Construction of an HIV-1 subtype C vector system

for

phenotypic drug resistance studies

By

Phadagi Muendi Tshililelwa

January 2013

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University of Venda

Student Number: 11607014

Dissertation submitted in partial fulfillment of the requirements for Master's degree in Microbiology

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DECLARATION

I, **MUENDI TSHILILELWA PHADAGI Student NO (11607014)**, hereby declare that the dissertation for the Master's 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 2013|01|28 ....

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I would like to first thank GOD ALMIGHTY for his grace that has enabled me to achieve my goals in life. I have achieved all this because of his favor, protection, guidance and tender mercy and to him be the Glory.

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I would also like to express my gratitude to **Technology Innovation Agency and National Research Foundation**, South Africa for financial support.

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DEDICATION

THIS MASTERS DISSERTATION IS DEDICATED TO THE FOLLOWING PEOPLE:

- To my understanding and hard caring husband **Khangwelo Sigidi,** he has been my pillar to lean on all the times of my studies. He has worked tirelessly for me to be what I am today.
- To my two lovely children **Wanga** and **Khangwelo Jnr**, your patience has been credited.
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List of Abbreviations

APOBEC-3G- Apolipoprotein B mRNA-editing, enzyme-catalytic, polypeptide-like 3G

APV- Amprenavir

ATV- Atazanavir

AIDS- Acquired Immunodeficiency Syndrome

ALV- Avian Leukemia virus

APV-Amprenavir

ART-Antiretroviral therapy

ASV- Avian sarcoma virus

ATP-Adenosine-5'-triphosphate

ATV- Atazanavir

AVS- Avian Sarcoma virus

AZT- Zidovudine

BFV- Bovine Foamy virus

BLV- Bovine Leukemia virus

CA- Capsid

$CaCl_2$- Calcium chloride

CDC- Center for Disease Control and Prevention

cDNA- Complementary DNA

CRF's- Circulating recombinant forms

CRS- Cis-acting repressive sequences

DNA- Deoxyribonucleic acid

ddC- Zalcitabine

ddI- Didanosine

DLV- Delavirdine

DRV- Darunavir

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DMEM- Dulbeco's Modified Eagles Medium

dNTP- Deoxynucleoside triphosphates

dsDNA- Double stranded deoxyribonucleic acid

EDTA- Ethylenediaminetetraacetic acid

EIAV- Equine Infectious Anemia virus

Env- Envelope gene

T20- Enfuvirtide

FCS- Fetal Calf Serum

FDA- Food and drug administration agency

FeLV- Feline Leukemia virus

FIV- Feline Immunodeficiency virus

f-APV- Fosamprenavir

Gag- Group associated antigen gene

HAART- Highly Active Antiretroviral Therapy

HBS- Hepes Buffered Saline

HIV-1- Human Immunodeficiency virus type 1

HIV-2- Human immunodeficiency virus type 2

HR1- heptad repeats sequence 1

HR2-Heptad repeats sequence 2

HSRV- Human Spuma virus

HTLV-1- Human T-cell Leukemia virus type 1

HTLV-2- Human T-cell Leukemia virus type 2

H_2O- Water

IL-2- Interleukin 2

IN- Integrase

INS- Inhibitory RNA sequences

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IE- Immediate early promoter enhancer

IDV- Indinavir

LTR- Long terminal repeats

3TC- Lamivudine

LB- Luria Bertanni

MDR- Multidrug resistance

MLV- Murine Leukemia virus

MSV- Murine Sarcoma virus

MA- Matrix

MPMV- Mason-pfizer monkey virus

mRNA- Messenger RNA

$MgCl_2$- Magnesium chloride

NC- Nucleocapsid

Nef- Negative factor

NRTIs- Nucleoside reverse transcriptase inhibitors

NNRTIs- Non-nucleoside reverse transcriptase inhibitors

NVP- Nevirapine

PCR- Polymerase Chain Reaction

PE-Psi elements

PBS- Phosphate buffered saline

Pol- Polymerase gene

PR- Protease

PRAMS- Protease resistance associated mutations

RNA- Ribonucleic acid

RSV- Rous Sarcoma virus

Rev- Regulator of viral protein synthesis

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RRE- Rev responsive element

RT- Reverse transcriptase

SIV- Simian Immunodeficiency virus

STLV- Simian T-cell leukemia virus

SFV- Simian Foamy virus

SSV- Simian Sarcoma virus

SRV-1- Simian retrovirus type 1

d4T- Stavudine

TAMS- Thymidine analogs

Tat- Transactivating protein

TAR- Tat responsiveness

tRNA- Transfer RNA

UNAIDS- United Nations AIDS Program

URF's- Unique recombinant forms

Vpr- Viral protein R

Vif- Virion infectivity factor

Vpu- Viral protein U

WHO- World Health Organization

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Abstract

Acquired immunodeficiency syndrome (AIDS) caused by human immunodeficiency virus (HIV) is one of the world's most deadly infectious disease. HIV-1 infections accounts for over 60% of global infections, yet majority of researchers are focused on subtype B viruses which only accounts for <12% of the global pandemic. UNAIDS has estimated 33.3 million HIV-1 infections globally in 2011, and South Africa accounts for 5.6 million of these infections. HIV-1 subtype C viruses predominate in Southern Africa and are rapidly spreading across the world accounting for more than 50% of infections worldwide. The focus on reverse transcriptase (RT) and protease (PR) gene as targets of antiviral therapy has led to the discovery of various major, minor and polymorphic mutations harbored thereof. There are mutations in HIV-1 subtype B strains that develop only under drug pressure leading to ARV drug resistance. These mutations have been observed in subtype C strains as polymorphisms. The aim of this study was to construct an HIV-1 subtype C vector system which could be used to evaluate the phenotypic significance of putative resistant mutations observed in HIV-1 subtype C polymerase gene sequences.

A mammalian derived plasmid pCMVGagPol(IndieC)RRE comprising unique ApaI and HpaI restriction sites was previously modified. Viral RNA from 14 drug naïve patient samples was reverse transcribed to cDNA using transcriptor reverse transcriptase thereafter amplified using nested PCR. The amplified 1650bp gene was cloned into pGEMT easy cloning vector and expanded in DH5α competent cells. Viral DNA was subsequently extracted from the pGEMT vector by double digestion using ApaI and HpaI restriction enzymes. A 1650 bp fragment was excised from pCMVGagPol(IndieC)RRE and replaced by a 1650 bp purified viral DNA to construct pCMVGagPol(Patient)RRE. Chimeric virus was expanded in DH5α cells followed by successive restriction digestion and colony PCR to confirm the cloned HIV-1 C *GagPol* gene. Orientation of the cloned viral DNA was confirmed by sequencing the vector and identifying the restriction enzyme positions.

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Of the analyzed sample sequences, the identified significant polymorphisms observed were K20R, M36L, I93L and L89M, these mutations are known to be associated with susceptibility to PI's in subtype B viruses.

The constructed pCMVGagPol(patient)RRE chimeric virus can be used for subsequent evaluation of mutations that are present in naïve and treatment exposed viruses but whose importance is not clear.

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CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1. HIV-1 epidemic

Human immunodeficiency virus (HIV) was first recognized in the early 1980's, it is believed to be a descendant of a Simian Immunodeficiency virus (SIV) because some of the strains have shown close relation to HIV-1 and HIV-2. HIV-1 has been identified as the etiologic agent causing acquired immunodeficiency syndrome (AIDS) (Zhu *et al*., 1998). In the past 20 years, AIDS has become the world's most deadly infectious disease. UNAIDS and World Health Organization (WHO) estimates that AIDS has killed over 25 million people since its discovery, making it one of the most destructive pandemic recorded in history. Despite recent improved access to antiretroviral treatment and care in many regions of the world, HIV has infected 33.3 million people in 2011 of which over half a million were found to be children (UNAIDS, 2010).

The continued rise in the world population of people living with HIV reflects the combined effects of high rates of new HIV infections and the beneficial impact of antiretroviral therapy. As of December 2008, approximately 4 million new HIV infections occurred. The latest epidemiological data indicate that globally the spread of HIV appears to have peaked since 1996, the annual HIV related mortality have peaked since 2004. An estimated 430 000 new HIV infection occurred among children under the age of 15 in 2008. Most of these infections arise from the transmission in the uterus, during delivery or through breastfeeding (UNAIDS, 2009). In 2010 an estimate of 22.5 million people living in Sub-Saharan Africa were found to be infected with HIV. Sub-Saharan Africa remains to be the region mostly affected by HIV and it accounts for about 67% of infections worldwide and 72% of the world's AIDS related deaths in 2008. HIV prevalence tends to peak at a younger age for women than men (Gouws *et al*., 2008).

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1.2. Taxonomy of HIV-1

Retroviruses represents a broad group of enveloped, icosahedral viruses with 2 positive stranded ribonucleic acid (RNA), they can reverse transcribe their genomic RNA to complementary cDNA by a unique replicative strategy involving enzyme reverse transcriptase (Hahn *et al*., 2000; Gualco *et al*., 2009; Ka *et al*., 2009). The result is a linear double stranded deoxyribonucleic acid (DNA), followed by integration of viral DNA into host genome. There are 2 taxonomic groups or divisions among retroviruses; these are simple and complex retroviruses. The complex retroviruses are subdivided into:

-Lentiviruses, to which belong HIV-1 and HIV-2, simian immunodeficiency virus (SIV), visna virus, feline immunodeficiency virus (FIV), and equine infectious anemia virus (EIAV)

- T-cell leukemia viruses, human T-cell leukemia virus type 1 (HTLV-1) and type 2 (HTLV-2), the simian T-cell leukemia virus (STLV) and bovine leukemia virus (BLV)

-and lastly spumaviruses represented by human spuma retrovirus (HSRV), simian foamy virus (SFV) and bovine foamy virus (BFV).

Simple viruses are subdivided into 4 subtypes:

-C type retrovirus of group A, rous sarcoma virus (RSV), avian leukemia virus (ALV), and avian sarcoma virus (ASV)

-C type retrovirus of group B, murine leukemia virus (MLV), feline leukemia virus (FeLV), murine sarcoma virus (MSV), and simian sarcoma virus (SSV)

-Lastly B type retroviruses, mason-pfizer monkey virus (MPMV), and simian retrovirus type 1 (SRV-1).

Lentiviruses bud concomitantly with the assembly of the viral core and their envelopes harbor characteristics knobs. T-cell leukemia viruses have morphological patterns common

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to lentiviruses. Spumaviruses have surface projections and a preformed core which does not fully condense after virion budding (Gelderblom *et al.*, 1989)

1.3. Morphology and structure of HIV-1

As shown in figure 1.1, HIV-1 has an envelope and is icosahedron in shape containing 72 external spikes (Gelderblom, 1991). These spikes comprise of 2 major viral envelope proteins, which is the glycoprotein gp120 and the transmembrane gp41. The gp41 subunit spans the membrane and anchors the glycoprotein complex to a virus particle while gp120 merely comprise the outer portion of the complex. Along the viral enveloped bilayer there are a series of host cell proteins that are acquired during virion budding (Arthur *et al.*, 1992). The overall diameter of the viral particle is about 100 nm (Gelderblom *et al.*, 1987)

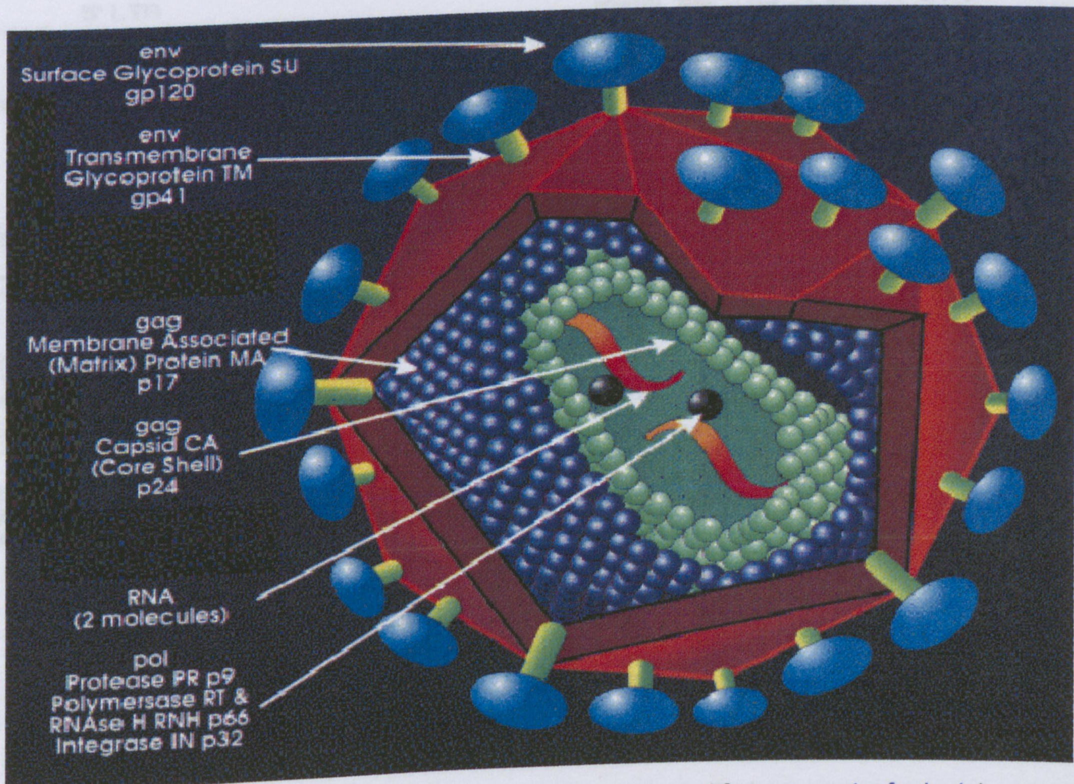

Figure 1.1: Schematic representation of HIV-1 architecture (Adapted from www.stanford.edu)

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The matrix protein (p17) is associated with the inner surface of the viral bilayer as shown in figure 1.2. Viral genomic RNA, accessory protein vpr, prolin-rich protein p9, enzymes reverse transcriptase (RT- p51, p66), protease (p11) and integrase (p31) are enclosed inside the conical capsid composed of 2000 copies of viral protein p24. The HIV-1 genome consists of an open reading frame encoding structural, regulatory, and accessory proteins. Two of identical elements termed long terminal repeats (LTRs) are generated at each end of the provirus during reverse transcription (Chen *et al.*, 1980; Lu *et al.*, 1993; Darlix *et al.*, 1995). The LTRs contains transcriptional enhancer sequences and promoters, these include cis-acting elements, polyadenylation signal sequences and TATA promoters among others.

gag:
p17 - myristylated capsid protein
p24 - major capsid protein
p9 - RNA-binding nucleocapsid protein
p7 - RNA-binding nucleocapsid protein

pol:
p10 - protease
p66/51 - reverse transcriptase (heterodimer)
p32 - integrase

env:
gp120 - envelope glycoprotein (external)
gp41 - envelope glycoprotein (transmebrane)

regulatory proteins:
tat: transactivating protein (transcription)
rev: regulator of viral protein synthesis
nef: "negative factor"

accessory proteins:
vif - virion infectivity factor
vpr - viral protein R (transactivator?)
vpu - viral protein U (virion release)

RNA sequence regions:
LTR - long terminal repeat
U3 - unique 3' region
R - terminal redundancy
U5 - unique 5' region
TAR - tat responsiveness
RRE - rev response element

Figure 1.2: Genomic organization of HIV-1 (Adapted from www.virology.com)

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HIV-1 genome contains 3 essential regions located between the 5'- and -3' of LTRs (refer figure 1.2):

1. Group associated gene (gag), situated at the 5' end of the genome and codes for gag polyprotein that is the precursor of 4 smaller structural proteins designated matrix (MA or p17), capsid (CA or p24), nucleocapsid (NC or p9) and p6. MA facilitate the nuclear transport of viral genome, CA forms the conical core of viral particles, NC is responsible for recognizing the packaging signal of HIV and p6 region mediates interactions between p55 Gag and accessory protein Vpr (Freed, 1998; Gallant *et al.*,2003; Tripathi and Agrawal, 2007).

