cell Infection

The viral stocks were produced from the infection of human cells, SupT1 and PMI, with the virus derived from transfection supernatant. Prior to transfection, these cell lines were split in 6 well plates at the concentration of 5million cells per well in 2 ml RPMI (supplements are on the page above). Two hundred nanogram of Hexadimethrine Bromide (Polybrene) was added to each well and the plate was incubated at 37°C for 30 min. In BL-3 conditions, 50-125ng p24 equivalents of virus was added to the corresponding well and the plate was incubated back to 37°C , 4.5% CO2, and 84% humidity for 4hours. Upon completion of the incubation, 2 ml well contents were transferred to corresponding 15 ml conical tubes then centrifuged at 3000rpm for 5min. For each tube the supernatant with noninfectious virus was discarded, and the infected cells were suspended in 5ml RPMI medium and the resultant cultures placed in a 25cm^2 flasks. The viral cultures were incubated at 37°C, 4.5% CO2, and 85% distilled water in BL-3 lab.

Every 3 days the cultures were split removing 3 ml of each culture replaced by 3ml of fresh medium. The removed 3 ml of cultures were separated by centrifugation from supernatant (Contained viral particles) and the infected cells. The supernatants were collected and stored at -80°C. Viral growth was determined by p24 using the different time lines of harvesting.

3.2.5. Construction of Packaging Vector Systems

The helper packaging plasmid vectors pCMVGagPol(MJ4)-RREr (HR3853) and pCMVGagPol(NL4.3)-RREr (HR3855) were created. They contain the simian cytomegalovirus (sCMV) immediate- early promoter as well as the RRE and poly (A) signal (Figure 3.3). The Gag/Pol regions were derived from pNL4-3ΔApaI-HpaI and pMj4ΔApa-7 (previously described) and this contain the previously described ApaI and HpaI sites. pCMV-RREr (HR190) is the backbone plasmid, which contains the simian CMV IE promoter and an 854bp fragment containing the HIV-RRE (Lewis *et al.*, 1990). After the plasmids were constructed, an additional ApaI site existing in the pCMV-RREr backbone was deleted by cleaving with BaHI and Bgl II followed by relegation to generate plasmids pCMVGagPol(MJ4)-RRErΔApaI (HR4010) and pCMVGagPol(NL4.3)-RRErΔApaI (HR4012).

pCMVGagPol(MJ4)-RREr and pCMVGagPol(NL4.3)-RREr were made in the following way: To insert the HIV-1 sequence into the plasmid vector, 1ug of pCMV-RREr was linearized with SacI and repaired at both ends by T4 DNA polymerase, which catalyzes the synthesis of DNA in the 5'-3' direction and it requires the presence of template and primer. It removes 3' overhangs to form blunt ends and fills in 5' overhang, to also form blunt ends. Two microgram DNA was dissolved in 5 ml 1X NEB buffer 2 (10mM Tris-HCl, 50mM NaCl, 10mM $MgCl_2$, 1mM Ditheothreitol, pH 7.9 at 25°C) or 1X T4 DNA Polymerase Reaction Buffer supplemented with 100uM dNTPs. T4 DNA Polymerase was added and incubated for 15 min at 12°C. The reaction was stopped by addition of EDTA to the final concentration of 10mM and heated at 75°C for 20 min. After repairing with T4 DNA polymerase, the reaction was subjected to Alkaline Phosphatase, Calf Intestinal (CIP). This removes 5' phosphates from DNA and dNTPs, prevents recircularization of cloning vectors and also dephosphorylates serine, threonine and tyrosine residues in proteins.

To generate pCMVGagPol(MJ4)-RREr plasmid, 5ug of pMj4ΔApa-7 was digested with 1ul of Nar1 and Stu1 endonuclease enzymes in 20ul reaction at 37°C overnight. To derive pCMVGagPol(NL4.3)-RREr, 5ug of pNL4-3ΔApaI$^+$ was digested with BssHII and PflmI overnight. The Nar1 - Stu1 and BssHII - PflmI fragments form different plasmids were individually purified using the Gel Purification Kit (Qiagen) and also repaired with T4 DNA polymerase (same as above). One microliter of each fragment was ligated to 5ul of CIP treated DNA pCMV-RREr) with 1ul T4 DNA ligase, in a 20ul reaction of 2ul ligation buffer, 1ul of 20mM DDT, 1ul of 20mM ATP and 1mg/ul BSA. Ligation was at 16°C for 4hours, but overnight works very well. Five microliter of the ligation mix was transformed in 100ul DH5a cells and 990ul SOC and heat shocked for 90 sec at 42°C and shaked at 37°C for 45 min. Three hundred microliters of transformation mix was spread on LBamp plates and incubated at 37°C overnight. Both the pCMVGagPol(MJ4)-RREr and pCMVGagPol(NL4.3)-RREr plasmids contain the polyadenylation signal replication defective.

The colonies were picked from two constructs, inoculated in 1.5 ml tubes with 50ul water and resuspended. The tubes were closed tight and boiled at 100°C for 5min. This helps the in the elimination of *E. coli* cells and burst open the plasmids, and the solution will remain with tons of plasmids. The solutions were spun down at 13000rpm for 5min. To screen the clone, 0.5ul of the H_2O/cell suspension was used as a template in PCR reaction. To obtain 1098bp of pCMVGagPol(MJ4)-RREr, sense primer (183) 5'-AATGACGCAAATGGGCGTTCCATT-3' and antisense (19) 5'-GTAAGAATGTATAGCCCTGTCAGC-3' were used. For pCMVGagPol(NL4.3)-RREr plasmid, sense primer(183) 5'-AATGACGCAAATGGGCGTTCCATT-3' and antisense (882) 5'-GCTACAACCATCCCTTCAGACA-3', and this gave out 403bp fragment. The reaction was carried out in a 20ul mixture of 2ul 10X Taq buffer (Invitrogen), 0.6ul 50mM $MgCl_2$, 1ul of 100ng/ul of the respective primers, 0.5ul of the cell suspension, and lastly 0.2ul of Taq DNA polymerase and the solution was taken to the volume of 20ul by dH_2O. The cycling conditions were 95°C for 45sec, 55°C for 45sec and 72°C for 2 min, 25 cycles. PCR was done to check if plasmid is not an antisense and the gene didn't bind to the opposite direction. The same colonies from the same master plates were inoculated into 5 ml tubes with LBamp media and the plasmids were grown at 37°C overnight for Miniprep DNA purification.

There is an extra ApaI site on the back of pCMVGagPol-RREr which makes the construct to have two ApaI sites instead of one which is on the gag/pol of either MJ4 or NL4-3 plasmids. To delete this site, the pCMVGagPol(MJ4)-RREr or the pCMVGagPol(NL4-3)-RREr was digestion with BamHI and Bgl II. This was then relegated with T4 DNA ligation and transformed in DH5a cells. Then, this gives only one ApaI and HpaI sites of the pCMVGagPol(MJ4)-RREr∆ApaI and pCMVGagPol(MJ4)-RREr∆ApaI

To construct pCMVGagPol(MJ4/NL4-3)-RREr∆ApaI (HR4014) and pCMVGagPol(NL4.3/MJ4)-RREr∆ApaI (HR4016), the chimeric vector plasmids, pCMVGagPol(MJ4)-RREr∆ApaI and pCMVGagPol(NL4.3)-RREr∆ApaI were digested with ApaI and Hpa1. The fragments ApaI-HpaI in both plasmids vectors have the unique ApaI - HpaI sites for the insertion of the patients DNA samples for resistance mutation analysis. One microgram of each vector plasmid was digested with 1ul of ApaI and HpaI in a 20ul reaction at 25°C for ApaI for 4hours and at 37°C for HpaI enzyme. The digestion mixtures were run on 0.8% agarose gel, and the bands containing the expected fragments were excised and DNA was purified by Gel purification kit (Qiagen) or Freeze Phenol method. The ApaI – HpaI fragment from each plasmid was sticky ligated to the opposite vector from each plasmid with T4 DNA ligase overnight. And these were transformed in DH5a cells.

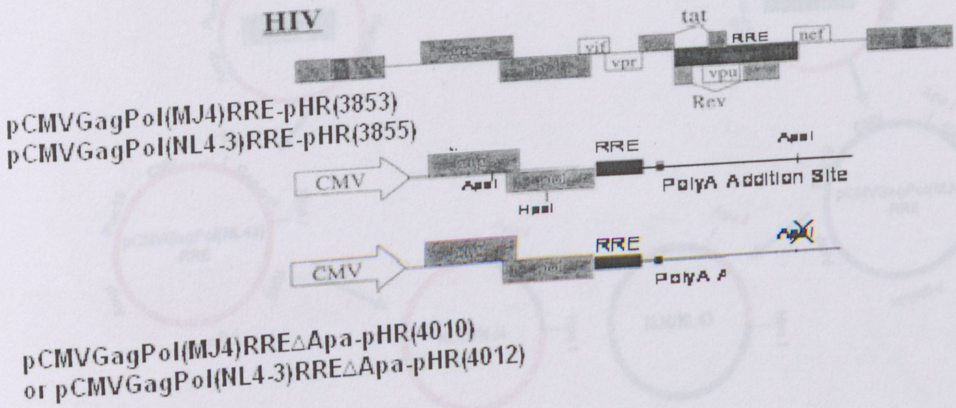

Figure 3.3: pCMVGagPol(MJ4)-RREr(pHR3853) or pCMVGagPol(NL4-3)-RREr (pHR3855)) and pCMVGagPol(MJ4)-RREr∆Apa (pHR4010) or pCMVGagPol(NL4-3)-RREr∆Apa (pHR4012) packaging constructs.

