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Studies on Human Immunodeficiency Virus Genetic Drug Resistance and Subtype Distribution in Northern South Africa.

By

Julius Nwobegahay
(11570077)

June, 2012

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Studies on Human Immunodeficiency Virus Genetic Drug Resistance and Subtype Distribution in Northern South Africa.

By

Julius Nwobegahay (BSc Honours - Calabar, Nigeria; MSc – Ibadan, Nigeria)

Thesis Submitted in Fulfilment of The Requirements For The Award of a Doctor of Philosophy Degree in Microbiology at the University of Venda.

Promoter: Professor Pascal O. Bessong

Department of Microbiology,

School of Mathematical and Natural sciences,

University of Venda, South Africa.

Co-Promoter: Dr. Gloria Selabe

HIV/AIDS and Hepatitis unit,

Medunsa campus,

South Africa.

June, 2012

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Declaration

I, Julius Nwobegahay, hereby declare that this thesis for the award of a Doctor of Philosophy degree in Microbiology at the University of Venda is my own work. It is being submitted for the degree of Doctor of Philosophy in Microbiology at the University of Venda, South Africa. It has not been submitted before for any degree examination at this or any other University. It is my own work in design and execution and all the reference materials contained therein have been duly acknowledged.

Julius Nwobegahay. Date

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Dedication

To God Almighty and to my late father – Mr William Ajua'mendem Mbekem

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Summary of the study

HIV/AIDS is still the world's leading infectious cause of death, despite global efforts for prevention, better access to treatment and management of patients. At the end of 2010, about 33.3 million people were living with HIV/AIDS in the world. Sub-Saharan Africa is the most affected region in the global AIDS epidemic, with 22.5 million adults and children infected with the virus. Even though the use of antiretroviral treatment has significantly reduced HIV-related mortality in the industrialised world, only a small number of people in need of treatment in the developing world actually have access to it. Global initiatives aiming at universal access to treatment are still ongoing

Southern Africa has the highest HIV prevalences, with very serious health and socioeconomic consequences. The nine countries with the highest HIV prevalence in the world are found in the sub region. In 2009, National adult HIV prevalence exceeded 10% in nine countries of Southern Africa (Botswana, Lesotho, Mozambique, Namibia, South Africa, Swaziland, Zambia, Malawi and Zimbabwe). This has a negative effect on health care delivery, especially in a region that already has limited human and health infrastructure. Financial resources which should have been used for development are now being spent on the procurement of antiretroviral drugs. South Africa is one of the countries in this region which has been hit hardest by this pandemic. According to the South African National HIV survey conducted in 2009, the estimated prevalence was 29.3% among antenatal women aged 15 to 49 years old. The survey also estimated that the provinces most affected were KwaZulu-Natal (39.5%), Mpumulanga (34.7%) and Free State (30.1%). Limpopo is the seventh, with a prevalence rate of 20.7%. Western Cape Province has the lowest prevalence (16.9%).

Rollout of antiretroviral therapy in sub-Saharan African countries with high HIV prevalence has been identified by the World Health Organization as a global public health priority. Hence, the use of highly active antiretroviral therapy has been rolled out in recent years, through the massive financial support from programs such as the US President's Emergency Plan for AIDS Relief and the Global Fund for AIDS, Tuberculosis and Malaria.

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Access to antiretroviral therapy is expanding in South Africa, including the Limpopo province. Antiretroviral drugs have been available to some communities for close to a decade in the Limpopo province (Northern South Africa). Within this time, there is the possibility of drug resistance emergence and transmission. In addition, resistance testing is not routinely performed prior to treatment initiation. Treatment is not accompanied by regular virological monitoring due to the high cost of viral load and drug resistance tests. As a result of this, patients stay on a standard first line therapy (which might be failing) for long periods, with the consequence of the emergence of high-level resistance.

There is paucity of information on the genetic resistance profile of patients in South Africa. Baseline HIV-1 genotypic drug resistance data are very important for resistance monitoring purposes, especially in a country such as South Africa, where large scale treatment programs have been initiated. It is very necessary to address the lack of knowledge about genotypic drug resistance in non B viral subtypes. The surveillance of antiretroviral drug resistance could be influenced by genetic diversity among HIV-1 subtypes. This information could impact on South African national policy on HIV prevention and monitoring, treatment, and vaccine research. Data generated will equally contribute immensely to the database of Southern African Treatment and Resistance Network, which is a group of African, European, and American researchers investigating issues peculiar to resistance and resistance development in HIV-1 subtype C which is becoming predominant worldwide.

Study subjects for these studies were recruited from the HIV/AIDS Wellness Clinic in Bela Bela and Phela O Phedishe (POP) HIV clinic in Mankweng Hospital, both in the Limpopo Province of South Africa. Bela Bela is located in the Waterberg district which has an estimated HIV prevalence of 27.5%. It is a holiday resort with hotels and camping sites. It is also a stop-over point for long distance truck drivers who patronize sex workers attracted to the luxury facilities of the area. The Bela Bela HIV Wellness Clinic receives patients from the urban and rural sectors of the area. The Clinic is run by the HIV/AIDS Prevention Group (HAPG) - a non-governmental organization established in 1996. On

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the other hand, the POP HIV clinic is a component of the Polokwane-Mankweng Hospital Complex. The HIV Clinic has nineteen feeder clinics situated in nearby villages. Mankweng is located in the Capricorn district which has an estimated HIV prevalence of 24.2%. The development of drug resistance is one of the major drawbacks in ART programmes. At the time of sample collection, antiretroviral treatment had been available to the Mankweng and Bela Bela communities for 4 and 8 years, respectively. Besides, these were the first communities to benefit from antiretroviral treatment in northern South Africa. In the intervening time, there is a possibility of resistance emergence and transmission to antiretroviral (ARV) drug naive populations in these two sites. There is a paucity of data from these sites on the prevalence of drug resistance mutations.

This work is focused on three main investigated themes, contained in four separate chapters. The first theme deals with the determination of the prevalence of genotypic drug resistance substitutions in newly diagnosed, drug naive HIV-infected individuals in two different communities - Bela Bela and Mankweng in Northern South Africa. This theme is treated separately for each community and contained in two different chapters. Demographic and socio-economic characteristics of infected individuals, HIV-1 subtype distribution, intragenetic diversity of the *protease* (PR) and *reverse transcriptase* (RT) gene sequences; and HIV-1 genetic recombination, were also examined. Drug resistance mutations were identified and described using the Stanford HIV Drug Resistance Interpretation Algorithm and the International AIDS Society-USA guidelines or the Calibrated Population Resistance (CPR) tool contained in the Stanford HIV Drug Resistance database. Demographic and socio-economic characteristics of the subjects were collected using a study questionnaire. This enabled the collection of information on age, sex, place of residence, probable place and date of infection, marital status, house hold income, level of education, and number of dependants. Subtyping of HIV was done by phylogenetic analysis. Recombination analysis was done using the Recombination Identification Program and REGA HIV-1 subtyping tools. REGA employs phylogenetic analysis and bootscanning methods to assign genetic subtypes and recombination patterns respectively. Predicted amino acids of the PR and RT genes were aligned using the

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BioEdit program. The intragenetic distance among the *protease* and *reverse transcriptase* sequences were determined by the Kimura 2- parameter model.

In Bela Bela where treatment became available to the community in 2001, major drug resistance mutations were detected in 2 out of 57 subjects (3.5%). One primary NNRTI mutation (Y181C) and one major PI mutation (L33F) were detected on two different patients. No primary NRTI mutation was recorded. The study group was made up of HIV-1 infected, treatment-naive subjects. The mean age of the subjects was 43.5 years (18 – 69 years). The most important risk factor for HIV transmission was sexual intercourse (88.6%). Phylogenetic analysis of the PR and RT genes showed that 56/57 (98.2%) of the viruses were HIV-1 subtype C on the PR; while 54/55 (98.2%) of the viruses were HIV-1 subtype C on the RT. One virus (08BBVCT31ZA) was HIV-1 subtype B on the PR and RT genes. Mean genetic distance for the PR sequences ranged from 0.0101 to 0.2035 and 0.0331 to 0.1377 for the RT sequences.

In Mankweng, 5 sequences (9.3%) harboured drug resistance mutations. Four of these (7.4%) were NRTI mutations and one (1.9%) was a PI mutation (M46I). No major NNRTI mutation was detected. The study group was also made up of HIV-1 infected, treatment-naive subjects. Phylogenetic analysis of the partial *pol* sequences showed that 52/54 (96.3%) of the viruses were HIV-1 subtype C. One virus (08MB08ZA) was HIV-1 subtype B, while another (08MB26ZA) was related to HIV-1 subtype J. HIV-1 subtype recombination analysis with REGA assigned the *pol* sequence to HIV subtype J (11_cpx) with a bootstrap value of 75%.

Apparently, the testing of patients for drug resistant viruses before the initiation of therapy may not be required in Bela Bela. However, the prevalence of drug resistance mutations observed in Mankweng is moderately high according to the World Health Organization classification for surveillance of transmitted resistance mutations, and relatively higher than previously reported from other parts of South Africa. According to WHO recommendations, this moderate drug resistance prevalence may necessitate routine drug resistance testing for all persons newly diagnosed with HIV or all persons

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beginning HIV treatment in this community. This level also requires a review of the treatment program monitoring data for this community and the investigation of potential problems with regards to factors such as: continuous access to services, drug supply, drug quality, prescribing practice, compliance toxicity and/or adverse events, drug sharing and treatment failures. However, resistance in this population does not reflect current transmission of resistant HIV, because many newly diagnosed persons have been infected many years prior to diagnosis. Subsequent surveillance studies are needed to confirm these findings and to generate data relevant to sentinel surveillance policy, and treatment initiation guidelines.

The second aspect examined in this study focused on the genetic drug resistance profile in a cohort of HIV infected patients entering antiretroviral treatment programmes at two sites in Northern South Africa. Specific issues examined were the baseline demographic and socioeconomic characteristics of HIV-1 infected individuals in the cohort, baseline genetic drug resistance mutation profile of the patients prior to therapy, baseline CD4+ and viral load measurements, HIV-1 subtypes, intragenetic diversity of the *protease* and *reverse transcriptase* sequences and recombination analysis.

Results obtained revealed that the mean age of 112 individuals in the cohort was 40.5 years (range 22 – 59 years). The proportion of women and men was 78/112 (69.6%) and 34/112 (30.4%) respectively. Seventy of the respondents (62.5%) were single compared to 26 (23.2%) who were married. The most important risk factor for HIV transmission was unprotected sex (98.2%). In 88.4% of the participants, the most probable place of HIV infection was South Africa and a further 24/112 (21.4%) reported to have been probably infected in 2008. Probably older infections (2000 – 2007) were reported in 65.2% of the subjects. The mean viral load was 5.4 Log 10 copies/ml (range 3.0 - 5.7). The mean CD4+ count was 138 cells/mm^3 (interquartile range 51-197). Twenty six patients (23.2%) were under treatment for tuberculosis. Overall, 1/112 (0.9%), 11/112 (9.8%), 48/112 (42.9%) and 52/112 (46.4%) were classified as AIDS stage I, II, III and IV respectively according to the WHO system. Two (2.5%) primary RT drug resistance mutations (K103N and M41L) were detected in 2 different subjects. Phylogenetic

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analysis of the PR and RT sequences showed that 79/80 viruses were HIV subtype C. One isolate (08BBCR06ZA) from Bela Bela clustered with pure A1 subtypes on the PR gene and pure C subtypes on the RT gene. Further analysis of the partial *pol* of 08BBCR06ZA (comprising the complete PR and 900 nucleotides of the RT) with recombination identification programs showed that the *pol* gene has a mosaic structure consisting of A1 sequences alternating with C sequences confirming that the isolate is a recombinant on the gene analyzed. Mean genetic distance for the PR sequences ranged from 0.0272 to 0.1311; and 0.0978 to 0.1014 for the RT sequences.

The results obtained from this baseline cohort study suggest that majority of the patients entering the ART programmes will obtain therapeutic benefit from the first line ART regimen. In addition, the level of resistant viruses appears to be low at the population level at the two treatment sites after eight years of access to ART. The identification of an A1/C recombinant may be a further indication of the gradual introduction of complex viruses in northeastern South Africa.

The third part of the study focused on the analysis of *integrase* gene sequences of viruses from treatment naive individuals in Northern South Africa. No *integrase* inhibitors are currently being used in South Africa, but with the rise in treatment failure, they could be introduced in the near future as salvage therapy. More understanding is needed on the significance of unknown mutational patterns associated with drug resistance loci in HIV-1 *integrase* gene. Furthermore, previous information gathered on circulating subtypes has largely been sought using the *gag* and partial *pol* genes. Given that HIV-1 is highly variable, it is necessary to attempt subtyping using the *integrase* (IN) gene so as to study the variability of this gene.

Integrase gene sequences from drug naive individuals from Bela Bela and mankweng communities were analysed for drug resistance substitutions, HIV-1 subtypes, intragenetic diversity, HIV-1 IN functional domains and HIV-1 genetic recombination. Using the Stanford HIV Drug Resistance Interpretation Algorithm and the International AIDS Society-USA guidelines, no major drug resistance mutations were detected in any

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of the subjects. However, 6/89 (7%) minor mutations and polymorphisms were detected. Phylogenetic analysis showed that 88/89 (99%) of the viruses were HIV-1 subtype C on the IN gene. One isolate (08BBVCT28ZA) did not cluster with any reference subtype. The IN gene (864bp, HXB2 location: 4230-5093) and a partial *pol* fragment of sample 08BBVCT28ZA (1127bp, HXB2 location: 2253-3379) was further analysed for recombination using REGA and RIP recombination tools. The results revealed that the sequence is a mosaic of A1, C, F1, and F2 subtypes on the IN gene and HIV-1 subtype C on the partial *pol* fragment (PR and RT genes). This suggest that the isolate is a recombinant on the IN gene and HIV-1 C on the partial *pol* gene region.

The analysis of IN gene sequences revealed that there are no major Raltegravir associated drug resistant HIV mutations among the treatment naive population in Bela-Bela and Mankweng, South Africa. The significance of the polymorphisms observed is not yet known; however, more studies need to be carried out in order to generate baseline data in anticipation of the eventual use of *integrase* inhibitors in South Africa. The identification of a recombinant virus further indicates the introduction of hitherto undocumented variants in a region where C viruses are known to overwhelmingly predominate.

Overall, there is a difference in the prevalence rate of drug resistance mutations among newly diagnosed persons and those about to start therapy in Mankweng community. A moderately high prevalence (9.3%; 95%CI: 0-16.99) of resistance mutations was observed in newly diagnosed persons; while a low prevalence (2.5%; 95%CI: 0077-0.0863) was observed in HIV-1 chronically infected individuals about to start therapy. Drug resistance mutations may decay over time and this could have influenced the low prevalence observed in the latter group. The prevalence of drug resistance mutations in Mankweng community could be high.

Overall, this study has elaborated on the data on genetic drug resistance in northern South Africa, providing new information on drug resistance levels among drug inexperienced patients, and previously undocument genetic variants. In conclusion, it is recommended

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that genetic studies are continued on a regular basis to update data on trends relevant for research and patient management.

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Scientific communications

The investigations contained in this thesis have led to the following scientific communications:

Peer-reviewed published articles:

1. **Nwobegahay J**, Bessong PO, Masebe TM, Mavhandu LG, Manhaeve C, Ndjeka NO, Selabe G (2011). Prevalence of drug resistance mutations in newly diagnosed drug-naive HIV-1 infected individuals in a treatment site in the Waterberg District, Limpopo province. South African Medical Journal 101(5):335-337

2. **Nwobegahay J**, Bessong PO, Masebe TM, Mavhandu LG, Iweriebor BC, Manhaeve C, Ndjeka NO, Selabe G (2010). Prevalence of antiretroviral drug resistance mutations and HIV-1 subtypes among newly-diagnosed drug-naive persons visiting a voluntary testing and counseling centre in northeastern South Africa. Journal of Health, population and Nutrition 29(4): 303 - 309.

3. **Nwobegahay J**, Masebe TM, Mavhandu LG, Iweriebor BC, Ndjeka NO, Manhaeve C, Bessong PO. Low Prevalence of Genetic Drug Resistance in a Cohort of HIV Infected Patients Entering Antiretroviral Treatment Programmes at Two Sites in Northern South Africa. Journal of Medical Virology (Accepted for publication).

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Conference proceedings:

1. **Nwobegahay J**, Masebe TM, Mavhandu LG, Iweriebor BC, Ndjeka NO, Manhaeve C, Bessong PO. Low Prevalence of Genetic Drug Resistance in a Cohort of HIV Infected Patients Entering Antiretroviral Treatment Programme at Two Sites in Limpopo Province, South Africa. Abstract Presented at the XVIII International AIDS conference, Vienna, Austria, July 18 – 23, 2010.

2. Bessong PO**, Nwobegahay J**, Masebe TM, Mavhandu LG, Iweriebor BC, Rikhotso M, Mangolele V. Genetic analysis of HIV-1 *integrase* gene from drug naive individuals in the Limpopo province of South Africa. Abstract Presented at the V South African AIDS conference, Durban, South Africa, June 7 – 10, 2011.

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Abbreviations

AIDS	Acquired immune deficiency syndrome
ATP	Adenosine triphosphate
APC	Antigen presenting cells
APOBEC3G	Apoliprotein B mRNA editing enzyme catalytic polypeptide-like 3G
ART	Antiretroviral therapy
cDNA	Complementary DNA
CCC	Catalytic core domain
CCR5	C-C Chemokine receptor type 5
CpA	Cytosine-adenosine dinucleotide
CXCR4	C-X-C chemokine receptor type 4
CNS	Central nervous system
CTD	C-terminal domain
DC	Dendritic cells
DC-SIGN	Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleotide triphosphates
env	Envelope
EDTA	Ethylene diamine tetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FAD	Food and drug administration
FDC	Follicular dendritic cell
FI	Fusion Inhibitors
GpT	Guanine-thymine dinucleotide
gag	Group-associated antigen
gp	Glycoprotein
HAART	Highly active antiretroviral therapy
HR1	Heptad repeat 1
HR2	Heptad repeat 2
HIV	Human immunodeficiency virus

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HIV-1	HIV type 1
HIV-2	HIV type 2
HLA	Human leucocyte antigen
ICAM-1	Intracellular adhesion molecule – one
IL-1	Interleukin-1
IN	*Integrase*
INF-α	Interferron alpha
kDa	Kilodaltons
LAV	Lymphadenopathy-associated virus
LTNP	Long term non-progressors
LTR	Long terminal repeat
MIP-1α	Macrophage inflammatory protein-1Alpha
MIP-1β	Macrophage inflammatory protein-1Beta
MHC	Major histocompatibility complex
MTCT	Mother to child transmission
M-tropic	Macrophage tropic
mM	Milli molar
ml	Millilitre
NK cells	Natural killer cells
NTD	N-terminal domain
NRTIs	Nucleoside *reverse transcriptase* inhibitors
NNRTIs	non-nucleoside analog *reverse transcriptase* inhibitors
PCR	Polymerase chain reaction
PR	*Protease*
PIC	Preintegration complex
PIs	*Protease* inhibitors
Pol	Polymerase gene
RANTES	Regulated upon activation, normal T-cell expressed and secreted
RNA	Ribonucleic acid
RNase H	Ribonuclease H
RT	*Reverse transcriptase*

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RT-PCR	Reverse transcription polymerase chain reaction
Rpm	Revolutions per minute
rev	Regulator of virion
REE	*Rev* response element
SIV	Simian immunodeficiency virus
SI	Syncytium-inducing
SIVcpz	Simian immunodeficiency virus found in chimpanzees
SIVsm	Simian immunodeficiency virus found in sooty mangabeys
SDF-1	Stromal-derived factor 1
tat	Transactivating regulatory protein
TH1	T-helper 1
TNF-α	Tumor necrosis factor alpha
TAR	Transactivation response elements
TAM	Thymidine analog mutations
TCR	T cell receptor complex
TLR	Toll-like receptor
T-tropic	T-cell line tropic
µl	Micro litre
V3 region	Third variable region
vif	Viral infectivity factor
vpr	Viral protein R
vpu	Viral protein U
WHO	World health organisation

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GENERAL INTRODUCTION AND LITERATURE REVIEW

1.1 Discovery and Classification of HIV

The first reports of homosexual patients suffering from previously rare diseases such as *Pneumocystis* pneumonia and Kaposi's sarcoma were published in 1981 (Centers for Disease Control and Prevention, 1981a; 1981b). It soon became clear that this new disease was affecting other population groups as well and the first cases were reported in injecting drug users. In 1983, the human immunodeficiency virus (HIV) was identified as the primary cause of acquired immune deficiency syndrome (AIDS) (Barré-Sinoussi, 1983; Broder and Gallo, 1984). HIV belongs to the family of Retroviridae and this family is divided into three sub groups: Spumavirus, Oncovirus and Lentivirus. HIV is a member of the Lentivirus sub-group. The simian immunodeficiency virus (SIV) also belongs to this sub-group. SIV has been isolated in more than twenty African primate species (Rambaut *et al.,* 2004). Two types of HIV infect humans: HIV-1 and HIV-2. HIV-1 is the virus that was initially discovered and termed lymphadenopathy-associated virus (LAV). It is more virulent, relatively easily transmitted and is the cause of the majority of HIV infections globally. HIV-2 is less transmissable than HIV-1 and is largely confined to West Africa (Rambaut *et al.,* 2004).

1.2 Transmission of HIV

There are three main routes of HIV transmission.

Sexual route – most HIV infections are acquired through unprotected sexual intercourse. Sexual transmission can occur when contaminated sexual secretions of one partner come into contact with the genital, oral, or rectal mucous membranes of another (CDC, 1999).

Blood and blood product route – this mode of transmission can account for infections in intravenous drug users, haemophiliacs and recipients of blood transfusions and blood

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products (CDC, 1999). It is also of concern for [illegible] receiving medical care in regions where there is prevalent substandard hygiene in the use of injection equipment. HIV can also be spread through the sharing of infected needles. Health care workers such as nurses, laboratory workers and doctors, have also been infected, although this rarely occurs. Tattooing, piercing and scarification procedures also present a risk of infection.

Mother-to-child transmission (MTCT) – the transmission of the virus from the mother to the child can occur during pregnancy, at child birth and through breastfeeding. In the absence of treatment, the transmission rate between the mother and the child is around 25%. However, where combination antiretroviral (ARV) drug treatment and caesarean section are available, this risk can be reduced to as low as 1% (Cooper *et al.,* 2002). Breast feeding also presents a risk of infection for the baby. HIV-2 is transmitted much less frequently by the MTCT and sexual route than HIV-1. HIV has been found at low concentrations in the saliva, tears and urine of infected individuals, but there are no recorded cases of infection by these secretions and the potential risk of transmission is negligible (CDC, 1999; Coovadia, 2004).

1.3. Morphology and structure of HIV

HIV viral particles have a diameter of about 100 nm and are surrounded by a lipoprotein membrane. Each viral particle contains 72 glycoprotein complexes, which are integrated into this lipid membrane and are each composed of trimers of an external glycoprotein gp120 (Hoffmann *et al.,* 2007) and a transmembrane spanning protein gp41 (Figure 1.1). The bonding between gp120 and gp41 is only loose and therefore gp120 may be shed spontaneously within the local environment (Hoffmann *et al.,* 2007). Glycoprotein gp 120 may also be detected in the serum as well as within the lymphatic tissue of HIV-infected patients. During the process of budding, the virus may also incorporate different host proteins from the membrane of the host cell into its lipoprotein layer, such as HLA class I and II proteins, or adhesion proteins such as intracellular adhesion molecule – 1 (ICAM-1) that may facilitate adhesion to other target cells. The matrix protein p17 is anchored to the inside of the viral lipoprotein membrane. The p24 core antigen contains two copies of HIV-1 RNA. The HIV-1 RNA is part of a protein-nucleic acid complex,

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which is composed of the nucleoprotein p15 and the RT p66. The viral particle contains all the enzymatic equipment that is necessary for replication: RT (p66), an IN (p32) and a PR (p11).