2. Polymerase gene (pol), encodes the viral associated enzymes, Reverse transcriptase (RT), protease (PR) and integrase (IN). Protease acts as a dimer, its activity is required for the cleavage of Gag and Gag-pol polyprotein precursors during maturation (Purohit *et al.*, 2007). RT assists in reverse transcribing viral RNA to complementary DNA. IN mediates the insertion of the HIV proviral DNA into genomic DNA of infected cells (Cherepanov, 2010). An exonuclease activity first trims two nucleotides from each 3' end of the linear viral DNA duplex, then a double stranded endonuclease activity cleaves the host DNA at the integration site finally a ligase activity makes a single covalent linkage at each of the proviral DNA ends (Gallant *et al.*, 2003).

3. Envelope gene (gp160) is expressed from mRNA's that are singly spliced. Env migrates through the golgi apparatus from the endoplasmic reticulum where it gets glycosylated. The glycosylation step is essential for viral infectivity. PR cleaves gp160 into gp120 and gp41 (Mavoungou *et al.*, 2005). Gp120 comprise of 9 highly conserved disulfide and 5 hypervariable regions called V1 through V5. An interaction between HIV and CD4 cells is mediated through specific domains of gp120. Gp41 contains an N-terminal that mediates fusion of the viral and cellular membranes allowing delivery of viral contents into host cell cytoplasm (Varmus *et al.*, 1989).

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1.3.1. HIV-1 genomes accessory proteins

Nef is not essential for replication in vitro; however it is required for in vivo HIV replication and pathogenicity. Its presence is essential for maintaining high viral load values and for the progression of AIDS disease. Nef is also known to stimulate the infectivity of HIV-1 virion; particles produced in its presence can be absorbed 10 times more than virions produced in its absence. Vif is a 23kD polypeptide which plays a vital role in supporting viral replication. Vif-deficient HIV-1 isolates do not replicate in CD4+ T cells or in macrophages however, they are able to enter a target cell and initiate reverse transcription (Garcia *et al.*, 1993; Aiken *et al.*, 1994; Das *et al.*, 2005).

Vpu is an integral membrane phosphoprotein localized in internal membranes of the cell. This gene overlaps the 5' end of the env gene (Strebel *et al.*, 1988). It is important for the virus budding process and also involved when CD4+-gp 160 complexes are degraded within the endoplasmic reticulum thus allowing the recycling of gp160 for formation of new virions (Crise *et al.*, 1990; Willey *et al.*, 1992; Bour *et al.*, 1995). Vpr gets incorporated into viral particles; its incorporation is mediated through the specific interactions with Gag p55. It also plays a crucial role in the ability of HIV to infect nondividing cells by facilitating nuclear localization of preintegration complex. Vpr tethers viral genome to the host nuclear pore complex, it also block cell division (Heinzinger *et al.*, 1994, Miller and Sarver, 1997). Vpr has the capacity to inhibit the progression of infected host cells from G2 to the M phase of the cell cycle (Fletcher *et al.*, 1996; Levy, 1993; Costin, 2007).

Vif is a basic protein typically about 23KD; it promotes the infectivity and not the production of viral particles. In its absence the viral particles would be defective. Vif is a cytoplasmic protein that exists in both soluble cytosolic form and membrane associated form. It is believed to prevent action of cellular APOBEC-3G protein which deaminates DNA: RNA heteroduplexes in the cytoplasm (Newman *et al.*, 2005). Rev is a necessary regulatory factor for HIV expression. It is a 19KD phosphoprotein found in the nucleolus. Rev acts by

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binding to RRE and promotes nuclear export, stabilization and utilization of viral mRNA. TAT is a transcriptional transactivator of HIV and is an essential viral regulatory factor for HIV-1 gene expression. Tat exists as Tat-1 exon or Tat-2 exon localized in the nucleus and acts by binding to TAR RNA elements activating transcription initiation and elongation from the LTR promoter preventing the 5' LTR AATAAA polyadenylation signal from causing premature termination of transcription and polyadenylation (Jeang, 1996; Siddappa *et al.*, 2006; Xiao *et al.*, 2000; Ponti *et al.*, 2008).

1.3.2. HIV-1 genomes structural elements

LTR (long terminal repeats) flanks the genome of the integrated provirus and it contains regulatory regions responsible for transcription initiation and polyadenylation. TAR, is a target sequence of viral transactivation. It is a binding site for tat and cellular proteins. TAR RNA forms a hairpin stem-loop structure with a side bulge necessary for tat binding and function.

Rev responsive element (RRE) is encoded within the env gene region of HIV-1 and is essential for Rev Function. Psi elements (PE) comprise of a 4 stem loop structures overlapping the Gag start codon. They are recognized by the cysteine histidine box which is a conserved motif with a sequence Cys×2Cys×4His×4Cys present in the Gag p7 MC protein (Zeffman *et al.*, 2000). SLIP, is a TTTTTT slippery site, followed by a stem loop structure responsible for regulating the 1-ribosomal frameshift out of the Gag reading frame into the pol reading frame. The Cis-acting repressive sequences (CRS) inhibit structural protein expression in the absence of rev. Finally inhibitory/instability RNA sequences (INS) inhibits expression posttranscriptionally (Brighty and Rosenberg, 1994).

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1.4. Genetic variability of HIV

HIV displays very high genetic variation; there are 2 types of HIV namely HIV-1 and HIV-2. HIV-1 is responsible for most of the AIDS infections worldwide (Reeves *et al*., 2002; Johnson *et al*., 2003). The different HIV-1 strains have been classified into 4 phylogenetic groups namely: M (major group), N (Non group M), P and O (Outlier) (Carr *et al*., 1998; Simon *et al*., 1998; Spira *et al*., 2003; Badreddine *et al*., 2007). The variation is accounted for by increased mutation rates, evolution of new resistant strains and huge population sizes. HIV-2 comprises 8 distinct groups A–H. Among the 4 HIV-1 groups (Figure 1.3) group M predominates the global epidemic with 9 subtypes, unique recombinant forms (URFs) and circulating recombinant forms (CRFs). The most prevalent HIV-1 subtype in the global epidemic is subtype C (Robertson *et al*., 2000; Osmanov *et al*., 2002).

Genetic recombination is believed to be one of the mechanisms that generate rapid diversification of HIV-1 populations by reasserting mutations (Jetzt *et al*., 2000; Negroni *et al*., 2001; Hu *et al*., 2003; Onafuwa *et al*., 2003). HIV-1 recombinants were estimated to contribute to 10-40% and 10-30% of infections in Africa and Asia respectively (Takebe *et al*., 2004). The global geographic distribution of various HIV subtypes and HIV-1 intersubtype recombinants is heterogenous, though the predominant clades are A, B and C. Clade A predominates in West and central AFRICA, B predominates in Europe and North/South America whereas C is prevalent in Southern Africa, Eastern Africa and India.

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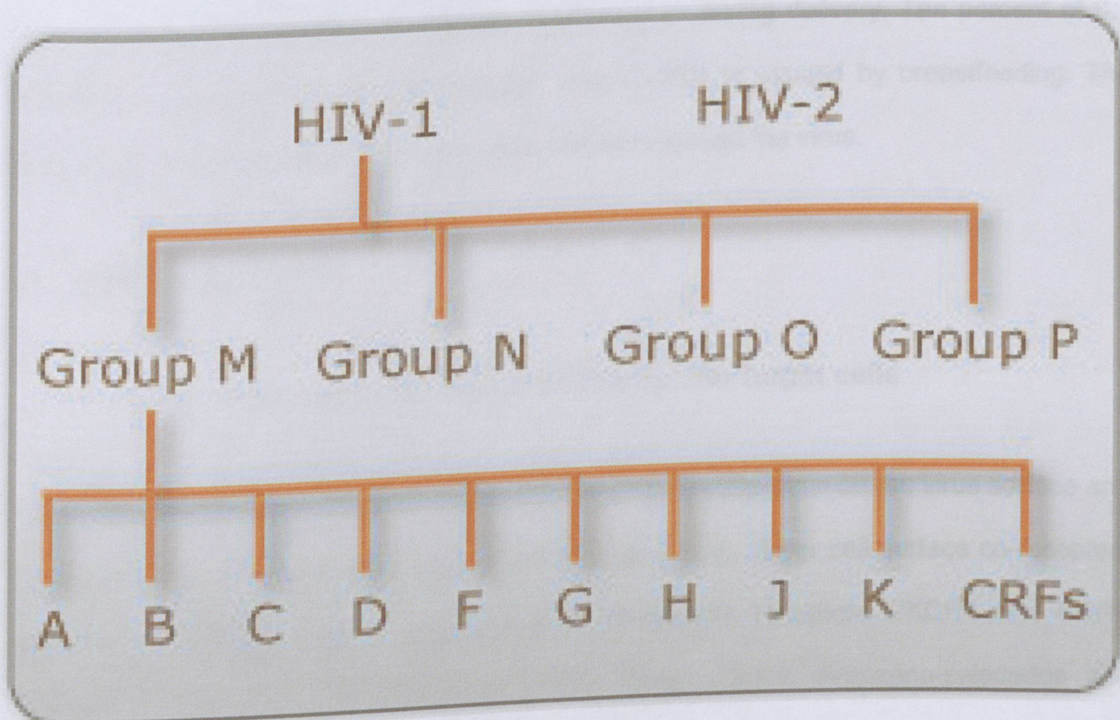

Figure 1.3: HIV phylogenetic groups and subtypes (Adapted from www.avert.org/hiv-types.html)

1.5. HIV-1 transmission routes

HIV can be spread in a number of ways namely: unprotected sexual intercourse with an infected partner, injection/transfusion of contaminated blood/blood products, sharing unsterilized injection equipment that has been used by an infected person and also through materno-fetal transmission. Majority of HIV infections are acquired through unprotected sexual relations worldwide. Sexual transmission can occur when infected secretions of one partner come into contact with the genital, oral or rectal mucous membrane of another, although the lower the viral load of an infected person the lower the chances of infection. The current and consistent use of latex condoms reduces the risk of infection. Sharing of used and unsterilized injections is also important transmission route given the high number of drug users worldwide. Without any form of drugs taken, about 15- 30% of mothers with

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HIV transmit the virus during the pregnancy and during delivery. Seventy five percent of the time, the virus is transmitted during late pregnancy or during delivery. Ten percent of the infections occur before the third trimester, and 10-15% is caused by breastfeeding. The transfusion of contaminated blood products can also spread the virus.

1.6. Life cycle of HIV-1

1.6.1. Virus binding, fusion and entry into target cells

HIV-1 infection is mediated by the interaction of gp120 glycoprotein on the virus surface and receptor CD4 molecule on the target host cell (Figure 1.4). Other cell surface co-receptors required for mediating HIV-1 entry are beta chemokine receptors CXCR4 and CCR5 (Alkhatib *et al.*, 1996; Bjorndal *et al.*, 1997; Dragic, 2001; Arenzana-seisdedos and Parmentier, 2006; Tibris, 2007). Upon cell entry the viral particle is uncoated in the host cell cytoplasm in preparation of proviral DNA synthesis (Trono , 1992; Moore *et al.*, 1993).

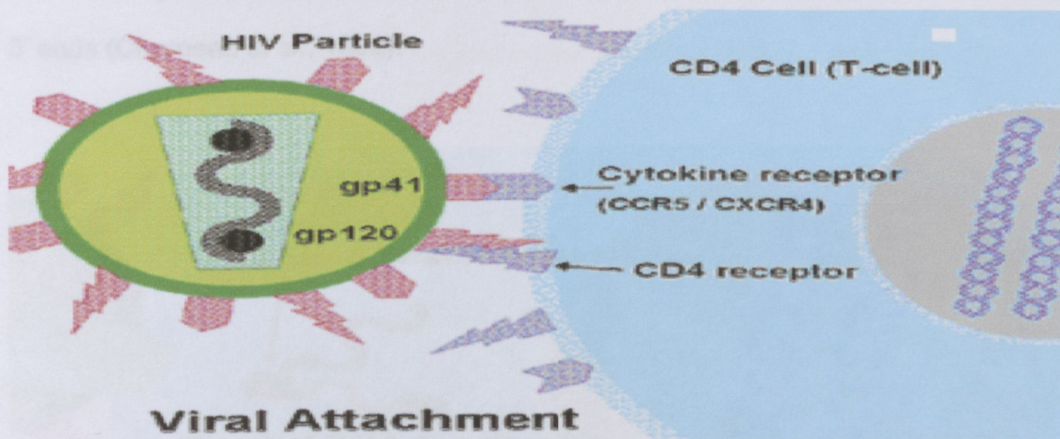

Figure 1.4: HIV-1 viral attachment to the CD4 receptor (Adapted from www.novinite.com/photo_106555.jpg)

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1.6.2. Reverse transcription of viral RNA

Upon entry, genomic RNA is reverse transcribed to a complementary DNA by enzyme reverse transcriptase (Figure 1.5). Host cell derived transfers RNA (tRNA) packaged into the virions serve to prime the synthesis of a negative DNA strand during the first phase of reverse transcription (Weiss *et al.*, 1992; Hottiger and Hubscher, 1996). Portion of the tRNA is complementary to sequences situated near the 5' end of viral genomic RNA called the primer binding site (Lori, 1992; Kohlstaedt *et al.*, 1992; Trono, 1992). RT elongates the nascent DNA from tRNA primer to the 5' end of the genomic RNA to form short DNA intermediates. During DNA synthesis, RNAse H activity of RT removes the RNA from RNA/DNA complex. After translocation of the negative strand, RT continues to copy the RNA genome until the complete synthesis of the –DNA strand, resulting in the formation of an RNA/DNA duplex. The RNA strand is then degraded by the RNAse H activity of RT and a polypurine tract at the 3' end remains resistant from degradation, this serves as a primer for the synthesis of a complementary DNA strand (Basavapathruni and Anderson, 2007). The resulting double stranded viral DNA (ds DNA) contains LTR elements at both the 5' and 3' ends (Charneau *et al.*, 1992).