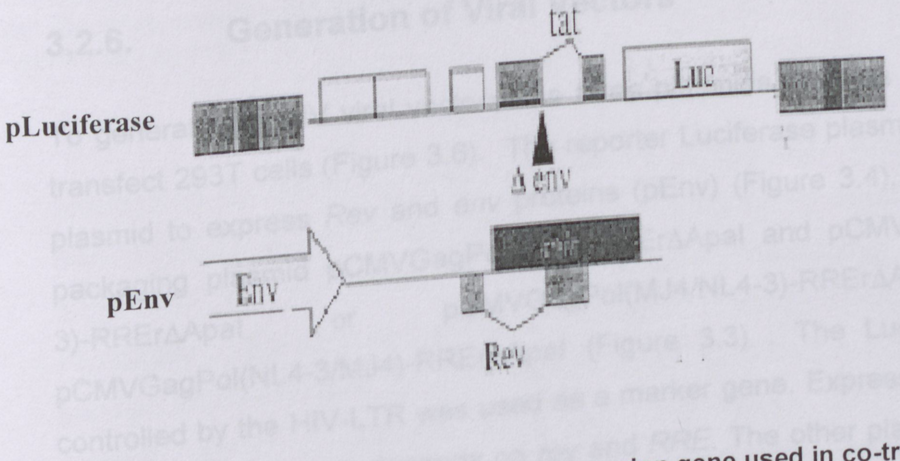

Figure 3.4: Luciferase reporter and Env expression gene used in co-transfection of 293T cells

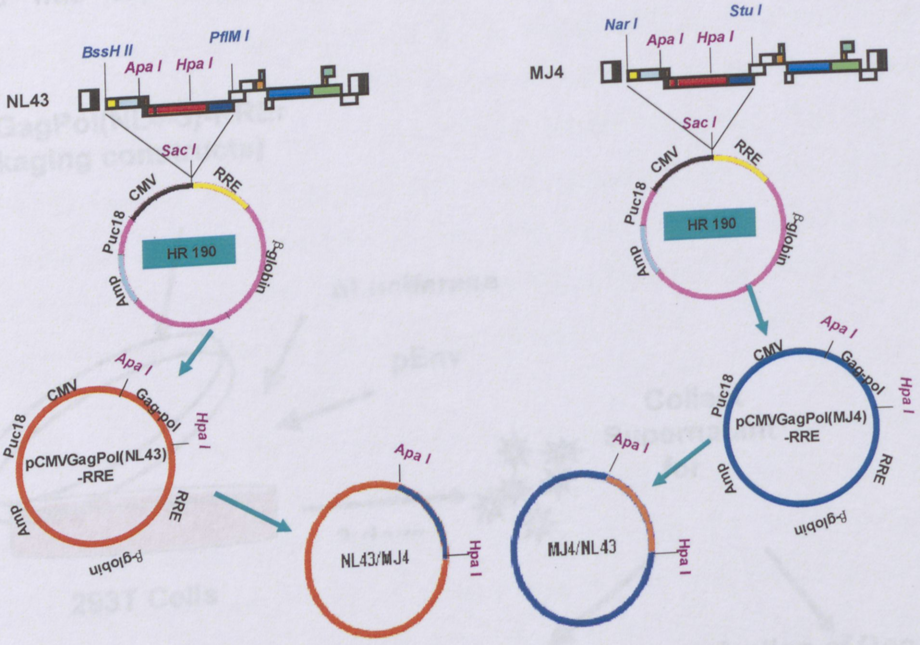

Figure 3.5: A schematic diagram showing the construction of viral vectors (packaging constructs)

3.2.6. Generation of Viral Vectors

To generate the HIV viral vectors, the three plasmids systems were used to transfect 293T cells (Figure 3.6). The reporter Luciferase plasmid (pLuc), the plasmid to express *Rev* and *env* proteins (pEnv) (Figure 3.4), and also the packaging plasmid pCMVGagPol(MJ4)-RRErΔApal and pCMVGagPol(NL4-3)-RRErΔApal or pCMVGagPol(MJ4/NL4-3)-RRErΔApal and pCMVGagPol(NL4-3/MJ4)-RRErΔApal (Figure 3.3) . The Luciferase gene controlled by the HIV-LTR was used as a marker gene. Expression of the *pol* gene of the constructs depends on *rev* and *RRE*. The other plasmid systems comprised (pCMVGagPol(MJ4)-RRer and pCMVGagPol(NL4-3)-RRer).

To detect viral-protein expression by the packaging plasmids, each packaging plasmid was separately co-transfected into human kidney 293T cells

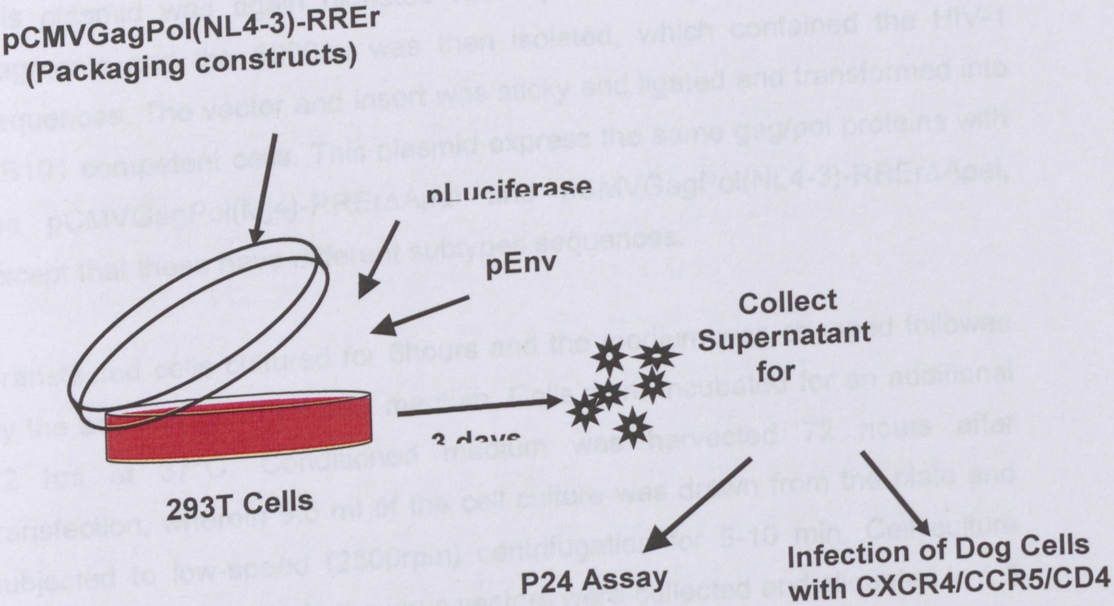

Figure 3.6: **$CaPO_4$ co-transfection:** 1X10^6 293T cells were co-transfected with the 15µg of the following packaging constructs: pCMVGagPol(NL4-3)-RREr, pCMVGagPol(MJ4)-RREr, pCMVGagPol-RRE, HIVpack. Twenty ug of the reporter gene pLuciferase and finally 5µg of the envelope plasmid for the cell tropism. Transfections were performed in fourplicate. And 72hours later the supernatants were collected for p24 assay and infection of T-cell lines. The maps of the plasmids are shown in fig 2 below.

propagated by IMDM supplemented supplemented with 10% Bovine Calf Serum (BCS), 0,5 ml of gentamycin, and 1.5mg/ml To detect viral-protein expression by the packaging plasmids, each packaging plasmid was separately co-transfected into human kidney 293T cells propagated by IMDM supplemented with 10% Bovine Calf Serum (BCS), 0,5 ml of gentamycin, and 1.5mg/ml selection antibiotic G418, using the Calcium phosphate method ($CaPO_4$). A total of 40ug of plasmid DNA was used in the transfection of a 10-cm diameter plate of 1X10^6 cells in the following proportion: 15ug of pCMVGagPol(Mj4)-RREr∆Apal and pCMVGagPol(NL4-3)-RREr∆Apal , 20µg of pLuc (Reporter gene), and 5ug of envelope plasmid as described earlier. pCMVGagPol-RREr (HR354) packaging constructs vector served as the controls. pCMVGagPol-RREr was constructed by cleaving pCMV to with SacI and BamH1 and the 4250 fragment was isolated. pSVGGagPol-RREr was cleaved with SacI and BamHI giving out approximately 6000bp fragment. And this plasmid was again digested with ApaLI to remove all the unwanted fragments and the 6000bp was then isolated, which contained the HIV-1 sequences. The vector and insert was sticky end ligated and transformed into HB101 competent cells. This plasmid express the same gag/pol proteins with the pCMVGagPol(Mj4)-RREr∆ApaI and pCMVGagPol(NL4-3)-RREr∆ApaI, except that these have different subtypes sequences.