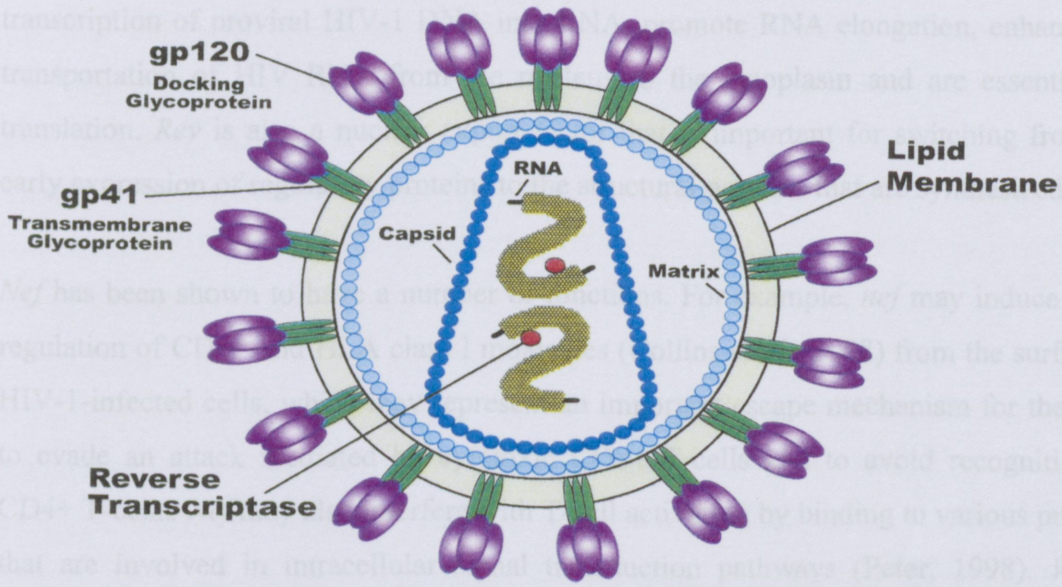

Figure 1.1: Structure of an HIV virion particle.
(www.niaid.nih.gov/factsheets/graphics/howhiv.jpg) (Accessed on 2-3-2011)

1.4. Genes and gene products of HIV

Most replication competent retroviruses depend on three genes: *gag* (group-antigen), *pol* (polymerase) and *env* (envelope) (Wong-Staal, 1991). The classified structural scheme of a retroviral genome is: 5′ LTR-*gag*-*pol*-*env*-LTR 3′. The LTR (Long Terminal Repeat) regions represent the two end parts of the viral genome, that are connected to the cellular DNA of the host cell after integration and do not encode for any viral proteins. The *gag* and *env* genes code for the nucleocapsid and the glycoproteins of the viral membrane respectively; the *pol* gene codes for the PR, RT and IN. In addition, HIV-1 contains six genes (*vif*, *vpu*, *vpr*, *tat*, *rev* and *nef*) in its 9kb RNA (Figure 1.2). *Nef*, *vif*, *vpr* and *vpu* were classified as accessory genes in the past, as they are not absolutely required for replication in vitro. The accessory genes, *nef*, *tat* and *rev*, are all produced early in the viral replication cycle. *Tat* and *rev* are regulatory proteins that accumulate within the nucleus and bind to defined regions of the viral RNA: TAR (transactivation-response

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elements), found in the LTR; and REE (*rev* response elements), found in the *env* gene, respectively. The *tat* protein is a potent transcriptional activator of the LTR promoter region and is essential for viral replication in almost all *in vitro* culture systems. Cyclin T1 is a necessary cellular cofactor for *tat* (Wei *et al.*, 1998). *Tat* and *rev* stimulate the transcription of proviral HIV-1 DNA into RNA, promote RNA elongation, enhance the transportation of HIV RNA from the nucleus to the cytoplasm and are essential for translation. *Rev* is also a nuclear export factor that is important for switching from the early expression of regulatory proteins to the structural proteins that are synthesized later.

Nef has been shown to have a number of functions. For example, *nef* may induce down regulation of CD4+ and HLA class I molecules (Collins *et al.*, 1998) from the surface of HIV-1-infected cells, which may represent an important escape mechanism for the virus to evade an attack mediated by cytotoxic CD8+ T-cells and to avoid recognition by CD4+ T-cells. *Nef* may also interfere with T-cell activation by binding to various proteins that are involved in intracellular signal transduction pathways (Peter, 1998). *Vpu* is important for the virus budding process because mutations in *vpu* are associated with persistence of the viral particles at the host cell surface (Peter, 1998). *Vpu* is also involved when CD4+-gp160 complexes are degraded within the endoplasmic reticulum and therefore allows recycling of gp160 for the formation of new virions (Cullen, 1998).

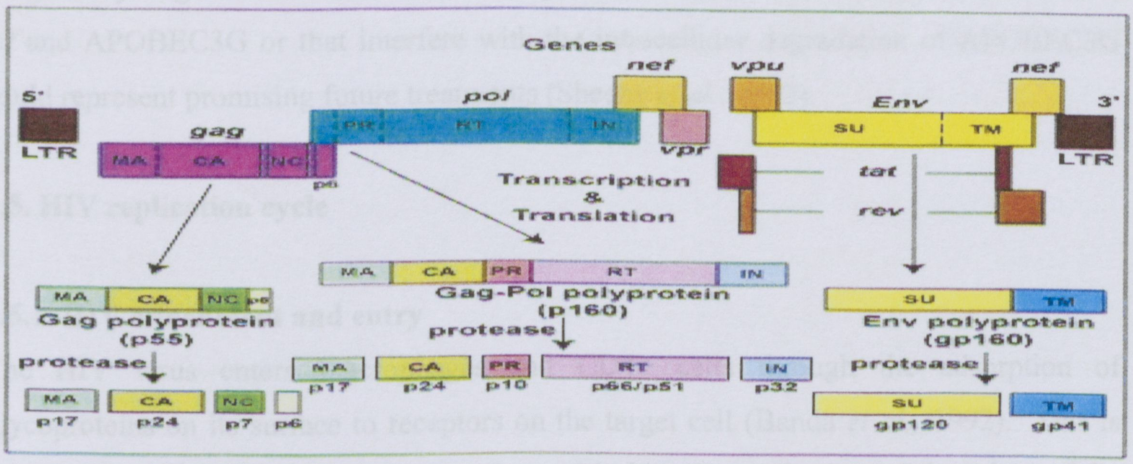

Figure 1.2: Schematic representation of genes and gene products of HIV (http://student.ccbcmd.edu/courses/bio141/lecguide/unit3/viruses/hivgenes.html) (Accessed on 3-3-2011).

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Some publications have highlighted an important role for *vif* in supporting viral replication (Mariani *et al.,* 2003). *Vif*-deficient HIV-1 isolates do not replicate in CD4+ T-cells, some T-cell lines (non-permissive cells) or in macrophages. *Vif*-deficient isolates are able to enter a target cell and initiate reverse transcription, but synthesis of proviral DNA remains incomplete. In vitro fusion of permissive and non-permissive cells leads to a non-permissive phenotype, suggesting that the replication of HIV depends on the presence or absence of a cellular inhibitor. This endogenous inhibitory factor was identified as APOBEC3G (apoliprotein B mRNA editing enzyme catalytic polypeptide-like 3G) (Sheehy *et al.,* 2002).

The antiviral activity of APOBEC3G is highly conserved among various species, whereas the blockage of APOBEC3G by *vif* is highly specific for HIV. HIV-1 *vif* does not complex to murine or rhesus APOBEC3G (Sheehy *et al.,* 2002). In the absence of *vif*, APOBEC3G is incorporated into newly formed viral particles and in subsequently infected target cells, synthesis of proviral DNA is blocked. On the contrary, in the presence of *vif*, APOBEC3G is complexed, degraded and not incorporated in newly formed virions. APOBEC3G is expressed in lymphocytes and macrophages representing the primary target cells of HIV infection. Specific inhibitors that block the interaction of *vif* and APOBEC3G or that interfere with the intracellular degradation of APOBEC3G could represent promising future treatments (Sheehy *et al.,* 2002).

1.5. HIV replication cycle

1.5.1. HIV attachment and entry

The HIV virus enters macrophages and CD4+ cells through the adsorption of glycoproteins on its surface to receptors on the target cell (Banda *et al.,* 1992). This is followed by the fusion of the viral envelope with the cell membrane and the relase of the HIV capsid into the cell. CD4+ is the primary receptor for HIV and entry into the susceptible cell begins with the interation of the trimeric envelope complex (gp160 spike) and both CD4+ and a chemokine co-receptor (CCR5 or CXCR4) on the cell surface

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(Figure 1.3). The attachment of gp120 to CD4+ is not only a crucial step for viral entry, but also interferes with intracellular signal transduction pathways and promotes apoptosis in CD4+ T-cells (Banda *et al.,* 1992). CD4+-independent viruses are easy to neutralize using the serum of HIV-infected patients, suggesting that the immune response selects against CD4+-independent viruses (Edwards, 2001).

Some chemokine receptors are co receptors for HIV entry. Experiments, using non-human cell lines transfected with human CD4+, showed that expression of human CD4+ on the cell surface of a non-human cell line was not enough to allow entry of HIV. Therefore, the existence of additional human co receptors necessary for viral entry was postulated. On the other hand, some laboratory HIV-1 isolates, as well as some HIV-2 and SIV isolates are able to infect human cells independently from CD4+; a milestone for the characterization of early events leading to HIV-1 entry was an observation by Cocchi and co-workers in 1995. CD8 T cells from HIV infected patients are able to suppress viral replication in co-cultures with HIV-infected autologous or allogenic CD4+ T-cells, and this is independent from their cytotoxic activity (Cocchi *et al.,* 1995). Cocchi (1995) identified the chemokines: Macrophage inflammatory protein-1Alpha (MIP-1α), Macrophage inflammatory protein-1Beta (MIP-1β) and regulated upon activation, normal T-cell expressed and secreted (Rantes) in supernatants from CD8+ T-cells derived from HIV-infected patients, and was able to show that these chemokines were able to suppress replication in a dose-dependent manner of some, but not all viral isolates tested. MIP-1α, MIP-1β and Rantes are ligands for the chemokine receptor CCR5. Later on, several groups were able to show that CCR5 is a necessary co-receptor for macrophage-tropic (M-tropic) HIV-1 isolates (Deng, 1996; Doranz *et al.,* 1996; Dragic *et al.,* 1998).

The chemokine receptor CXCR4 (fusin) had earlier on been described as being the co-receptor used by T-cell-tropic (T-tropic) HIV isolates (Feng *et al.,* 1996). M-tropic HIV-1 isolates are classically those viruses that are more easily propagated in macrophage cultures, are unable to infect T-cell lines (i.e., immortalized T-cells), but are able to easily infect primary T-cells from peripheral blood samples. Conversely, T-cell-tropic HIV-1 isolates have classically been identified as being those that are easily propagated in T-cell

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lines, and grow poorly in macrophages, but are also able to easily infect primary T-cells from peripheral blood samples. It is worth noting that both M-tropic and T-tropic HIV-1 variants can easily infect primary human non-immortalized T-cells in vitro (Feng *et al.,* 1996). Chemokines (chemotactic cytokines) and their receptors have been characterized with respect to their role in promoting the migration (chemotaxis) of leucocytes and their pro-inflammatory activity. T-tropic HIV-1 isolates mainly infect activated peripheral blood CD4+ T cells and cell lines and use CXCR4 for entry into the CD4+ target cell. M-tropic isolates are able to infect CD4+ T cells, monocytes and macrophages, and depend on the use of CCR5 and CD4+ for viral entry. Gp120 primarily binds to certain epitopes of CD4+. Binding to CD4+ induces conformational changes in gp 120 that promote a more efficient interaction of the V3 loop of gp120 with its respective co receptor (Feng *et al.,* 1996). Membrane fusion is dependent on gp120 co receptor binding. Gp41, as the transmembrane part of the envelope glycoprotein gp160, is crucial for the fusion of the viral and host cell membrane (Feng *et al.,* 1996).

Employing transfected cell lines, besides those expressed by CCR5 and CXCR4, other chemokine receptors, such as CCR3, CCR2, CCR8, CCR9, STRL33, Gpr 15, Gpr1, APJ and ChemR23, were identified and shown to be used for entry by certain HIV isolates (Deng, 1997; Liao *et al.,* 1997). APJ may represent a relevant co receptor within the central nervous system (CNS). Despite this broad spectrum of potentially available co receptors, CCR5 and CXCR4 seem to represent the most relevant co receptors of HIV-1 in vivo. The expression of co receptors on CD4+ T-lymphocytes depends on their activation level. CXCR4 is mainly expressed on naive T-cells, whereas CCR5 is present on activated and effector/memory T-cells. During the early course of HIV-1 infection, predominantly M-tropic HIV-1 isolates are detected. M-tropic HIV-1 isolates are preferentially transmitted regardless of whether or not the donor predominantly harbors T-tropic isolates. The blockade of CCR5, therefore, seems to represent a promising target of therapeutic intervention. In vitro, monoclonal antibodies to CCR5 (D7 and others) are able to block the entry of CCR5-using HIV isolates into CD4+ T-cells and macrophages (Veazey *et al.,* 2005).

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HIV infects many immune cells such as CD4+T cells, macrophages and microglial cells. HIV-1 entry into macrophages and CD4+T cells is mediated through interaction of the virion envelope gp120 with the CD4+ molecule on the target cells and also with chemokine co-receptors. M-tropic strains of HIV-1 or non-syncythia-inducing strains NSI use the β-chemokine receptor CCR5 for entry and are thus able to replicate in macrophages and CD4+T cells. This CCR5 co-receptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype (Rambaut *et al.,* 2004). Macrophages play an important role in many critical aspects of HIV infection. They are most likely to be the first cells infected by HIV and maybe the source of HIV production when CD4+T cells become depleted in the patient. In the Central Nervous System (CNS), macrophages and microglial cells are the cells infected by HIV. In the tonsils and adenoids of HIV-infected patients, macrophages fuse into multinucleated giant cells that produce large amounts of virus (Rambaut *et al.,* 2004).

T-tropic isolates or syncytium-inducing (SI) strains replicate in primary CD4+ T cells as well as in macrophages and use the α-chemokine receptor, CXCR4, for entry. Dual tropic HIV-1 strains are thought to be transitional strains of the HIV-1 virus and thus are able to use both CCR5 and CXCR4 as co-receptors for viral entry. The α-chemokine, stromal-derived factor 1 (SDF-1), a ligand for CXCR4, suppresses replication of T-tropic HIV-1 isolates (Rambaut *et al.,* 2004). It does this by down regulating the expression of CXCR4 on the surface of these cells. HIV strains that use only the CCR5 receptor are known as R5, while those that use only CXCR4 are termed X4, and those that use both, X4R5. However, the use of co-receptor alone does not explain viral tropism, as not all R5 viruses are able to use CCR5 on macrophages for a productive infection and HIV can also infect a subtype of myeloid dendritic cells, which probably constitute a reservoir that maintains infection when CD4+T cell numbers have declined to extremely low levels (Rambaut *et al.,* 2004).

Certain individuals are resistant to some strains of HIV. For example, people with the CCR5-Δ32 mutation (Rambaut *et al.,* 2004); these people are resistant to infection with R5 virus as the mutation prevents mature protein from being expressed on the surface of

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the cell. Sexual intercourse is the major mode of HIV transmission. Both X4 and R5 HIV are present in the seminal fluid which is passed from partner to partner. The virions can then infect numerous cellular targets and spread into the whole organism. However, a selection process leads to a predominant transmission of the R5 virus through this pathway (Rambaut *et al.,* 2004).

Spermatozoa may selectively carry R5 HIV as they possess both CCR3 and CCR5 but not CXCR4 on their surface and genital epithelial cells preferentially sequester X4 virus (Rambaut *et al.,* 2004). In patients infected with subtype B HIV-1, there is often a co-receptor switch in late-stage disease and T-tropic variants appear that can infect a variety of T-cells through CXCR4. These variants then replicate more aggressively with more virulence that causes rapid T-cell depletion, immune system collapse, and opportunistic infections that mark the onset of AIDS (Rambaut *et al.,* 2004). In the course of infection, viral adaptation to the use of CXCR4 instead of CCR5 may be a key step in the progression to AIDS. Studies with subtype B individuals have determined that between 40 and 50% of AIDS patients can harbor viruses of the SI and presumable the X4, phenotype (Rambaut *et al.,* 2004). However, many HIV infected patients progress to AIDS without the appearance of X4 viruses. Furthermore, HIV-1 subtype C, now the most common subtype in sub-Saharan Africa, is mainly made up of R5 virus strains, but still causes AIDS with the same frequency as other subtypes (Rambaut *et al.,* 2004).

1.5.2. Reverse transcription, integration, transcription and translation

Shortly after the viral capsid enters the cell, the RT enzyme releases the single-stranded (+) RNA genome from the attached viral proteins and copies it into a complementary DNA (cDNA) molecule (Zheng *et al.,* 2005). The RT also has ribonuclease H activity that degrades the viral RNA during the synthesis of cDNA, as well as performs DNA-dependent DNA polymerase activity that copies the sense cDNA strand into an antisense DNA. Together, the cDNA and its complement form a double-stranded viral DNA that is then transported into the cell nucleus. The integration of the viral DNA into the host cell's genome is carried out under the influence of the IN enzyme (Zheng *et al.,* 2005). IN catalyzes three important reactions – 3' processing, strand transfer and integration (Malet

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et al., 2008). During 3'-end processing, the terminal guanine-thymine dinucleotide (GpT) is cleaved from the 3' end of each long terminal repeat, producing cytosine-adenosine dinucleotide (CpA) 3'-hydroxyl ends. The reaction occurs in the cytoplasm, within a nucleoprotein complex known as the preintegration complex (PIC) (Malet *et al.,* 2008). The PIC is then transported through the nuclear pore to the nucleus, where strand transfer occurs. During strand transfer and integration, IN transfers both newly exposed 3'-extremities of the viral DNA into the target DNA via a single step transesterification. Thereafter, the viral genome is inserted and covalently linked into the host genome to form a provirus (Malet *et al.,* 2008).

During viral replication, the integrated DNA provirus is transcribed into messenger RNA (mRNA), which is then spliced into smaller pieces. These small pieces are transported from the nucleus into the cytoplasm, where they are translated into the regulatory proteins *Tat* (which encourages new virus production) and *Rev* (Pollard and Malim, 1998). The newly produced Rev protein binds to viral mRNAs and allows unspliced RNAs to leave the nucleus. Full-length mRNA is then used to produce gag and env structural proteins (Pollard and Malim, 1998).

1.5.3. Assembly and release

The final step of the viral cycle, assembly of new HIV-1 virions, begins at the plasma membrane of the host cell. The *Env* polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by cellular PR and processed into the two HIV envelope glycoproteins gp41 and gp120 (Bushman *et al.,* 1990). These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV PR cleaves viral polyproteins into individual structural HIV proteins and enzymes. The various structural components then assemble to produce a mature HIV virion (Bushman *et al.,* 1990).

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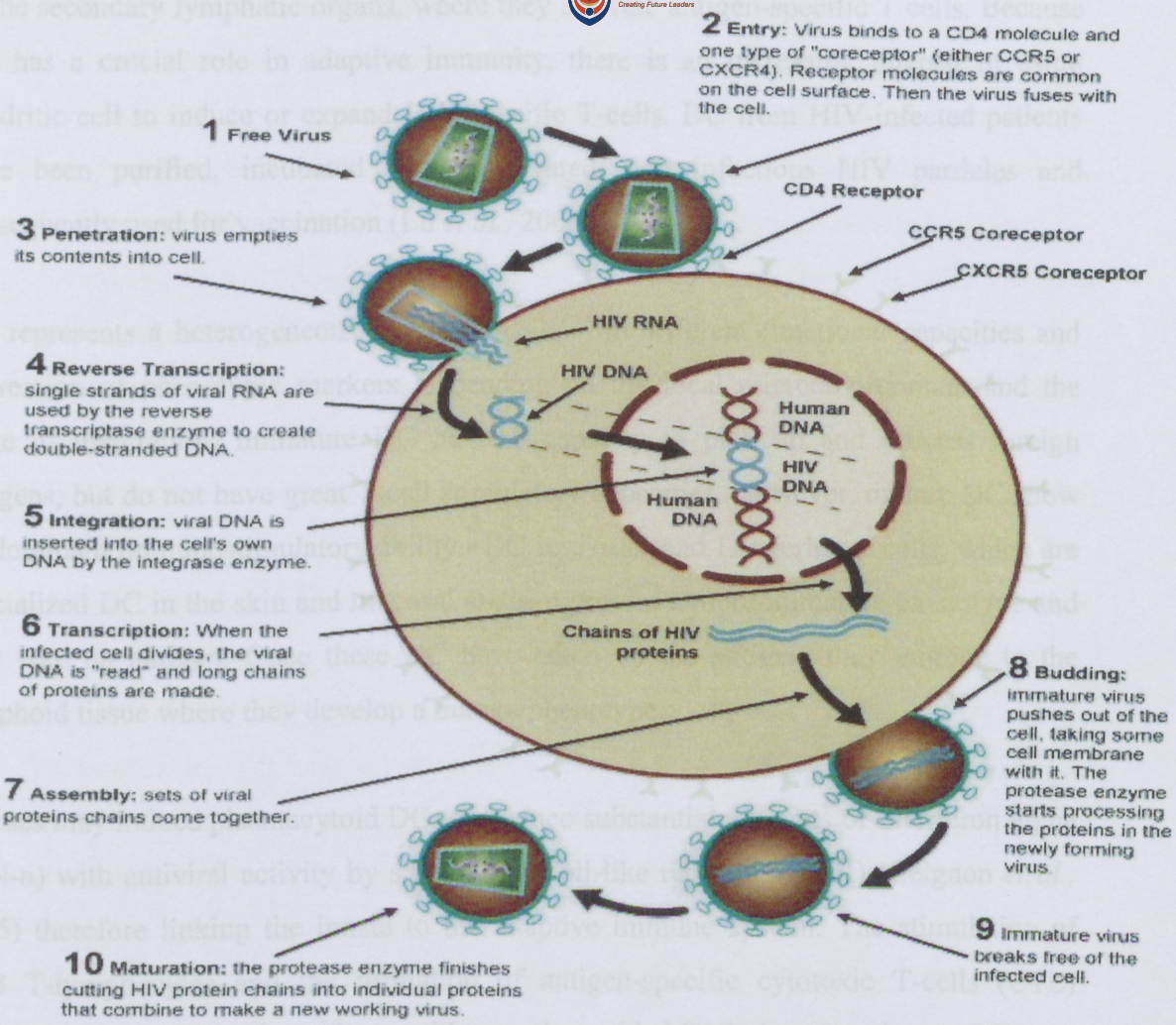

Figure 1.3: HIV replication cycle. (http://www3.niaid.nih.gov/topics/HIVAIDS/Understanding/Biology/hivReplicationCycle.htm) (Accessed on 5-3-2011).

1.6. HIV and the immune system

Dendritic cells (DCs), macrophages and B cells represent the main antigen presenting cells (APC) of the immune system. DCs are the most potent inducers of specific immune responses and are considered essential for the initiation of primary antigen-specific immune reactions. DC precursors migrate from the bone marrow towards the primary lymphatic organs and into the submucosal tissue of the gut, the genitourinary system and the respiratory tracts. They are able to pick up and process soluble antigens and migrate

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to the secondary lymphatic organs, where they activate antigen-specific T cells. Because DC has a crucial role in adaptive immunity, there is an increasing interest in using dendritic cell to induce or expand HIV-specific T-cells. DC from HIV-infected patients have been purified, incubated with inactivated, non infectious HIV particles and subsequently used for vaccination (Lu *et al.,* 2004).

DC represents a heterogeneous family of cells with different functional capacities and expression of phenotypic markers, depending on the local microenvironment and the stage of maturation. Immature DC has the capacity to pick up and process foreign antigens, but do not have great T-cell stimulatory capacities. However, mature DC show predominant immunostimulatory ability. DC in tissues and Langerhans' cells, which are specialized DC in the skin and mucosal areas, represent a more immature phenotype and may take up antigen. Once these DC have taken up the antigen, they migrate to the lymphoid tissue where they develop a mature phenotype.

Viruses may induce plasmacytoid DC to produce substantial amounts of Interferon alpha (IFN-α) with antiviral activity by stimulating Toll-like receptors (TLR) (Beignon *et al.,* 2005) therefore linking the innate to the adaptive immune system. The stimulation of CD8 T-lymphocytes and the formation of antigen-specific cytotoxic T-cells (CTL) depend on the presentation of a peptide together with MHC class I antigens. DC may become infected with viruses, for example influenza. Viral proteins are then produced within the cytoplasm of the cell, similar to cellular proteins, then degraded to viral peptides and translocated from the cytosol into the endoplasmic reticulum, where they are bound to MHC class I antigens. These peptide- Major histocompatibility complex (MHC) class I complexes migrate to the DC surface. An alternative way, where DC acquire exogenous antigens for MHC class I presentation to infected cells is termed cross presentation and may be necessary for both classical and pharmacytoid DC in HIV immunity.