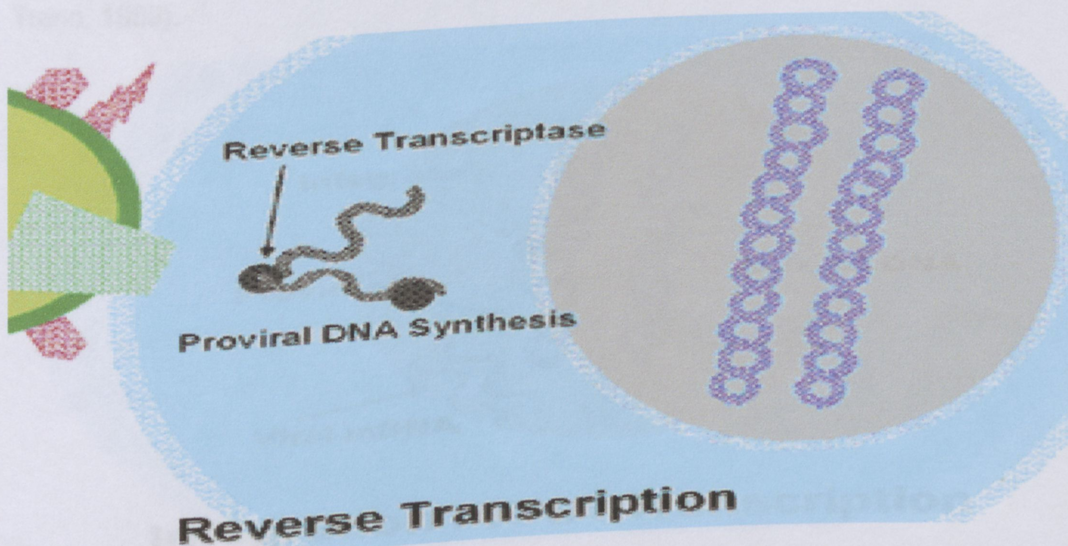

Figure 1.5: HIV-1 proviral DNA synthesis (Adapted from www.novinite.com/photo_106555.jpg)

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1.6.3. Integration into host DNA and viral expression

After reverse transcription,double stranded DNA remains associated with the enzymes RT and integrase forming a preintegration complex. Translocation of this complex to the nucleus of the host cell requires ATP. The linearized copy of HIV genome is inserted into the chromosomal DNA with the aid of an endonuclease activity of enzyme integrase (figure 1.6). Since the HIV provirus is covalently integrated into host cell genetic material, viral DNA stays permanently associated with host cell chromosome (Vink *and Plasterk,* 1993). The integrated viral genome contains LTRs at its 5' and 3' ends. The 5' LTR plays a role in the regulation of viral gene expression whereas 3' LTR is crucial for termination of transcription. Viral gene expression is divided into 2 phases, early phase of expression and a late structural phase. The early phase of expression is characterized by cytoplasmic expression of the 2kb viral mRNAs encoding tat, rev and nef. The accumulation of rev causes a switch from early to late phase of viral gene expression. Rev exerts its regulatory activity posttranscriptionally by activating expression of the unspliced and singly spliced transcripts encoding gag, pol, env as well as vif, vpr, and vpu. Rev binds to RRE to promote the stability, nuclear export and translocation of mRNA-RRE (Cullen, 1991; Cullen, 1992; Trono, 1995).

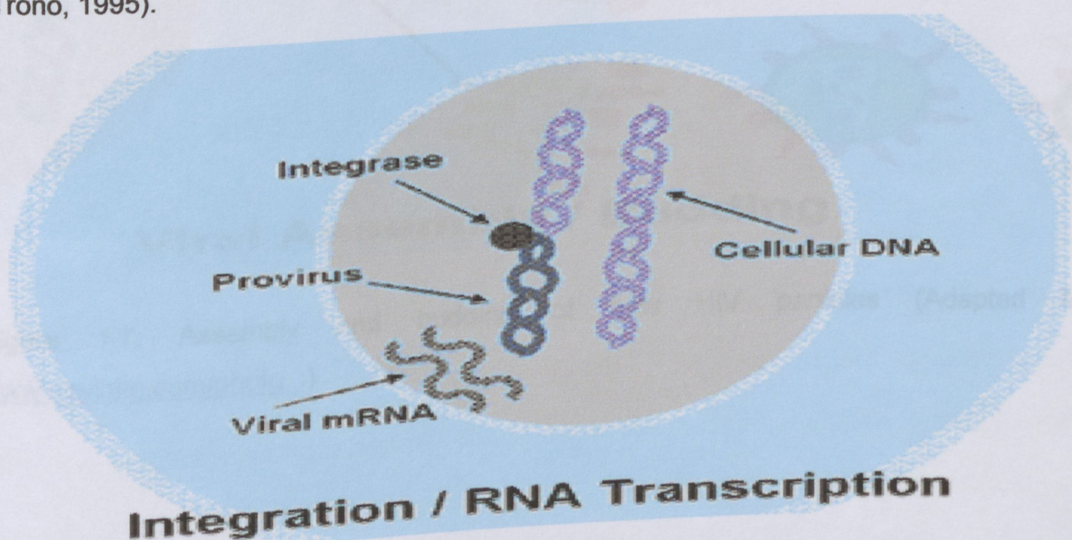

Figure 1.6: HIV-1 nuclear localization and integration (Adapted from www.novinite.com/photo)

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1.6.4. Assembly and budding of viral particles

HIV-1 gag and pol gene products are transported to the cell membrane via cytoplasmic pathways, env glycoproteins are transported via secretory pathway. The gag polyprotein interacts with gag-pol precursor molecules, vpr and genomic RNA facilitating their incorporation into nascent virions. RNA binding nucleocapsid is required for viral RNA encapsidation through its interactions with the nucleic acid packaging signal (Lever *et al.*, 1989). The myristylation of gag and gag-pol is required for attachment of these proteins to the plasma membrane (Dorfman *et al.*, 1994; Li *et al.*, 1997). The overall assembly process is initiated by P17, once assembled the viral core buds through the plasma membrane acquiring parts of the cell lipid membrane (figure 1.7). After release of the virion from the cell surface, gag and pol polyproteins are fully processed within the virion by the viral protease leading to maturation of the viral particle (Kohl *et al.*, 1988; Yu *et al.*, 1992).

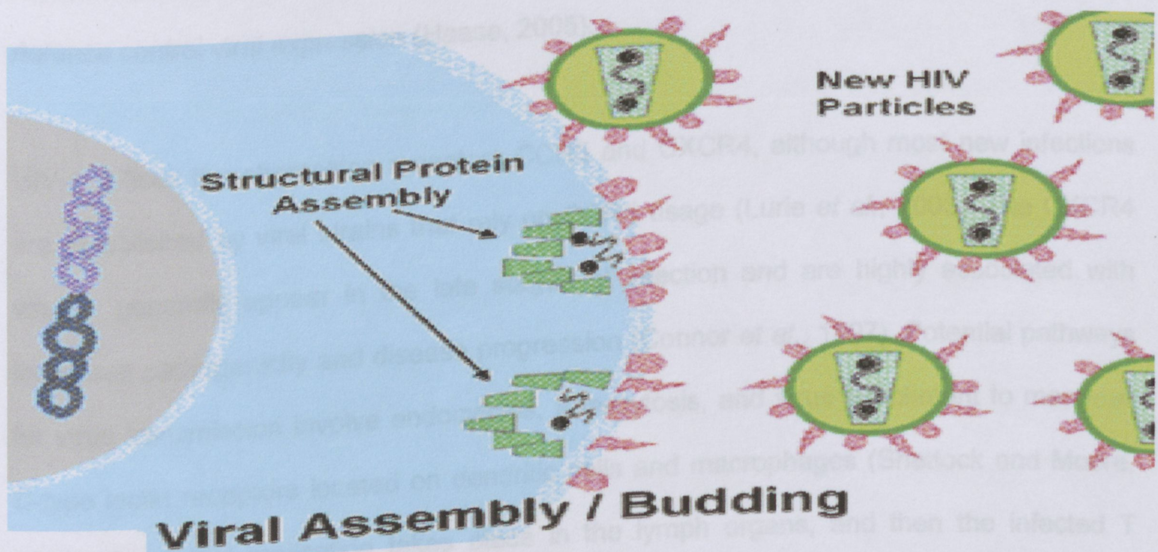

Figure 1.7: Assembly and budding of new HIV particles (Adapted from www.novinite.com/photo .)

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1.7. Pathogenesis of HIV-1

Infections by HIV-1 are characterized by complex interactions with the host and a chronic course of disease. Common features of disease include long and variable incubation periods, persistent viral replication, neurologic manifestation and destruction of specific immunologic cells. HIV-1 effectively counteracts innate, adapted and intrinsic immunity despite its genome size and few genes thereof. This virus basically takes advantage of cellular pathways while neutralizing and hiding from the different components of the human immune system (Barre-Sinoussi, 1996; Coffin, 1996; Emerman and Malim, 1998). In the early steps of HIV infection, HIV-1 gains entry to cells without causing immediate lethal damages although entry process in turn facilitates viral replication (Cicala *et al*, 2002; Balabanian *et al*., 2004). Several studies suggest that after HIV-1 breaches the mucosal barrier there's a window period in which viral propagation is not yet established and host defence control viral expression (Haase, 2005).

HIV-1 utilizes the chemokine receptors CCR5 and CXCR4, although most new infections are established by viral strains that rely on CCR5 usage (Lurie *et al*., 2003). The CXCR4 viruses generally appear in the late stages of infection and are highly associated with increased pathogenicity and disease progression (Connor *et al*., 1997). Potential pathways for virus transmission involve endocytosis, transcytosis, and virus attachment to mannose C-type lectin receptors located on dendritic cells and macrophages (Shattock and Moore, 2003). The initial replication takes place in the lymph organs, and then the infected T lymphocytes migrate into the bloodstream where secondary amplification results in massive infection of susceptible cells. Clinical symptoms can manifest during primary HIV-1 infection. A pronounced depletion of activated as well as memory CD4+ T cells located in the gut-associated lymphoid tissues has been seen in individuals identified early after infection (Mehandru *et al*., 2004). Different depletion mechanisms have been proposed with an emerging consensus favouring generalized immune activation as cause for constant

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depletion of the CD4+ cell reservoir (De Boer *et al;* 2003). Immune activation predicts disease progression (Giorgi *et al.*, 1999).

During the initial phase of HIV infection most infected individuals develop influenza or mononucleosis-like illness called acute HIV infection. The most common symptoms include fever, lymphadenopathy, pharyngitis, rash, myalgia, malaise, mouth and oesophagal sores. The follow up stage, HIV acts within lymphoid organs where most of the virus particles are trapped in follicular dendritic cells. During this phase, $CD4^{+}CD45RO^{+}$T cells carry most of the proviral load. When CD4 cells level drop below 200 cells/µl cell mediated immunity is lost (Desrosiers and Letvin, 1987). HIV-1 infection leads to impairment of a variety of CD4+ T cells functions including T-cell formation, autologous mixed lymphocyte reactions, expression of IL-2 receptors and IL-2 production. T cell proliferation response to a range of stimuli (ie antigens such as tetanus toxoid, influenza, *Candida albicans* and *Cryptococcus neoformans*) are diminished in all stages of HIV disease. Direct killing of $CD4^{+}$ cells may deplete certain functional subsets of $CD4^{+}$ T cells eliminating that particular immune function. Even if not killed, the infected cells's function may be compromised.

The cytopathic effects of monocytes/macrophage on HIV infection are less than those observed when CD4+ T cells are infected. The numbers of infected monocytes in the blood stream are quite low compared to CD4+ cells in the blood and macrophages in the tissues. Monocyte/macrophage dysfunction is caused by exposure to HIV proteins (Spear *et al.*, 1990).

Basically, infection with HIV-1 infects CD4+ T-lymphocytes and monocytes/macrophages, but also astrocytes cells of the central nervous system (brain microglial cells) as targets. The infection spreads to the lymphatic tissue that contains follicular dendritic cells that may also act as storage place for latent viruses. With time, virus replication leads to a slow and progressive destruction of the immune system.

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1.8. Treatment of HIV-1

Studies on the life cycle of the virus have identified several opportunities for therapeutic intervention. RT has been the most studied chemotherapeutic target of HIV-1. Antiretroviral treatment helps in viral suppression thereby reducing morbidity and mortality. However these drugs do not eradicate the infection. The RT inhibitors are classified into 2 major groups based on structural considerations (table 1.8): nucleoside RT inhibitors (NRTI) and non-nucleoside RT inhibitors (NNRTIs).