Transfected cells cultured for 6hours and the medium was changed followed by the addition of 10 ml new medium. Cells were incubated for an additional 72 hrs at 37°C. Conditioned medium was harvested 72 hours after transfection, wherein 9.5 ml of the cell culture was drawn from the plate and subjected to low-speed (2500rpm) centrifugation for 5-10 min. Cell culture supernatants that contain the virus vectors were collected and aliquoted in 1.5 ml eppendorf tubes and stored at -80°C. Some of the supernatants were used in the determination of the protein p24 by ELISA as earlier described, and was also used to infect the dog cells CF2/CD4/CXCR4.

3.2.7. Infection with the HIV Vectors and Analysis of Marker Gene Expression

3.2.7.1 Single-Round Luciferase Virus

Luciferase encoded by the firefly luciferase gene is sensitive reporter enzyme for the study of transcriptional regulation. The enzyme catalyzes, in the presence of ATP the oxidation of luciferin with concomitant emission of yellow-green light, which can be conveniently measured by scintillation counters or luminometers. Light emission peaks in several seconds at 560 nm when the reaction is conducted at pH 7.8 (Analytical Biochemistry. 80:496,1977). The rapid appearance and decay of the light flash require consistent timing of the light measurement to obtain reliable data. This provides the basis for an assay system many times more sensitive than the bgalactosidase (GAL) or other reporter gene systems (PNAS USA 82:7870, 1984 & Molecular Cell Biology 7:725, 1987). The luciferase-based reporter assay is thus well suited for those cell systems exhibiting low transfection efficiency.

The harvested supernatants were used to determine the infectivity and the Luciferase activity of the virus vectors. The following assay was done to achieve these: Titrating Single-Round Entry Luciferase Virus Stock the cell lines CD4/CCR5/CF2 and CD4/CXCR4/CF2 (Dog cells) were washed with 4 ml PBS per 10cm dish and 2ml of PBS/5mM EDTA was added in the dish and incubated at 37°C for 3-5min or until the cell detach from the dish (In tissue culture/ BL-2). The 2ml of DMEM medium with 5mM MgSO4 was added to the dish to quench the remaining EDTA. Ten milliliter of DMEM/BGS/PS medium was added and mixed by pipetting up and down, and then transferred to 15ml tube. The cells were then counted using the hemacytometer and then diluted by DMEM/BGS/PS to the concentration of $1X10^4$ cells per ml (only 10 ml of diluted cells per titration of 2 virus stocks in a one 96-well plate). The calls were transferred to BL-3 containment suite. Hundred microliter of DMEM/BGS/PS was added in 96-well tissue culture treated luminescence plate. The SRLV stock (25ul) was transferred to the first 4 wells (Column 1 row A-D for one virus and E-H for the second virus), mixing was done by using the multi-channel pipetman, and 25ul was transferred to the next column (5 fold dilution). Serial dilution was done for a total of 11 dilutions. The last 25ul from the 11^{th} dilution was discarded, and the wells in column 12 were served as the cell control, no virus was added. A 100ul of the target cells ($1X10^4$ cells/ well) was added to all the wells and the plate was incubated at 37°C for 48hours. The pipette tips between each virus stock dilutions were discarded to prevent contamination. After two days incubation, the medium was removed from the plate by using 200ul tips connected to a vacuum source, followed by addition of 30ul 1X Passive Lysis buffer (Promega Cat # E1941) and lyze the cells at room temperature for 30min.

The plate can be stored at -70°C out of the BL-3 suite if the luciferase activity is not measured immediately. Hundred microliter of Luciferase Buffer (19ml dH2O, 300ul of 1M MgSO4, 300ul 1M KPO4 buffer (pH 7.8), 100ul 200mM DTT and 200ul 100mM ATP) was added to each well just before measurement. The plate was read by GloMax Luminometer with one injector that injects 50ul of D-Luciferin per well. After the reading, the TCID50 was calculated using the TCID50 macro and wells with relative luciferin unit less than 10 times background were considered negative for calculation.

3.2.8. Viral Inhibition Susceptibility Test

To test the viral reverse transcriptase and protease inhibition and susceptibility to drugs, the following antiviral drugs were used: Nucleoside Reverse Transcriptase Inhibitor (NRTI): Zidovudine (AZT), Non-Nucleotide Reverse Transcriptase Inhibitor (NNRTI): Nevarapine (NVP), and Protease Inhibitor (PI) Saquinavir (SQV) (Roche Farma. Madrid, Spain). These were the drugs used to test if the vector and proviral system for the packaging vector constructed works, in the presence of the drugs. The drug concentration was 10-fold diluted, from 10uM to 0.0000001uM. The stock concentration used was 100mM dissolved in DMSO and diluted with IMDM. 5uL viral stock was used to infect dog's cell in the. The aim of this was to test the putative drug resistance mutation from primary drug naïve patients.

3.2.8.1. Drug Susceptibility Assay

pCMVGagPol(NL4-3)-RRErΔApaI (10ug), pLuciferase (15ug), and *env* plasmid (2.5ug), were co-transfected in 293T cells propagated in IMDM supplemented with 10% Bovine Calf Serum (BCS), 0,5 ml of gentamycin, and 1.5mg/ml selection antibiotic G418. To determine the susceptibility to PRI Saquinavir (SQV), since PRIs block the maturation of viruses, 16–18 hr after transfection 293T cells were trypsinized and plated into 96-well plates ($5X10^4$ cells/well) containing PRI dilutions. Viral stocks (5ul) generated in the presence of PRIs were harvested at 48 hr after transfection and were used to infect CF2 cells in the absence of drug.

To test for Reverse Transcriptase inhibitors, transfection was done in the absence of drugs using the same amount of vector DNA. The susceptibility and inhibition of the virus populations (5μl) to antiretroviral drugs were determined by infecting the Dog cells (Cf2) using 96-well culture plate in the presence or absence of drugs. The drugs, Nevarapine and Zidovudine were added in the infection process together with 5μl of the virus stocks from transfection. Target cells were lysed 48 hr after infection to assess virus replication, and luciferase activity was measured with the Renilla Luciferase Assay (Promega) using a 96-well plate luminometer (Orion, Berthold, Oak Ridge, TN).

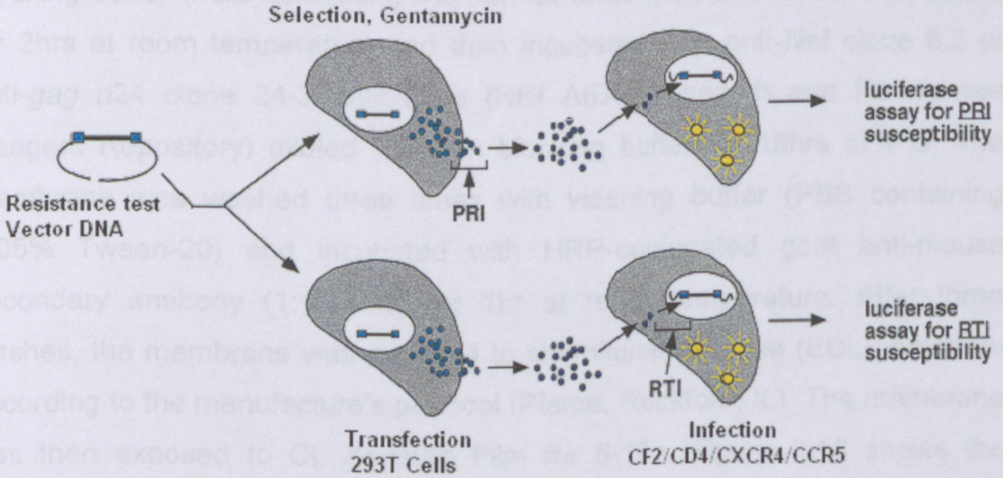

Figure 3.7: Procedure of transfection and the drug susceptibility assay. The packaging plasmid NL4-3 was transfected in 293T cells with the presence of the drugs. (A) The vector virus was co-transfected in 293T cells together with the Saquinavir (SQV), Protease Inhibitor, pLuciferase, and envelope plasmid for the co receptor usage. Medium was changed 6hr after transfection replaced by fresh medium and incubated for 48hr. The supe was collected post transfection and 5µl was used for T-cell infection (See above). The luciferase activity was measured as well as the IC-50 of the drug. (B) The NRTI Zidovudine (AZT) and the NNRTI Nevarapine (NVP).

Each drug was prepared by eight serial ten fold dilutions. The Dog cells (CF2) cells were infected in the presence of the ten different RTIs dilutions to assess the susceptibility to RT inhibitors (RTIs).