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1.6.1. Interaction of dendritic cells and B/T cells

B and T-lymphocytes may be regarded as the principle effector cells of antigen specific immune responses. However, their function is under the control of dendritic cells. DC is able to pick up antigen in the periphery. These antigens are processed and expressed on the cell surface, together with co-stimulatory molecules that initiate T-cell activation. B-cells may recognize antigen after binding to the B-cell receptor. Recognition of antigen by T-cells requires previous processing and presentation of antigenic peptides by DC. T-cells express different T-cell receptors (TCR) that may bind to the peptide: MHC class I on the cell surface of dendritic cells to allow activation of CD8 T-cells, or to the peptide: MHC class II molecules, to activate CD4+ T-cells. The ability of DC to activate T-cells also depends on the secretion of stimulatory cytokines such as IL-12, which is a key cytokine for the generation and activation of T-helper one (TH1) and natural killer (NK) cells. Only a few DC and small amounts of antigen are sufficient to induce a potent antigen-specific T-cell response, thus demonstrating the immunostimulatory potency of DC. The expression of adhesion molecules and lectins, such as DC-SIGN, support the aggregation of DC and T-cells and promote the engagement of the T-cell receptor (TCR). Dendritic cell-specific intracellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) is a type C lectin that has also been shown to bind to lentiviruses, such as SIV and HIV-1 and -2 by interaction of gp120 with carbohydrates (Geijtenbeek *et al.,* 2000).

Lymphatic tissue is the primary site of viral replication, and is extensive in the primary stages of the disease (Embretson *et al.,* 2003; Pantaleo, 1993). During the initial stage of HIV-1 infection, there is a burst of virus into the plasma, followed by a relative decline in viraemia. During this time, a strong HIV-1 specific cytotoxic T-cell response is generated, which coincides with the early expression of plasma viremia in most patients. Virions are trapped by the follicular dendritic cell (FDC) network within the lymphoid tissue, Macrophages, and activated and quiescent DC4+ T-cells are the main targets of infection. Permanent viral reservoirs, mainly in macrophages and latently infected CD4+ T-cells, are established in the early phase of infection and probably represent the major obstacle so far to successful eradication of HIV. During the whole course of infection with HIV-1, the lymphoid tissue represents the principle site of HIV-1 replication. The

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frequency of cells containing proviral DNA is 10x higher in lymphoid tissue than in circulating peripheral mononuclear cells in the blood, and the difference in viral replication in lymphoid tissue exceeds that in the peripheral blood by about 10-100x. After entry of HIV-1 into a quiescent CD4+ T-cell and after completion of reverse transcription, the viral genome is represented by proviral unintegrated HIV DNA. In vitro experiments have shown that HIV-1 preferentially integrates into active genes (hot spots) (Schroder *et al.,* 2002). The activation of CD4+ T-cells is necessary for the integration of the HIV DNA into the host cell genome and is therefore a prerequisite for the synthesis of new virions. In this regard, the micromilieu of the lymphoid tissue represents the optimal environment for viral replication. The close cell-cell contact between CD4+ T-cells and antigen-presenting cells, the presence of infectious virions on the surface of the FDC, and an abundant production of proinflammatory cytokines such as IL-1, IL-6 or TNF-α promotes the induction of viral replication in infected cells and augments viral replication in cells already producing the virus. It is worth noting that both IL-1 and TNFα induce NFkB, which binds to the HIV-1 LTR to promote proviral transcription. Chronic activation of the immune system is a hallmark of progressive HIV infection and predicts disease outcome.

1.6.2. Immune response to HIV

CD8 T-cells recognize their antigen (peptide) in context with HLA class I molecules on antigen-presenting cells, whereas CD4+ T-cells require the presentation of antigenic peptides in context with HLA class II molecules. The generation of an HIV-specific immune response is therefore dependent on the individual HLA pattern. Antigen-presenting cells may bind HIV peptides in different ways within grooves on the HLA class I molecules. Therefore, CD8 T-cells can be activated in an optimal or suboptimal way or may not be activated at all. Using large cohorts of HIV-1 infected patients, in whom the natural course of disease (fast versus slow progression) is known, HLA patterns were identified that were associated with a slow versus fast disease progression. These studies suggest that the HLA type could be responsible for the benign course of disease in about 40% of patients with a long term non-progressive course of disease (Carrington *et al.,* 1999). Homozygosity for HLA Bw4 is regarded as being protective.

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Patients who display heterozygosity at the HLA Class I loci characteristically show a slower progression of immunodeficiency than patients with homozygosity at these loci (Carrington, 1999). An initial study demonstrated that HLA B14, B27, B51, B57 and C8 are associated with a slow disease progression; however, the presence of HLA A23, B37 and B49 were associated with the rapid development of immunodeficiency (Kaslow *et al.,* 1996). All patients with HLA B35 had developed symptoms of AIDS after eight years of infection. Previous studies suggest that discordant couples with a mismatch at the HLA class I have a protective effect towards heterosexual transmission (Lockett *et al.,* 2001).

In vitro studies in HLA B57-positive patients demonstrate that these patients display HLA B57-restricted CTL directed against HIV-1 peptides. However, it is possible that the identification of protective HLA alleles or HLA-restricted peptides in HIV-1 infected patients with a benign course of disease does not necessarily indicate that the same alleles or peptides are crucial for the design of a protective vaccine. Kaul and co-workers were able to show that CD8+ T-cells from HIV-1-exposed but uninfected African women recognize different epitopes than CD8+ T cells from HIV-1 infected African women (Kaul, 2001). This suggests that the epitopes, that the immune system is directed against during a natural infection, might be different from those that are protective against infection. In addition, the individual HLA pattern may affect the adaptive immune response and the evolution of viral escape mutations (Friedrich *et al.,* 2004; Leslie *et al.,* 2004). CTL from patients with HLA B57 and B58 may force the virus to develop certain mutations in *gag* that enable the virus to escape CTL response. However, these mutations result in a reduced replicative competence. If such a virus is transmitted to another individual with a different HLA background, the virus may back mutate to the original genotype and regain its full replicative competence. Similar MHC alleles as in monozygotic twin lead, at least during the early phase of the infection, to similar HIV-specific CTL-specificities (Draenert *et al.*, 2006). HLA class II antigens are crucial for the development of an HIV-1-specific CD4+ T-cell response. Rosenberg (1997) was the first to show that HIV-1-infected patients with a long-term non-progressive course of disease had HIV-1-specific CD4+ T-cells that could proliferate against HIV-1 antigens

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(Rosenberg *et al.,* 1997). The identification of protective or unfavorable HLA class II alleles is less well elaborated than the knowledge about protective HLA class I alleles. Cohorts of vertically infected children and HIV-infected adults demonstrate a protective effect of HLA DR13 (Keet *et al.*, 1999). Killer cell immunoglobulin like (KIR) receptors represent ligands that bind to HLA class I antigens and by functioning as either activating or inhibiting receptors they regulate the activation status of NK cells. Polymorphisms of KIR genes were shown to correlate with slow or rapid progression of HIV disease, especially when the analysis includes known HLA class I polymorphisms (Fauci *et al.,* 2005; Martin *et al.,* 2007) thereby indicating a relevant function of N cells in HIV immunity. During HIV infection, NK cells may not only be decreased, but may also show a diminished cytolytic activity. Various genetic polymorphisms have been identified that have an impact on the course of HIV disease (Fauci *et al.,* 2005; Martin *et al.,* 2007).

1.6.3. HIV-specific cellular immune response

Cytotoxic T-cells (CTL) are able to recognize and eliminate virus-infected cells. Several studies have demonstrated that CTL are crucial for the control of HIV replication and have a substantial impact on disease progression once infection is established (Goulder *et al.,* 1997). However, there is little evidence to assume that CTL play a major role in primary protection. In comparison to HIV-1-infected patients with a rapid decline in CD4+ T-cell numbers, patients with a long term non-progressive course of disease (LTNP=Long term non-progressors) have high quantities of HIV-1 specific CTL precursors with a broad specificity towards various HIV-1 proteins. The different capacities of certain HLA alleles to present viral peptides more or less efficiently and to induce a generally potent immune response may explain why certain HLA alleles are associated with a more rapid or a slowly progressive course of disease. Individuals have been described, who developed CTL escape mutants after years of stable disease and the presence of a strong CTL response. The evolution of CTL escape mutants was associated with a rapid decline in CD4+ T-cells in the patients, indicating the protective role of CTL. HIV-specific CTL responses have been detected in individuals exposed to, but not infected by HIV-1(Goulder *et al.,* 1997).

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1.6.4. TH1/TH2 immune response

Depending on the secretion pattern of cytokines, CD4+ T-cells may be differentiated into T-helper 1(TH1) and T-helper 2 (TH2) cells. TH1 CD4+ T-cells primarily produce interleukin-2 (IL-2) and interferon gamma (INFγ), which represents the cytokines that support the effector functions of the immune system (CTL, NK-cells, macrophages). TH2 cells predominantly produce IL-4, Il-10, Il-5 and Il-6, which represent the cytokines that favor the development of a humoral immune response. Since TH1 cytokines are critical for the generation of CTLs, an HIV-1-specific TH1 response is regarded as being a protective immune response. Studies on HIV-exposed but non-infected individuals have shown, that following in vitro stimulation of HIV-1 *env* antigens (gp120/gp160) and peptides, T-cells from these individuals secrete IL-2 in contrast to non-exposed persons (*Clerici et al.,* 1991).

1.6.5. HIV-1 specific humoral immune response

The association between an HIV-1-specific humoral immune response and the course of disease is less well characterized. In SIV, injection of an antibody cocktail consisting of various neutralizing antibodies is able to prevent SIV infection after a mucosal virus challenge (Ferrantelli *et al.,* 2004), indicating that primary protection is mainly dependent on a broad humoral immune response. This suggests that HIV-specific antibodies are necessary for a preventive vaccine strategy. On the other hand, B-cell depletion by a monoclonal antibody directed against B-cells in monkeys with already established SIV infection, does not affect the course of plasma viraemia (Schmitz *et al.,* 2003). A slow progression of immunodeficiency was observed in patients with high titers of anti-p24 antibodies (Hogervorst *et al.,* 1995), persistence of neutralizing antibodies against primary and autologous viruses (Montefiori *et al.,* 1996), and lack of antibodies against certain gp120 epitopes (Wong, 1993).

Long-term non-progressors with HIV tend to have a broad neutralizing antibody activity towards a range of primary isolates and show persistence of neutralizing antibodies against autologous virus (Saha *et al.*, 2001). It is still unclear whether the presence of neutralizing antibodies in LTNP represents part of the protection or whether it merely

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reflects the integrity of a relatively intact immune system (Saha *et al.*, 2001). Individuals that have a substantial risk of HIV-infection, but are considered exposed, non-infected, represent individuals with lack of a detectable antibody response to HIV-1. It has been shown that these individuals may demonstrate a local (mucosal) IgA response against HIV-1 proteins that are not detected by the usual antibody testing methods (Saha *et al.*, 2001). Thus, local IgA, rather than systemic IgG, may be associated with protection against HIV-1 infection.

1.7. Pathogenesis of HIV infection

Infection with HIV-1 is associated with a progressive depletion of the CD4+ T-cell count and an increase in viral load. The stage of infection can be determined by measuring the patient's CD4+ T-cell count, and the level of HIV in the blood. HIV infection has basically three stages: acute infection, latency stage and AIDS (Figure 1.4).

1.7.1. Acute infection

The initial infection with HIV generally occurs after transfer of body fluids from an infected person to an uninfected individual. The first stage of infection, the primary, or acute infection, is a period of rapid viral replication that immediately follows the individual's exposure to HIV leading to an abundance of virus in the peripheral blood with levels of HIV commonly approaching several million viruses per mL (Piatak *et al*, 1993).

This response is accompanied by a marked drop in the numbers of circulating CD4+ T cells. This acute viremia is associated in virtually all patients with the activation of $CD8^+$ T cells, which kill HIV-infected cells, and subsequently with antibody production, or seroconversion. The $CD8^+$ T cell response is thought to be important in controlling virus levels, which peak and then decline, as the CD4+ T cell counts rebound to around 800 cells/mm³ (the normal blood value is 1200 cells/mm³). A good $CD8^+$ T cell response has been linked to slower disease progression and a better prognosis, though it does not eliminate the virus (Pantaleo *et al.*, 1997).

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During this period (usually 2–4 weeks post-exposure) most individuals (80 to 90%) develop an influenza or mononucleosis-like illness called acute HIV infection, the most common symptoms of which may include fever, lymphadenopathy, pharyngitis, rash, myalgia, malaise, mouth and esophagal sores, and may also include, but less commonly, headache, nausea and vomiting, enlarged liver/spleen, weight loss, thrush, and neurological symptoms. Infected individuals may experience all, some, or none of these symptoms. The duration of symptoms varies, averaging 28 days and usually lasting at least a week (Kahn and Walker, 1998).

Because of the nonspecific nature of these symptoms, they are often not recognized as signs of HIV infection and diagnosis is often difficult at this stage. As a consequence, these primary symptoms are not used to diagnose HIV infection, as they do not develop in all cases and because many are caused by other more common diseases. However, recognizing the syndrome can be important because the patient is much more infectious during this period (Daar *et al.*, 2001).

1.7.2. Chronic asymptomatic HIV infection (clinical latency)

A strong immune defense reduces the number of viral particles in the blood stream, marking the start of the infection's clinical latency stage. Clinical latency can vary between two weeks and 20 years. During this early phase of infection, HIV is active within lymphoid organs, where large amounts of virus become trapped in the follicular dendritic cells (FDC) network (Burton *et al.*, 2002)

The surrounding tissues that are rich in CD4+ T cells may also become infected, and viral particles accumulate both in infected cells and as free virus. Individuals who are in this phase are still infectious (Clapham and McKnight, 2001).

1.7.3. Late stage of HIV (AIDS)

AIDS, the final stage of HIV infection shows as symptoms of various opportunistic infections. When CD4+ T cell numbers decline below a critical level of 200 cells/mm^3, cell-mediated immunity is lost, and infections with a variety of opportunistic microbes can appear. The first symptoms often include moderate and unexplained weight loss,

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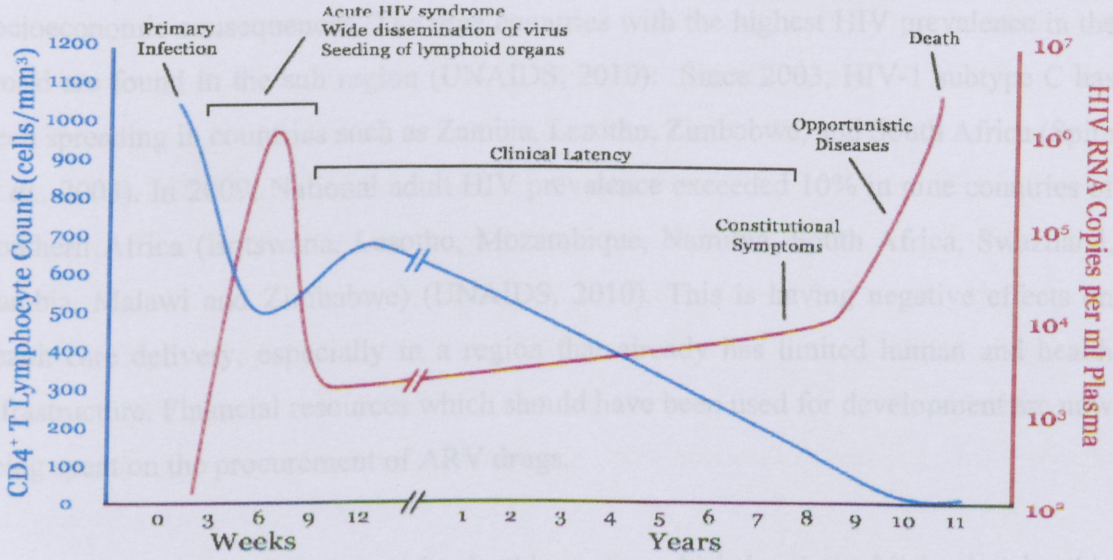
recurring respiratory tract infections (such as sinusitis, bronchitis, otitis media, and pharyngitis), prostatitis, skin rashes, and oral ulcerations (Kahn and Walker, 1998).

Common opportunistic infections and tumors, most of which are normally controlled by robust CD4+ T cell-mediated immunity then start to affect the patient. Typically, resistance is lost early on to oral Candida species and to Mycobacterium tuberculosis, which leads to an increased susceptibility to oral candidiasis (thrush) and tuberculosis. Later, reactivation of latent herpes viruses may cause worsening recurrences of herpes simplex eruptions, shingles, Epstein-Barr virus-induced B-cell lymphomas, or Kaposi's sarcoma.

Pneumonia caused by the fungus *Pneumocystis jirovecii* is common and often fatal. In the final stages of AIDS, infection with cytomegalovirus (another herpes virus) or Mycobacterium avium complex is more prominent. Not all patients with AIDS get all these infections or tumors, and there are other tumors and infections that are less prominent but still significant (Kahn and Walker, 1998).



____ CD4+ T-cell count (cells/mm³); ____HIV RNA copies per mL of plasma.

Figure 1.4: A generalized graph of the relationship between HIV copies (viral load) and CD4+ counts over the average course of untreated HIV infection; any particular individual's disease course may vary considerably. (Fauci *et al.* Ann Intern Med, 1996.)

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1.8. The epidemiology of HIV/AIDS

In 2009, UNAIDS estimated that approximately a total of 33.3 million people were living with HIV in the world. Of this total, between 32.6 million were adults; 17.2 million were women; while 3.4 million were children under the age of 14 years (UNAIDS, 2010). In the same year, UNAIDS estimated that approximately 2.6 million people were newly infected with HIV. Adults represented 2.2 million; while children under the age of 14 years represented 370,000. Furthermore, about 1.8 million AIDS-related deaths were recorded in 2009 (UNAIDS, 2010). Sub-Saharan Africa still remains the most affected region in the global AIDS epidemic. UNAIDS estimated that about 22.5 million adults and children were living with HIV in this part of the world by the end of 2009. In this region, about 1.8 million adults and children were newly infected with HIV in 2009; while about 1.3 million deaths were recorded in adults and children (UNAIDS, 2010). The majority (60%) of people living with HIV in sub-Saharan Africa are women (UNAIDS, 2010).

Southern Africa has the highest HIV prevalences, with very serious health and socioeconomic consequences. The nine countries with the highest HIV prevalence in the world are found in the sub region (UNAIDS, 2010). Since 2003, HIV-1 subtype C has been spreading in countries such as Zambia, Lesotho, Zimbabwe, and South Africa (Spira *et al.,* 2003). In 2009, National adult HIV prevalence exceeded 10% in nine countries of Southern Africa (Botswana, Lesotho, Mozambique, Namibia, South Africa, Swaziland, Zambia, Malawi and Zimbabwe) (UNAIDS, 2010). This is having negative effects on health care delivery, especially in a region that already has limited human and health infrastructure. Financial resources which should have been used for development are now being spent on the procurement of ARV drugs.

South Africa is one of the countries in this region which has been hit hardest by this pandemic. According to the South African National HIV survey conducted in 2009, the estimated prevalence is 29.3% among antenatal women aged 15 to 49 years old (Department of Health, 2009). The survey also estimated that the provinces most affected are KwaZulu-Natal (39.5%), Mpumulanga (34.7%) and Free State (30.1%). Limpopo is

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the seventh, with a prevalence rate of 20.7%. Western Cape Province has the least prevalence (16.9%) (Department of Health, 2009).

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1.9 Genetic variability of HIV

It is now known that HIV-1 and HIV-2 have different evolutionary origins (Rambaut *et al.,* 2004). HIV-1 is most closely related to SIVcpz, which is found in some sub-species of chimpanzee (Pan troglodytes troglodytes and Pan troglodytes schweinfurthii) that inhabit parts of equatorial western and Central Africa, respectively. HIV-2 on the other hand is most closely related to SIVsm, which is found in a high prevalence in sooty mangabey monkeys (Cercocebus atys) (Rambau t *et al.,* 2004). The sooty mangabey monkeys are mostly found in regions of West Africa. Molecular phylogenies reveal that there have been cross-species transmissions to humans, because there is mixing of HIV and SIV lineages. HIV differs from many viruses in that it has very high genetic variability (Rambaut *et al.,* 2004). This diversity is a result of its fast replication cycle, with the generation of 10^9 to 10^{10} virions every day, coupled with a high mutation rate of approximately 3×10^{-5} per nucleotide base per cycle of replication and recombinogenic properties of *reverse transcriptase*. This complex scenario leads to the generation of many variants of HIV in a single infected patient in the course of one day. This variability is compounded when a single cell is simultaneously infected by two or more different strains of HIV. When simultaneous infection occurs, the genome of progeny virions may be composed of RNA strands from two different strains. This hybrid virion then infects a new cell where it undergoes replication. As this happens, the *reverse transcriptase*, by jumping back and forth between the two different 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 (Rambaut *et al.,* 2004).

The closely related SIV exhibits a somewhat different behavior. In its natural hosts, (African Green Monkeys (AGM) and Sooty Mangabays (SM) the retrovirus is present in high levels in the blood, but evokes only a mild immune response, does not cause the development of simian AIDS, and does not undergo the extensive mutation and

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recombination typical of HIV (Rambaut *et al.*, 2004). By contrast, infection of heterologous hosts (rhesus or cynomologus macaques) with SIV results in the generation of genetic diversity that is on the same order as HIV in infected humans; these heterologous hosts also develop simian AIDS (Rambaut *et al.*, 2004).

Four groups of HIV-1 have been identified on the basis of differences in *env*: M, N, O and the recently discovered strain which has been designated as HIV-1 group P (Plantier *et al.*, 2009). Group M is the most prevalent and is subdivided into nine subtypes (clades), based on the whole genome, which are geographically distinct. The most prevalent are subtypes B (found mainly in North America and Europe), A and D (found mainly in Africa), and C (found mainly in Africa and Asia); these subtypes form branches in the phylogenetic tree representing the lineage of the M group of HIV-1 (Plantier *et al.*, 2009). Coinfection with distinct subtypes gives rise to circulating recombinant forms. Most HIV-1 research is focused on subtype B; few laboratories focus on the other subtypes. The genetic sequence of HIV-2 is only partially homologous to HIV-1 and more closely resembles that of SIV than HIV-1 (Figure 1.5).

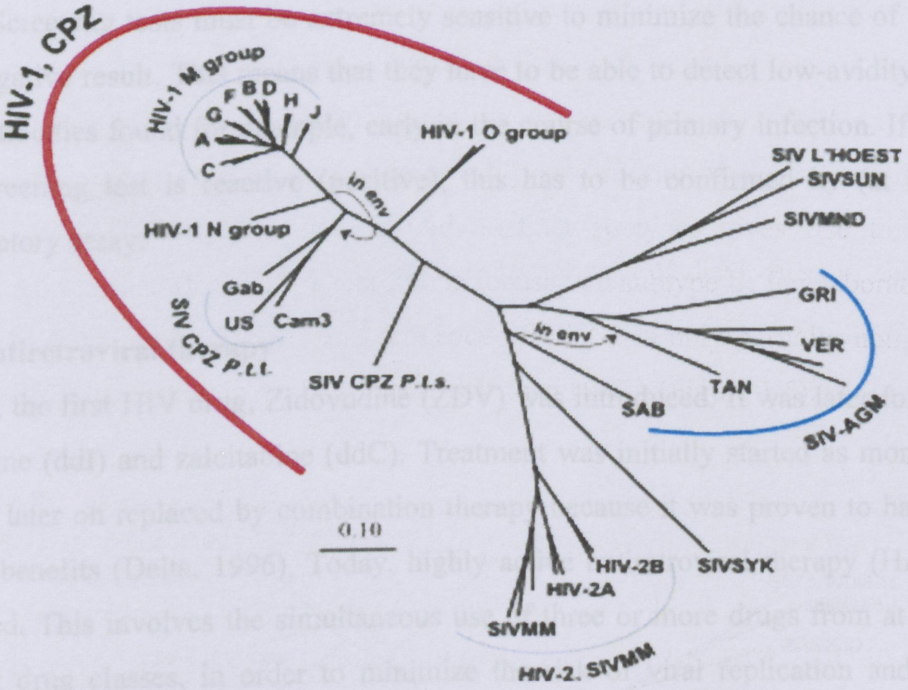

Figure 1.5: Phylogenetic tree of SIV and HIV.
(http://www.hiv.lanl.gov/content/sequence/HIV/COMPENDIUM/1999/1/intro.pdf)
(Accessed on 6-3-2011)

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1.10. The diagnosis of HIV

The diagnosis of HIV infection is normally made indirectly, through the demonstration of virus-specific antibodies (Gurtler, 1996). These markers of a humoral immune response against the agent are found in virtually 100% of HIV infected individuals. Their presence means the diagnosis of chronic active HIV infection. Cases in which infected individuals persistently fail to have detectable antibodies against HIV are exceedingly rare and play hardly any role in clinical practice (Connick, 2005). Direct diagnosis of HIV infection is also possible through the demonstration of infectious virus, viral antigen (p24 antigen ELISA) or viral nucleic acid (nucleic acid testing). In order to determine the infectious status of a patient, direct virus detection is necessary only under certain circumstances, such as a suspected primary or vertically transmitted infection.