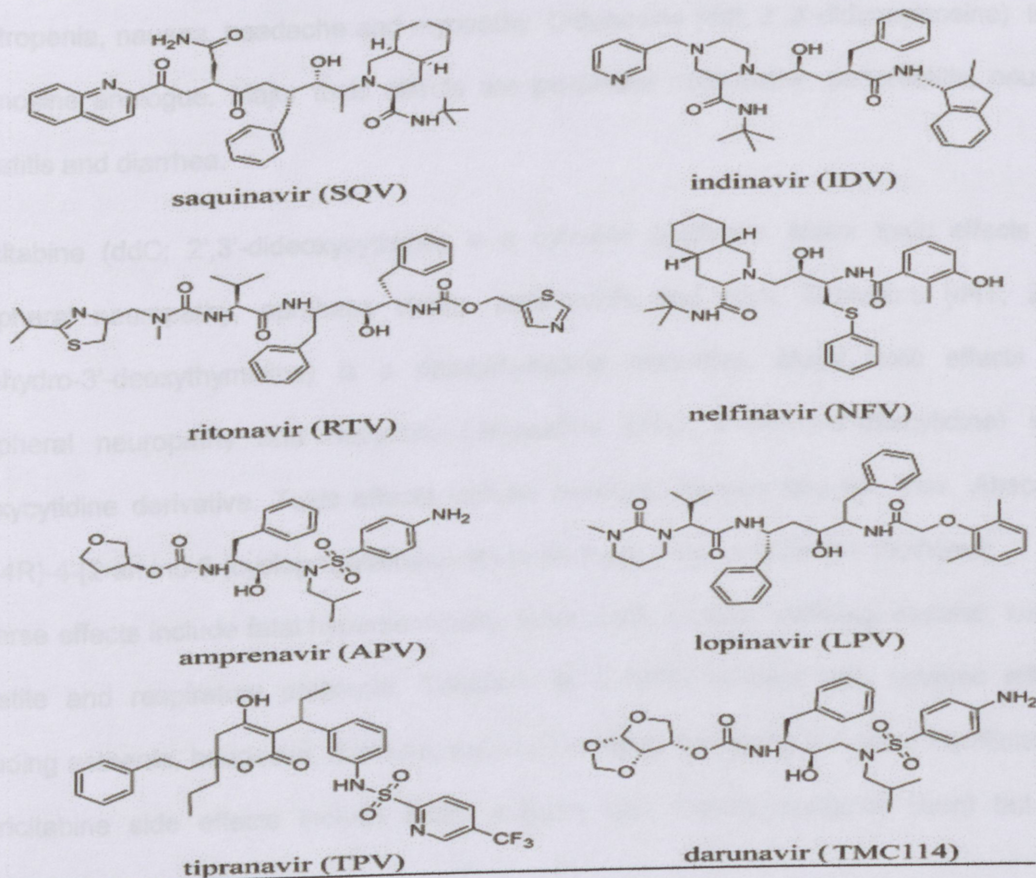

Figure 1.8: The 2D chemical structures of the eight FDA-approved drugs (Hou et al., 2008)

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1.8.1. NRTIs

These are first converted to triphosphates to be effective and also mimic the natural substrates of the enzyme. The phosphorylated NRTIs compete with naturally occurring dNTPs, their incorporation into proviral DNA blocks further elongation of the proviral DNA (Clarke and Mousa, 2009). Since they lack the 3'-OH, they prematurely terminate the transformation of the viral RNA into dsDNA. Zidovudine (AZT, 3'-azido-2',3'-dideoxythymidine) is a backbone of combination regimens. Major toxic effects are anaemia, neutropenia, nausea, headache and myopathy. Didanosine (ddI; 2',3'-dideoxyinosine) is an adenosine analogue. Major toxic effects are peripheral neuropathy, pancreatitis, nausea, hepatitis and diarrhea.

Zalcitabine (ddC; 2',3'-dideoxycytidine) is a cytosine analogue. Major toxic effects are peripheral neuropathy, aphthous ulcers, pancreatitis and rash. Stavudine (d4T; 2',3'-didehydro-3'-deoxythymidine) is a deoxythymidine derivative. Major toxic effects are peripheral neuropathy and hepatitis. Lamivudine (3TC; 2'-deoxy-3'-thiacytidine) is a deoxycytidine derivative. Toxic effects include anaemia, nausea and hair loss. Abacavir: (1S,4R)-4-[2-amino-6-(cyclopropylamino)-9H-purin-9-y1]-2-cyclopentene-1-methanol; adverse effects include fatal hypersensitivity, fever, rash, nausea, vomiting, malaise, loss of appetite and respiratory problems. Tenofovir is a NRTI inhibitor with adverse effects including asthenia, headache, diarrhea, nausea, vomiting, flatulence and renal insufficiency. Emtricitabine side effects include lactic acidosis with hepatic steatosis (rare) but life threatening.

1.8.2. NNRTIs

These are non-competitive inhibitors of RT; they specifically inhibit HIV-1 RT by binding at different sites on the enzyme's RNA and DNA-dependent polymerase activity. They inhibit movement of protein domains of RT needed to carry out the process of DNA synthesis

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(Temesgen *et al.*, 2006). They are quite small molecules that bind to the hydrophobic pocket close to the catalytic domain of the RT (Merluzzi *et al.*, 1990; De Clercq, 1992). Nevirapine (NVP, 11 cyclopropyl-5,11-dihydro-4-methyl-6H-dipyrido[3,2-L:2',3'-E][1,4]diazepin-6 belongs to the dipyridodiazeperone class of compounds that inhibit the cytochrom P450 leading to several drug-drug interactions. Side effects include rash, Stevens-Johnson syndrome, symptomatic hepatitis and fatal hepatic necrosis.

Delavirdine (DLV, 1-[3-[(1-methyl-ethyl) amino]-2-pyridinyl]-4-[[5-(methylsulfonyl) amino]-1H-indol-2-y1]carbonyl]piperazine, also inhibits cytochrome P450. Side effects include rash, increased transaminase levels and headache. Efavirenz:(5)-6-chloro-4-(cyclopropylethynyl)-1-4-dihydro-4-(trifluoromethyl)-2H-3,1-benzoxazin-2-one functions the same way as nevirapine and delavirdine. Side effects include rash, central nervous system symptoms, increased transaminase levels, false-positive cannabinoid test.

1.8.3. Protease inhibitors (PIs)

Several protease inhibitors have been clinically used on HIV infected people. They are believed to prevent viral replication by inhibiting the activity of HIV-1 protease (Ma *et al.*, 2007) an enzyme used to cleave nascent proteins for final assembly of new virions. These include: Saquinavir, a protease inhibitor with side effects such as GI intolerance, nausea and diarrhea, headache, elevated transaminase enzymes, abdominal pains and dyspepsia. Ritonavir, it is a PI with GI intolerance, nausea, vomiting, diarrhea, paresthesias, hyperlipidemia, hepatitis, asthenia and taste perversion as side effects. Indinavir (IDV) may lead to nephrolithiasis, GI intolerance, nausea, indirect hyperbilirubinemia, headache, asthenia, blurred vision, dizziness, rash, metallic taste, thrombocytopenia, alopecia and hymolytic anemia. Nelfinavir causes diarrhea, hyperlipidemia, hyperglycemia, serum transaminase elevation. Amprenavir (APV) adverse effects include GI intolerance, nausea, vomiting, diarrhea, rash, oral paresthesias, hyperlipidemia, transaminase elevation,

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hyperglycemia, fat maldistribution, possible increased bleeding episodes in patients with hemophilia.

Lopinavir treatment generates GI intolerance, nausea, vomiting, diarrhea, paresthesias, hyperlipidemia, and hyperglycemia. Atazanavir (ATV): Adverse effects include indirect hyperbilirubinemia, hyperglycemia, fat maldistribution, possible increased bleeding episodes in patients with hemophilia. Fosamprenavir (f-APV): side effects include skin rash, diarrhea, nausea, vomiting, headache, hyperlipidemia, transaminase elevation and hyperglycemia. Tipranavir inhibitor side effects are hepatotoxicity, skin rash and hyperlipidemia. Darunavir (DRV) side effects include hyperlipidemia, hyperamylasemia, transaminase elevation, and headache and GI symptoms.

1.8.4. Fusion/ Entry inhibitors

They prevent fusion of viral and target cell membrane. The two types are Maraviroc and Enfuvirtide. They are synthetic peptides that mimics the C-terminal HR2 (Heptad repeat sequence 2) domain of gp41 and competitively binds to HR1 (Heptad repeat sequence 1). The heptad repeat is a type of tandem repeat sequence in which a group of 7 amino acids occurs many times in a protein sequence. The interactions between HR1 and HR2 is blocked and the conformational change of gp41 necessary for fusion of the virion to cell membrane is inhibited (Westby *et al.*, 2006). These drugs prevent entry of HIV-1 into CD4+ cells. The CCR5 co-receptor functions by binding to CCR5 molecule which then is unable to bind to gp120 subunit. The conformational changes leading to insertion of the gp41 fusion peptide is prevented and viral entry is stopped (Qadir and Malik, 2010).

1.8.5. Integrase inhibitors

Integrase inhibitors interfere with integrase enzyme, the enzyme is vital for the virus to insert genetic material into host genome (Steigbigel *et al.*, 2008). Raltegravir is an FDA approved integrase inhibitor in use.

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1.8.6. Maturation inhibitors

This type of drugs (Alpha interferon) inhibit the final step in gag processing through which the capsid is cleaved thereby blocking conversion of the polyprotein into mature capsid proteins (Salzwedel *et al.*, 2007)

1.9. HIV-1 Drug resistance

Since the discovery of AZT as an effective antiretroviral agent against HIV-1, drug therapy has been used widely in the treatment of AIDS. Larder *et al.* (1989) was the first researcher to recognize the loss of therapeutic effect of AZT as a result of acquisition of resistance. It was demonstrated that HIV-1 isolates from patients with the advanced disease become less sensitive to the drug during the course of the treatment. High level of resistance to certain drugs may develop over a short period of therapy in the presence of a single mutation, this indicates that the particular antiretroviral drug has low genetic barrier like nevirapine and 3TC (Geretti and Easterbrooke, 2001). The emergence of drug resistance is the most commom reason behind treatment failure, insufficient compliance, drug side-effects or drug-drug interactions can lead to viral rebound (WHO, 2010). The resistance patterns have been described for all ARV drugs. The different HIV-1 subtypes differ in the sequence of mutations leading to drug resistance and some naturally occurring polymorphisms might modulate resistance (Lurie *et al.*, 2003; McCutchan *et al.*, 2005). Drug resistance mutations have been primarily characterized for subtype B viruses in countries where ART has been readily available and 10% of new infections are drug resistant (Hemelaar *et al.*, 2006).

The 2 main biochemical mechanisms leading to NRTIs resistance are sterical inhibition and phosphorylysis via ATP/pyrophosphate. Sterical inhibition is caused by mutations (M184V,

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Q151M, L74V, or K65R) enabling RT to recognize structural differences between NRTIs and dNTPs (Naeger, 2001; Clavel and Hance, 2004). Incorporation of NRTIs is prevented in favor of dNTPs. Phosphorylysis leads to excision of NRTIs already incorporated in the growing DNA chain. This is possible in the presence of the following mutations which cause resistance on their own or in the presence of secondary mutations or polymorphisms: M41L, D67N, K70R, L210W, T215Y, and K219Q (Meyer, 2000). The genotypic and phenotypic patterns emerging in HIV-1 infected patients treated with protease inhibitors are complex and cross resistance between structurally different compounds occur frequently. Most of the protease inhibitors possess high genetic barrier to emerge primary resistance. The majority of drug resistance protease mutations identified are conservative changes involving gain/loss of a methylene group.

1.9.1. Resistance to NRTIs

Lamivudine's high degree of resistance can develop following a single mutation (Schuurman *et al*., 1995). Lamivudine specific mutation M184V also reduces viral replication capacity by 40-60% (Sharma and Crumpacker, 1999; Miller, 2003). Continuous monotherapy with 3TC delays virological and immunological deterioration (Castagna, 2006). FTC has some resistant patterns as 3TC, failure of treatment is associated with development of M184V mutation. Thymidine analog or TAMs include the mutation M41L, D67N, K70R, L210W, T215Y and K219Q, these mutations were initially observed on zidovudine therapy. They can also be selected for stavudine (Loveday, 1999). Three or more of the TAMs are associated with a reduction in the sensitivity to stavudine. Didanosine's virologic response depends on the number of specific TAMs, mutations T215Y/F, M41L and L210W, D67N and K219Q have been found to be associated with a reduced efficacy (Marcelin *et al*., 2005). The response was not dependent on mutations M184V and K70R.

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Tenofovir is active in the presence of mutations D67N, K70R, T215Y/F or K219Q/E, however if 3 or more including either M41L or L210W is present, a virologic response can be expected (Antiniou *et al*., 2003). Lamivudine associated mutation M184V induces re-sensitization to AZT resulting in 50-60% reduction of $IC_{50.}$ Multidrug resistance (MDR) to all nucleoside analogs with the exception of lamivudine is established in 1 of the following combinations, mutation T69S plus an insertion of 2 amino acids (SS, SG or SA) between positions 69 and 70, plus an AZT associated mutation or Q151M plus a further MDR mutation V75I/ F77L or F116Y. MDR mutation Q151M alone leads to intermediate resistance to AZT, d4T, ddI and abacavir (Masquelier, 2001).

1.9.2. Resistance to NNRTI

A single mutation can confer a degree of resistance to 1 or more NNRTIs. Mutations K103N and Y188L leads to 20-30 fold increase in resistance to NNRTIs (Petropoulus *et al*., 2000). Mutation V106A leads to a 30 fold increase in nevirapine resistance and intermediate efavirenz resistance (Grossman, 2004). A98G, K101E and V108 lead to low grade resistance to all available NNRTIs. Intermediate resistance to efavirenz and delavirdine and low grade results to nevirapine results from the L101I mutation. Y181C/I causes nevirapine resistance, G190A is associated with a high degree of nevirapine resistance and intermediate resistance to efavirenz and delavirdine (De Mendoza *et al*., 2002; Shafer, 2002).

1.9.3. Resistance to PIs

G48V emerges under unboosted saquinavir and leads to a 10-fold decrease in the susceptibility to saquinavir, if combined with L90M it results to high degree of decreased susceptibility to saquinavir (Jacobsen *et al*., 1995). According to the retrospective study carried out by Marcelin *et al*., (2005) mutation 10F/I/M/R/V, 15A/V, 20I/M/R/T, 24I, 62V,

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73ST, 82A/F/S/T, 84V and 90M were identified as those most strongly associated with the virological response. Virologic failure on nelfinavir is associated with the emergence of mutation L90M. This mutation also leads to a moderate reduction in replicative capacity. Unboosted indinavir and/or ritonavir selected for the major mutation V82A (T/F/S) which in combination with other mutations leads to cross-resistance to other PIs (Shafer, 2002). Unboosted amprenavir or fosamprenavir, the selected mutations are I54L/M, I50V or V32I and I47V (Chapman *et al.*, 2004).

Tipranavir shows good efficacy against viruses with multiple PI mutation (De Meyer *et al.*, 2006), a reduced sensitivity can be observed in the presence of protease inhibitor resistance associated mutations (PRAMs). These PRAMs include L33I/V/F, V82A/F/L/T, I84V and L90M. Darunavir showed good activity in vitro and in vivo against most PI resistant viruses. About 11 baseline mutations were found to be associated with reduced response to darunavir, V11L, V32I, L33L, I47V, I50V/L, I54L/M, G73S, L76V, I84V and L89V (De Meyer *et al.*, 2003; De Meyer *et al.*, 2007). PI's polymorphisms that are likely to be associated with development of resistance are K20R, M36I and I93L among others (www.hivdb.stanford.edu/).