3.2.8.2 Western Blot of viral protein expression

The protein p24 expression from pCMVGagPol(NL4-3)-RRErΔApaI viral vector was determined by Western Blotting. Cells, 293T (10^6) were transfected and the supernatants were used to infect dog cells CF2. On day 3, the cells were washed twice by PBS and lysed in 100µl lysis buffer for 30 min at room temperature. The cell lysate from 2.5 x 10^6 (25µl) was then mixed with an equal volume of 2x gel loading buffer and boiled for 5min before loaded onto 12% polyacrylamide gel. Protein from the gel was transferred to the polyvinylidene difluoride membrane (PVDF, Immobilon, Millipore, Bedford, MA) by a semi-dry electroblotter. The membrane was then blocked by the blocking buffer (PBS containing 3% non-fat dried milk and 0.05% Tween-20) for 2hrs at room temperature and then incubated with anti-Nef clone 6.2 or anti-*gag* p24 clone 24-3 antibodies (NIH AIDS Research and References Reagent Repository) diluted 1:500 in blocking buffer for 18hrs at 4°C. The membrane was washed three times with washing buffer (PBS containing 0.05% Tween-20) and incubated with HRP-conjugated goat anti-mouse secondary antibody (1:150000) for 1hr at room temperature. After three washes, the membrane was exposed to chemiluminescence (ECL) substrate according to the manufacture's protocol (Pierce, Rockford, IL). The membrane was then exposed to CL Xposure Film for 5-10s. Figure 3.18 shows the results of this analysis.

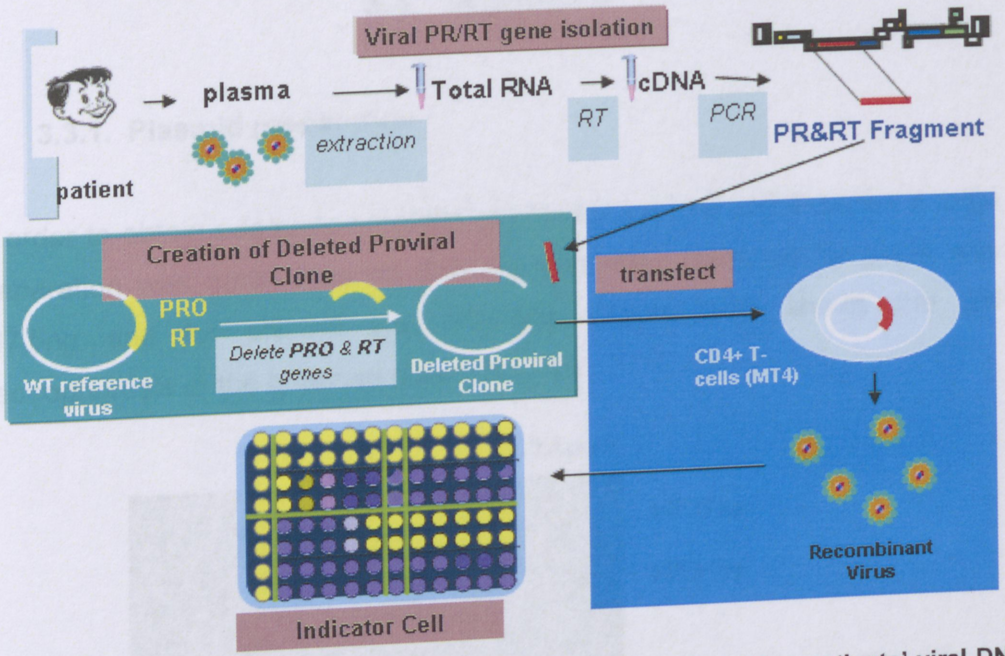

Figure 3.8: Schematic showing the overall procedure of evaluating patients' viral DNA containing drug resistance mutations for resistance phenotypic evaluation making use of proviral clones.

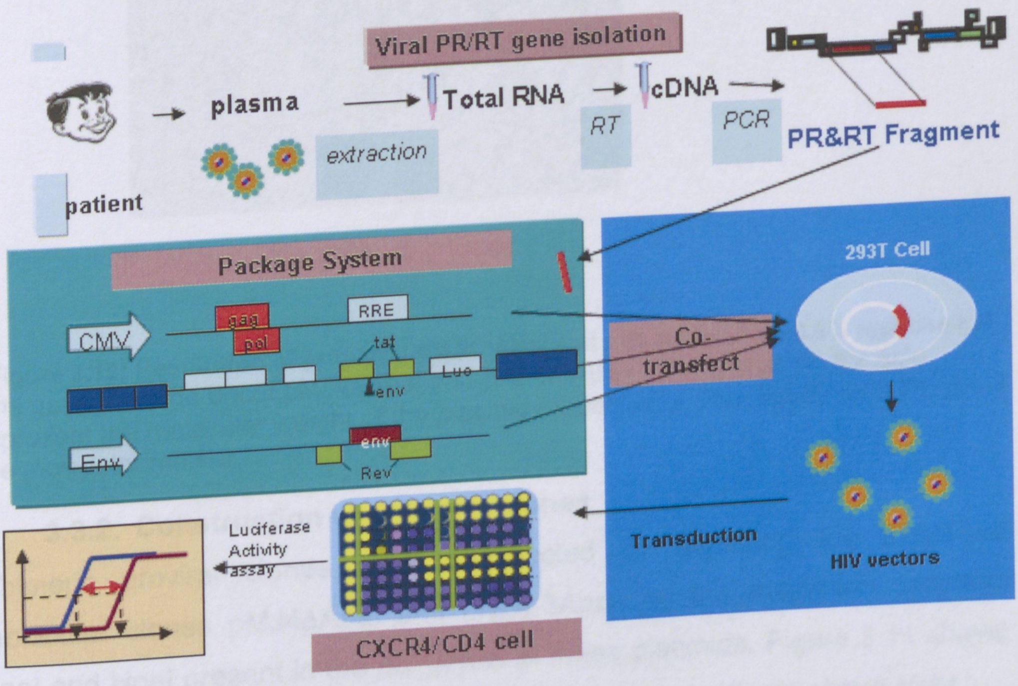

Figure 3.9: Schematic showing the overall procedure of evaluating patients' viral DNA containing drug resistance mutations for resistance phenotypic evaluation making use packaging systems.

3.3 RESULTS

3.3.1. Plasmid preparation

In order to obtain adequate amounts of pMJ4ΔApaI and pNL4-3Apa+ a large preparation was done. Plasmid DNA concentration obtained for pMJ4 was 267.5ng and for pNL4-3 was 233.4ng. Figure 3.10 shows the gel electrophoresis of the obtained plasmids DNA.

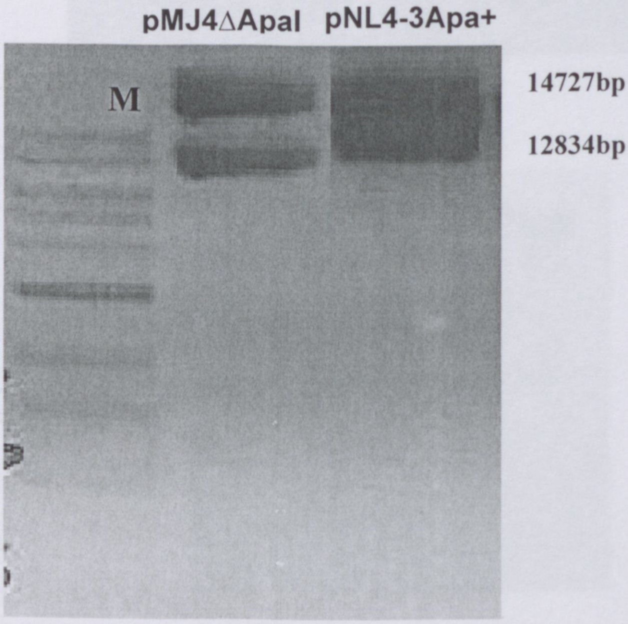

Figure 3.10: Resolution of prepared pMJ4ΔApaI and pNL4-3Apa+ on 0.8% agarose gel
The gel shows the uncut pMJ4ΔApaI and pNL4-3Apa+. The arrows on the side represent the molecular weight of the plasmid respectively. The first lane on the gel is the molecular marker.

3.3.2. Construction of chimeric clones

Chimeric proviral clones were constructed by digesting the infectious molecular clones pMJ4ΔApaI and pNL4-3Apa+ by the restriction enzymes ApaI and HpaI present in the *pol* genes of these plasmids. Figure 3.11 shows the digestion products of pMJ4ΔApaI and pNL4-3Apa+ with ApaI and HpaI.