HIV antibody diagnosis requires at least two different assays: a screening test and at least one confirmatory test. In order to exclude inadvertent mix-ups of samples, a second blood sample is generally tested. Most screening tests are based on the ELISA principle (enzyme linked immunosorbent assay) or other, closely related test formats (UNAIDS, 1997). Screening tests must be extremely sensitive to minimize the chance of yielding a false negative result. This means that they have to be able to detect low-avidity and low-level antibodies found for example, early in the course of primary infection. If the result of a screening test is reactive (positive), this has to be confirmed by (at least) one confirmatory assay.

1.11. Antiretroviral therapy

In 1987, the first HIV drug, Zidovudine (ZDV) was introduced. It was later followed by didanosine (ddI) and zalcitabine (ddC). Treatment was initially started as monotherapy, but was later on replaced by combination therapy because it was proven to have higher clinical benefits (Delta, 1996). Today, highly active antiretroviral therapy (HAART) is employed. This involves the simultaneous use of three or more drugs from at least two different drug classes, in order to minimize the risk of viral replication and thus the emergence of drug resistance, which is common with mono- or dual therapy (Richman *et al.,* 1994). Since the introduction of combination antiretroviral (ARV) therapy, the

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number of deaths due to AIDS has decreased and the quality of life of HIV-infected individuals has increased (Palella *et al.*, 1998) ARV therapy results in a drastic decrease in viral load and an increase in CD4+ T-cell counts (Mohri *et al.*, 2001) However, treatment is life long because HIV infects long-lived cells like microglia cells and establishes latency in resting memory CD4+ T-cells (Wong *et al.*, 1997). Today, 23 ARV drugs are licensed for clinical use. They make up five different classes; nucleoside/nucleotide analog *reverse transcriptase* inhibitors (NRTI), non-nucleoside analog *reverse transcriptase* inhibitors (NNRTI), *protease* inhibitors (PI), *integrase* inhibitors (INI) and fusion (entry) inhibitors (FI). These drugs target different sites of the HIV life cycle shown on Figure 1.6.

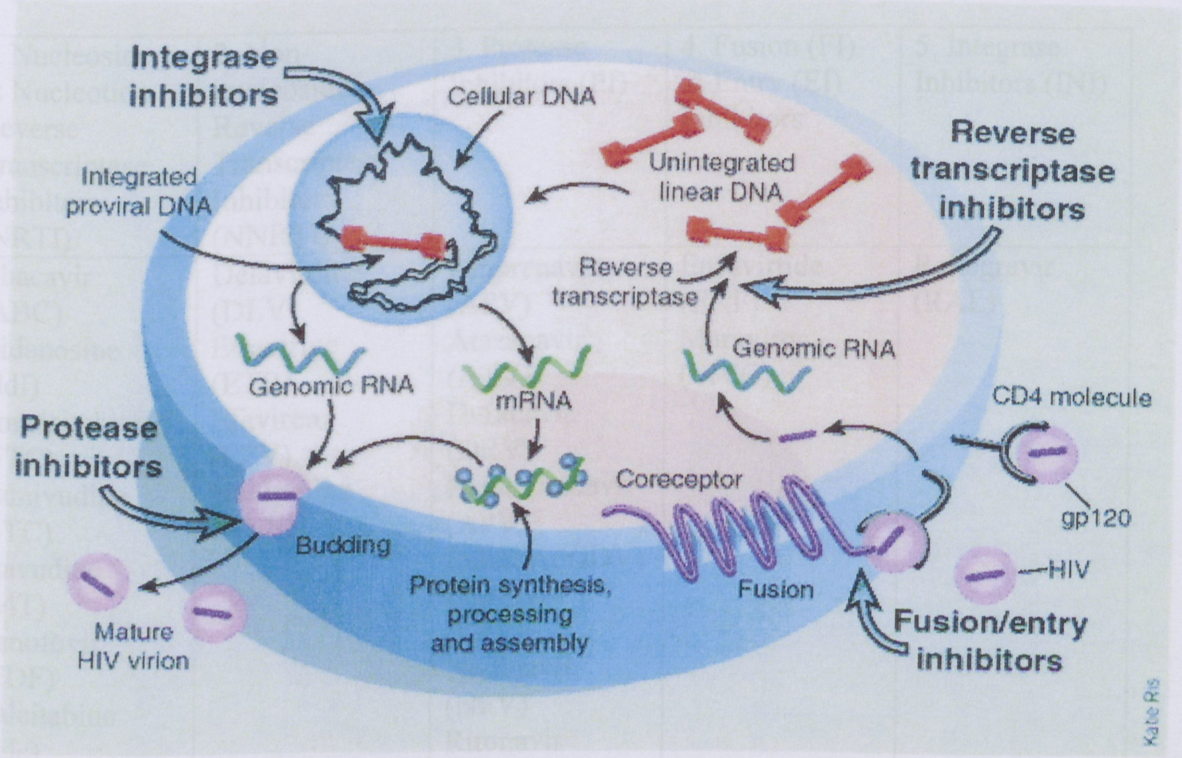

Figure 1.6: Targets for ARV chemotherapy in the life cycle of HIV. (www.biology.arizona.edu/IMMUNOLOGY/tutorials/AIDS/treatment.html) (Accessed on 8-3-2011)

1.11.1. Antiretroviral drugs

Successful ARV therapy reduces the viral load in plasma to undetectable levels. However, if the virus rebounds to detectable levels, this is an indication of treatment

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failure. This situation could be due to adherence problems, low potency of the drugs, disturbance in drug metabolism or absorption (drug pharmacokinetics), or the emergence of mutated, drug-resistant virus. One of the major challenges in the treatment of HIV is the development of resistance to ARV drugs. The emergence of resistant viral strains is one of the main reasons for failure of ARV therapy (Hoffmann *et al.,* 2007). Five main groups of ARVs are used today against HIV. These are NRTI, NNRTI, PI, INI and FI (Table 1.1). However, the most commonly used drugs are the NRTI, NNRTI and PI.

Table 1.1: FDA approved antiretroviral drugs

1. Nucleoside & Nucleotide Reverse Transcriptase Inhibitors *(NRTI)*	2. Non-nucleoside Reverse Transcriptase Inhibitors *(NNRTI)*	3. Protease Inhibitors (PI)	4. Fusion (FI) & Entry (EI) Inhibitors	5. Integrase Inhibitors (INI)
Abacavir (ABC) Didanosine (ddI) Emtricitabine (FTC) Lamivudine (3TC) Stavudine (d4T) Tenofovir (TDF) Zalcitabine (ddc) Zidovudine (ZDV)	*Delavirdine (DLV)* Etravirine (ETR) Efavirenz (EFV) Nevirapine (NVP)	*Amprenavir (APV)* Atazanavir (ATV) Duranavir (DRV) Fosamprenavir (APV) Indinavir (IDV) Lopinavir (LPV) Nelfinavir (NFV) Ritonavir (RTV) Saquinavir mesylate (SQV) Tipranavir (TPV)	*Enfuvirtide (ENF)* Maraviroc (MVC)	*Raltegravir (RAL)*

(http://www.niaid.nih.gov/daids/dtpdb/FDADRUG.asp) (Accessed 8-3-2011)

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1.11.2. Mechanisms of action of antiretroviral drugs

NRTIs are analogues of normal nucleosides. They are pro-drugs and only become effective after conversion to triphosphates by host cellular enzymes (Hoffmann *et al.*, 2007). When phosphorylated NRTI is incorporated into the proviral DNA, further elongation of the proviral DNA is blocked and leads to interruption of the chain (premature termination of HIV DNA synthesis). Basically, phosphorylated NRTIs compete with natural deoxynucleoside triphosphates (dNTPs) for incorporation into the newly synthesized DNA chains and thereby cause chain termination.

Non-nucleoside analog *reverse transcriptase* inhibitors are small molecules that bind to the hydrophobic region close to the catalytic domain of the RT. They block polymerization of viral DNA. NNRTIs are inactive against HIV-2 (Hoffmann *et al.*, 2007).

Protease inhibitors bind to the active site of the *protease* and block the cleavage of viral polyproteins by *protease* into structural and enzymatic components. This leads to the production of non-infectious viral particles (Hoffmann *et al.*, 2007).

Fusion (entry) inhibitos generally act on the transmembrane protein gp 41 and prevent the fusion of viral and cell membranes of susceptible cells (Enfuvirtide) or prevent co-receptor binding (Maraviroc). By interfering during the early phase of the HIV life cycle, fusion inhibitors prevent the replication of HIV. Maraviroc is an entry inhibitor (CCR5 receptor antagonist). CCR5 is a chemokine co-receptor which is found on the surface of some human cells (macrophages and CD4+ T cells). It is an important co-receptor for most HIV stains and essential in the entry process of the virus into the host cell. Maraviroc binds to CCR5 and blocks HIV protein gp120 from binding with the co-receptor (Dorr *et al.*, 2005). HIV therefore becomes unable to enter susceptible cells. Enfuvirtide is an HIV fusion inhibitor and it binds to gp41 and prevents the creation of an entry pore for the viral capsid (Jamjian and McNicholl, 2004).

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Integrase inhibitors block the action of *integrase* thereby preventing the integration of synthesized viral DNA into the host cell DNA to form the provirus (Hoffmann *et al.,* 2007). The IN gene catalyzes three important reactions – 3' processing, strand transfer and integration (Malet *et al.,* 2008). In 3'-end processing, the terminal GpT is cleaved from the 3' end of each LTR, producing CpA 3'-hydroxyl ends. During strand transfer and integration, IN transfers both newly exposed 3'-extremities of the viral DNA into the target DNA via a single step transesterification. Raltegravir is a strand transfer inhibitor and blocks this second reaction (Mouscadet *et al*., 2009).

1.11.3. HIV antiretroviral drug resistance mutations

Antiviral drug resistance is defined as the presence of viral mutations that reduce drug susceptibility compared with the susceptibility of wild-type viruses (Shafer, 2002). HIV-1 drug resistance can be acquired (developing in a person receiving ART) or transmitted (occurring because a virus with drug-resistance mutations was transmitted to a drug-naive person). Antiviral resistance can be mediated either by changes in the molecular target of therapy (the primary mechanism observed in HIV-1) or in other viral proteins that indirectly interfere with a drug's activity. The development of resistant viruses, even with the use HAART is one of the primary reasons for the failure of ARV therapy (Hoffmann *et al.,* 2007). The high turnover of HIV-approximately 10 million new viral particles per day; and the high error rate of HIV RT, is responsible for the development of resistant variants. This leads to a high mutation rate and a steady production of new viral strains, even without treatment. There is selection of resistant strains as the dominant species in the presence of ARV drugs. 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 ARVs poses a serious challenge to the containment of the course of HIV-1 infection and management of patients. The demonstration of drug resistant variants in drug-naive individuals and the transmission of resistant and multi-drug resistant strains only go on to make the situation worse.

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In general, resistance mutations are described using a number and two letters. The number shows the position of the codon (a group of three nucleotides which defines a specific amino acid in the protein sequence). The letter preceding the number corresponds to the amino acid specified by the codon at this position in the wild-type virus; meanwhile the letter after the number describes the amino acid that is produced from the mutated codon. For example M184V will indicate a mutation in codon 184 of the *reverse transcriptase* gene leading to a valine for methionine substitution in the *reverse transcriptase* enzyme.

The phenomenon of cross-resistance is common with some drug classes (Clavel and Hance, 2004). Cross-resistance refers to resistance to drugs to which a virus has never been exposed. This is caused by mutations that have been selected for by the use of another drug. At the beginning of the evolution of resistance to nucleoside analogues or *protease* inhibitors, viruses may have only a low level of cross-resistance to alternative agents within each of these two classes of drugs. However, these strains may need to add only one or a few more mutations to this preexisting scaffolding for high-level cross-resistance to develop. So in patients infected with strains that have low levels of cross-resistance, the switch to apparently active alternative drugs can be accompanied by rapid selection for highly resistant variants, at the expense of minimal evolutionary changes.

1.11.3.1. NRTI Resistance mutations

The US Food and Drug Administration (FDA) has approved seven nucleoside (zidovudine, didanosine, zalcitabine, stavudine, lamivudine, abacavir and emtricitabine) and one nucleotide analog (tenofovir disoproxil fumarate) (Shafer, 2002).

RT is a heterodimer consisting of p66 and p55 subunits (Shafer, 2002). The p66 subunit contains the DNA-binding groove and active site; while the p55 subunit displays no enzymatic activity. The general shape of the polymerase domain of the p66 subunit can be compared to a human hand with the subdomains referred to as fingers, palm and thumb (Shafer, 2002). A large number of the NRTI resistance mutations appear in the so called "fingers" and "palm" subdomains of the RT enzyme. The mutations confer

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resistance by two biochemical mechanisms; sterical inhibition and phosphoroylysis (Gotte, 2004).

- In sterical inhibition, mutations enable the RT to recognize structural differences (discriminate) between NRTIs and deoxynucleotide triphosphates (dNTPs) during polymerization. The incorporation of NRTIs is then prevented in favor of dNTPs. The K65R, L74V, Q151M, and M184V mutations use this mechanism.
- In Phosphoroylysis, mutations promote the hydrolytic removal of the chain-terminating NRTI and thus enable continued DNA synthesis. This occurs through ATP or pyrophosphate, leading to the removal of NRTIs already incorporated in the growing DNA chain. The hydrolytic removal requires a pyrophosphate donor, which in most cells is usually ATP. The thymidine analog mutations (TAMs); M41L, D67N, k70R, L210W, T215Y/F and K219Q/E, use this mechanism. It is also referred to as nucleotide excision or primer unblocking (Shafer, 2002). The exact location of the mutations on the RT enzyme determines the mechanism used.

NRTI multi-drug resistance mutations.

Drug resistance mutations selected for one drug can also cause cross-resistance for some, or all, the other drugs in the same class. Three pathways exist to multi-drug resistance; the TAM pathway, the 69 insertion complex pathway and the 151 complex pathway.

The Thymidine Analog Mutations (TAMs) are selected by Zidovudine (ZDV) and Stavudine (d4T) and accumulate in a step-wise manner and can cause cross-resistance to the other NRTIs if three or more of them are expressed. The M41L and T215Y/F are known as primary mutations; while the D67N, K70R, L210W and K219Q are defined as secondary mutations (Miller *et al.,* 2004). The TAMs are also sometimes known as nucleoside-associated mutations (NAMs), to take into account that such changes can also reduce susceptibility to non-thymidine analogs (Miller *et al.,* 2004).

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The 69 insertion complex consists of a substitution on codon 69 (typically Y69S) and an insertion of two or more amino acids. It is associated with resistance to all NRTIs when present with one or more TAMs at codons 41, 210, or 215 (Miller *et al.,* 2004).

The 151 pathway consists of the Q151M mutation and mutations A62V, V75I, F77L and F116Y. It confers resistance to all the NRTIs except Tenofovir DF (Miller *et al.,* 2004). The Q151M complex is relatively rare in HIV-1 but it can confer resistance to most analogues and it is markedly more frequent in HIV-2 than in HIV-1 (Clavel and Hance, 2004).

1.11.3.2. NNRTI resistance mutations

There are four FDA-approved NNRTIs: nevirapine, delavirdine, efavirenz and Etravirine. NNRTI resistance mutations are clustered around the hydrophobic binding pocket in close proximity to the active site, and reduce the affinity of the NNRTI to the RT and thus leads to loss of antiviral activity of NNRTI and therefore treatment failure (Ren *et al.,* 2000). The most prominent mutations include the K103N, Y181C/I and Y188C changes. Resistance to Nevirapine is often associated with the Y181C mutation, but other mutations such as Y188C, K103N, G190A, and V106A, also occur. Resistance to Effavirenz is characterized by the K103N mutation, but the Y188L mutation is also observed (Clavel and Hance, 2004). The phenomenon of cross-resistance is very common amongst the NNRTIs and a single mutation can give cross resistance to all drugs used in this class. This is partly due to the fact that most NNRTI resistance mutations confer resistance to multiple drugs and also because a single drug may select for multiple different NNRTI resistance mutations even if only one or two predominant mutations are detected during genotyping.

1.11.3.3. *Protease* Inhibitors resistance mutations

There are eight FDA-approved PIs: amprenavir, indinavir, lopinavir (manufactured in combination with ritonavir) nelfinavir, ritonavir, saquinavir, atazanavir and tipranavir. Resistance to PI is due to amino acid changes in the active site of the HIV-1 *protease* or in surrounding regions involved in the binding of the PI. The PIs have multiple,

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overlapping patterns of resistance. An interesting fact about PI resistance is that it usually develops slowly because several minor mutations must first accumulate. Therefore, there are major (primary) and minor (secondary) mutations in PIs. The major mutations lead to phenotypic resistance. They occur early in the process of resistance to one drug. Minor mutations are located outside the active site and usually occur after major mutations. Minor mutations are common at polymorphic sites of non-B subtypes (Hoffmann *et al.,* 2007). High level resistance to most PI therefore requires the accumulation of multiple mutations (the genetic barrier), either by a complete change of the resistance profile or the successive addition of mutations on an unchanged background (Back and Berkhout, 1997).

1.11.3.4. *Integrase* Inhibitor resistance mutations

The only FDA approved *integrase* inhibitor is Raltegravir. Very little is known about resistance development in this drug class because it is new. Nonetheless, primary resistance data shows two mutational pathways associated with mutations on positions 148 or 155 on the *integrase* gene (Cahn and Sued, 2007).

1.11.3.5. Fusion/entry Inhibitor resistance mutations.

The two FDA approved fusion inhibitors and entry inhibitors are Enfuvirtide and Maraviroc, respectively. Enfuvirtide (T-20) is mostly associated with mutations in the heptad repeat 1(HR1) region of the gp41 envelope gene. These mutations prevent the FIs from acting on the transmembrane protein gp 41 so as to prevent the fusion of viral and cell membranes of susceptible cells. A single amino acid substitution in gp41 can reduce the efficacy of T-20. On the other hand, maraviroc mutations are clustered in the third variable region (V3) of the gp120 enzyme (Westby *et al.,* 2007). HIV-1 isolates resistant to enfuvirtide have been identified in patients receiving it and also by culturing HIV-1 in the presence of increasing enfuvirtide concentrations (Shafer, 2002). Sequences of resistant isolates developing during in vitro passage experiments have demonstrated mutations in positions 36 to 38 of gp41, whereas sequences from patients receiving enfuvirtide have demonstrated either single or double mutations between positions 36 and 45. In site-directed mutagenesis experiments, isolates with a single mutation display one-

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to 21-fold reductions in susceptibility, whereas isolates with two mutations display 15- to 500-fold reductions in susceptibility (Shafer, 2002).

In general, drug resistance increases over time (drug resistance evolution). Single mutations hardly lead to complete resistance to ARVs. However, the M184V (which results in complete resistance to Lamivudine) mutation is an exception to this rule. Single mutations in the hydrophobic pocket of the RT also provide strong resistance to NNRTIs, but the viral strains in patients in whom regimens using these drugs are not longer working often incur additional mutations, indicating that the level of resistance provided by single mutations is not optimal. ARV drug resistance to RTIs via the accumulation of TAMs or Q151M complex mutations and resistance to PIs are always gradual processes, leading to progressive increases in the level of resistance (Clavel and Hance, 2004).

1.11.4. Evolution of HIV-1 drug resistance

Drug resistance evolution within a patient depends on the generation of genetic variation in the virus and on the selection of drug-resistant variants during therapy (Shafer, 2002). The genetic variability of HIV-1 is due to the inability of HIV-1 RT to proofread nucleotide sequences during replication. This variability is further enhanced by the high rate of HIV-1 replication, the accumulation of proviral variants during the course of HIV-1 infection and genetic recombination when viruses of different sequence infect the same cell (Shafer, 2002). All these lead to the evolution of many genetically distinct variants (quasispecies) within the patient, immediately after primary infection. Drug resistance development depends on the extent to which virus replication continues during drug therapy, the ease of acquisition of a particular mutation (or set of mutations), and the effect of drug resistance mutations on drug susceptibility and viral fitness. In some cases, certain mutations selected during drug therapy give rise to measurable phenotypic resistance by themselves, whereas other mutations compensate for the diminished replicative activity associated with drug resistance or cause resistance only when present in combination with other mutations (Shafer, 2002). In a single day, about 10^4 and 10^5 single point mutation can occur in an untreated HIV-1-infected individual and double mutants also occur commonly (Shafer, 2002). Therefore, most drug resistance mutations

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are probably present prior to the start of therapy. However, viruses containing sufficient drug resistance mutations to replicate in the presence of multiple drugs do not appear to exist in previously untreated persons infected with wild-type viruses. Indeed, once potent HIV-1 suppression is achieved in previously untreated persons, it usually persists indefinitely if therapy is not interrupted (Shafer, 2002).

1.11.5. Antiretroviral drug resistance in treated and untreated populations

In South Africa, some studies show that drug resistance among untreated or naive patients may not be common. No known drug resistance mutations were reported in 2002 against Zidovudine (Pillay *et al.,* 2002). Apart from minor mutations such as L10I, K20R, M36I, M63P and V77I in the *protease*, no major *protease* resistance mutations were found in 72 patients from KwaZulu-Natal (Gordon *et al.,* 2003). In the same study, no primary or secondary 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. In another study carried out by Bessong *et al.,* in 2005, 2 out of 40 *protease* sequences harbored resistance substitutions M46L and G73S; and among 35 RT sequences, one virus contained the Y318F substitution related to resistance (Bessong *et al.,* 2005).

In untreated populations, previous studies have shown that the prevalence of resistant strains could be as high as 20% in countries where most patients are under treatment. On the other hand, in countries were therapy is limited resistant viruses are rare in the naive populations (Pires *et al.,* 2004). Before 2004, antiretrovirals were available only through private health establishments in South Africa; and due to the scarcity and high cost, the majority of patients could not afford them. In early 2004, a national ARV delivery programme was initiated to provide drugs to those who had a CD4+ count less than 200 cells/mm^3 or with 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 ARVs, with Nevirapine replacing Efavirenz for women of child bearing age in this group. The second-line therapy comprises Zidovudine, Didanosine and Lopinavir/Ritonavir (South African Department

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of Health, 2004). Currently, the revised regimen is comprised of Tenofovir, Lamivudine and Efavirenz/nevirapine. Stavudine is still recommended where side effects are tolerated (Department of Health, 2010). The 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). Therefore, information regarding the presence of resistant viruses could be important in tailoring combination regimens or to decide whether to prescribe resistance testing prior to initiation of therapy.

1.12. Rationale of the study

Over the years, data has accumulated on the amino acid changes that are related to the development of resistance in HIV-1 subtype 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, which differ from subtype B in the envelope gene by as much as 30%. For example, HIV-1 subtype 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; Santos and Soares, 2010). The non-subtype B viruses prevalent in the African epidemics are only now becoming the targets of widespread antiretroviral therapy. As treatment efforts mature, there is the expectation that differences in resistance patterns may emerge between divergent subtypes (Kantor and Katzenstein, 2002; Santos and Soares, 2010) and hence the need to study them.

Antiretroviral therapy is increasing rapidly in South Africa and most patients are still using the first line therapy. Transmitted resistance can reverse the effectiveness of first line antiretroviral therapy at the population level. Persons with transmitted drug resistance begin antiretroviral therapy with a lower genetic barrier to resistance, a higher risk of virologic failure, and a higher risk of developing resistance even to those drugs in their regimen that were originally fully active (Shafer *et al*., 2007). Studies on resistance transmission can therefore supply information to support public health bodies in South Africa in designing education and prevention programs to minimize the development and

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transmission of drug-resistant viruses and to further support the rational use of ARV drugs by treatment programs. Information regarding the presence of resistant viruses is also important in tailoring combination regimens or to decide whether to prescribe resistance testing prior to initiation of therapy.

Access to antiretroviral therapy is rapidly expanding in South Africa, including the Limpopo province. Studies on antiretroviral genetic drug resistance have been done in places such as Pretoria and Cape Town. For example, Pillay *et al.* (2008) reported a prevalence of 4.2% in a surveillance study of antiretroviral drug resistance among drug-naive HIV-1 infected individuals in Gauteng province in 2002 and 2004. In another study carried out by Orrell *et al.* (2009) in Cape Town, the prevalence of primary resistance in naive patients was 2.5%. Jacobs *et al.* (2008) also reported a prevalence of 3.6% in a study on HIV-1 treatment-naive patients from Cape Town. However, there is paucity of data in Northern South Africa (Limpopo province), which has an HIV prevalence rate of 20.7% (Department of Health, 2009) and with an expanding antiretroviral treatment program. This is one of the first provinces where therapy was first initiated in private health establishments in early 2000.