1.9.4. Resistance to Fusion inhibitors (FI's)

The loss of efficacy was shown to be accompanied by emergence of mutations at T-20 binding site, a heptad repeat 1 (HR1) region of GP41. Mutations in gp41 region of amino acids 36-45 have been identified in in-vitro assays: these include G36S, V38M, V38A, Q39H, Q40H, and N43D. The decrease in susceptibility is higher for double mutations than single mutations eg. G36S + L44M, N42T + N43K, N42T + N43S or Q40H + L45M (Sista *et al.*, 2004). These mutations hinder binding of FI's to transmembrane gp41 thereby prevent fusion of viral and cell membranes of susceptible cells.

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1.9.5. Resistance to Integrase inhibitors

Raltegravir resistance emerges because of signature mutation N155H or Q148K/R/H. Secondary mutations observed with N155H included V1511, T97A, G163R, L74M and E92Q. The 2 pathways known leading to raltegravir resistance are Q148H/R/K selecting E138K and G140S/A, the second pathway Y143R/C together with L74A/I, E92Q, T97A, I203M and S230R (Temesgen and Siraj, 2008).

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2. Rationale of the study

South Africa has a population of 49.9 million people of which 5.6 million are estimated to be infected with HIV-1. Limpopo province ranks seventh with a prevalence of 21.9% infections of the antenatal clinic attendees (DOH, 2010; SSA, 2010; UNAIDS, 2010). The HIV epidemic in South Africa is mainly driven by HIV-1 subtype C viruses. With the introduction of highly active antiretroviral therapy (HAART), it has become possible to control the infections to a certain extent. However, there are mutations in the polymerase gene (*pol*) that influence drug resistance (Hogg *et al.*, 1998). These mutations have been known to confer drug resistance against first line and second line ARV used in South Africa.

There are mutations on subtype B strains that develop under drug pressure and lead to resistance to ARV. These mutations have been observed in subtype C strains as polymorphisms. There is information on phenotypic assays designed for studying the meaning of mutations in HIV-1 subtype B genes and scanty data on subtype C assays. The question is, are the polymorphisms observed in subtype C viruses important in resistance development?

Drug susceptibility testing is known to measure the ability of an HIV-1 isolate to grow in the presence of a drug and is performed using assays in which the degree of virus replication can be measured at different drug concentrations. The measurement of phenotypic HIV drug susceptibility for the management of HIV infections has now become practical via rapid, high-throughput automated assays based on recombinant DNA technology. It is important that appropriate systems be constructed to evaluate putative resistant mutations found in HIV-1 subtypes responsible for a greater part of the epidemic such as subtype C which accounts for more than 60 % of infections worldwide.

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3. Objectives of the study

3.1. General objective

The general objective of this study was to construct an HIV-1 subtype C vector for evaluation of polymorphic mutations observed in the *GagPol* genes of HIV-1 subtype C infected patients.

3.2. Specific objectives of the study were:

1. To design and optimize a PCR protocol to amplify HIV-1 *GagPol* gene from viral RNA.

2. To construct a chimeric virus comprising patient HIV *GagPol* and pCMVGag/pol(IndieC)RRE plasmid.

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CHAPTER TWO: MATERIALS AND METHODS

2.1. Ethical considerations

This study was a component of a broader study in HIV drug resistance, and bioassay development. Ethical clearance was obtained from the Health, Safety and Research Ethics committee of the University of Venda, and permission to use public health institutes was obtained from the Department of Health Limpopo Province, South Africa.

2.2. Characteristics of study population

Samples used in this study were chosen from the archived RNA and plasma samples stored at -80°C in the Department of Microbiology, University of Venda. The samples were obtained from HIV positive volunteers from Thohoyandou, Musina and Bela Bela health care settings. These were participants in the broarder HIV drug resistance study. Demographic data collected included sex, age, use of antiretrovirals, stage of infection, CD4 count and viral loads.

2.3. Optimizing PCR conditions to amplify partial *GagPol* gene

2.3.1. Viral RNA extraction

Viral RNA was extracted from the stored plasma samples using viral RNA mini kit according to manufacturer's instructions (Qiagen,Valencia, USA). Briefly 140µl plasma was aliquoted into microcentrifuge tubes. RNA-AVE (560µl) was added to the plasma and pulse vortexed then incubated for 10 minutes at room temperature. After incubation, the mixture was spun at 3000rpm for 1 minute and 560µl of absolute ethanol was added, pulse vortexed and subjected to a short spin. From the mixture, 630µl was added to the extraction column and spun at 8000 rpm/minute; the same was done with the remaining mixture. The filtrate was

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discarded; the column placed back in a collection tube and 500µl of buffer AW1 was added and centrifuged at 14000rpm followed by addition of 500µl of AW2 and spun again. In both wash rounds the filtrate was discarded. The column was then placed in a clean eppendorf tube and 60µl of buffer AVE was added, to elute the RNA from the column. This was incubated for a minute then spun at 8000rpm for 1 minute before storage at -80ºC for future use.

2.3.2. cDNA synthesis

The cDNA was synthesized from the extracted RNA using transcriptor reverse transcriptase following the manufacturers' instructions (Roche, Mannheim, Germany). The significance of synthesizing cDNA is to reverse transcribe viral RNA to DNA. The previously isolated RNA as well as other reagents were removed from the freezer and allowed to thaw on ice. To synthesize cDNA; nuclease free water was added to 0.4µM of Inrevl primer and 10µl of the RNA in PCR tubes. The mixture was short spun and heated in thermocycler for 10 min at 65°C. The cycler was paused to allow addition of the following reagents at final concentrations,10U transcriptor RT, 20U Rnase inhibitor, 200µM dNTP mix and 2µl of 10X RT buffer to each PCR tube respectively, in a final concentration of 20µl. The cycler was resumed with the cDNA synthesis programme for an hour at 55°C.

2.3.3. First round and nested polymerase chain reaction

All PCR amplifications were perfomed in final volume mixture of 50µl containing 1X PCR buffer, 200µM of dNTP's, 1.5mM magnesium chlroride, 2U Taq polymerase, 10µl of the cDNA and 0.2µM of each of the primers. Nuclease free water (Qiagen, Valencia, USA) was added to make the final volume to 50µl and subjected to 30 cycles in a thermal cycler (Bio-Rad, Hercules, California, USA). The following set of primers were used for first and nested round PCR respectively:

1395 (5'-TGGCAAGGAAGGGCACATAGCCAAAAAATTG-3') and

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1353 (5'-TTAGGAGTCTTTCCCCATATTACTATGCTTT-3');

1389 (5'-AAATTGCAGGGCCCCTAGG-3') and

1396 (5'-CTCTGTTAACTGTTTTACATCATTAGTGTGGG-3').

The nested PCR primers contained restriction enzyme sites for ApaI and HpaI for subsequent digestion reaction. The cycling conditions were as follows: 95ºC for 2 min (1 cycle); 95ºC for 1min, 60ºC for 1min, 72ºC for 2 min (30 cycles) and final extension at 72ºC for 10 minutes. The cycling conditions for nested PCR were the same as in the first round PCR step, except that the annealing temperature for nested PCR was 57°C.

2.3.4. Gel electrophoresis and purification of viral DNA

Polymerase chain reaction products were analysed by electrophoresis on 1% agarose gel (Sigma- Aldrich, Steinheim, Germany). This was performed to verify expected band size of DNA and specificity of reaction and contamination. The gel was stained with ethidium bromide and visualized under UV transluminator. The PCR products were purified using the Qiaquick PCR purification kit (Qiagen, Valencia, USA) following the manufacturers' instructions. Briefly 250µl of buffer PBI was added to 45µl of PCR sample and mixed. Purification columns were placed in a clean 2ml collection tubes and PCR mixture was poured onto the column in order to bind the DNA. The tubes were centrifuged and flow-through discarded. The column was washed with 750µl of buffer PE, centrifuged and flow-through discarded. The column was centrifuged again to remove excess buffer and then moved to a clean 1.5ml eppendorf tube. Thirty micro liters of elution buffer was added and centrifuged to elute the DNA.

2.4. Ligation of viral DNA to pGEM-T vector and verification of transformation

Ligation reaction was perfomed using pGEM-T vector following manufacturer's instruction. Viral DNA was cloned into pGEM-T easy vector to allow action of the restriction endonucleases before transferring the cleaved viral DNA to the actual plasmid. To clone the

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amplified viral DNA into pGEM-T vector system; the control insert, vector and viral DNA were briefly centrifuged and reactions set up as shown in table 2.1.

Table 2.1: Ligation protocol using pGEM-T vector and 2X Rapid ligation buffer (Invitrogen)

Reagent	Standard Reaction	Positive Control	Background Control
2X Ligation buffer	5µl	5 µl	5 µl
PGEM-T vector (50ng/µl)	1 µl	1 µl	1 µl
PCR product (Undigested)	3 µl	-	-
Control insert (4ng/µl)	-	2 µl	-
T4 DNA ligase (5 weiss units/µl)	1 µl	1 µl	1 µl
Nuclease H_2O	-	1 µl	3 µl

The reaction mixtures were briefly centrifuged then incubated at 4°C overnight. Fifteen milliliters round bottom tubes were prechilled on ice and 50µl of DH5α cells (10^6 – 10^7 cfu/µg) were mixed with 2µl of ligation reaction and incubated on ice for 30 minutes. Cells were heat shocked for 40 – 45 seconds at 42°C and quickly placed back on ice for 1-2 minutes. Nine hundred microliters of super optimal broth with catabolite repression (SOC) was added and incubated at 37°C with slow shaking (150rpm) for an hour. The transformed cells were spread on pre-warmed LB plates containing ampicillin (100µg/ml) and incubated at 37°C overnight. Two spread volumes were done to allow evaluation at 100µl should 200 µl give overgrowth of colonies. Of the positively identified colonies, master plates were inoculated to allow growth of specific colonies. Sixteen colonies each from the different plates were resuspended in nuclease free water, boiled at 100 °C to break the DH5α competent cells to release the transformed plasmid which was then used as a template for

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PCR. Nested primers were used in the amplification of the inserted partial *GagPol* gene to verify that ligation and transformation were successful.

2.5. HIV-1 molecular clones

A mammalian expression vector pCMVGagPol(IndieC)RRE from the Department of Microbiology, University of Venda was chosen to be used as a vector (Lewis *et al*., 1990). It was previously modified to include unique ApaI and HpaI restriction sites and has a Subtype C backbone. The choice was due to the fact that Cytomegalovirus comprise of an immediate early promoter enhancer (IE) and a polyadenylation signal from the bovine growth hormone and Col E1 origin of replication. CMV has a large genome size, it can easily be manipulated in vitro, has slow replication kinetics in tissue culture and finally it increases expression thus makes it a useful plasmid vector.

2.6. Digestion of pGEM-T vector/viral DNA and pCMVGagPol(IndieC)RRE

Viral DNA insert was extracted from the pGEM-T vector by double restriction digestion.The pGEM-T vector carrying viral DNA insert was digested with ApaI (10U/µl) and HpaI (10U/µl) restriction enzymes. The 2 restriction enzymes were to generate ends that recognize their complementary pairs on the plasmid [pCMVGagPol(indieC)RRE] during ligation. HpaI generates blunt ends whereas ApaI generates sticky or cohesive ends. Digestion mixture (50µl) comprising 40µl purified DNA, 2µl 10X multicore buffer, 2µl 10X BSA and 1µl of each of the restriction enzymes and 5µl of nuclease free water H_2O. Digestion was done for 4 – 5 hrs at 37ºC. pCMVGagPol(IndieC)RRE was double digested to linearize and also trim the ends to match those of the digested viral DNA. Digestion products were resolved by 0.8% agarose electrophoresis. The expected products were excised from the gel with a sterile scalpel and transferred to a clean eppendorf tube. Digestion products were purified using agarose gel extraction kit following the manufacturer's instructions (Qiagen, Valencia, USA).

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2.7. Ligation of viral DNA into pCMVGag/polRRE

The digested viral DNA released from the pGEM-T vector was cloned into the pCMVRRE vector using T4 DNA ligase enzyme (5 weiss units/µl) following manufacturer's instructions (Invitrogen). The linearized RRE vector (4µl) was added to 10µl insert in the presence of PEG 4000 and 10X BSA ligase buffer and 1 unit of T4 ligase enzyme. The reaction was incubated at 22°C overnight.

2.8. Transformation of DH5α cells

DH5α competent cells were transformed with chimeric pCMVGagPol(patient)RRE as follows: fifteen milliliters round bottom tubes were prechilled on ice and 50µl of DH5α cells (10^6 – 10^7 cfu/µg) was mixed with 10µl of ligation reaction and incubated on ice for 30 minutes. Cells were heat shocked for 40 – 45 seconds at 42°C and quickly placed back on ice for 1-2 minutes. Nine hundred microliters of SOC was added and incubated at 37°C with slow shaking (150rpm) for an hour. The transformed cells were spread on pre-warmed LB plates containing ampicillin (100µg/ml) and incubated at 37°C overnight.

2.9. Minipreparation and plasmid purification

In order to purify plasmid from DH5α cells a qiagen kit was used following manufacturer's instructions. To isolate plasmid from transformed competent cells, five tubes per reaction containing 5ml of LB mixed with ampicillin (100µg/ml) were inoculated with a bacterial colony. After overnight incubation at 37C with shaking (225rpm), the culture was spun down. Plasmid was prurified using a Qiagen kit as follows. The supernatant was discarded and pellets resuspended in 250µl of Qiagen P1 buffer, vortexed until the pellet was fully resuspended. Two hundred and fifty microlitres of buffer P2 was added and mixed by gentle

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shaking, 350µl of buffer N3 was added and thoroughly mixed followed by centrifugation at 13000 rpm for 10 minutes. The supernatant were transferred to appropriate columns in order to bind the DNA. The columns were spun for 1 minute at 13 000rpm then washed with 750µl buffer PE. To remove residual buffer the column was spun an additional minute. To elute the DNA, 50µl of buffer EB was added directly onto the membrane, allowed to stand for a minute then centrifuged for another minute at same speed. Purified plasmids were stored at -20˚ C.