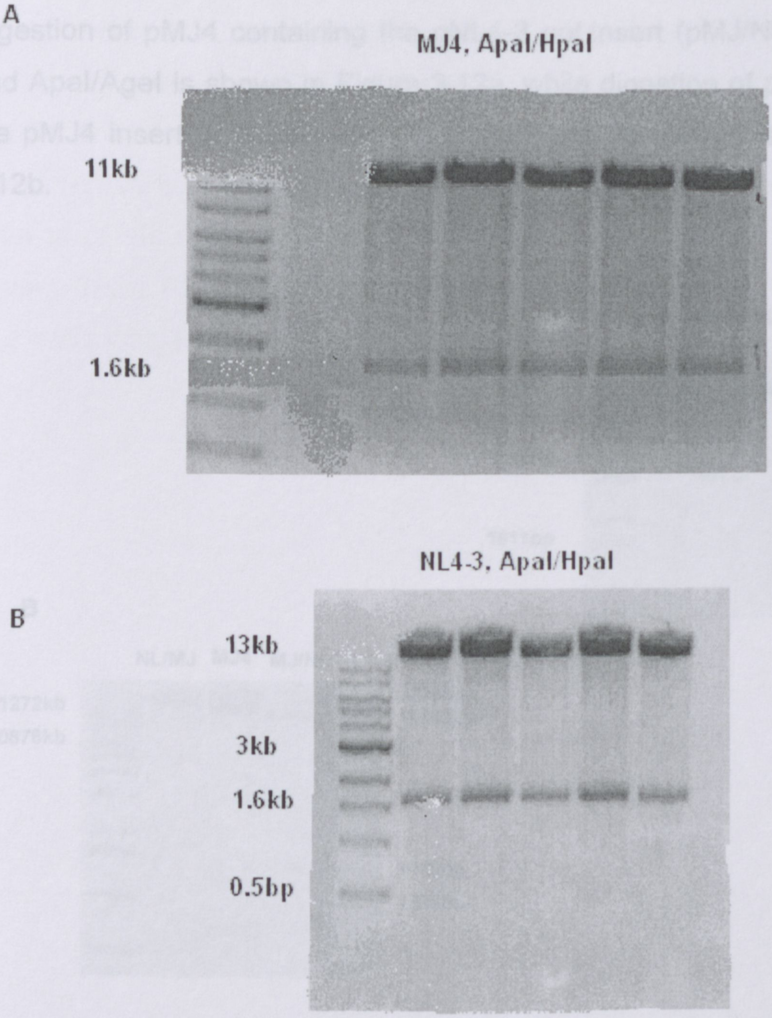

Figure 3.11: Digestion products (1.6 kb) from pMJ4 (A), and pNL4-3 (B)
Five micrograms of pMJ4 (A) and pNL4-3 (B) were digested with 1ul of ApaI and HpaI restriction enzymes. The reaction was carried out in 50ul volume with restriction buffers for 4hrs with ApaI at 25°C and overnight with HpaI at 37°C. The quality of the digestion was checked by 0.8% agarose electrophoresis stained with ethidium bromide and 9ul of each plasmid was added in 5 wells. They both popped out two fragments; pMJ4 11kb and 1.6kb, and pNL4-3 13kb and 1.6kb respectively. The ladder or maker used was a 1kb ladder with the highest molecular weight being 10kb.

A portion of *pol* gene of pMJ4 proviral clone (1.6kb) was replaced by the corresponding equivalent fragment from pNL4-3. Likewise, a *pol* gene fragment of pNL4-3 was replaced by a *pol* fragment from pMJ4 to form the chimeric proviral clones. The infectious clones would then contain unique ApaI and HpaI sites flanking the *pol* region of the plasmid within which most mutations coding for resistance to anti-HIV drugs have been described. The ligation was successful as shown by subsequent restriction digestions. Digestion of pMJ4 containing the pNL4-3 *pol* insert (pMJ/NL) with ApaI/pfmlI and ApaI/AgeI is shown in Figure 3.12a, while digestion of pNL4-3 containing the pMJ4 insert (pNL/MJ) with ApaI/pfmlI, and ApaI/AgeI is shown in Figure 3.12b.

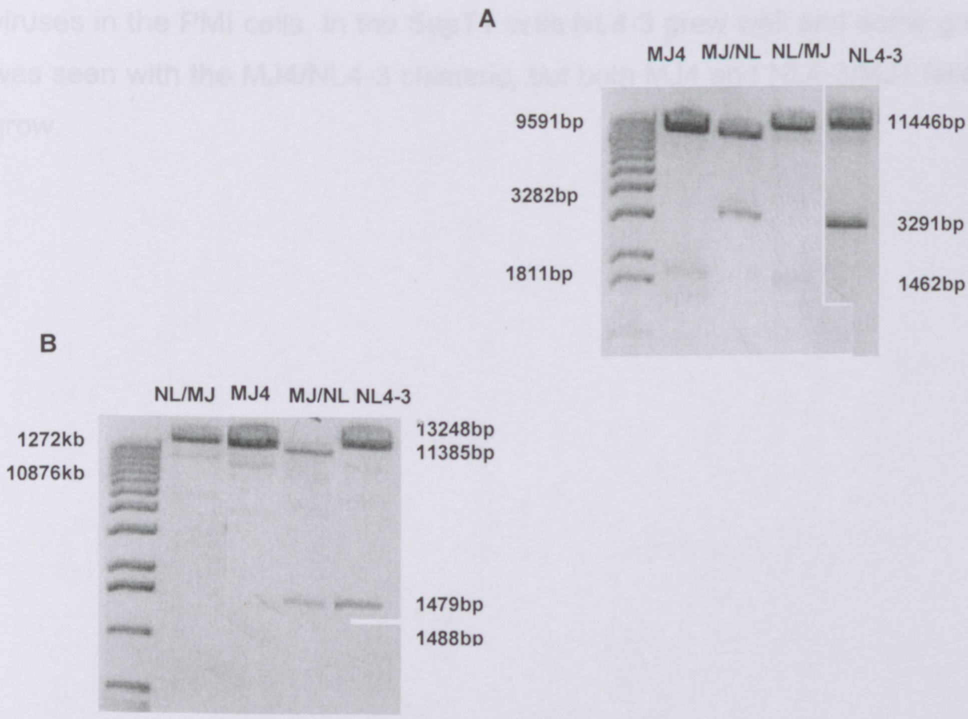

Figure 3.12: Restriction digestion profile of constructed chimeric clones. The gel electrophoresis of the digestion products of the molecular clones pMJ4 and pNL4-3, and the constructed chimeras pMJ/NL and pNL/MJ (**A**) shows the digestion with ApaI and PflmI and (**B**) are the digestion products of ApaI and AgeI. This was done to confirm cloning of the Chimeric constructs.

3.3.3. Transfection and Infectivity of chimeric clones

Constructed chimeric clones were transfected into 293T cells by the CaPO4 method. Transfection was assessed by viral p24 antigen production estimation. Evaluation with an in-house p24 antigen assay gave a p24 antigen concentration of 0.188ng/ml, 0.225ng/ml, 0.188ng/ml, 0.316ng/ml for pMJ4ΔApaI, pNL4-3Apa+, pMJ/NL and pNL/MJ after 3 days of transfection respectively.

To determine whether the viral particles released by the 293T producer cell line were infectious, culture supernatants were used to infect the target cells SupT1 and PMI1 cells. After 14 days of culture, successful infection was monitored by p24 antigen analysis. Figure 3.13a and 3.13b show the growth kinetics of the chimeric clones. The results show little growth for any of the viruses in the PMI cells. In the SupT1 cells NL4-3 grew well and some growth was seen with the MJ4/NL4-3 chimeric, but both MJ4 and NL4-3/MJ4 failed to grow.