Antiretroviral drugs have been available to some communities for close to a decade in the Limpopo province (Northern South Africa). Within this time, there is the possibility of drug resistance emergence and transmission. In addition, resistance testing is not routinely performed prior to treatment initiation. Many patients enter treatment without testing and remain on standard therapy for long periods, with the consequence of the emergence of high level resistance and treatment failure in both first and second line drug regimens. Raltegravir may be introduced in South Africa in the near future as part of salvage therapy. Despite this, only a few studies in South Africa have focused on the IN gene in terms of viral genetic variability and resistance (Marconi *et al.,* 2008; Fish *et al.,* 2010). No data on HIV-1 IN drug resistance is available from the north-eastern South Africa, where HIV prevalence is relatively higher. It is equally important to provide data sequence on drug targets in the evolving dynamic HIV landscape of treatment options and genetic variability.

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Furthermore, since genetic drug resistance testing is not yet the standard of care for each patient, a population-based, antiretroviral drug resistance study in the Northern Province with an expanding treatment program was therefore necessary in order to generate data and to inform policy on the treatment program, as well as resistance monitoring, critical in patient management.

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PREVALENCE OF DRUG RESISTANCE MUTATIONS IN NEWLY DIAGNOSED DRUG NAIVE HIV-1 INFECTED INDIVIDUALS IN A TREATMENT SITE IN THE WATERBERG DISTRICT, LIMPOPO PROVINCE, SOUTH AFRICA

ABSTRACT

Information on the presence of antiretroviral (ARV) drug resistance viruses in drug naive populations is relevant in guiding treatment guidelines. The present study was carried out to determine the prevalence of resistance mutations in drug naive HIV infected individuals at the Bela Bela treatment site. Drug naive HIV-1 infected individuals were sequentially recruited between February 2008 and December 2008 from among individuals visiting the voluntary counseling and testing (VCT) services of the Bela Bela HIV/AIDS Wellness Clinic. Viral subtyping was done by phylogenetic analysis; drug resistance mutations were determined using the Stanford HIV Drug Resistance Interpretation and the International AIDS society-USA guidelines. A drug resistance mutation prevalence of 3.5% {95%; CI=0.019796-0.119077} comprising Y181C and L33F was observed. Phylogenetic analysis of the PR and RT genes showed that 56/57 (98.2%) of the viruses were HIV-1 subtype C on the PR; while 54/55 (98.2%) of the viruses were HIV-1 subtype C on the RT. One isolate (08BBVCT31ZA) was subtype B on the PR and RT genes. The prevalence of drug resistance mutations in drug naive persons may be low in Bela Bela after 8 years of access to antiretroviral treatment (ART), and resistance testing prior to treatment initiation may not be needed.

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2.1. INTRODUCTION

Highly active antiretroviral therapy (HAART) has dramatically transformed the health of patients infected with HIV/AIDS since its introduction. The length and quality of life of patients has considerably improved (Tang and pillay, 2004; Gatanaga *et al.,* 2007). However, one of the major drawbacks of HAART is the development of drug resistance which usually accompanies the use of ARV drugs (Gatanaga *et al.,* 2007; Turner and Wainberg, 2006). Patients with ARV drug resistance have a relatively higher risk of virologic failure as they start ART with a lower genetic barrier to resistance (Shafer *et al.,* 2007). In addition, harbouring resistance viruses prior to treatment initiation complicates alternative treatment algorithms.

In the developed world, HAART has been used with considerable success and treatment is accompanied by regular virologic monitoring including viral load measurements and drug resistance testing to guide patient management (Gupta and Pillay, 2007; Richman *et al.,* 2009). On the other hand, the use of HAART in resource-poor settings still poses a lot of problems. Virological monitoring is less frequently done due to cost and dearth of qualified personnel. Many countries have therefore resorted to the WHO public health approach to ART delivery (Gupta and Pillay, 2007). The objective of this approach is to reach out to as many patients as possible, minimizing morbidity and mortality. The approach has therefore seen the use of standard first line therapy consisting of two nucleoside/nucleotide *reverse transcriptase* inhibitors and a non-nucleoside *reverse transcriptase* inhibitor, and second-line therapy comprising of boosted PI with at least one NRTI (Gupta and Pillay, 2007). An important consequence of this approach is the probable emergence of high-level resistance during standard first-line therapy because most of the patients stay on a virologically failing regimen for longer periods of time.

South Africa has one of the highest HIV/AIDS prevalences in the world, and according to the South African National antenatal sentinel HIV and Syphilis survey conducted in 2008, the estimated prevalence is 29.3% (Department of Health, 2009). Limpopo Province has a prevalence rate of 20.7% (Department of Health, 2009). Prior to 2004,

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ARVs were available only through private health establishments in South Africa; and due to the scarcity and prohibitive high cost majority of patients could not afford treatment. In early 2004 a national ARV delivery programme was initiated to provide drugs to those who had a CD4+ count less than 200 cells/mm³ or with a clinically defined AIDS condition. Other psychosocial considerations for eligibility required patients to demonstrate reliability, no active alcohol or other substance abuse, no untreated active depression, disclosure of HIV status to at least one friend or family member or have joined a support group, accepted their HIV positive status, have insight into the consequences of HIV infection, understood the purpose of ART, and be able to visit the treatment centre on a regular basis.

The 2004 national drug regimen guideline stipulated 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. The second-line therapy comprised of Zidovudine, Didansone and Lopinavir/Ritonavir (Department of Health, 2004). Currently, the revised regimen is made up of Tenofovir, Lamivudine and Efavirenz/nevirapine. Stavudine is still recommended where side effects are tolerated (Department of Health, 2010).

The 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 (Turner and Wainberg, 2006; Pillay *et al.,* 2008). 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. In South Africa, some studies (Pillay *et al.,* 2008; Orrell *et al.,* 2009) have described drug resistant mutations in drug naive populations. Using the threshold survey, Pillay *et al.,* (2008) reported an overall resistance prevalence of less than 5% in 2002 and 2004 among drug-naive HIV-1 infected individuals in Gauteng province. However, despite the low prevalence reported, it is important to monitor the emergence of drug resistance in drug naive populations, particularly in regions such as the Limpopo Province where such data is scarce.

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2.1.1. Objectives of the study

The main objective of this study was to determine the prevalence of drug resistance mutations in newly diagnosed treatment naive HIV-1 infected individuals in a treatment site in the Waterberg District of South Africa.

The specific objectives of this study were:

1. To determine the types and scope of genetic drug resistance mutations among drug naive HIV-1 subjects in the Waterberg district.
2. To phylogenetically determine the HIV-1 subtypes prevalent in the Waterberg district of South Africa.
3. To determine the intragenetic diversity of the PR and RT genes from HIV-1 infected individuals in the Waterberg district of South Africa.

2.2. MATERIALS AND METHODS

2.2.1. Ethical approval

Approval of the study protocol was obtained from the Health, Safety, and Research Ethics Committee of the University of Venda, South Africa. Permission was obtained from the Limpopo Provincial Department of Health and authorities of the HIV/AIDS prevention Group (HAPG) at Bela Bela. Signed informed consent was obtained from all study participants.

2.2.2. Study site

Study subjects were recruited from the HIV/AIDS Wellness Clinic in Bela Bela, Waterberg District, South Africa (Figure 2.1). Treatment at the Wellness Clinic began in 2001; three years before the roll out of ARVs in South Africa in public health facilities. The Wellness Clinic established in 1996 is run by the HIV/AIDS Prevention Group (HAPG), a non-governmental organisation (Ndjeka and Manhaeve, 2006). Bela Bela is a holiday resort with hotels and camping sites. It is also a stop-over point for long distance truck drivers who patronize sex workers attracted to the luxury facilities of the area. Bela Bela Centrum is about 120 km from Gauteng Province to the South and Northwest Province to the Southwest. It is surrounded by localities of poor socioeconomic status,

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and has a population of about 63,000 inhabitants (Ndjeka and Manhaeve, 2006). The Bela Bela HIV wellness Centre receives patients from the urban and rural sectors of the town. The centre also runs a home-based service and a voluntary counselling and testing facility. According to the South African National antenatal sentinel HIV and Syphilis survey conducted in 2008, the estimated HIV prevalence in Bela Bela was 27.5% (Department of Health, 2009).



Figure 2.1: Map showing Limpopo province and location of the study site – Bela Bela, indicated by the arrow. (www.routes.co.za/lp/Bela Bela/) (Accessed 10-3-2011).

2.2.3. Study population, sample collection and plasma preparation

A total of 79 HIV positive individuals without prior exposure to ARV visiting the HIV Voluntary Counseling and Testing facility of the Bela Bela Wellness Clinic were recruited sequentially between February 2008 and December 2008.

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Five ml of venous blood was collected into EDTA vacutainer tubes from each consenting subject. Demographic data such as age, sex, place of residence, probable date and place of infection, and marital status were obtained by questionnaire administration. Samples were stored at 4°C for a maximum of 48 hours prior to RNA extraction. However, a few samples, due to transportation logistics, were stored at 4° C for 4 days prior to RNA isolation. Whole blood was spun at 3000 rpm for 3 minutes. Plasma was aspirated aseptically and stored at -80° C for subsequent RNA extraction. Data on viral load and CD4+ count measurements were not available.

2.2.4. Viral RNA isolation, RT-PCR and nested PCR

Viral RNA was isolated using the viral mini RNA kit (Qiagen, Hilden, Germany), stored at -80°C until used. Two different protocols were used at different periods to amplify the HIV polymerase gene. With protocol 1, a one-tube RT- PCR was performed with AMV RT (Roche) and Taq DNA polymerase (Invitrogen), followed by a nested PCR as previously described (Bessong *et al.,* 2006). Five microlitres of RNA template was added to 45 µl of reaction mix. The reaction mix consisted of the following as final concentrations: 1X buffer, 0.1 mM dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 1.5mM $MgCl_2$, 0.2µM each of *Pol* 1C and RT-RV primers, 0.0125U Fast start Taq Polymerase, 0.024U AMV RT (Roche Diagnostics, Mannheim, Germany) and 0.05U RNase inhibitor (Roche Diagnostics, Mannheim, Germany). The first round primers were *Pol* 1C (5'-GAA GGA CAC CAA ATG AAA GAC TGC AC-3'; HXB2 location 2044→2069) and RT-RV (5'-*TAT* TTC AGC *TAT* CAA GTC TTT TGA TGG GTC A-3'; HXB2 location 3506←3536). Amplification conditions were: 1 cycle of 42°C for 60 minutes, 1 cycle of 95°C for 3 minutes. This step was followed by 35 cycles of 95°C for 1 minute, 58°C for 1 minute, and 68°C for 2 minutes and a final extension at 68°C for 10 minutes. The second round (nested) PCR consisted of 5 µl of the first round PCR product in 100 µl final volume. The reaction mix consisted of the following as final concentrations: 1X PCR buffer, 0.2mM dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 1.5mM $MgCl_2$, 0.2µM each of *Pol* 2R and *Gag* P1 primers, 0.00625U Fast start Taq Polymerase and 73.5 µl of PCR grade water. The nested primers were *Gag* P1 (5'-CAA GGG *GAG* GCC AGG GAA TTT-3'; HXB2 location 2112→2132) and *Pol* 2R

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(5'-TGA TGG GTC ATA ATA TAC TCC A[illegible]XB2 location 3492←3515). The PCR cycling conditions for the second round were: 1 cycle of 95°C for 2 minutes, 30 cycles of 95°C for 1 minute, 58°C for 1 minute, and 68°C for 2 minutes, followed by a final extension cycle at 68°C for 10 minutes and a hold at 4°C. This generated approximately a 1.4kb product (HXB2 location 2253-3713) comprising the complete PR and at least 900bp of the RT gene.

In the second protocol, cDNA was synthesized using the Thermoscript RT-PCR system (Invitrogen) as described by Pillay *et al.,* (2008). Four µl of RNA template was used for cDNA synthesis and the reaction mix consisted of the following as final concentrations: 1x cDNA synthesis buffer, 0.005M DTT, 0.5U RNase out, 0.0625U Thermoscript RT (Roche Diagnostics GmbH, Mannheim, Germany), DEPC treated water and 0.1U/ µl E. coli RNase H. Cycling conditions were: 1 cycle of 65°C for 5 minutes, 1 cycle of 55°C for 60 minutes, 85°C for 5 minutes, 37°C for 20 minutes and hold at 4°C. In first round PCR, five microlitres of synthesized cDNA was added to 45 µl of first round reaction mix. The reaction mix consisted of the following as final concentrations: 1X buffer 1 plus $MgCl_2$, 0.1mM dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 0.2µM each of G25Rev and IN3 primers, 0.025U/ µl DNA Polymerase (Expand long template enzyme), and 37 µl of DEPC water. The first round primers were G25 *REV* (5′-GCAAGAGTTTTGGCTGAAGCAATGAG-3′; HXB2 location 1867→1892) and IN3 (5′-TCTATVCCATCTAAAAATAGTACTTTCCTGATTCC-3′; HXB2 location 4212←4246). The second round (nested) PCR consisted of 5 µl of the first round PCR product in 100 µl final volume. The reaction mix consisted of the following as final concentrations: 1X buffer 1 plus $MgCl_2$, 0.1mM dNTPs (Roche Diagnostics GmbH, Mannheim, Germany), 0.2µM each of AV150 and *Pol* M4 primers, 0.025U/ µl DNA Polymerase (Expand long template enzyme), and 79.5 µl of DEPC water. The primers were AV150 (5′-GTGGAAAGGAAGGACACCAAATGAAAG-3′; HXB2 location: 2036→2062) and *Pol* M4 (5′-CTATTAGCTGCCCCATCTACATA-3′; HXB2 location 3870←3892). The PCR cycling conditions for the first and second round were identical: 1 cycle at 94°C for 2 minutes, 10 cycles at 94°C for 10 seconds, 50°C for 30 seconds, and 68°C for 2 minutes; 25 cycles at 94°C for 15 seconds, 50°C for 30 seconds, and 68°C for

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2 minutes plus 20 seconds cycle elongation for each successive cycle with a final step at 68°C for 7 minutes. This generated approximately a 1.7kb (HXB2 location 2253-3842) product comprising the complete PR and at least 1200bp of the RT gene.

2.2.5. Detection of amplified products (agarose gel electrophoresis).

The amplicons from the nested PCR were detected and verified for desired size and specificity by running a 1% agarose (ethidium bromide stained) gel electrophoresis, at a constant voltage of 100V for 40 minutes. DNA molecular weight marker X (Roche Diagnostics GmbH, Mannheim, Germany) was used to verify PCR fragment size.

2.2.6. Purification of PCR product, Sequencing and editing.

PCR amplicons were purified with QIAquick PCR purification kit (Qiagen, Hilden, Germany) and direct population-based sequencing performed on both strands with the BigDye Terminator v3.0 kit on ABI Prism 377 (Applied Biosystems) using Taq DNA polymerase. The sequencing primers are shown in table 2.1. Generated nucleotide sequences were edited manually using SeqMan II (DNASTAR) software version 7.

Table 2.1: Sequencing primers for the partial *pol* gene (combined PR and RT).

Pol protocol 1	
Primer name and HXB2 location	Primer sequence
Pol 1F (2546→2572)	5′-TTTTCCCATTAGTCCTATTGAACCTGT-3′
Pol 1R (3016←3041)	5′-CATGCTACTCTGGAATATTGCTGGTG-3′
Pol 2F (2932→2957)	5′-CTGCATTCACCATACCTAGTATAAAC-3′
Pol 2R (3492←3515)	5′-TGATGGGTCATAATATACTCCATG-3′
Pol protocol 2	
Primer name and HXB2 location	Primer sequence
Pol Mo (2251→2272)	5′-TCCCTCAGATCACTCTTTGGCA-3′
Pol Mg (2798←2823)	5′-ATTGAACTTCCCAGAAGTCTTGAGTT-3′
Pol M1 (3016←3041)	5′-CATGCTACTCTGGAATATTGCTGGTG-3′
Pol M2 (3105→3124)	5′-GATTTGTATGTAGGATCTGA-3′
Pol M4 (3870←3892)	5′-CTATTAGCTGCCCCATCTAGATA-3′
Pol M8 (3302←3323)	5′-CTGTATATCATTGACAGTCCAG-3′

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2.2.7. Viral genetic subtyping

HIV subtyping was done by phylogenetic analysis. The complete *protease* and partial *reverse transcriptase* genes of test viruses were separately aligned using Clustal X with representative subtype (year 2000) reference sequences (group M subtypes A-D, F-H, J and K, excluding the recombinant sequences) obtained from GenBank. Previously described PR and RT sequences from Limpopo Province were included in the analysis. Thereafter, neighbour joining phylogenetic trees were generated with the PHYLIP programme, making use of the maximum likelihood model to take care of differences in the transition and transversion rates. 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. Predicted amino acids for the PR and RT genes were aligned using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Briefly, consensus amino acid sequences for the RT and PR gene regions were created and then compared to the global subtype C and the global subtype B consensus sequences obtained from GenBank. The mean genetic distances among the PR and RT sequences were determined by the Kimura 2- parameter model using the Bio Edit and Clustal W software packages.

2.2.8. Genetic drug resistance determination

Due to the broad scope of viral mutations, drug resistance mutations were identified using the Stanford HIV Drug Resistance Interpretation Algorithm (http://hivdb.stanford.edu/) and the International AIDS Society-USA Guidelines (Johnson *et al.*, 2008). The aim of using two algorithms was to describe as broad as possible the significance of observed substitutions. The Bayesian analysis was used to calculate the 95% confidence interval, in order to estimate the confidence limits around the prevalence estimate.

2.3. RESULTS

2.3.1. Demographic data

A total of 79 HIV positive individuals were sequentially recruited for this study. The mean age was 43.5 years (18 – 69 years). The proportion of women and men was 57% and 40.5% respectively. Seventy-seven percent of the study subjects were single and 23%

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were married. The most important risk factor for HIV transmission was sexual intercourse (88.6%). However, the questionnaire was not designed to determine whether it was heterosexual or homosexual. In 90% of the participants, the most probable place of HIV infection was South Africa. Twenty-three percent of the study subjects reported to have been infected in 2008, while the rest estimated that they were infected between 2000 and 2007. The highest level of education for most of them was grade 6 and above (53.2%), but neither university students nor graduates were recorded within the participants. A monthly income range of R1000 – R5000 (125-625 USD) was common to most (27.8%) of the subjects, while households with 1-5 persons were reported by 63.3% of the respondents. Details of the demographics of the study population are shown in Table 2.2.

2.3.2. Viral DNA amplification, sequencing and drug resistance mutations

Viral DNA was obtained for 62 out of 79 (78%) individuals (Figure 2.2). Reliable nucleotide sequences were obtained for 57 PR genes and 55 RT genes. Genetic subtyping and resistance analysis were done on these sequences.

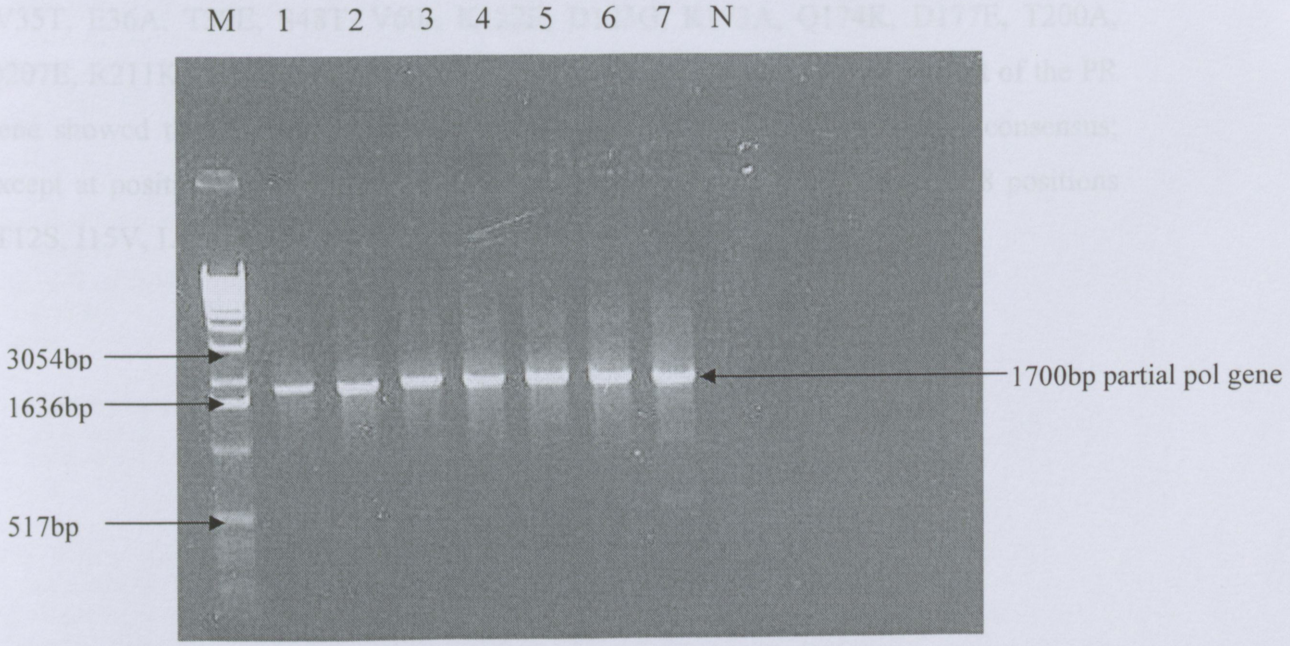

Figure 2.2: A representative gel electrophoresis photo of the amplified partial *pol* (PR and RT) gene. Lane M: Molecular weight marker X (12.2kbp); lanes 1-7: test samples and lane N: Negative control.

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Using the Stanford and IAS guidelines, major drug resistance mutations were detected in 2 out of 57 subjects (3.5%) {95%; CI=0.019796-0.119077}. One primary NNRTI mutation (Y181C) and one major PI mutation (L33F) were detected on two different patients: 08BBVT15ZA and 08BBVCT71ZA, respectively. No primary NRTI mutation was recorded. The detected mutations, nucleotide substitutions, and their potential significance are shown in Table 2.3. The frequencies of the mutations on the PR and RT genes are depicted in Figure 2.3.

2.3.3. HIV genetic subtypes

Phylogenetic analysis of the PR and RT genes showed that 56/57 (98.2%) of the viruses were HIV-1 subtype C on the PR; while 54/55 (98.2%) of the viruses were HIV-1 subtype C on the RT. One isolate (08BBVCT31ZA) was subtype B on the PR and RT genes (Figures 2.4 and 2.5). Mean genetic distance for the PR sequences ranged from 0.0101 to 0.2035 and 0.0331 to 0.1377 for the RT sequences. Considering the first 300 amino acids in the RT gene, sequence alignment showed that the consensus sequences of the test viruses was identical to the global subtype C consensus; except at 2 positions (V60I and Q174K). It differed from the global subtype B consensus at 17 positions (V35T, E36A, T39E, S48T, V60I, K122E, D123G, K173A, Q174K, D177E, T200A, Q207E, R211K, V245Q, A272P, K277R, and T286A). Amino acid alignment of the PR gene showed that the test consensus was identical to the global subtype C consensus; except at position I13T. It differed from the global subtype B consensus at 8 positions (T12S, I15V, L19I, M36I, R41K, H69K, L89M, and I93L).