2.10. Sequencing of pCMVGagPol(patient)RRE

Direct population-based sequencing was performed on both strands with the BigDye terminator kit on ABI Prism 377 (Applied Biosystems) using Taq DNA polymerase and nested PCR primers for sequencing. The samples that were successfully amplified were sequenced to detect polymorphic mutations harbored and to confirm the amplified region of interest. Nucleotide sequences were edited using Seqman II (DNASSTAR) software. The sequences were investigated for drug resistance mutations using the Stanford HIV-1 Drug Resistance Database Algorithm (http://hivdb.stanford.edu/). The database assigns a numerical score to each sequence with regard to individual ARV i.e. susceptible, potential low level, low level, intermediate or high level resistance.

2.11. Viral genetic subtyping

HIV subtyping was done by phylogenetic analysis, Protease (PR) and Reverse transcriptase (RT) genes of amplified viral DNA were aligned using ClustalX with reference subtypes sequences (group M subtypes A-D, F-H, and J. The refferal sequences were obtained from GenBank. Neighbour joining phylogenetic trees were generated with the PHYLIP programme. Trees were rooted with the HIV-1 group O reference strain (L20571). The reliability of isolate clusters was assessed by a bootstrapping of 1000 replicates.

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2.12. Data analysis

The analysis of drug resistant mutations was done using the Stanford HIV drug resistance interpretation programme. This interactive programme compares codons of query sequences with resistance coding nucleotides. Mutations that on their own may result in resistance are referred to as major (primary) mutations, while mutations that reduce susceptibility in association with other mutations are referred to as minor (secondary) mutations. The different viral growth pattern was accounted for by the presence of various mutations harbored within the *GagPol* gene. Comparison of viral growth patterns was done in order to determine the degree of resistance with respect to the different mutations thereof. The student t-test was used to determine the level of significance and a *P* value of 0.05 was regarded as statistically significant.

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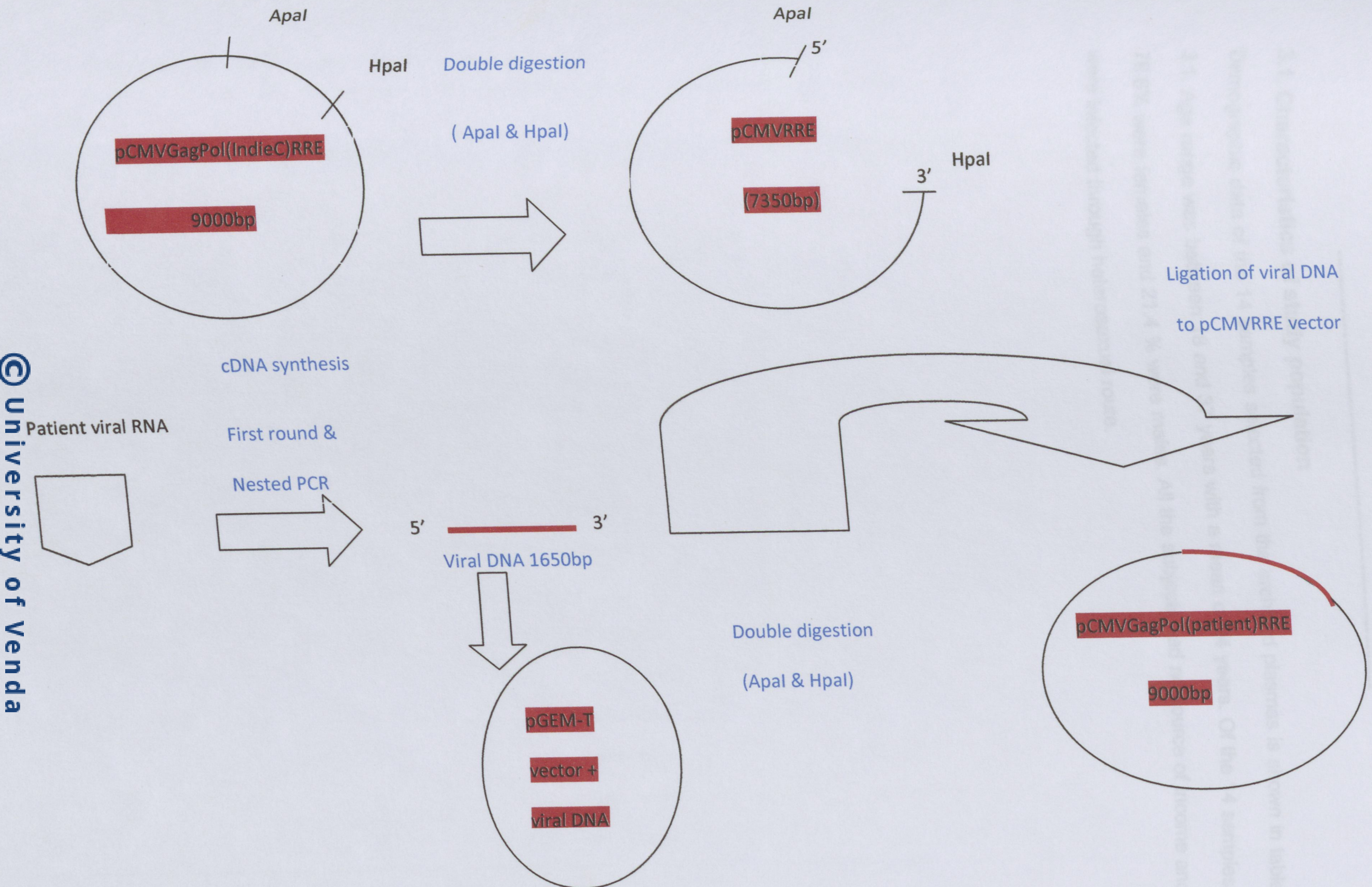

Figure 2.1: Schematic presentation of pCMVGagPol(patient)RRE construction

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CHAPTER THREE: RESULTS

3.1. Characteristics of study population

Demographic data of the 14 samples selected from the archived plasmas is shown in table 3.1. Age range was between 18 and 37 years with a mean of 24 years. Of the 14 samples, 78.6% were females and 21.4 % were males. All the subjects had no source of income and were infected through heterosexual route.

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Table 3.1. Demographics and disease profiles of the study subjects

Subject code	Age	Gender	Route of infection	HIV-1 subtype (Pol gene)
V11-01	31	F	Heterosexual	C
V11-02	36	M	Heterosexual	C
V11-03	19	M	Heterosexual	C
V11-04	18	F	Heterosexual	C
V11-05	20	F	Heterosexual	C
V11-06	21	F	Heterosexual	C
V11-07	22	M	Heterosexual	C
V11-08	19	F	Heterosexual	C
V11-09	37	M	Heterosexual	C
V11-10	23	F	Heterosexual	C
V11-11	20	F	Heterosexual	C
V11-12	21	F	Heterosexual	C
V11-13	20	F	Heterosexual	C
V11-14	25	F	Heterosexual	C

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3.2. Establishment of amplifying partial *GagPol* PCR

3.2.1. First round and nested polymerase chain reaction

RNA was extracted from all the samples and nested PCR was done to amplify an expected 1650 bp of pol gene. Of the 14 samples, 12 (85,7%) were successfully amplified. For the other samples, 2 bands of lower molecular weight (800bp) were obtained. The 2 samples were not included in any further analysis. A representative agarose gel of amplified products is presented in figure 3.1.

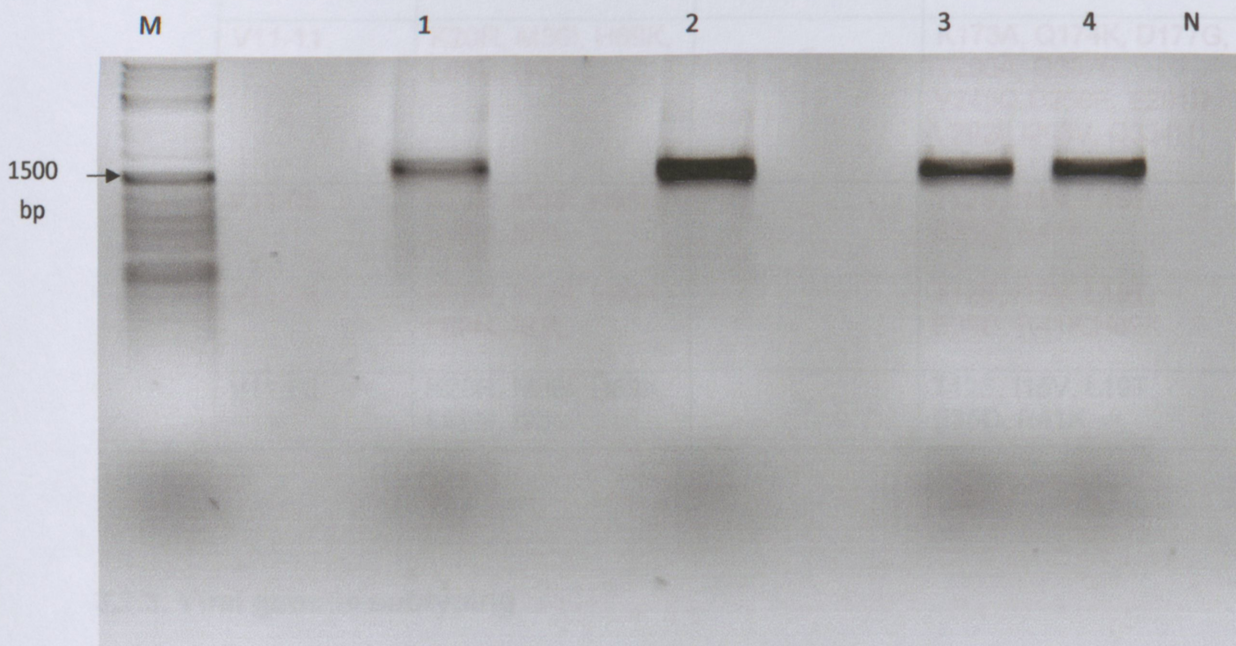

Figure 3.1: Representative gel of nested PCR. Lane M is the molecular weight marker. Lanes 1- 4 contain the expected amplified viral DNA; lane N is the negative control. The expected DNA fragment of 1650bp is shown.

3.2.2 .Sequencing of amplified viral DNA

The 12 amplified viral DNA was sequenced using nested PCR primers, edited using Seqman and blasted in the HIV drug resistance database to analyse mutations. However, several polymorphisms and minor mutations associated with drug resistance were observed

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(table 3.2). Of the sequenced samples, 6/12 protease regions were successfully sequenced and 3/6 (highlighted in red) were fully sequenced in both RT and PR regions. There were no major resistance mutations observed in any of the sequences .

Table 3.2: Polymorphic mutations harbored in PR and RT gene regions of HIV-1

Sample Code	PR mutations	RT mutation	Polymorphisms observed
V11-12	K20R, M36I, H69K, L89M, I93L	_	T12S, I15V, L19T, E35D, R41K
V11-06	K20R, M36I, H69K, L89M, I93L	_	T12S, I15V, L19T, E35D, R41K
V11-11	K20R, M36I, H69K, L89M, I93L	_	K173A, Q174K, D177G, T200A, Q207E, V245Q,D250E, E291D, V292I, I293V, Q334H, G335D
V11-05	K20R, M36I, H69K, L89M, I93L	_	T12S, I15V, L19T, E35D, R41K
V11-08	K20R, M36I, H69K, L89M, I93L	_	T12S, I15V, L19T, E35D, R41K,H69K
V11-02	K20R, M36I, H69K, L89M, I93L	_	T12S, I15V, L19T, E35D, R41K

3.2.3. Viral genetic subtyping

Phylogenetic analysis of the PR and RT genes showed that 3/3 (100%) of the aligned sequences were HIV-1 C as shown in figure 3.2 and 3.3 respectively. Only 3 samples were fully sequenced, edited and included for phylogenetic analyses. The remaining samples yielded short fragment sizes after sequencing and some were bad sequences.

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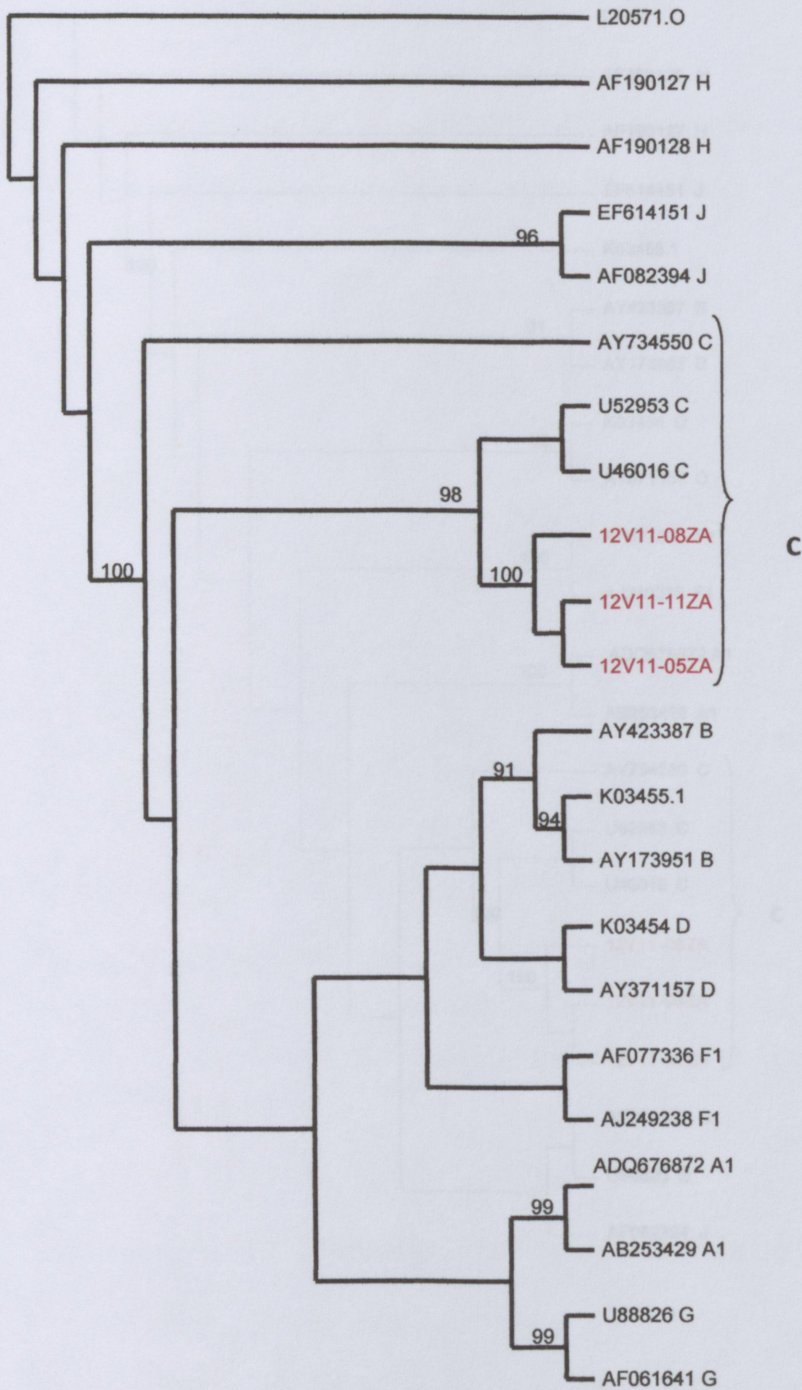

Figure 3.2: Phylogenetic relationship of 3 Limpopo HIV-1 C protease nucleotides. The tree shows test sequences in red clustering with described reference HIV-1 C PR sequences obtained from GenBank.