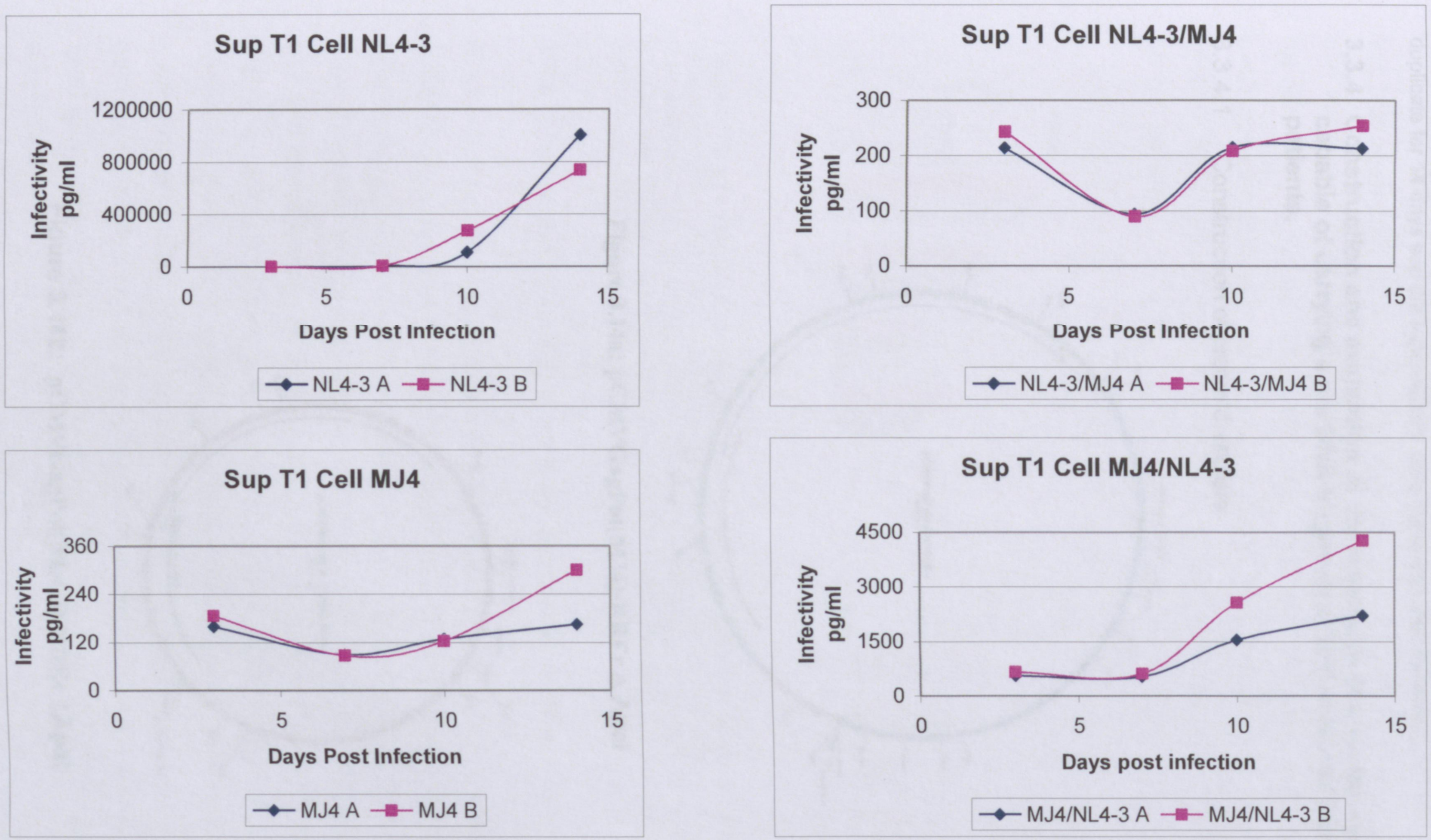

Figure 3.13a: Growth kinetics of pMJ4ΔApaI, pNL4-3Apa+, and pMJ/NL and pNL/MJ in SupT1 cells

SupT1 cell infection using the harvested supernatants of viral particles produced from transfection by pMJ4ΔApaI, pNL4-3Apa+, and pMJ/NL and pNL/MJ. Infection was done in duplicate for 14 days and the supernatants were harvested after three days. The Y-axis labeled infectivity represents pg of p24 in the supernatant.

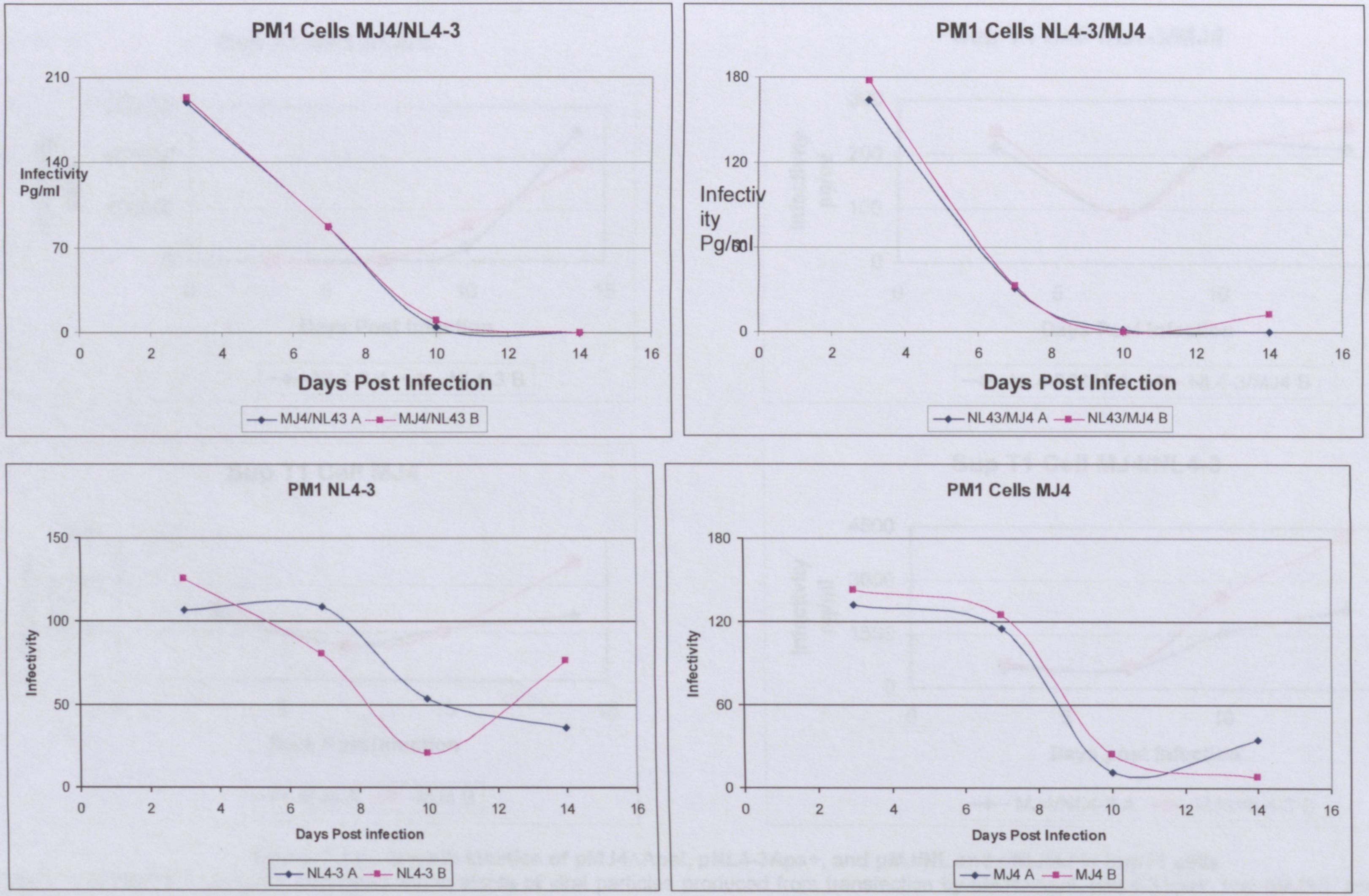

Figure 3.13b: Growth kinetics of pMJ4ΔApal, pNL4-3Apa+, and pMJ/NL and pNL/MJ

PM1 cell infection using the harvested supernatants of viral particeles produced from transfection by pMJ4ΔApaI, pNL4-3Apa+, and pMJ/NL and pNL/MJ. Infection was done in duplicate for 14 days and the supernatants were harvested after three days.

3.3.4 Construction and evaluation of the infectivity of a vector system capable of carrying a *pol* DNA fragment of HIV obtained from patients.

3.3.4.1 Construction of plasmid vectors

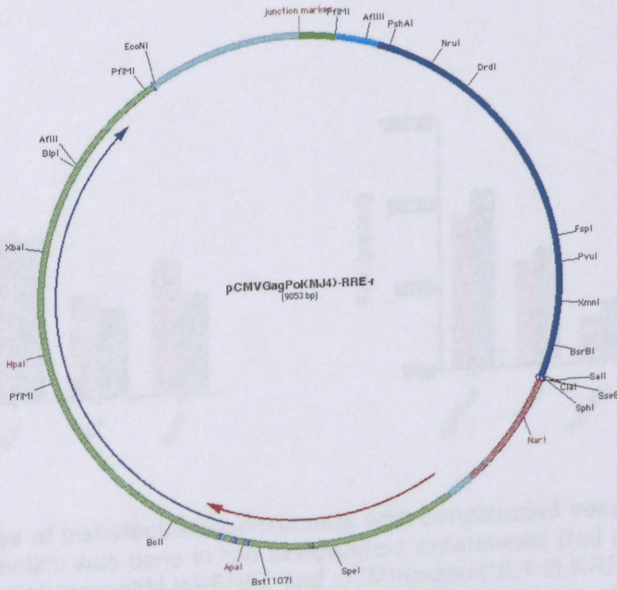

Figure 3.14a: pCMVGagPol(MJ4)-RREr Δ ApaI

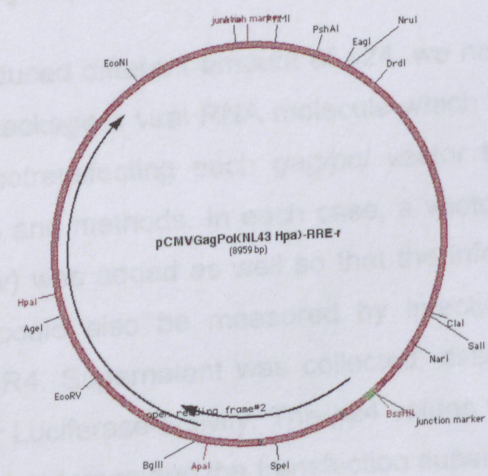

Figure 3.14b: pCMVGagPol(NL4-3)-RREr ΔApaI

3.3.5 Transfection of vector systems

We first tested the *gag/pol* expression plasmids that were constructed for the p24 assay. 293T cells were transfected with MJ4, NL4-3, MJ/NL, and NL/MJ. As controls, two vectors known to express lots amount of viral particles were used as well (HR354 and HIVpack (HR3861)). Figure 3.15 shows that all four vectors tested expressed reasonable p24 levels although lower than the control vectors.