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Table 2.2: Characteristics of the study population (drug naive subjects from Bela Bela

Characteristics	Patients (N= 79) Number (%)
Sex	
Males	32 (40.5)
Females	45 (57.0)
N/A	02 (2.5)
Marital status	
Single	61 (77.2)
Married	09 (11.4)
Widow	03 (3.8)
Divorced	01 (1.3)
N/A	05 (3.3)
Probable place of infection	
South Africa	71 (90.0)
Other countries	01 (1.3)
Unknown	03 (3.8)
N/A	04 (5.1)
Risk factor for transmission	
Sex	70 (88.6)
Blood & blood product route	01 (1.3)
Unknown	01 (1.3)
N/A	07 (9.0)
Probable Year of infection	
Before 2000	02 (2.5)
2000 – 2007	44 (55.7)
2008 (newly diagnosed)	16 (20.3)
Unknown	08 (10.1)
N/A	09 (11.4)
Household income (SAR)	
<R1000	15 (19.0)
R1000 – R5000	22 (27.8)
>R5000	00 (0)
N/A	42 (53.2)
Number of dependents	
1-5 persons	50 (63.3)
≥ 6 persons	05 (6.3)
N/A	24 (30.4)
Level of education	
Not educated	01 (1.3)
≤ Grade 5	17 (21.5)
Grade 6 & above	42 (53.2)
University	00 (0)
N/A	19 (24.1)

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Table 2.3: Drug resistance associated mutations, frequency, coding nucleotides and HIV-1 subtypes from HIV infected patients in Waterberg District, South Africa

Protease gene (n=57)	Frequency	Coding nucleotides		HIV-1 subtype	Comment	*Reverse transcriptase* (n=55)	Frequency	Coding nucleotides		HIV-1 subtype	Comment
		Wild type	mutant					Wild type	mutant		
L10I/F	2 (3.5)	CTC	ATT/CTT	C	Minor mutation to Atazanavir/ritonavir according to Stanford & IAS	T69N (NRTI mutation)	1 (1.8)	ACT	AAT	C	Secondary mutation (Stanford); Primary mutation (IAS).
K20R	19 (33.3)	AAG	AGG	C	Polymorphism (Stanford); minor mutation (IAS).	K100D (NNRTI mutation)	1 (1.8)	TTA	GTA	C	Secondary mutation(Stanford); undocumented(IAS)
L23F	2 (3.5)	CTA	TTT	C	Highly unusual minor mutation with unknown significance (Stanford); undocumented by IAS	K101Q (NNRTI mutation)	1 (1.8)	AAA	CAG	C	Secondary mutation(Stanford); undocumented(IAS)
D30G	1 (1.7)	GAT	CGT	C	Minor mutation (Stanford); undocumented (IAS).	V118I (NRTI mutation)	3 (5.5)	GTT	ATT	B & C	Secondary mutation(Stanford); undocumented(IAS)
L33F	1 (1.7)	TTA	AGA	C	Major mutation to *protease* inhibitors (Stanford); but a minor mutation by IAS	E138A (NNRTI mutation)	1 (1.8)	*GAG*	GCA	C	Polymorphism (stanford); but a Secondary mutation to etravirine (IAS)
M36I	46 (80.7)	ATG	ATA	C	Minor mutation to atazanavir, ritonavir,indinvir,nelfinavir (Stanford & IAS).	V179D (NNRTI mutation)	1 (1.8)	GTT	GAT	C	Minor mutation according to Stanford & IAS
L63S	7 (12.3)	CTC	TCT/AGT	C	Minor mutation (Stanford); undocumented (IAS)	**Y181C** (NNRTI mutation)	1 (1.8)	*TAT*	GTC	C	Primary mutation to efavirenz/ etravirine/nevirapine(Stanford & IAS).
A71L/T	2 (3.5)	GCT	TGT/AAA	C	Minor mutation to Atazanavir/ritonavir (Stanford & IAS).	L210V (NRTI mutation)	1 (1.8)	TTG	AAA	C	Secondary mutation(Stanford); undocumented(IAS)
T74S	4 (7.0)	ACA	TCA	C	Minor mutation (Stanford); undocumented by IAS						
L89M	46 (80.7)	CTG	ATG	C	Polymorphism (Stanford); undocumented (IAS)						
I93L	54 (94.7)	ATT	CTT	C	Common polymorphism (Stanford); undocumented (IAS).						

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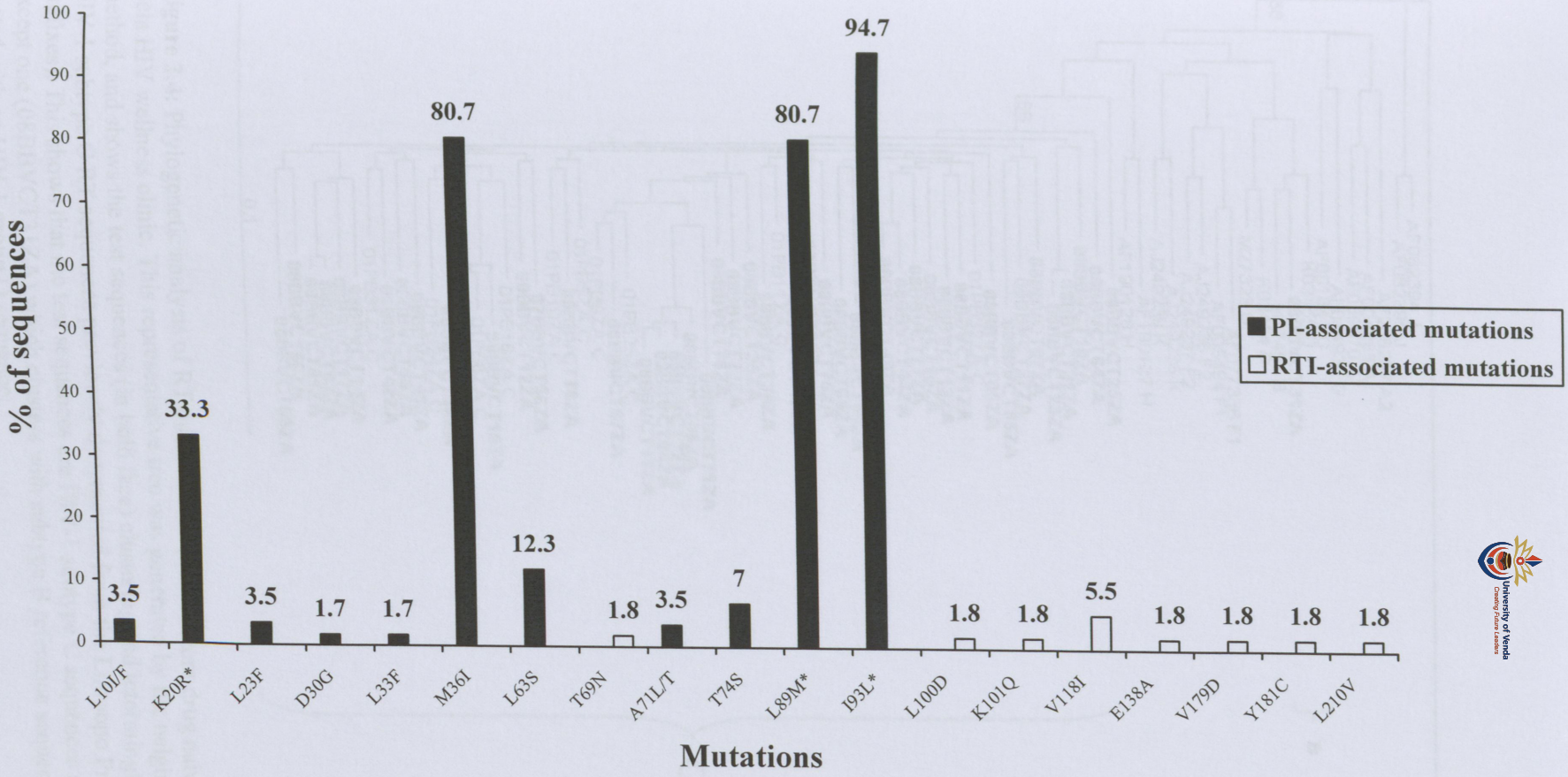

Figure 2.3: Distribution of resistance associated mutations on the *protease* and *reverse transcriptase* genes among HIV-1 viruses from South Africa. * K20R, L89M and I93L are considered as polymorphisms in HIV-1 subtype C, but which may influence resistance development.

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Figure 2.4: Phylogenetic analysis of RT nucleotide sequences from drug naive patients from Bela Bela HIV wellness clinic. This representative tree was generated by the neigbhour-joining method, and shows the test sequences (in bold face) clustering and intermingling with reference HIV-1 subtype C RT sequences previously documented from the Limpopo Province (with 01PB prefixes). This shows that the test sequences are HIV-1 subtype C sequences on the RT gene; except one (08BBVCT31ZA) which clusters with subtype B reference sequences. The tree was rooted with an HIV-1 group O sequence.

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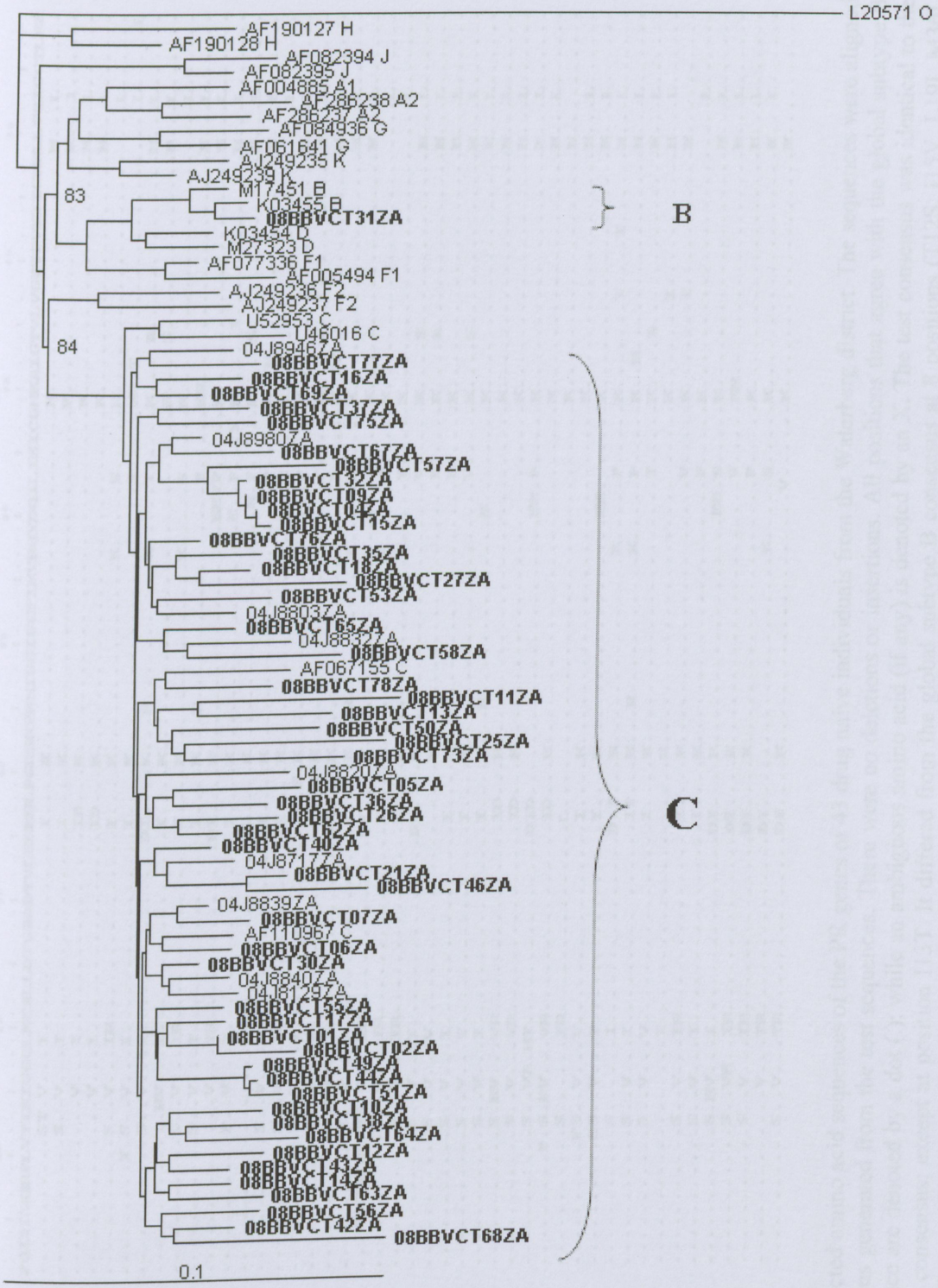

Figure 2.5: Phylogenetic analysis of PR nucleotide sequences from drug naive patients from Bela Bela HIV wellness clinic. This representative tree shows the test sequences (in bold face) intermingling with reference HIV-1 subtype C PR sequences (with 04J prefixes) previously documented. This shows that the test sequences are HIV-1 subtype C sequences at least on the PR gene; except one (08BBVCT31ZA) which clusters with subtype B reference sequences.

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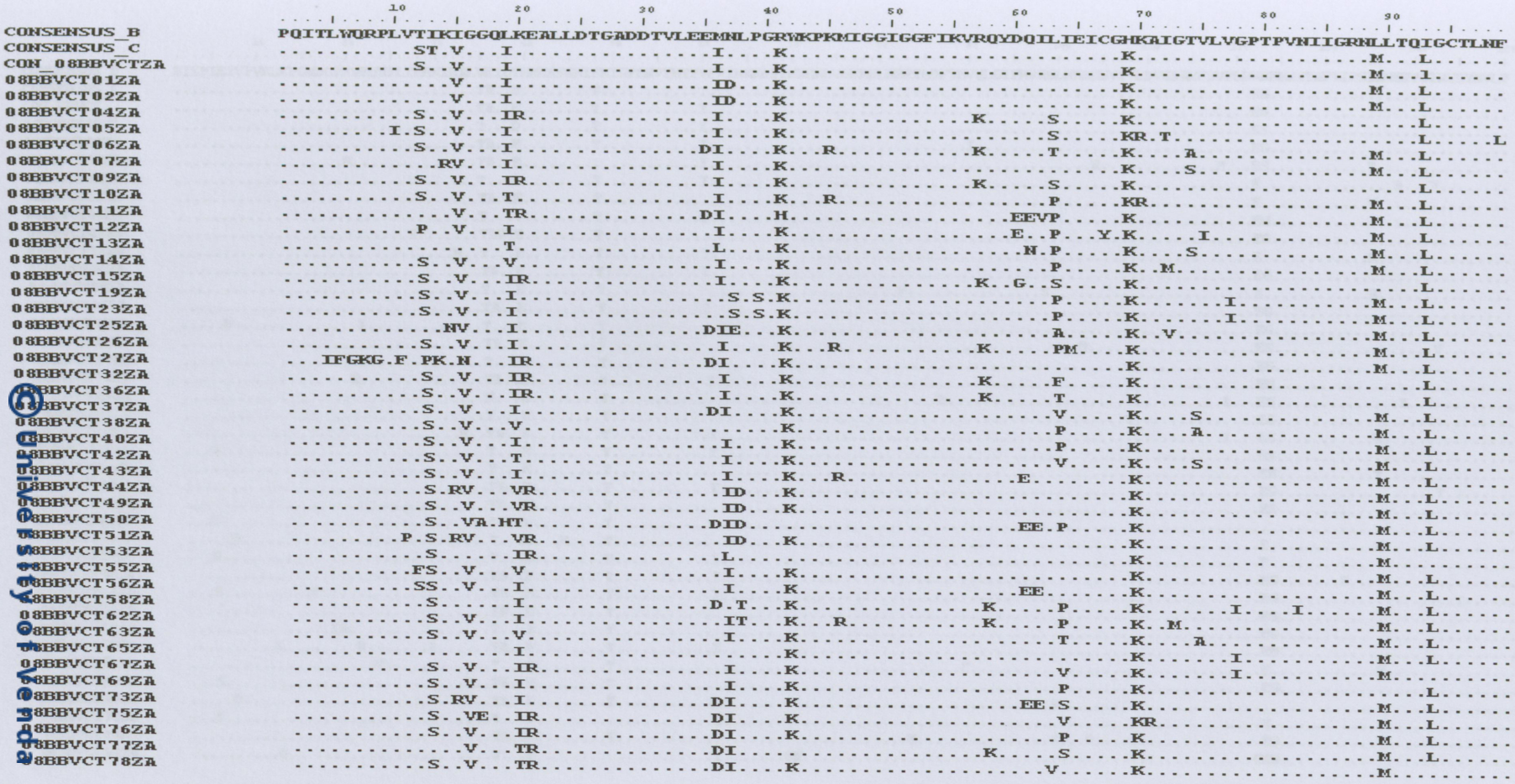

Figure 2.6: Predicted amino acid sequences of the PR genes of 43 drug naive individuals from the Waterberg district. The sequences were aligned against a consensus generated from the test sequences. There were no deletions or insertions. All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid (if any) is denoted by an X. The test consensus was identical to the global subtype C consensus; except at position I13T. It differed from the global subtype B consensus at 8 positions (T12S, I15V, L19I, M36I, R41K, H69K, L89M, and I93L).

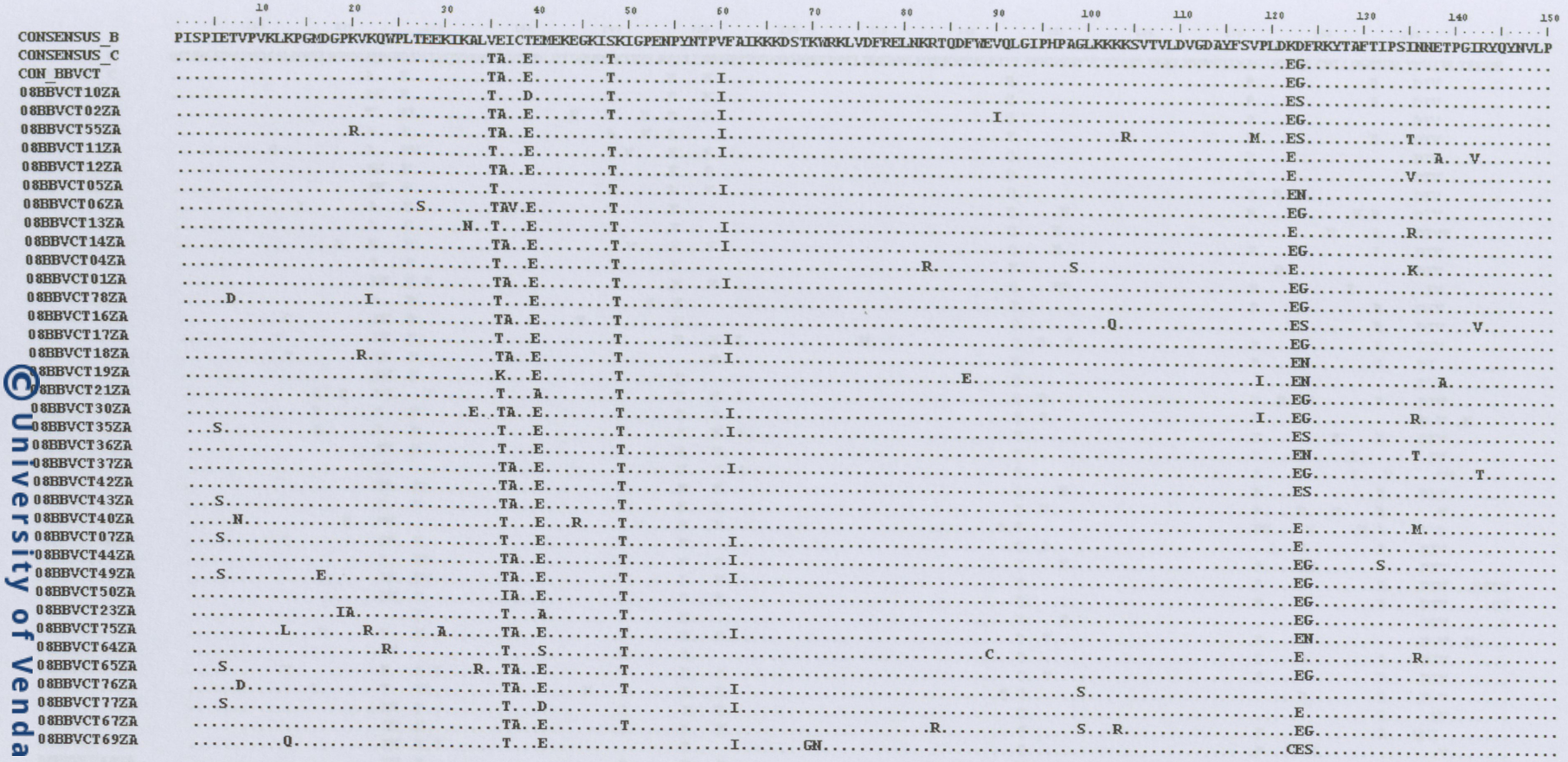

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Figure 2.7: Amino acid alignment of RT sequences (residues 1-150). The test consensus was generated from the HIV database (GenBank) using some 37 viruses from this study. The global subtype B and C consensuses were obtained also from HIV database program. The entire consensuses were aligned using BioEdith software. All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid (if any) is denoted by an X. The test consensus was identical to the global subtype C consensus; except at 2 positions (V60I and Q174K). It differed from the global subtype B consensus at 17 positions (V35T, E36A, T39E, S48T, V60I, K122E, D123G, K173A, Q174K, D177E, T200A, Q207E, R211K, V245Q, A272P, K277R, and T286A).

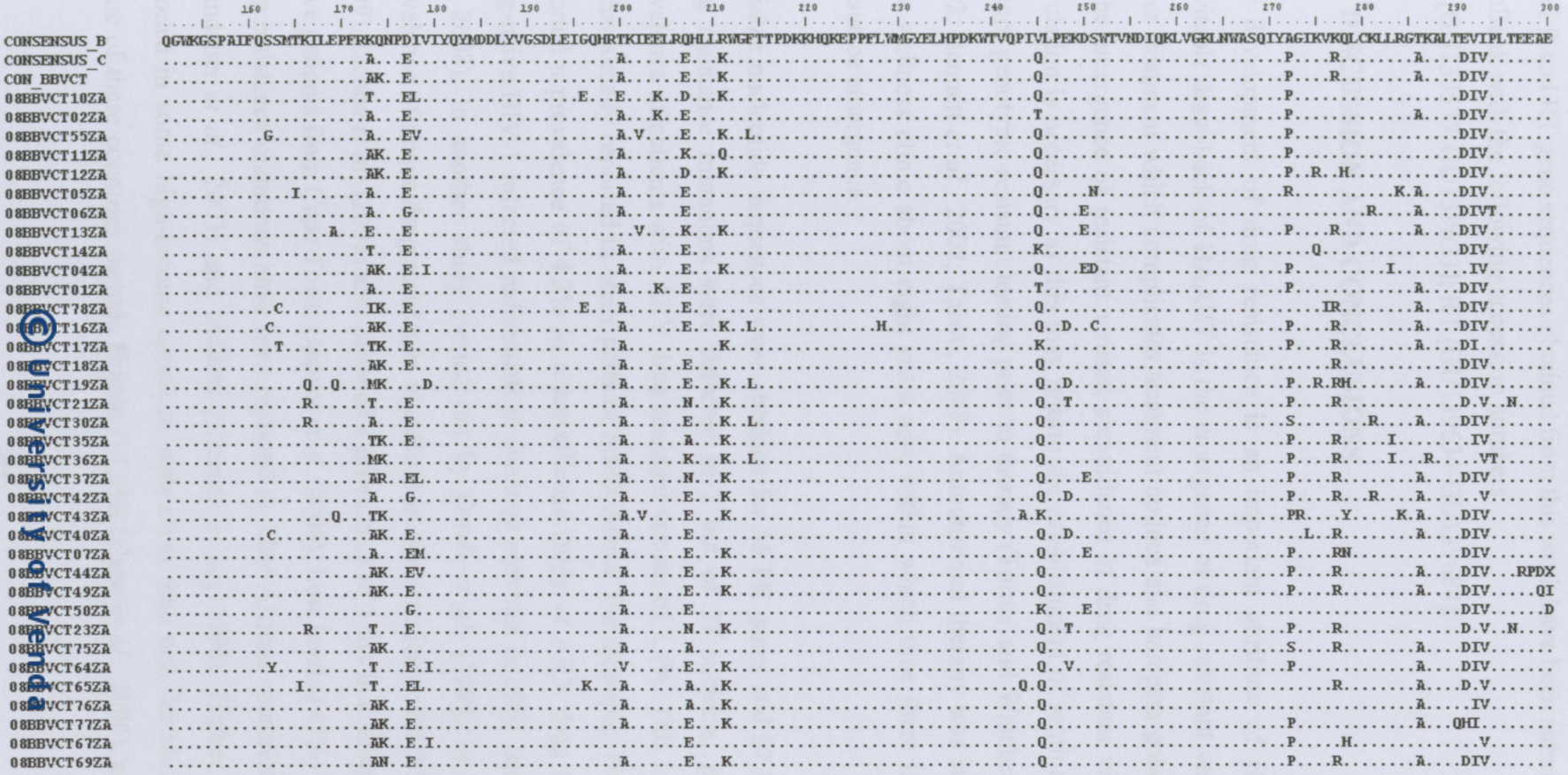

Figure 2.8: Amino acid alignment of RT sequences (residues 151-300). The test consensus was generated from the HIV database (GenBank) using some 43 viruses from this study. The global subtype B and C consensuses were obtained also from HIV database program. The entire consensuses were aligned using BioEdith software. All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid (if any) is denoted by an X.

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2.3.4. GenBank Sequence accession numbers

The PR and RT gene sequences obtained from this work have been submitted to GenBank with the following accession numbers:
PR [GU139577-GU139633]; RT [GU139634-GU139688].