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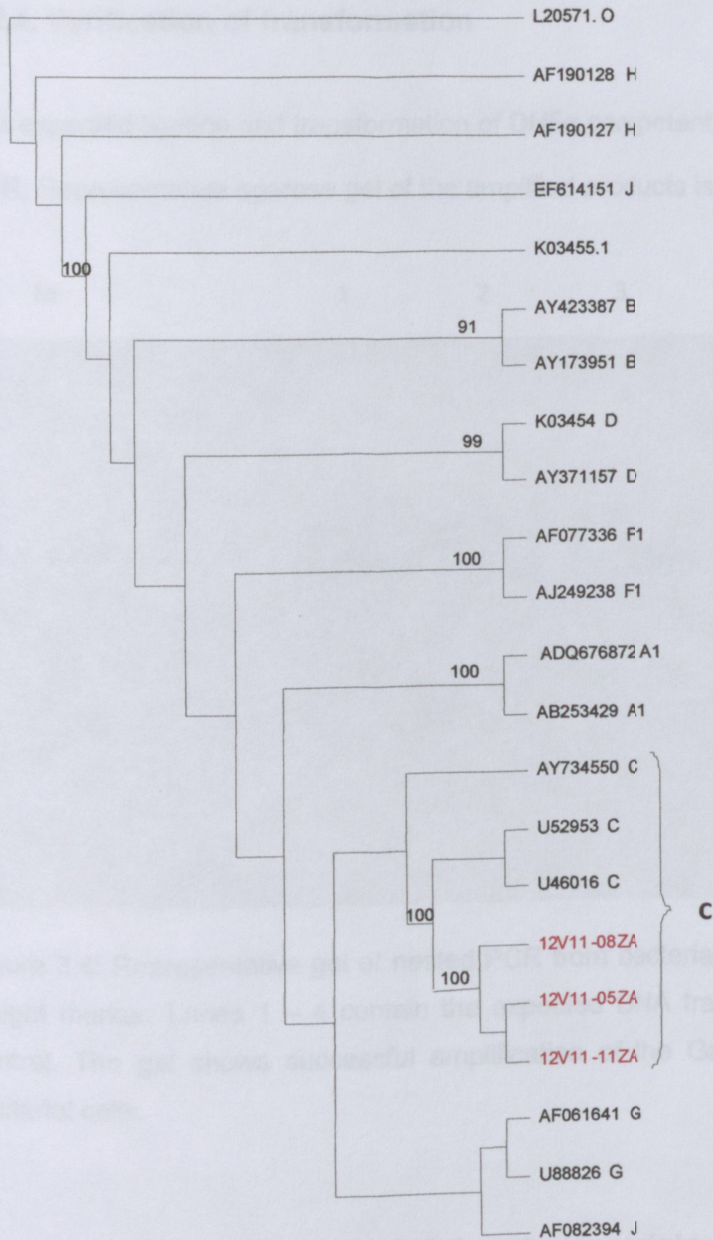

Figure 3.3: Phylogenetic relationship of 3 Limpopo HIV-1 C reverse transcriptase nucleotide sequences. The tree shows test sequences in red clustering with described RT sequences obtained from GenBank.

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3.2.4. Verification of transformation

The expected ligation and transformation of DH5α competent cells was verified by colony PCR. Representative agarose gel of the amplified products is presented in figure 3.4.

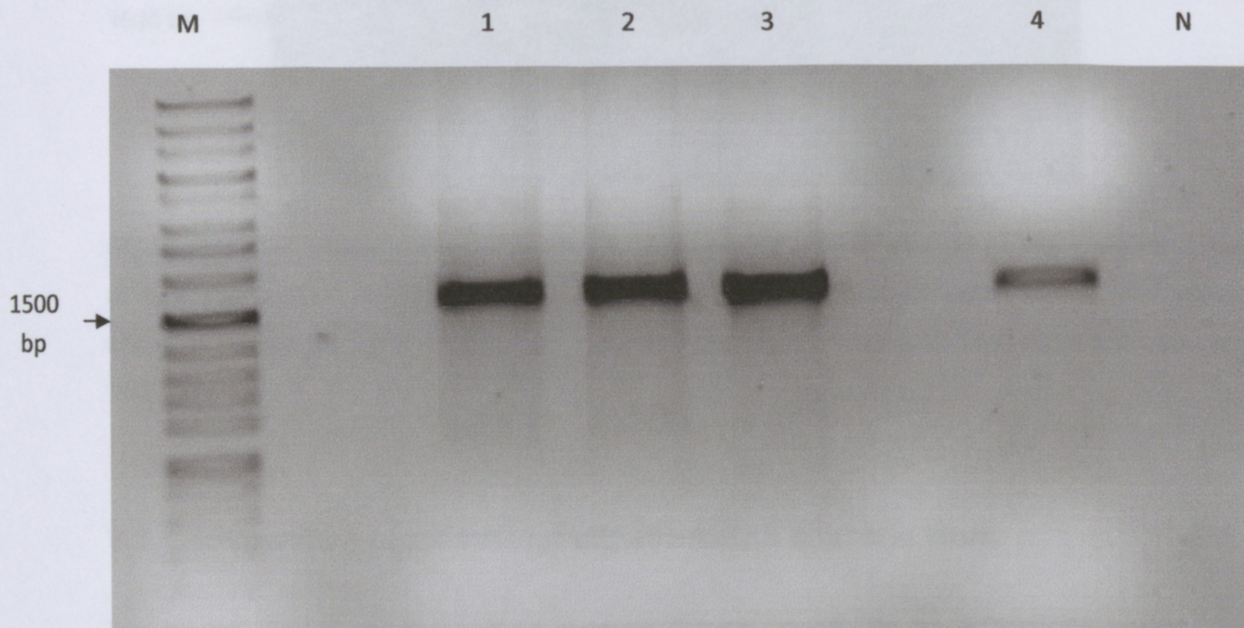

Figure 3.4: Representative gel of nested PCR from bacterial cells. Lane M is the molecular weight marker. Lanes 1 – 4 contain the expected DNA fragment. Lane N is the negative control. The gel shows successful amplification of the GagPol gene (1650bp) from the bacterial cells.

3.2.5. Double digestion of pGEM-T vector containing viral DNA

The pGEMT vector containing viral DNA was double digested with Apal and Hpal enzymes to extract the partial GagPol gene. The digestion reaction was run on 0.8% agarose and bands of interest were purified. A representative agarose gel of digested products is presented in figure 3.5.

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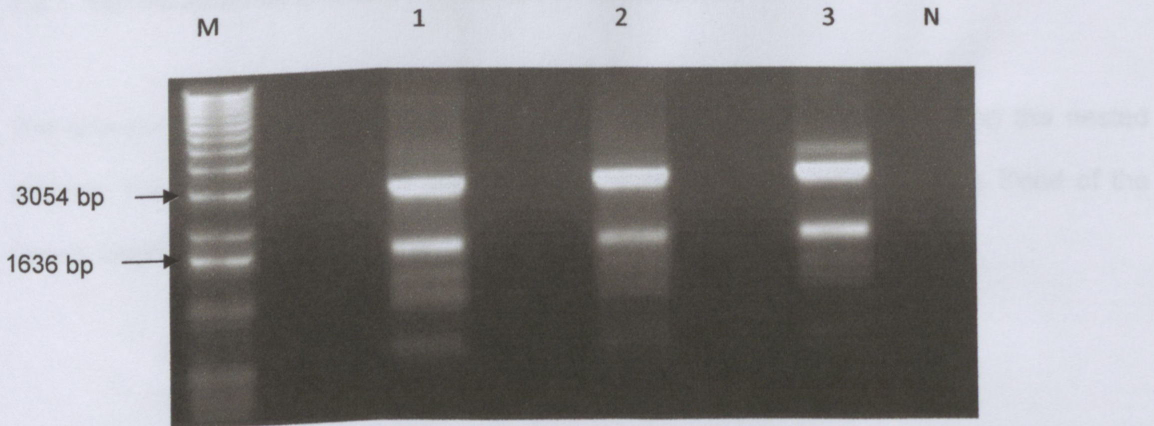

Figure 3.5: Representatives gel of the double digested viral DNA from pGEM-T vector. Lane M is the molecular weight marker X. Lanes 1 – 3 contain double digested pGEM-T vector showing vector and patient viral DNA. Lane N is negative control. The expected size was 1627bp.

3.3. Construction of pCMVGagPol(patient)RRE chimeric virus

The partial *GagPol* gene of HIV-1 subtype C was cloned into the pCVMRRE vector. The recombinant clone was confirmed for the presence of *GagPo*l gene by double restriction digestion using Apal and Hpal enzymes. A representative agarose gel is presented in figure 3.6.

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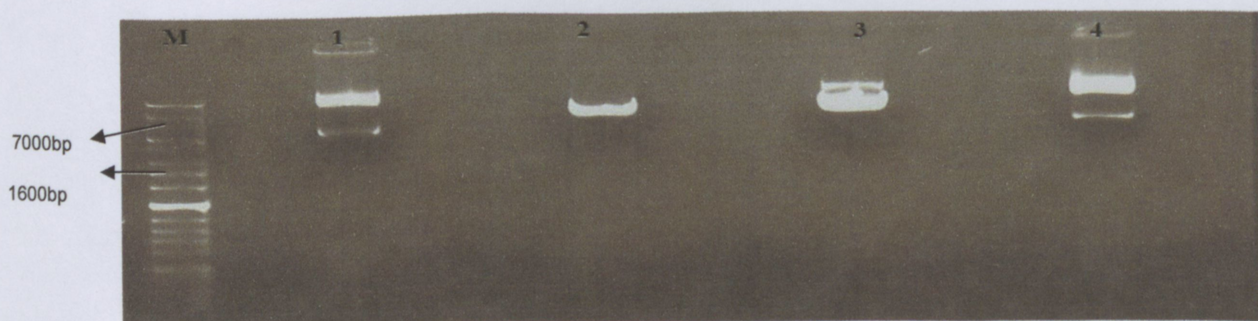

Figure 3.6: Representatives gel of the digested chimera viruses. Lane M is the molecular weight express marker. Lane 1 and 4 contain double digested products, lane 2 is uncut DNA and lanes 3 contain single digest (Hpal) of the chimeric virus. Expected size band was 1627bp.

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3.3.1. Verification of cloned-in viral DNA orientation

The orientation of the cloned viral DNA was confirmed by sequencing using the nested primers. The restriction sites of one of the enzyme used was located at the 3'end of the cloned fragment as expected (Figure 3.7)

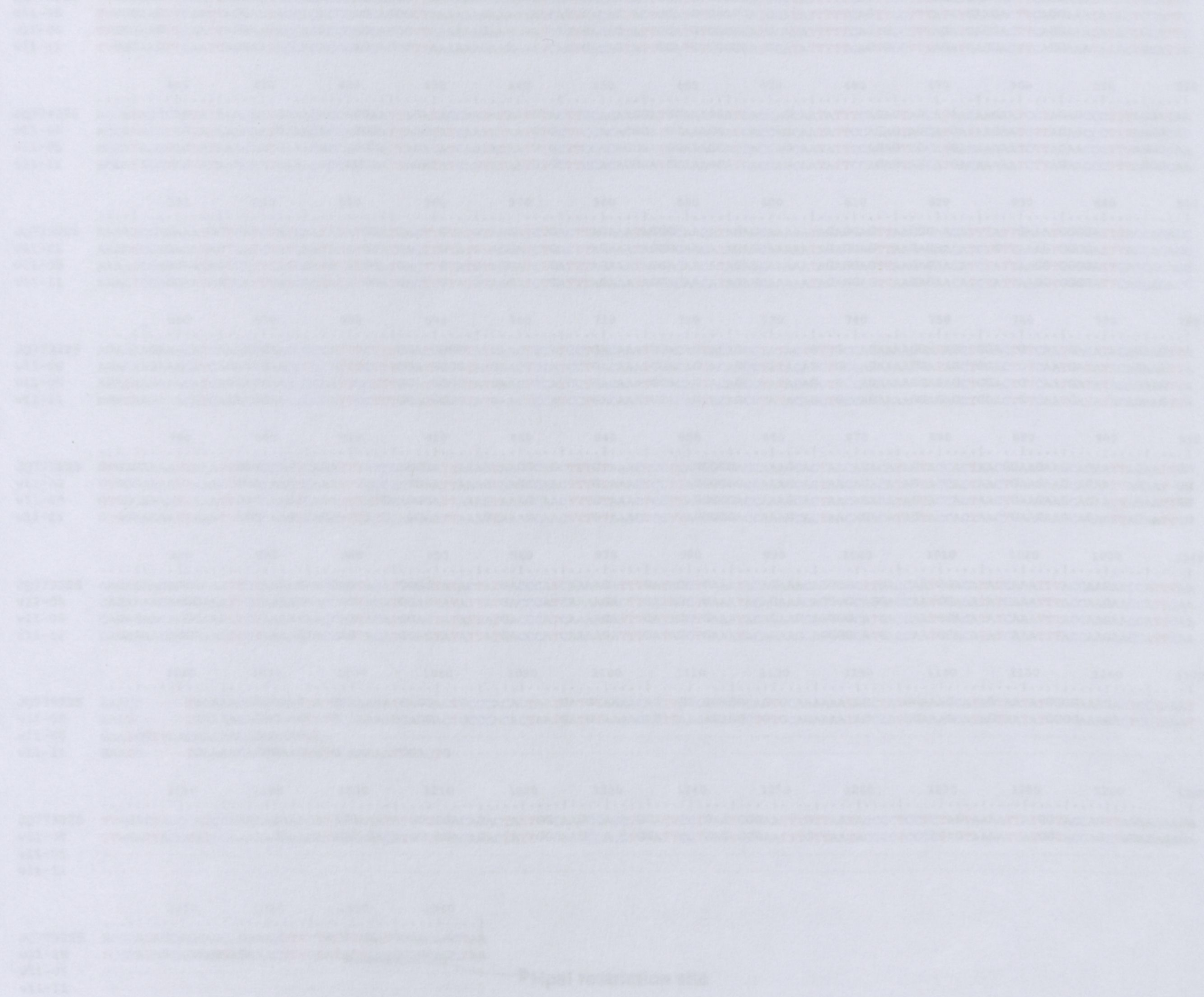

Figure 3.7: Alignment of the reverse transcriptase gene region of 3 limpopo HIV-C strains containing Hpal restriction site in the cloned viral DNA