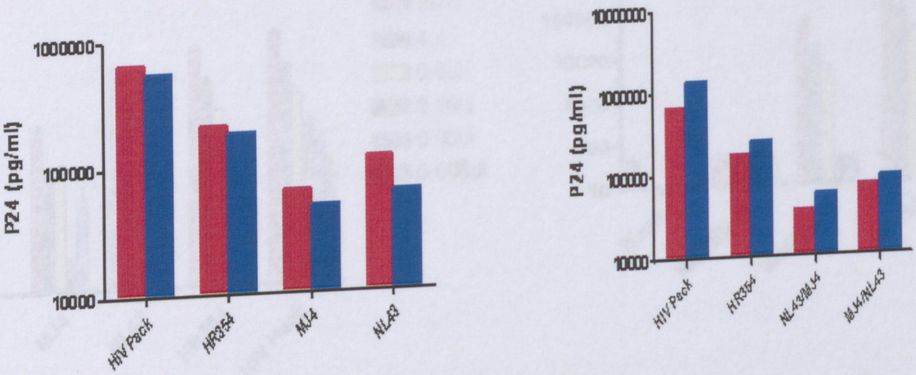

Figure 3.15: p24 values of transfection experiments with constructed vector systems. For each vector transfection was done in two independent experiments (red and blue bars). MJ4 and NL4-3 are pCMVgagpol(MJ4)-RREr and pCMVgagpol(NL4-3)-RREr respectively. HIVpack and HR354 are positive controls. NL4-3/MJ4 and MJ4/NL4-3 are chimeric vectors (Construction data not showed).

3.3.6 First round infectivity of particles produced viral particles

Since all of the vectors produced different amount of p24, we now tested the ability of the constructs to package a viral RNA molecule which is capable of expressing Luciferase by cotransfecting each *gag/pol* vector together with pLuc described in materials and methods. In each case, a vector expressing *env* and *Rev* proteins (pEnv) was added as well so that the infectivity of the resulting plasmid vectors could also be measured by infecting dog cells containing CD4/CCR5/CXCR4. Supernatant was collected; assayed for p24 levels and also assayed for Luciferase activity. The p24 values were used to normalize the infection with Luciferase and the transfection supernatants were used to infect dog cells containing CD4/CCR5/CXCR4 and Luciferase activity was measured.

The expression of vectors in figure 3.16 was similar to that of figure 3.15, that the values of the vectors are still lower than those of the controls. NL4-3 and MJ/NL are still the ones with the better infectivity compared to the MJ4 and NL/MJ. This is similar to the infectivity of the chimeric proviral clones. The p24 adjusted Luciferase (figure 3.17) showed the similar results for the infectivity in different folds and MJ4 is still very low. (Some of the experiments were done in the collaboration with Dr Hua cheng, Hamrek lab, UVA).

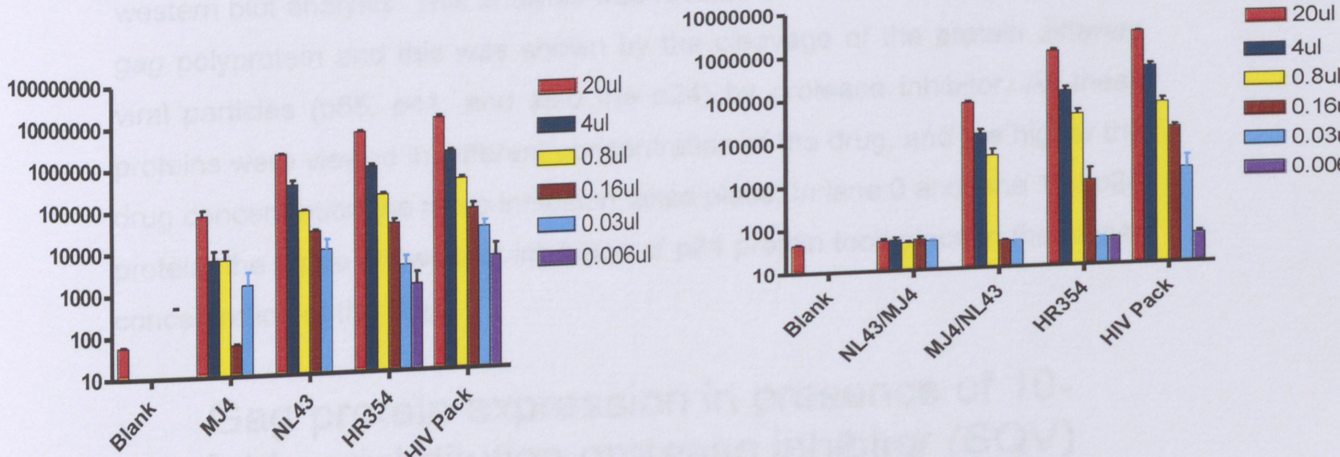

Figure 3.16: Shows the p24 of four vectors measured in five serial dilutions.

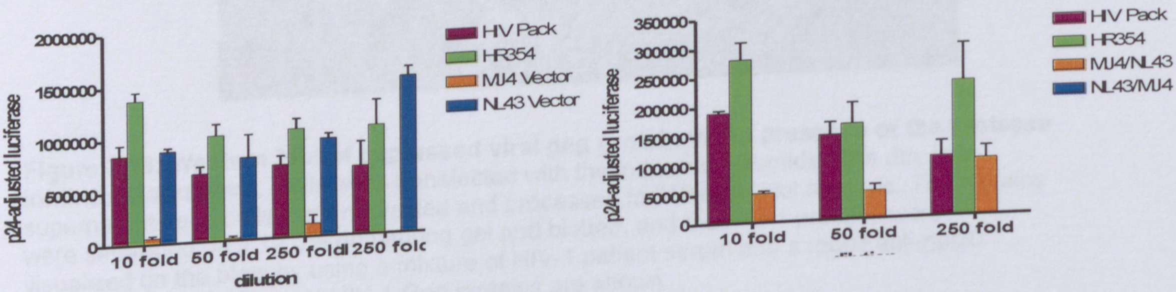

Figure 3.17: p24 antigen adjusted Luciferase activity

3.3.7 Gag expression in the presence of protease inhibitor

Since when pCMVGagPol(NL4-3)-RRErΔApaI showed better expression of gag/pol and lucifesase assay, it was then used to test for the expression of the gag protein p24 in the presence of the protease inhibitor (saquinavir). The vector was cotransfected with the expression vector (pEnv), pLuc and the drug Saquinavir. The transfection supernatant was collected and analyzed by western blot analysis. This analysis was reveled that the vector expressed the *gag* polyprotein and this was shown by the cleavage of the protein different viral particles (p55, p41, and also the p24) by protease inhibitor. All these proteins were viewed in different concentration of the drug, and the higher the drug concentration the more inhibition takes place. In lane 0 and lane 1 of p24 protein, the figure shows that inhibition of p24 protein took place in the higher concentration of the drug.

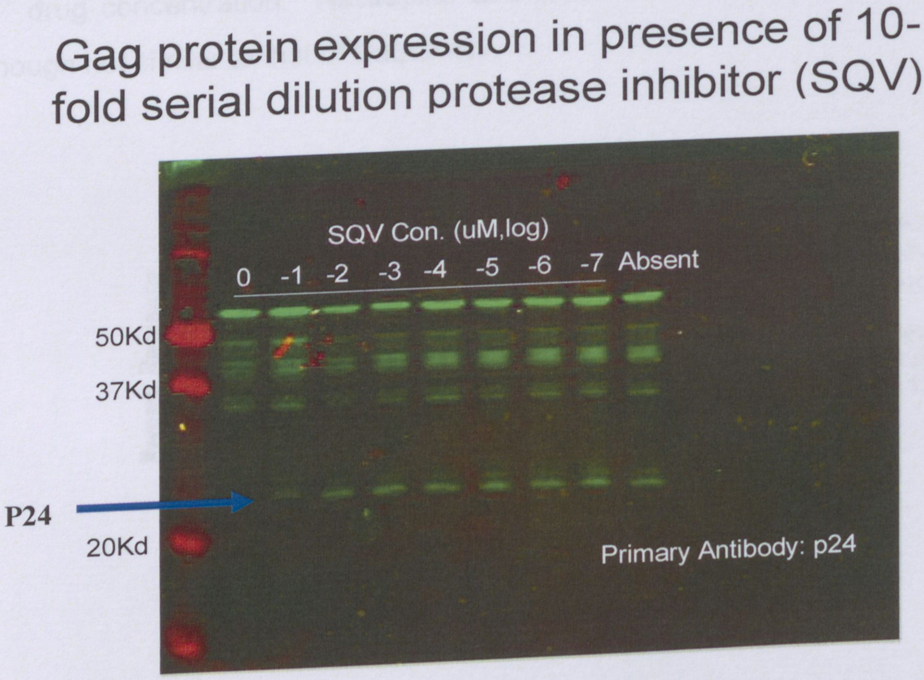

Figure 3.18: Western blot of expressed viral gag protein in the presence of the protease inhibitor Saquinavir. Cells were transfected with the indicated plasmids. One day later, supernatants and cells were harvested and processed for Western blot analysis. The proteins were separated on a 12.0% denaturing gel and blotted, and the HIV-1 proteins were visualized on the blots by using a mixture of HIV-1 patient serum and a rabbit anti-gpl20 antiserum. The positions of HIV-1 Gag proteins are shown.