2.4. DISCUSSION AND CONCLUSIONS

The development of drug resistance is an important attribute of HIV biology. An inevitable draw-back of HAART is the emergence of drug resistant variants in patients under treatment which complicates treatment options and hampers good prognosis. Due to the emergence of resistant viruses, surveillance of drug resistant viruses among the population is necessary as this may inform the establishment of sentinel surveillance or prompt genotypic resistant testing prior to therapy (Turner and Wainberg, 2006; Shafer, 2002; Bennett *et al.,* 2008; Taiwo, 2008). Antiretroviral therapy was started at the Bela Bela wellness clinic about eight years ago, within which time there is a possibility of resistance emergence.

Reliable nucleotide sequences were obtained for 55 PR genes and 57 RT genes. Major drug resistance mutations were detected in 2 out of 57 subjects; giving an overall prevalence of patients with ARV drug resistant viruses of 3.5%. This low rate is similar to that already reported in other parts of South Africa. For example, Pillay *et al.* (2008) reported a prevalence of 4.2% in a surveillance study of ARV drug resistance among drug-naive HIV-1 infected individuals in Gauteng province in 2002 and 2004 (Pillay *et al.,* 2008). In another study carried out by Orrell *et al.* (2009) in Cape Town, the prevalence of major drug resistance mutations in naive patients was 2.5% (Orrell *et al.,* 2009). Jacobs *et al.* (2008) also reported a prevalence of 3.6% in a study on HIV-1 drug-naive patients from Cape Town (Jacobs *et al.,* 2008). Low levels (<5%) or the absence of drug resistance mutations have been reported in other African countries such as Zambia (Handema *et al.,* 2003) and Malawi (Kamoto *et al.,* 2008). Higher rates have been reported in some high-income countries with more than one decade of ART history. Some of these countries include Britain (14.2%) (Cane *et al.,* 2006), and USA (25.2%)

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(Smith *et al.,* 2007). Unlike in high-income countries, low rates of ARV drug resistance are expected in low-income countries because most patients are starting therapy on highly potent regimens. In high-income countries, ART scale-up began with resistance associated monotherapy and one-class dual therapy (Fischl *et al.,* 1987). In the present study, one primary NNRTI mutation (Y181C) was recorded on the RT gene, while no primary NRTI mutation was recorded. This mutation occurred in a 40 years old, married female patient. The Y181C causes high level resistance (50- to 100-fold) to Nevirapine and Delavirdine and low level resistance to efavirenz. At the time of the investigation, the South African national drug regimen guideline stipulated the use of Stavudine, Lamivudine and Efavirenz as the first-line therapy for those who have never been exposed to ARVs, with Nevirapine replacing Efavirenz for women of child bearing age. It is not clear whether the patient who harboured the Y181C detected in this study had been enrolled in a Prevention of mother-to-child transmission programme, in which Nevirapine is used.

One major PI mutation (L33F) was detected on the PR gene in the present study. This mutation is classified as a major mutation according to the Stanford Drug Resistance Interpretation Algorithm, and a minor mutation by the IAS-USA program. The L33F mutation is selected by Fosamprenavir/ritonavir, Duranavir/ritonavir, Lopinavir/ritonavir, Atazanavir/ritonavir, and Tipranavir/ritonavir and contribute to resistance to these drugs. These drugs are boosted with low doses of Ritonavir so as to prevent the virus from escaping PI treatment by the initial selection of only one or two primary mutations in the PR. In South Africa, the PI containing regimen is used for children less than 3 years of age, and in the second line regimen for adults (Department of Health, 2004). It is difficult to tell the spread of the Y181C and L33F mutations and what effect they will have on treatment in this population.

The present study indicates that the prevalence of drug resistant HIV among the drug naive population in Bela Bela, South Africa is low after eight years of free ART. Population based sequencing was used in this study and this could have however, contributed to an underestimation of drug resistance prevalence. Patients may harbour

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populations of minor resistant viruses which are usually not detected by population sequencing. This prevalence study also used chronically infected patients, which could mean that resistance acquired may have disappeared over time and this might have also led to an underestimation of the results.

Apparently, the testing of patients for drug resistant viruses before the initiation of therapy may not be required. However, subsequent periodic studies are required in Bela Bela and in other treatment sites to monitor the emergence and spread of drug resistant viruses. Data in this regard is important to inform policy on sentinel surveillance, and treatment algorithms. A high prevalence could indicate the need for regular sentinel surveillance or the need for baseline genotypic drug resistance testing before treatment.

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CHAPTER THREE

PREVALENCE OF ANTIRETROVIRAL DRUG RESISTANCE MUTATIONS AND HIV-1 SUBTYPES AMONG NEWLY DIAGNOSED DRUG-NAIVE PERSONS VISITING A VOLUNTARY TESTING AND COUNSELLING CENTRE IN NORTH-EASTERN , SOUTH AFRICA

ABSTRACT

Data on antiretroviral drug resistance among drug naive persons is important in developing sentinel surveillance policies. This study was conducted to determine the prevalence of antiretroviral drug resistance mutations among drug naive HIV infected individuals attending a voluntary testing and counseling centre at the Mankweng Hospital in northeastern South Africa. A total of 79 drug naive HIV positive individuals were sequentially recruited between February 2008 and December 2008. Drug resistance mutations were determined using the calibrated population resistance tool. Viral DNA was obtained from 57/79 (72%) individuals. Reliable nucleotide sequences were obtained for 54 *reverse transcriptase* (RT) and 54 *protease* (PR) gene regions from 54 individuals. Overall, 5 sequences (9.3% %) harboured drug resistance mutations (95% confidence interval 0 to 16.99%). Four of these (7.4%) were nucleoside *reverse transcriptase* inhibitor mutations (D67G, D67E, T69D, and T215Y) and one (1.9%) was a *protease* inhibitor mutation (M46I). No major non-nucleoside *reverse transcriptase* resistance mutation was detected. Phylogenetic analysis of the partial *pol* sequences showed that 52/54 (96.3%) of the viruses were HIV-1 subtype C. One isolate (08MB08ZA) was HIV-1 subtype B, while another (08MB26ZA) is related to HIV-1 subtype J. HIV-1 subtype recombination analysis with REGA assigned the *pol* sequence to HIV subtype J (11_cpx) with a bootstrap value of 75%. The prevalence of drug resistance mutations observed in the studied population is moderately high according to the World Health Organization

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classification for surveillance transmitted resistance mutations, and relatively higher than previously reported from other parts of South Africa. In addition, this is apparently the first report of an HIV-1 subtype J-like virus from northeastern South Africa.

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3.1. INTRODUCTION

Antiretroviral treatment rollout in resource-poor settings such as in many African countries is often associated with lack of virological monitoring (Gupta *et al.*, 2009). Some patients develop extensive ARV drug resistance as a consequence of this. The efficacy of first-line regimens becomes compromised and this could eventually lead to poor responses to second-line regimens (Gupta *et al.*, 2009). In the developed world, the use of HAART has been accompanied by frequent virologic monitoring; including viral load and drug resistance testing to guide treatment (Gupta and Pillay, 2007; Richman *et al.*, 2009).

Laboratory equipment necessary for monitoring patients on HAART are lacking in most resource-poor countries. More financial resources are being tailored towards the prevention and treatment of diseases, whereas almost no funding is available to improve the existing laboratory structure. Patient management becomes difficult and inefficient under such circumstances and this is affecting HAART roll-out. Viral load, ARV drug resistance testing and CD4+ monitoring is not yet the standard of care. Hence, many patients stay on standard first line therapy for longer periods, with a high risk of developing ARV drug resistance and subsequent treatment failure (Shafer *et al.*, 2007; Gatanaga *et al.*, 2007; Turner and Wainberg, 2006). Other setbacks to laboratory monitoring are the lack of adequately trained laboratory personnel, the high cost of commercial drug resistance, viral load assays and CD4+ tests. Even where CD4+ tests are largely available, regular supply of reagents remains a challenge (Stephenson *et al.*, 2002)

The prevalence of HIV/AIDS in South Africa is quite high. The South African National HIV and Syphilis survey conducted in 2007 estimated the prevalence to be about 29.3% (Department of Health, 2009). In 2004, the South African ARV drug regimen guideline recommended 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. The second-line therapy comprised of

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Zidovudine, Didanosine and Lopinavir/Ritonavir (Department of Health, 2004). Currently, the revised regimen is comprised of Tenofovir, Lamivudine and Efavirenz/nevirapine. Stavudine is still recommended where side effects are tolerated (Department of Health, 2010).

It is estimated that 500,000 individuals (adults and children) were receiving ARV in South Africa by mid 2008, with about 34,000 of this in Limpopo Province (northern South Africa) (Adam and Johnson, 2009). Low prevalence of ARV drug resistance has been reported in some parts of South Africa (Pillay *et al.,* 2008; Orrell *et al.,* 2009). However, despite the low prevalence, it is still necessary to monitor the prevalence of drug resistance in drug naive populations, particularly in regions such as the north east of South Africa where such data is limited. The only resistance data available for northern South Africa were obtained from samples collected in 2001 from Waterberg district (Bessong *et al.,* 2005; Bessong *et al.,* 2006) before the availability of ARV through the public health sector in 2004. These studies showed the absence of resistance mutations in the samples studied. In addition, since 2004 access to ARV has escalated in northern South Africa (from an estimated 300 in 2001 to 34,000 in 2008) (Orrell *et al.,* 2009); and it is important to address drug resistance development through periodic studies.

3.1.1. Objectives of the study

The main objective of this study was to determine the prevalence of antiretroviral drug resistance mutations and HIV-1 subtypes among newly diagnosed drug-naive persons visiting a voluntary testing and counselling centre in north-eastern , South Africa.

The specific objectives of this study were:

1. To determine the types of genetic drug resistance mutations among drug naive HIV-1 subjects in the Capricorn district.
2. To phylogenetically determine the prevalence of HIV-1 subtypes in the Capricorn district of South Africa.
3. To determine the intragenetic diversity of the *protease* and *reverse transcriptase* genes in HIV-1 infected individuals in Capricorn district, South Africa.

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4. To determine the recombination patterns (if any) of HIV-1 viruses from Capricorn district, Limpopo province, South Africa.

3.2. MATERIALS AND METHODS

3.2.1. Ethical approval

Approval of the study protocol was obtained from the Health, Safety, and Research Ethics Committee of the University of Venda, South Africa. Permission to conduct the study was obtained from the Limpopo Provincial Department of Health, and the Mankweng Hospital. Signed informed consent was obtained from each study participant.

3.2.2. Study site

Study participants were recruited from the Phela O. Phedishe (POP) HIV clinic in Mankweng (Figure 3.1). This clinic is part of the Polokwane-Mankweng hospital complex and is a referral hospital situated in Mankweng township, East of Polokwane city (Capricon district of the Limpopo province). The Phela O. Phedishe HIV clinic has nineteen feeder clinics situated in nearby villages. This clinic has about 2500 patients and some of them come from neighbouring countries such as Zimbabwe, Botswana, Lesotho, Mozambique and Zambia. According to the South African National antenatal sentinel HIV and Syphilis survey conducted in 2008, the estimated HIV prevalence in the Capricorn district was 24.2% (Department of Health, 2009).

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Figure 3.1: Map showing Limpopo province and location of the study site – Mankweng, indicated by the arrow. (www.routes.co.za/lp/Bela Bela/) (Accessed 10-3-2011).

3.2.3. Study population, sample collection and plasma preparation

A total of 79 individuals without prior exposure to ARV and who were newly diagnosed for HIV antibodies at the HIV Voluntary Counseling and Testing Clinic in Mankweng Hospital, South Africa were recruited sequentially between February 2008 and December 2008. Participants were apparently healthy individuals at the time of HIV testing. The Mankweng Hospital is a component of the Polokwane-Mankweng Hospital Complex, a regional referral hospital for the Limpopo Province, Northern South Africa. The VCT centre in Mankweng Hospital caters for 19 health centres in the rural precincts of Mankweng in the Capricorn district.

Blood and demographic data collection, plasma preparation and storage were carried out as described in section 2.2.3. Data on viral load and CD4+ count were not available.

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3.2.4. Viral RNA isolation, RT-PCR and nested PCR.

This was performed as described in section 2.2.4.

3.2.5. Detection of amplified products (agarose gel electrophoresis).

This was performed as described in section 2.2.5.

3.2.6. Purification of PCR product, sequencing and editing

This was performed as described in section 2.2.6.

3.2.7. Viral genetic subtyping

HIV subtyping was done by phylogenetic analysis. Polymerase sequences (880 bp) comprising the complete *protease* and partial *reverse transcriptase* genes of test viruses were aligned using ClustalX with representative subtype (year 2000) reference sequences (group M subtypes A-D, F-H, J and K, excluding recombinant sequences) obtained from GenBank. Previously described sequences from Limpopo Province were included in the analysis. Neighbor joining phylogenetic trees were generated with the PHYLIP programme, making use of the maximum likelihood model to take care of differences in the transition and transversion rates. Trees were rooted with an HIV-1 group O strain (L20571). The reliability of the trees was assessed by a bootstrapping of 1000 replicates. REGA HIV-1 subtyping tool version 2.0 was used to analyze the sequences for recombination. The tool employs phylogenetic analysis and bootscanning methods to assign genetic subtypes and recombination patterns respectively (http://www.bioafrica.net/rega-genotype/html/subtypinghiv.html). Predicted amino acids of the PR and RT genes were aligned using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Consensus amino acid sequences of the PR and RT regions were created and then compared to the global subtype C and the global subtype B consensus sequences obtained from GenBank (year 2000). The mean genetic distances among the PR and RT sequences were determined by the Kimura 2- parameter model.

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3.2.8. Genetic drug resistance determination

Drug resistance mutations were examined according to the calibrated population resistance (CPR) tool version 5.0 beta contained in the Stanford HIV Drug Resistance database (http://cpr.stanford.edu/cpr/servlet/CPR). The CPR tool analyzes submitted sequences for resistance mutations based on the World Health Organization (WHO) surveillance transmitted drug resistance mutation list of 2009 (Bennett *et al.*, 2009). In addition, the tool also detects unusual, bad and hypermutated sequences and performs subtype assignment.

3.3. RESULTS

3.3.1. Study subject demographics

A total of 79 HIV positive individuals were sequentially recruited for this study. The mean age was 38.5 years (18 – 59 years). The proportion of women and men was 73.4% and 25% respectively. 70.9% of the study subjects were single and 22.8% were married. The most important risk factor for HIV transmission based on responses to questionnaires was sexual intercourse (97.5%). However, the questionnaire was not designed to determine whether it was heterosexual or homosexual. In 89.9% of the participants, the most probable place of HIV infection was South Africa. About 47% of the study subjects reported to have been infected in 2008, while the rest estimated that they were infected between 2000 and 2007. The highest level of education for most of the participants was grade 6 and above (79.7%), but neither university students nor graduates were recorded within the participants. Details of the demographics and socio-economic characteristics of the study population are shown in Table 3.1.

3.3.2. Viral DNA amplification and drug resistance mutations

Viral DNA was obtained for 57 out of 79 (72%) individuals. Reliable nucleotide sequences were obtained for 54 RT genes and 54 PR genes, on which genetic subtyping and drug resistance mutational analyses were performed. Using the CPR analysis for surveillance drug resistance mutations, 5 of the 54 sequences (9.3%) had resistance mutations (0 to 16.99, 95% confidence interval). These include 4 NRTI, (D67E, D67G,

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T69D, T215Y) harboured by sequences 08MB38ZA, 08MB26ZA, 08MB27ZA and 08MB29ZA respectively, and 1 PI resistance mutation (M46I) observed in sequence 08MB62ZA. No NNRTI mutation was detected. No sequence harboured more than 1 resistance mutation.

3.3.3. HIV genetic subtypes

Phylogenetic analysis of the partial *pol* sequences showed that 52/54 (96.3%) of the viruses were HIV-1 subtype C. One isolate (08MB08ZA) was HIV-1 subtype B; while isolate 08MB26ZA was observed to be related to HIV-1 subtype J reference sequences (Figure 3.2). Bootscanning analysis of 08MB26ZA with the REGA HIV subtyping tool showed that its *pol* gene has a mosaic structure and related to CRF_11 (Figure 3.3). The STAR genotyping feature within the CRP analysis also assigned 08MB26ZA to CRF_11. Furthermore, this sample harboured the only D67G NRTI mutation observed in the study. Pure subtypes were assigned to all the other sequences after bootscanning analysis. Considering the first 300 amino acids in the RT gene, sequence alignment showed that the consensus of the test viruses was identical to the global subtype C consensus. It differed from the global subtype B consensus at 18 positions (V35T, E36A, T39E, S48T, K122E, D123G, K173A, D177E, T200A, Q207E, R211K, V245Q, A272P, K277R, T286A, E291D, V292I and I293V) (Figures 3.4 and 3.5). Amino acid alignment of the PR gene showed that the test consensus was identical to the global subtype C consensus; except at position T13I. It differed from the global subtype B consensus at 8 positions (T12S, I15V, L19I, M36I, R41K, H69K, L89M, and I93L). Mean genetic distance for the PR sequences ranged from 0.0239 to 0.2519 and 0.0273 to 0.1527 for the RT sequences.

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Table 3.1: Demographic and socio-economic characteristics of the study population.

Characteristics	Patients (N= 79) Number (%)
Sex	
Males	20 (25)
Females	58 (73.4)
Data not available	01 (1.3)
Marital status	
Single	56 (70.9)
Married	18 (22.8)
Widow	01 (1.3)
Divorced	02 (2.5)
Data not available	02 (2.5)
Probable place of infection	
South Africa	71 (89.9)
Other countries	00 (0)
Data not available	08 (10.1)
Risk factor for transmission	
Sex	77 (97.5)
Data not available	02 (2.5)
Probable Year of infection	
Before 2000	00 (0)
2000 – 2007	40 (50.6)
2008 (recently infected)	36 (47.4)
Data not available	03 (3.8)
Household income (SAR)	
<R1000	59 (75)
R1000 – R5000	18 (22.8)
>R5000	00 (0)
Data not available	02 (2.5)
Number of dependents	
1-5 persons	58 (73.4)
≥ 6 persons	04 (5.0)
Data not available	17 (21.5)
Highest level of education	
Not educated	02 (2.5)
≤ Grade 5	04 (5.1)
Grade 6 & above	63 (79.7)
University	06 (7.6)
Data not available	04 (5.1)

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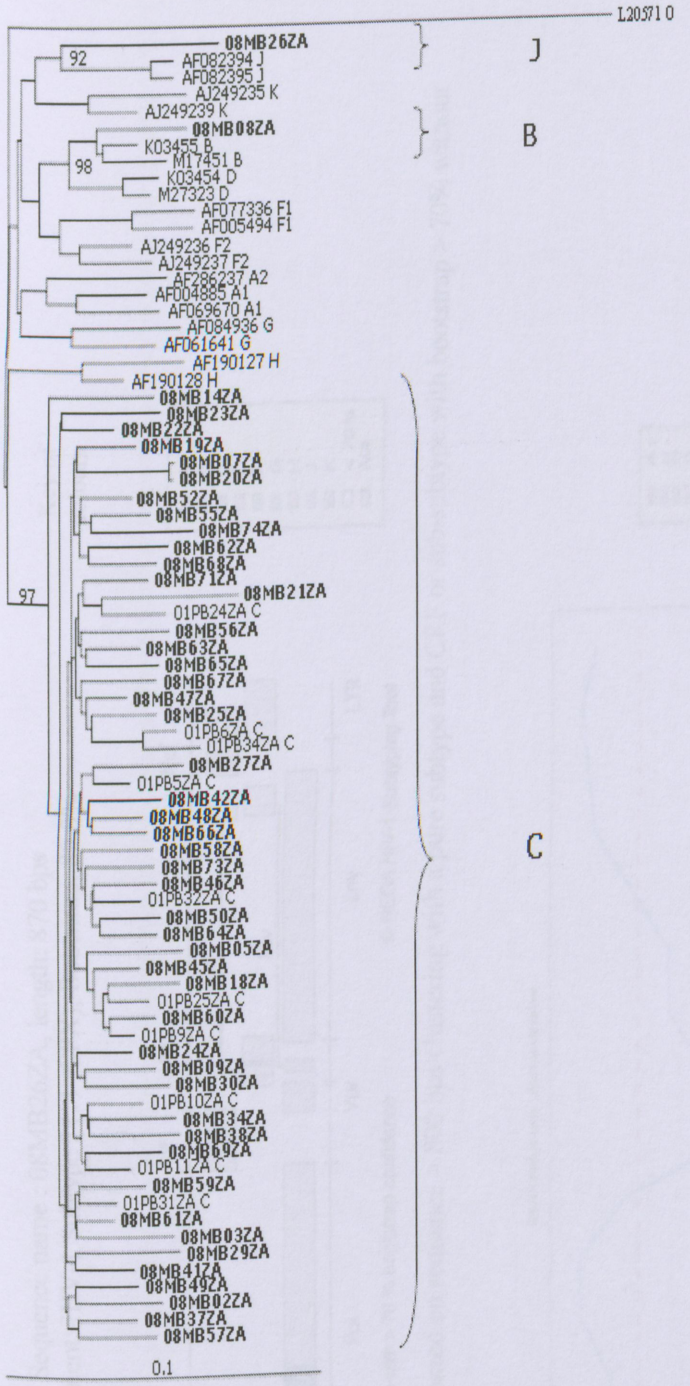

Figure 3.2: Phylogenetic analysis of partial HIV *pol* sequences from drug naive individuals from northeastern South Africa. This representative tree shows most of the test sequences (in bold face) clustering and intermingling with reference HIV-1 subtype C sequences previously obtained from northeastern South Africa. This shows that majority of the test sequences are HIV-1 subtype C sequences on the *pol* gene. One sequence clustered with subtype B, and another is closely related to subtype J. The tree was rooted with an HIV-1 group O sequence.

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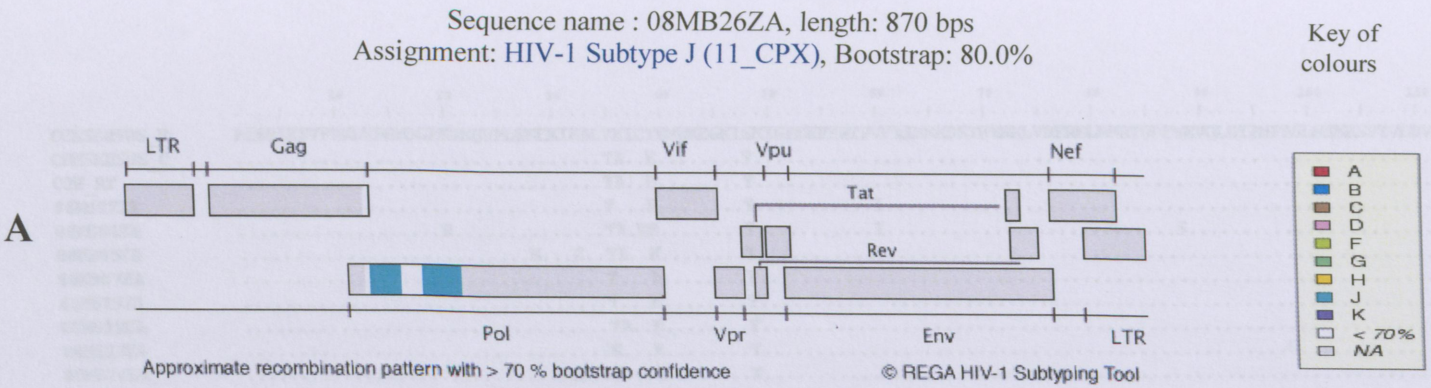

A

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Motivation: Subtype assigned based on sequence > 800 bps clustering with a pure subtype and CRF or sub-subtype with bootstrap > 70% without recombination in the bootscan.

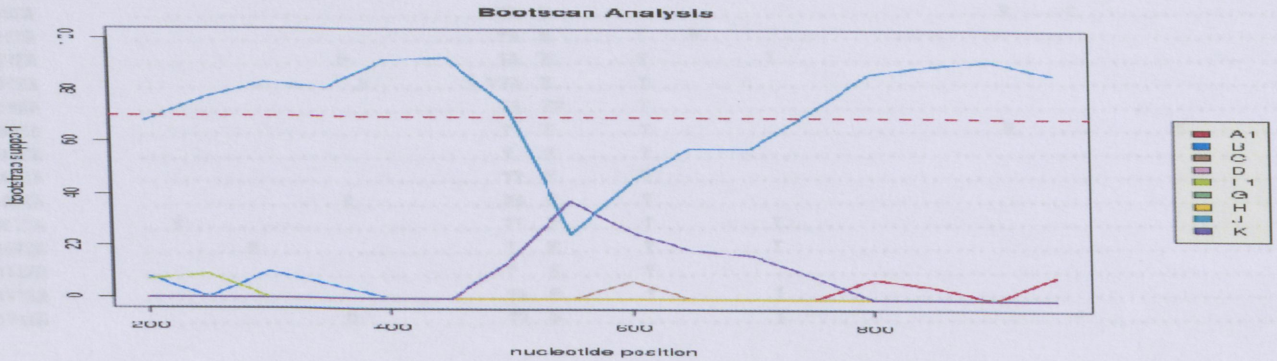

Figure 3.1: Subtype assignment and HIV-1 subtype recombination analysis of 08MB26ZA (870bp).
Panel A shows the subtype assignments of the partial *pol* based on phylogenetic analysis, while panel B shows the recombinant profile based on bootscanning analysis. The combined analysis shows that the isolate is an HIV-1 subtype J-like virus.