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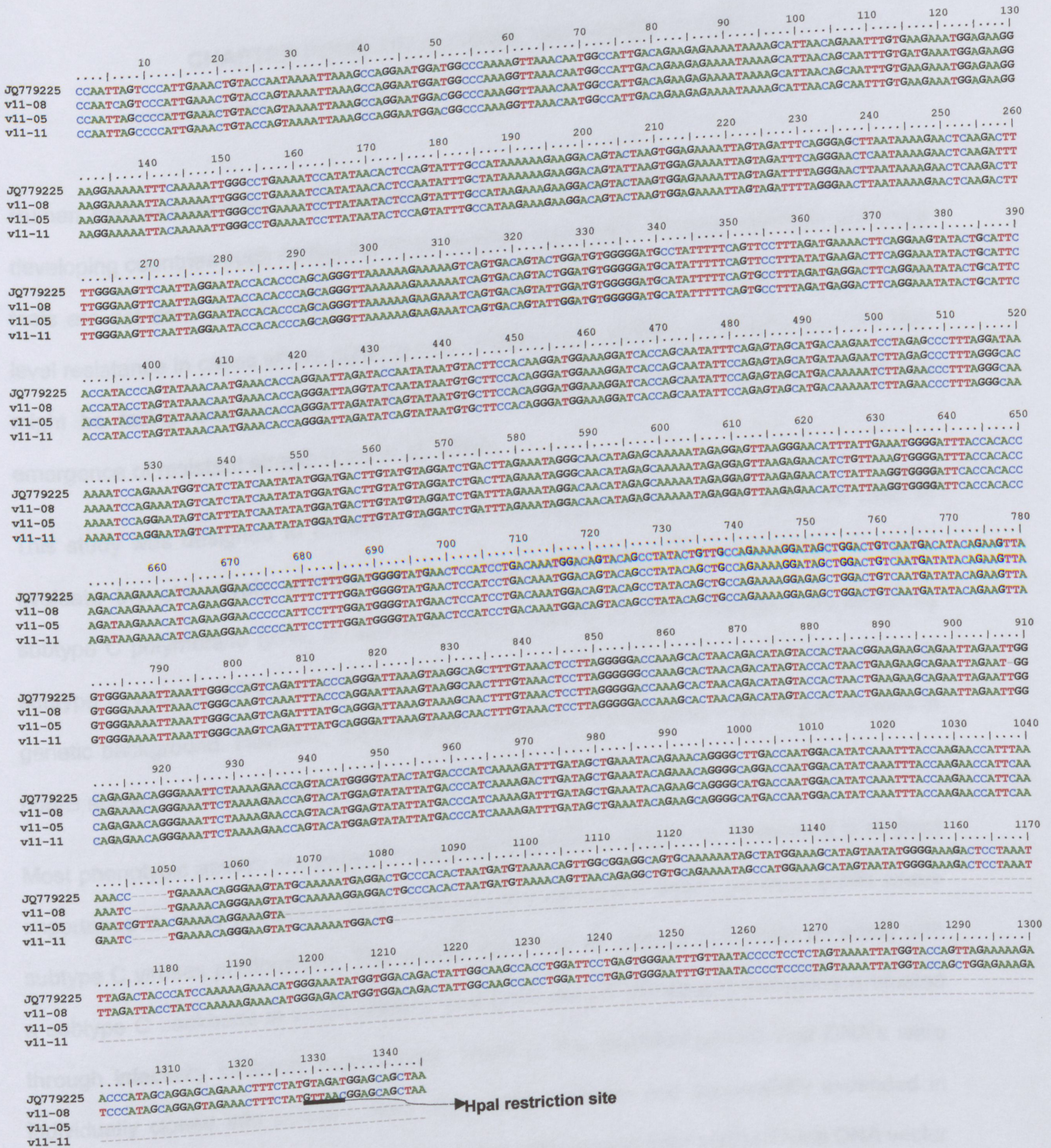

Figure 3.7: Alignment of the reverse transcriptase gene region of 3 limpopo HIV-C strains containing Hpal restriction site in the cloned viral DNA

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CHAPTER FOUR: DISCUSSION AND CONCLUSIONS

Human Immunodeficiency virus remains the world's most destructive disease to date. In developing countries such as South Africa, regular virological monitoring and drug resistance tests are not done due to cost efficiency. Consequently, this leads to emergence of high level resistance in cases where patients stay on prescribed therapy despite the fact that they might be failing therapy. This could result in the evolution of new recombinants and emergence of resistant strains (Carr et al, 1998).

This study was designed to construct an HIV-1 C vector system which could be used to evaluate the phenotypic significance of putative resistant mutations observed in HIV-1 subtype C polymerase gene. In Southern Africa, majority of HIV-1 infections are driven by subtype C viruses. ARV drugs are designed and developed mainly against the subtype B genetic background. However, the observed mutations are naturally occurring mutations in non-B subtypes whose clinical relevance is unknown.

Most phenotypic assays are based on subtype B genetic background, however it is of great importance to study subtype C viral mutations in a subtype C based assay in areas where subtype C viruses predominate. The current study was an attempt to develop an assay with a subtype C backbone in which primary viral DNA can be cloned and mutations evaluated through infectivity inhibitory experiments. Three of the amplified patient viral DNA's were individually cloned into pGEM-T easy clone vector system and successfully expanded in DH5α competent cells. Viral DNA was extracted with success from pGEM-T/viral DNA vector and then cloned into pCMVRRE vector after excision of a product of the same size. The constructed pCMVGagPol(patient)RRE vector was double digested to confirm the presence of the cloned HIV-1 *GagPol* gene and investigate the presence of restriction enzyme's sites (Apal and Hpal). The properties of the generated vector are as follows: the construct enabled the insertion and excision of the 1650bp fragment from patient samples comprising

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unique Apal and Hpal restriction sites, and can be easily replicated in competent cells. The constructed chimeric virus can be used to insert patient viral DNA, co-transfect in 293T cells with plasmids carrying luciferase and envelope genes. The harvested viral stocks from trasfection step can be used in phenotypic drug resistance studies. The protocols for designing the pCMVGagPol(patient)RRE were repeated several times to ensure reproducibility and viral DNA orientation in the vector was confirmed by location of restriction enzyme sites. Several studies have been aimed at developing HIV-1 pseudoviruses and their observations are comparable to our current research findings however their main focus was on constructing vectors of subtype B assays. (Mochizuki et. al., 1999; Chugh, 2003; Chugh and Seth, 2004). Chung and Seth (2004) successfully constructed a gagprotease C plasmid for use in DNA vaccine studies. The in-vitro expression studies of their chimeric virus detected up to 110pg/ml of secreted antigen in transfected COS-7 cell supernatants.

The commercially available phenotypic tests include antivirogram (Tibotec-Virco), phenosense (Monogram Biosciences) and Phenoscript (Viralliance). The antivirogram assay generates homologous recombinant virus by introducing the PR and RT region of HIV-1 amplified from patient plasma. In the phenosense assay, the recombinant PR and RT region uses luciferase gene for fluorescence. Phenoscript is an in-vitro single cycle recombinant virus assay where 3 regions PR, RT and env are employed. PCR products are separately co-transfected into CD4 cells along with different ARV drugs to be tested. These commercial tests are expensive as opposed to the in-house methodology that has proven to be cheaper and effective although it is time consuming.

The protocol for amplifying HIV-1 subtype C partial *GagPol* gene was optimized. Of the 14 selected samples 12 were successfully amplified (1650 bp) whereas a product size of 800bp was obtained for other samples (2/14), and such samples were considered negative and excluded from the analysis. This could be attributed to the primer annealing at unexpected sites. The gene region in this study was chosen because the viral protease gene provides native protease that processes gag gene products from pr55 precursor. The RT gene's main

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function is to reverse transcribe viral RNA into DNA hence the 2 genes harbor most of the known mutations that render ARV drugs ineffective. With the expression of gag gene, it has been shown that self assembly of pr55 molecule triggers formation of new viral particles (Nermut *et al.*, 1998). This was studied with baculovirus, vaccinia, yeast and mammalian expression vector system (Gheysen *et al.*, 1989; Wagner *et al.*, 1992).

Phylogenetic analysis was used to confirm subtype designation of 3/12 amplified HIV isolates. The PR and RT reliable gene sequences were analyzed. Comparisons of the PR and RT regions showed that the 3 samples used in this study were HIV-1subtype C and they intermingled with HIV-1 C viruses from other countries although more samples needed to be sequenced to deduce the finding.

The significant genetic drug resistance mutations observed in 6/12 samples were as follows: K20R, M36I, L89M and I93L (common polymorphisms on the protease gene in subtype C strains). These amino acid changes reduce susceptibility of the virus to PI's in subtype B isolates. K20R is frequently observed in subtype B treated strains and highly associated with resistance to IDV, RTV and LPV. Mutations M36I and I93L are weakly associated with PI resistance in B viruses when present with other mutations. L89M is a common polymorphism associated with decreased PI susceptibility also in B viruses. These observations suggest that the administration of the above mentioned PI's will impact negatively on the morbidity and mortality of HIV/AIDS.

The presence of these polymorphisms may influence resistance development and patient failing therapy. These could also impact on the development or evolution of new resistant strains in subtype C treated patients (Brenner *et al.*, 2003; Vergne *et al.*, 2006). No major drug resistance mutations were observed in any of the analyzed samples, unlike in studies done in other parts of South Africa. For example, Orrel *et al*, (2008); Jacobs *et al* (2008) and Pillay *et al* (2008) reported a 2.5%, 3.6% and 4.2% prevalence rates of major drug resistance mutations in Free State, Cape Town and Gauteng province respectively. Study

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conducted by Bessong *et al* (2005) showed no primary resistance mutations in PR and RT regions, however polymorphisms associated with drug resistance in B viruses were observed in the analyzed C viral strains. An observation was that K20R, M36I and I93L are in high prevalence in C viruses and are associated with reduced virologic response to atazanar-vir/ ritonavir containing regimens (Bessong, 2008). In 2010, Wainberg and Brenner documented that enzymatic and virological data supports the concept that polymorphisms in non B subtypes can affect HIV-1 susceptibility to antiretroviral drugs.

Distinct limitation encountered in the current study was lack of clinical data (viral load and CD4 measurements). This may have enabled us to know whether it is possible to amplify samples from drug experienced patients with low viral loads.

The constructed pCMVGagPol(patient)RRE chimeric virus can be used for subsequent evaluation of mutations that are present in naïve and treatment exposed viruses but whose importance is not clear.

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CHAPTER FIVE: REFERENCES

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1.8. Treatment of HIV-1

Studies on the life cycle of the virus have identified several opportunities for therapeutic intervention. RT has been the most studied chemotherapeutic target of HIV-1. Antiretroviral treatment helps in viral suppression thereby reducing morbidity and mortality. However these drugs do not eradicate the infection. The RT inhibitors are classified into 2 major groups based on structural considerations (table 1.8): nucleoside RT inhibitors (NRTI) and non-nucleoside RT inhibitors (NNRTIs).

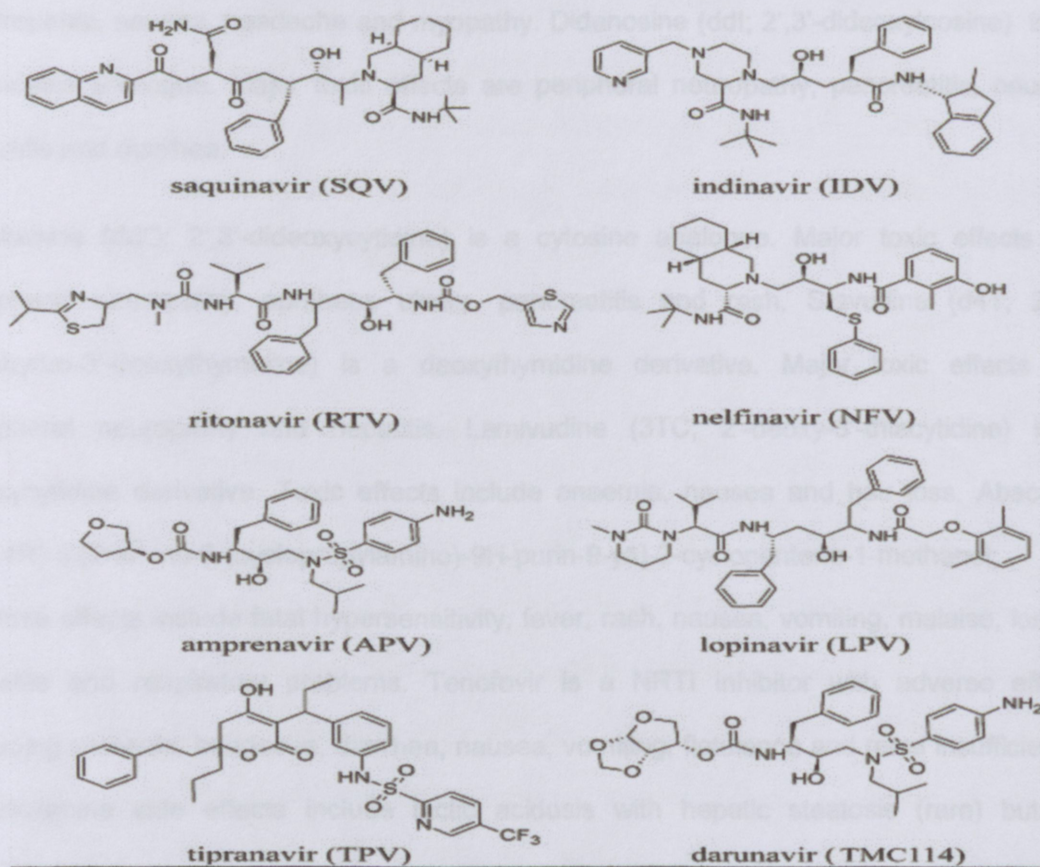

Figure 1.8: The 2D chemical structures of the eight FDA-approved drugs (Hou et al., 2008)

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