3.3.8 Evaluation of the susceptibility of vector system to selected protease and reverse transcriptase inhibitors

To demonstrate the usefulness of this phenotypic assay for measuring the inhibition of viral replication by antiretroviral drugs, we determined by drug (saquinavir, nevirapine, and zidovudine) titration the concentration of protease and reverse transcriptase inhibitors needed to inhibit the replication of the pCMVgappol(NL4-3)-RREr∆ApaI vector. Three plasmids were cotransfected with the protease inhibitor and the supernatant used to infect the dog cells, whereas in *RT* susceptibility assay, the transfection was done without the presence of the drugs. Drugs were added later in the infection process. The Luciferase was measured as well as the IC-50 of the drug in both the *PR* and *RT*. The drug concentration was versus the percentage response and the results showed that the viral particles were inhibited by all the drugs. The *PR* inhibitor showed that at 50% the viral particles were completely inhibited with 10^{-1} drug concentration. Nevirapine and Zidovudine also showed inhibition although not similar to that of Saquinavir.

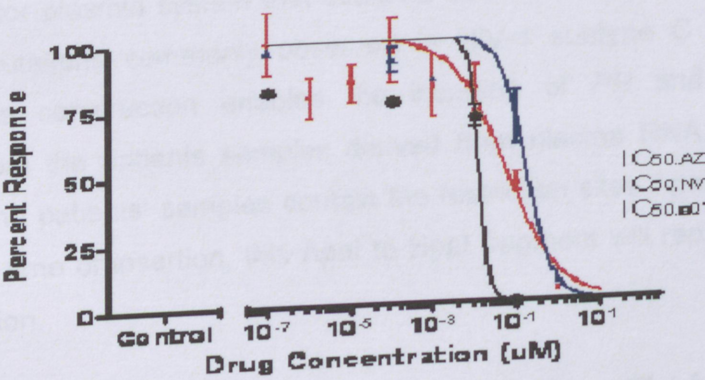

Figure 3.19: Inhibitor curve for NL4-3 vector (pCMVGagPol (NL4-3)-RREr) in the presence of AZT, NVP and SQV. pCMVGagPol (NL4-3)-RREr) transduces the Luciferase reporter gene into 293T target cells. Reporter gene transduction is inhibited with *PR* and *RT* inhibitors in concentration-dependent manner. This drug susceptibility testing was performed with viral stock from transfection supernatant of the viral vectors. Susceptibilities to a panel of three drugs zidovudine (AZT), nevirapine (NVP) and saquinavir (SQV) are shown. Fold differences in drug susceptibility were determined by the IC_{50} of the sample virus.

3.4 DISCUSSION

Due to the development and expansion of resistant viruses in a significant fraction of HIV infected individuals receiving antiretroviral therapy and the availability of alternative treatment possibilities, there is a growing need to determine the sensitivity or resistance of HIV to antiviral agents. Methods that are currently used to assess virus resistance include genotypic and phenotypic test systems. Genotypic analysis determines mutations at particular positions in the *RT* and *PR* genes that correspond to a specific level of functional drug resistance of the particular enzyme. These tests are rapid to perform but may be difficult to interpret when multiple mutations occur. Phenotypic tests measure enzyme function. Thus, phenotypic analysis potentially reflects more precisely the sensitivity of the virus to the antiviral compound. Conventional phenotypic analyses, however, need several weeks to months and require high titer culture infectious HIV (Japour *et al*., 1993; Kellam and Lader, 1994; Hertogs *et al*., 1998).

This study describes the construction of the chimeric proviral clones and the retroviral vector plasmid system that could be used for testing putative drug-resistance mutations commonly observed in HIV-1 subtype C drug-naïve patients. This construction enables the insertion of *PR* and *RT* gene fragments from the patients samples derived from plasma RNA. The PCR products of the patients' samples contain the restriction sites ApaI and HpaI, which by the time of insertion, this ApaI to HpaI fragment will replace that in the construction.

When the proviral chimeric clones pMJ4/NL4-3 and pNL4-3/MJ4 were constructed, the 1.6kb fragment used as an insert in each plasmid, respectively, will be the one substituted by the patients'. Cloning of the plasmids was successful and that was confirmed by digestion with restriction enzymes ApaI, PflmI, and AgeI (Figure 3.11), and this showed that the vector ligated to the correct insert, no relegation of the vectors. Digestion also revealed that the sizes of the constructs are of correct sizes and the molecular weight of the bands on the agarose gel comparing to the plasmids maps.

Overall, the results with virus produced from the proviral clones were disappointing. No growth was observed in the PM1 cells from any of the constructs although PM1 is known to grow both CCR5 and CXCR4 dependent viruses. The fact that NL4-3 did not grow at all suggests that something was wrong with the PM1 cells. In SupT1 cells, only the NL4-3 control virus grew robustly, however, it was not expected that these cells would support MJ4 or MJ4/NL4-3 growth as these viruses are CCR5 dependent and SupT1 contain only CXCR4. A small amount of growth was seen with the NL4-3 virus carrying the MJ4 ApaI-HpaI cassette (NL4-3/MJ4). It is not known why this chimera grew more poorly than the wild type NL4-3.

In the case of the viral vector system, good p24 production was obtained from all four of the *Gag/Pol* plasmids. However it was disappointing, once again, that the only clone that worked efficiently to deliver luciferase to the target cells was the wild type NL4-3, although wild type MJ4 showed some activity, but very low infectivity. It was surprising to find that MJ4/NL4-3 worked better than MJ4, while NL4-3/MJ4 appeared totally devoid of any infectivity. In these cases, coreceptor usage cannot explain these differences as all viruses received the same CXCR4 dependent envelope protein.

There are several reasons one can postulate as to why the vector system gave such disappointing results. It may be that the use of a clade B luciferase vector is not compatible with the Clade C *Gag/Pol* protein in that it fails to get packaged properly. While this might explain the poor result obtained with the MJ4 clone, it does not explain the fact that NL4-3/MJ4 was totally devoid of infectivity and that MJ4/NL4-3 seems to work better than MJ4. These latter results suggest that there might be something intrinsically wrong with the MJ4 sequence. We are presently in the process of repeating this strategy with a different Clade C infectious clone, in the hope that the system can be made to work.

The vector expressed the majority of viral *gag* polyprotein and the drug seemed to be working very well because the inhibition observed was good.

The expression of *gag* was enhanced by the coexpression of pEnv with the *Rev* gene in it. Analysis of the protein components of the pCMVGagPol(NL4-3)-RRErΔApaI viral particles indicated that HIV-1 *PR* was capable of cleaving the gag polyprotein to yield the mature core protein.

The results with viral inhibition susceptibility test showed that the viral particles produced by our constructed vector can be inhibited by the NRTIs, NNRTIs, and the PIs respectively. Inhibition was seen in all drugs in different concentration, and this means that the vector system works.

The 50% inhibitory concentrations (IC50s) obtained from a wide range of different virus inocula demonstrated that titration of virus stocks was not necessary (data not shown). The inhibition percentage was calculated as [1-(luciferase activity in the presence of drug/luciferase activity in the absence of drug)]. The dose-response curves were obtained plotting these percentages against the log10 of drug concentration. The IC50 was calculated by nonlinear regression curve, using a four-parametric sigmoidal dose-response equation with variable slope, fitting with the GraphPad Prism software (2.01 version, 1996). Changes in drug susceptibility were determined by dividing the IC50 for the test sample by the IC50 for the reference virus that was tested in the same experiment.

In conclusion, the assay can also be used to monitor the frequency of drug-resistant virus in newly infected individuals (Boden *et al.*, 1999; Hecht *et al.*, 1998; Little *et al.*, 1999), to characterize baseline variation in susceptibilities to various drugs in treatment-naïve patients, and get better understanding on why current treatment fail (Havlir *et al.*, 2000; Parkin *et al.*, 1999). Finally, the assay can be used to screen for or assist in the development of new drugs that are active against resistant strains of HIV-1.

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