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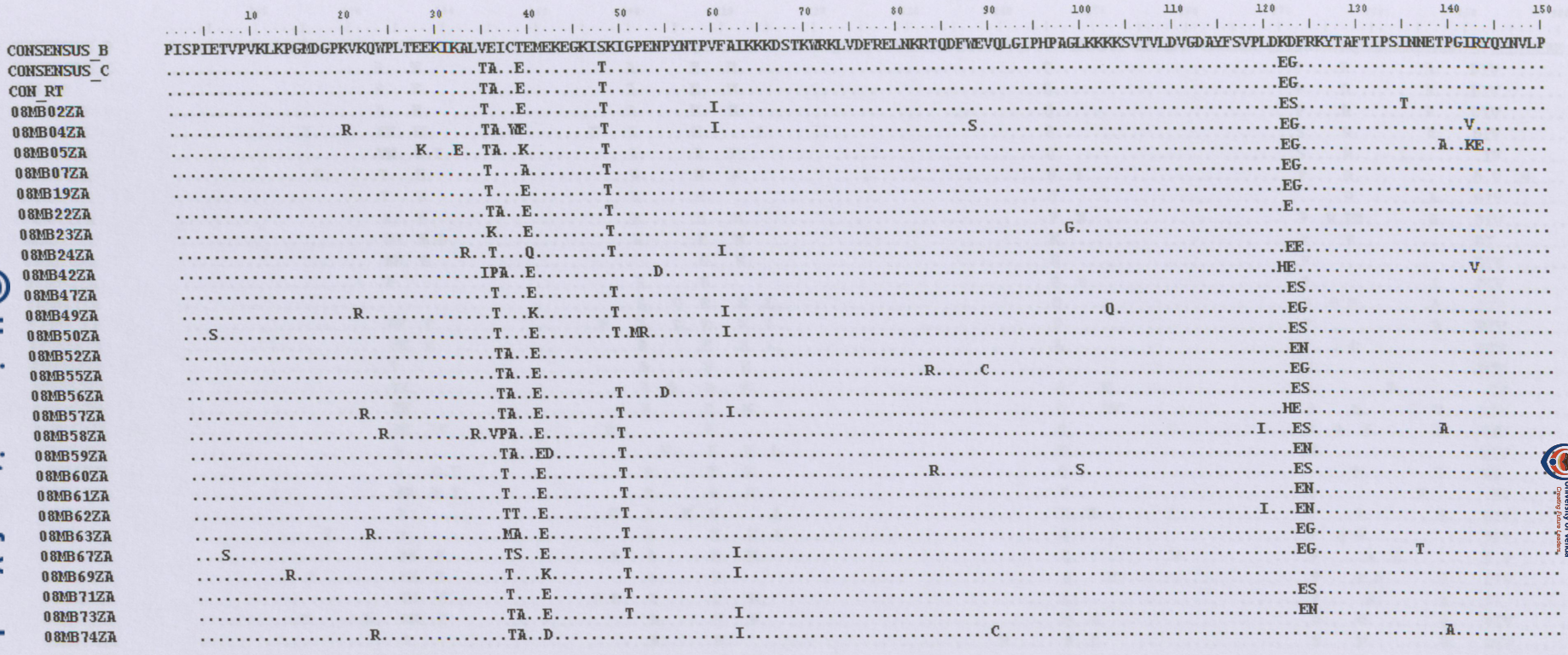

Figure 3.4: Amino acid alignment of RT sequences (residues 1-150). The test consensus was generated from the HIV database (GenBank) using some 27 viruses from this study. The global subtype B and C consensuses were obtained also from HIV database program. The entire consensuses were aligned using BioEdith software. All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid (if any) is denoted by an X. The test consesnsus was identical to the global subtype C consensus. It differed from the global subtype B consensus at 18 positions (V35T, E36A, T39E, S48T, K122E, D123G, K173A, D177E, T200A, Q207E, R211K, V245Q, A272P, K277R, T286A, E291D, V292I and I293V)

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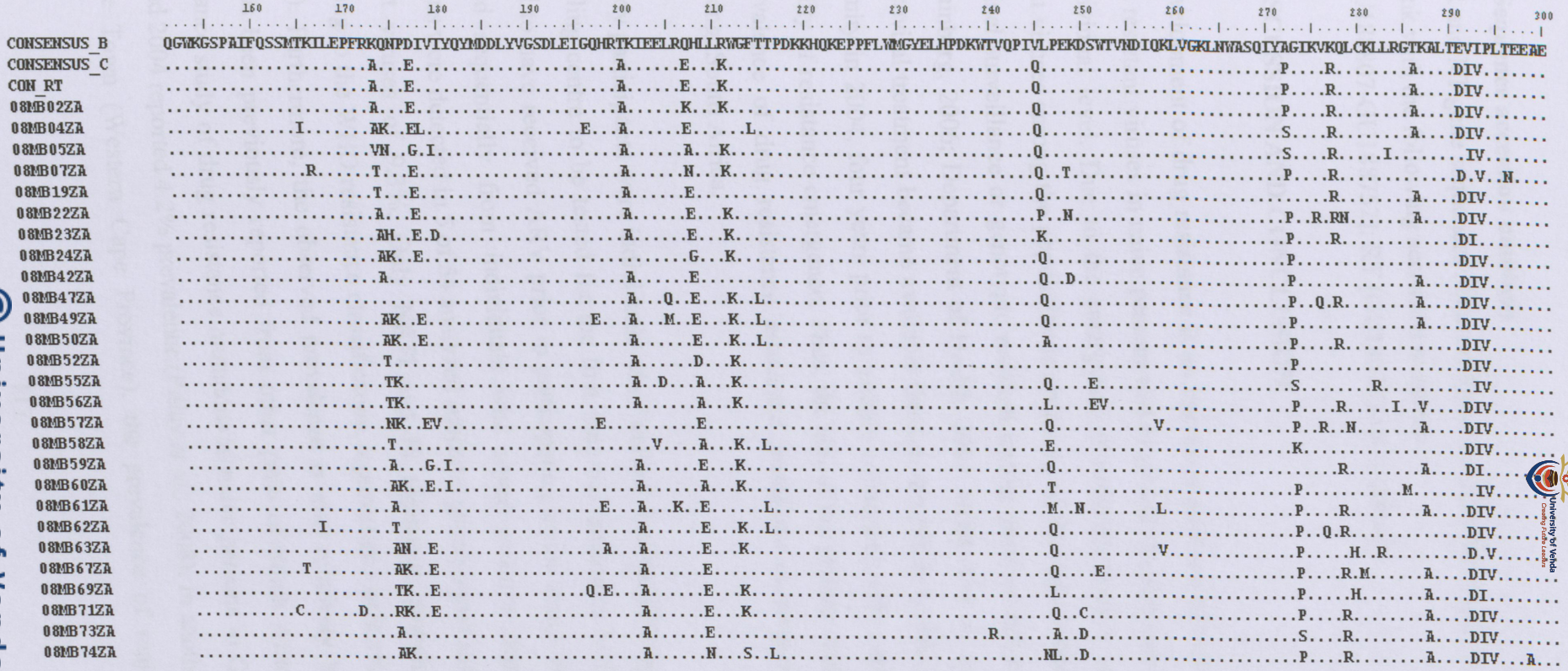

Figure 3.5: Amino acid alignment of RT sequences (residues 151-300). The test consensus was generated from the HIV database (GenBank) using some 43 viruses from this study. The global subtype B and C consensuses were obtained also from HIV database program. The entire consensuses were aligned using BioEdith software. All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid (if any) is denoted by an X.

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3.3.4. Sequence accession numbers

The PR and RT gene sequences obtained from this work have been submitted to GenBank with the following accession numbers:
PR [GU188807-GU188752]; RT [GU188753-GU188806].

3.4. DISCUSSION AND CONCLUSION

The development of drug resistance is an important attribute of HIV biology. Detection of drug resistant viruses in naive persons makes patient management difficult at least on the individual level. Due to the emergence of resistant viruses, surveillance of drug resistant viruses among the population is necessary as this may inform the establishment of sentinel surveillance or genotypic resistant testing prior to therapy initiation (Turner and Wainberg, 2006; Department of Health, 2004; Bennett *et al.,* 2008; Taiwo, 2008). Antiretroviral treatment became available through the public health sector at Mankweng community in 2004, four years prior to sample collection, within which time there is a possibility of resistance emergence. Thus, the aim of the present study was to determine the prevalence of drug resistance associated mutations in drug-naive individuals in Mankweng, South Africa.

The study participants were individuals who 'walked' into the HIV voluntary testing and counselling centre to be tested for the first time for antibodies to HIV. None of them claimed to have received ARV prior to participating in the study. Blood samples were collected sequentially from individuals who tested positive. Resistance associated mutations were detected in 5 of 54 analyzed subjects; giving a prevalence of patients with resistant viruses of 9.3%. Only NRTI and PI resistance mutations were detected. According to the WHO resistance classification, a prevalence of 9.3% is moderately high (5-15%). Furthermore, the observed prevalence is also relatively higher compared to what has been previously reported from other parts of South Africa. For example, a surveillance study of drug resistance mutations in naive persons in Gauteng Province in 2002 and 2004 reported 4.2% prevalence (Pillay *et al.,* 2008). In another study carried out in Cape Town (Western Cape Province), the prevalence of major drug resistance

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mutations in naive patients was 2.5%. Similarly, a prevalence of 2.3% has been reported from the Free State (Huang *et al.,* 2009). Low levels (<5%) or the absence of drug resistance mutations have been reported in other southern African countries such as Zambia (Handema *et al.,* 2003), and Malawi (Kamoto *et al.,* 2008). The prevalence observed in the present study is similar to what has been reported in some high-income countries with more than a decade of ART use. Some of these countries include Britain, where a 14.2% resistance level (Cane *et al.,* 2006), and the United States where up to 25% has been documented (Smith *et al.,* 2007). Unlike in high-income countries, low prevalence of drug resistance mutations are expected in low-income countries in the early phase of treatment roll-out because most patients are starting therapy on highly potent regimens. In high-income countries, ART use began with resistance associated monotherapy and one-class dual therapy (Fischl *et al.,* 1987).

In the present study, all the observed resistance mutations were detected in different individuals, with 2008 being the probably year of infection. Substitutions D67E, D67G, and T69D were detected in 3 women, while T215Y and M46I were detected in 2 men. Several polymorphisms such as K20R, M36I and I93L common in HIV-1 subtype C viruses and associated with drug resistance site were also observed and has been previously reported (Bessong *et al.,* 2005; Bessong *et al.,* 2006). According to the WHO drug resistance algorithm, substitution D67E has the potential to cause low level resistance to Zidovudine, Stavudine, and Didanosine, while D67G causes low level resistance to Zidovudine, and a potential for low level resistance to Abacavir, Stavudine, and Didanosine. In the same vein, T69D causes low level resistance to Didanosine, with a potential for low level resistance to Stavudine. Substitution T215Y causes intermediate resistance to Zidovudine and Stavudine, and low level resistance to Abacavir, Didanosine, and Tenofovir. The only *protease* inhibitor mutation observed, M46I, causes intermediate resistance to Nelfinavir, with a potential for low level resistance to Ritonavir boosted doses of Atazanavir, Fosamprenavir, Indinavir, and Lopinavir.

At the time of the current study, a combination regimen of Stavudine, Lamivudine and Efavirenz was the recommended first line regimen, with Nevirapine replacing Efavirenz

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for women of child bearing age. A PI containing regimen is used for children less than 3 years of age and in second line regimen for adults (Department of Health, 2010). It is interesting to note that no non-nucleoside *reverse transcriptase* inhibitor resistant mutations were detected in the present study despite their use. Nevertheless, most of the patients investigated would benefit from the current first line treatment regimen based on their viral genetic profiles. However, it is difficult to predict the spread of the mutations observed and the implications on treatment outcomes at the population level. The resistance mutations described herein were observed after 4 years of the availability of treatment in the public health sector in the study area. It should also be noted that although access to treatment in the public sector began in 2004, antiretrovirals were available through the private sector prior to 2004 albeit, at a higher cost and limited access.

Accepting that the study participants had no prior exposure to antiretrovirals, the mutations may have been acquired from transmitted viruses. Although the participants were apparently healthy, the absence of viral load and CD4+ count measurements does not allow a good estimation of their immunologic and virologic state. Mutations may revert over time in the absence of drug pressure, leading to a possible underestimation of transmitted resistance viruses. About 25% of the samples could not be amplified probably because of very low viral loads, since most of the patients were asymptomatic.

The identification of subtype C viruses as the predominant subtype is in agreement with previous genotyping studies in northern South Africa (Bessong *et al.,* 2005; Bessong *et al.,* 2006). However, the presence of subtype B, and CRF_11 related viruses have not been previously reported. Since only the partial *pol* gene of the CRF_11-like sample was analyzed, a full-length genomic analysis of the virus would be required to reveal its complete mosaic nature.

Population based sequencing was used in this study and this could have however, contributed to an underestimation of drug resistance prevalence. Patients may harbour populations of minor resistance viruses which are usually not detected by population

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sequencing. This prevalence study also used chronically infected patients, which could mean that resistance acquired may have disappeared over time and this might have also led to an underestimation of the results. Furthermore, HIV drug prevalence in this population of newly diagnosed persons does not reflect transmitted resistance within the sampling period (February 2008 – December 2008). A direct estimate of the incidence could be obtained by restricting the focus to recently infected persons.

In conclusion, a moderately high prevalence of resistance mutations was observed in Mankweng, northern South Africa. Apparently, this is the first time that genetic drug resistance data is presented for Capricorn district. According to WHO recommendations, this moderate drug resistance prevalence may necessitate routine drug resistance testing for all persons newly diagnosed with HIV or all persons beginning HIV treatment in this community (WHO, 2003; Myatt and Bennett, 2008). This level also requires a review of the treatment program monitoring data for this community and the investigation of potential problems with regards to factors such as: continuous access to services, drug supply, drug quality, prescribing practice, compliance toxicity and/or adverse events, drug sharing and treatment failures. However, resistance in this population does not reflect current transmission of resistant HIV, because many newly diagnosed persons have been infected many years prior to diagnosis. Subsequent surveillance studies are needed to confirm the findings of this report and to generate data relevant to sentinel surveillance policy, and treatment initiation guidelines.

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CHAPTER FOUR

PREVALENCE OF GENETIC DRUG RESISTANCE IN A COHORT OF HIV INFECTED PATIENTS ENTERING ANTIRETROVIRAL TREATMENT PROGRAMMES AT TWO SITES IN NORTHERN SOUTH AFRICA

ABSTRACT

South Africa has one of the highest burdens of HIV infections, and access to treatment is expanding. Infection with drug resistant viruses influences the outcome of antiretroviral treatment (ART). This study was carried out to determine genetic resistance profile in a cohort of patients prior to therapy initiation at two treatment sites in northern South Africa. These two communities were among the first to benefit from antiretrovirals. In northern South Africa, resistance data are limited and resistance testing prior to therapy is not done.

One hundred and twelve antiretroviral drug naive HIV positive individuals who qualified and were preparing to enter treatment programmes at Bela Bela and Mankweng sites were recruited consecutively between February 2008 and December 2008. Reliable sequences were obtained for eighty *protease* (PR) and *reverse transcriptase* (RT) genes. These gene sequences were analyzed for genetic drug resistance using the calibrated population resistance (CPR) tool for transmitted drug resistance. Viral genetic subtypes were determined by phylogenetic analysis and recombination sequences by bootscanning.

Two primary RT drug resistance mutations (K103N and M41L) were detected in 2 different individuals (2.5%; 95%CI: 0077-0.0863). Twenty three sequences (29%) haboured at least one secondary mutation in the RT gene; while all had at least one minor mutation in the PR gene. Phylogenetic analysis of the PR and RT genes showed that 79 out of 80 viruses were HIV-1 subtype C, and one virus was an A1/C recombinant.

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The observations suggest that after 4 and 8 years of access to ART in Mankweng and the Bela Bela communities respectively, drug resistance mutations in the naive population is low, and resistance testing prior to therapy initiation, at least at the population level, may not be required. In addition, the identification of an A1/C recombinant may be an indication of the introduction of complex viruses in northeastern South Africa.

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4.1. INTRODUCTION

The HIV/AIDS pandemic is a major public health crisis. An estimated 33.0 million adults and children are infected with HIV worldwide. Sub-Saharan Africa is one of the regions that have been hardest hit by the pandemic; with an estimated 22.5 million adults and children living with the virus (UNAIDS, 2010). Access to ART in South Africa has expanded since 2004 when treatment became available in the public health sector.

Prior to 2010, the South African national antiretroviral treatment guideline recommended a Stavudine, Lamivudine, Efavirenz combination as the first-line regimen for adults who have never been exposed to antiretrovirals, with nevirapine replacing efavirenz for women of child bearing age. The second-line regimen comprised Zidovudine, Didanosine and Lopinavir/Ritonavir. In 2010, Tenofovir was introduced for those with poor tolerance to Stavudine in the first line regimen. It also recommends eligibility for treatment of pregnant women and patients with HIV/TB co-infection with a CD4+ count of less than 350 cells/mm^3 (Department of Health, 2004; Department of Health, 2010).

Antiretroviral therapy significantly reduces the morbidity and mortality of HIV infected individuals. However, the baseline genotypic drug resistance profile of patients influences the outcome of therapy. Patients with drug resistance have limited therapeutic options and can respond poorly to first-line treatment regimens (Little *et al.*, 2002; Bergroth *et al.*, 2009). The determination of a treatment 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 *et al.*, 2002; Turner *et al.*, 2006; Taiwo *et al.*, 2008). 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. The aim of the present study was to determine the genetic drug resistance mutations in a cohort of patients entering a first line treatment programme in northern South Africa where data on HIV drug resistance is very limited, and where resistance testing prior to treatment initiation is not done. Information on the prevalence of drug resistance could inform policy on the need for

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genotypic resistance testing before initiation of treatment, and influence the policy on drug resistance sentinel surveillance.

4.1.1. Objectives of the study

The main objective of this study was to determine the prevalence of genetic drug resistance in a cohort of HIV infected patients entering antiretroviral treatment programmes at two sites in North-eastern South Africa. The specific objectives of this study were:

1. To determine the baseline genetic drug resistance mutation profile of the patients prior to therapy.
2. To determine the baseline CD4+ and viral load measurements in the cohort.
3. To phylogenetically determine the HIV-1 subtypes.

4.2. MATERIALS AND METHODS

4.2.1. Ethical approval

This was obtained as described in sections 2.2.1 and 3.2.1

4.2.2. Study sites

Study subjects were recruited from the HIV/AIDS Wellness Clinic in Bela Bela and Phela O Phedishe (POP) HIV clinic in Mankweng Hospital - both in the Limpopo Province of South Africa. Bela Bela is located in the Waterberg district, which has an estimated HIV prevalence of 27.5% (Department of Health, 2009). It is a holiday resort with hotels and camping sites. It is also a stop-over point for long distance truck drivers who patronize sex workers attracted to the luxury facilities of the area (Ndjeka and Manhaeve, 2006). The Bela Bela HIV Wellness Clinic receives patients from the urban and rural sectors of the area. The Clinic is run by the HIV/AIDS Prevention Group (HAPG) - a non-governmental organization established in 1996. The POP HIV clinic is a component of the Polokwane-Mankweng Hospital Complex. The HIV Clinic has nineteen feeder clinics situated in nearby villages. Mankweng is located in the Capricorn

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district which has an estimated HIV prevalence of 24.2% (Department of Health, 2009). At the time of sample collection, antiretroviral treatment has been available to the Mankweng and Bela Bela communities for 4 and 8 years respectively. In the intervening time, there is a possibility of resistance emergence and transmission to naive populations in these two sites. There is a paucity of data from these sites on the prevalence of drug resistance mutations.

4.2.3. Study population, sample collection and plasma preparation

One hundred and twelve antiretroviral drug naive HIV positive individuals who qualified and were preparing to enter treatment programmes at Bela Bela and Mankweng sites were recruited consecutively between February 2008 and December 2008. Blood samples were collected from all study participants before the initiation of treatment. Viral load and CD4+ counts were determined by branched DNA technology and flow cytometry (Beckon Dickinson), respectively. Demographic data collection, plasma preparation and storage were carried out as described in section 2.2.3.

4.2.4. Viral RNA isolation, RT-PCR and nested PCR.

This was performed as described in section 2.2.4.

4.2.5. Detection of amplified products (agarose gel electrophoresis).

This was performed as described in section 2.2.5.

4.2.6. Purification of PCR product, sequencing and editing

This was performed as described in section 2.2.6.

4.2.7. Viral genetic subtyping and recombination analysis

HIV subtyping was performed as described in section 2.2.7. Putative recombinant *pol* sequences were analyzed by the REGA HIV-1 subtyping Tool version 2.0 (www.bioafrica.net/subtypetool/html/), and the recombinant identification program (RIP) (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html) to confirm recombination. The REGA tool employs bootscanning while RIP uses the similarity plot to identify

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recombination patterns. Consensus sequences of the PR and RT were aligned using BioEdit (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). With the global subtypes B and C consensus sequences (year 2000) obtained from Genbank, and compared for similarities. This comparison is necessary because traditional HIV drugs were designed using a subtype B (the prevalent subtype in North America and Europe) backbone. However, HIV-1 subtype C is driving the epidemic worldwide and is also the predominant subtype in South Africa. Significant amino acid substitutions in the global subtype C concensus could have implications in diagnostics, treatment and vaccine development.

4.2.8. Genetic drug resistance determination

This was performed as described in section 3.2.8.

4.3. RESULTS

4.3.1. Study subject demographics

One hundred and twelve patients entering the ART programme were recruited for the study. The mean age was 40.5 years (range 22 – 59 years). The proportion of women and men was 78/112 (69.6%) and 34/112 (30.4%) respectively. Seventy of the respondents (62.5%) were single compared to 26 (23.2%) who were married. The most important risk factor for HIV transmission was unprotected sex (98.2%). However, the questionnaire was not designed to capture heterosexual or homosexual orientations. In 88.4% of the participants, the most probable place of HIV infection was South Africa and a further 24/112 (21.4%) reported to have been probably infected in 2008. Probably older infections (2000 – 2007) were reported in 65.2% of the subjects. The mean viral load was 5.4 Log10 copies/ml (range 3.0 - 5.7). The mean CD4+ count was 138 cells/mm^3 (interquartile range 51-197). Twenty six patients (23.2%) were under treatment for tuberculosis. Overall, 1/112 (0.9%), 11/112 (9.8%), 48/112 (42.9%) and 52/112 (46.4%) were classified as AIDS stage I, II, III and IV respectively according to the WHO system. The average weight of all the subjects was 57.5 kg (range 36.5 – 78.5). Details of the characteristics of the study participants are shown in Table 4.1.

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4.3.2. Viral DNA amplification and sequencing

Viral DNA was obtained for 93 out of 112 samples (83%), and all viral DNA was sequenced on both strands by population based sequencing. Reliable nucleotide sequences were obtained for 80 PR and RT genes. Nineteen DNA samples could not be amplified; while 13 nucleotide sequences were eliminated because they were of poor quality.

4.3.3. Drug resistance mutations, HIV subtypes, CD4+ counts and Viral Load

Two primary drug resistance mutations, M41L (an NRTI mutation) and K103N (an NNRTI mutation) were each detected in two different female subjects giving an overall prevalence of 2.5% (95% CI: 0.0077-0.0863). K103N mutation was detected in 08MC02ZA (Mankweng treatment site); while M41L mutation was detected in 08BBCR29ZA (Bela Bela treatment site). No major PR resistance mutation was found among the analyzed sequences. The frequencies of the observed PR and RT resistance associated mutations for the entire study population are depicted in Figure 4.1. Several amino acid substitutions at resistance associated sites and polymorphisms were also observed particularly in the PR gene. Details of the resistance associated mutations from the two treatment sites are shown in Tables 4.2 and 4.3.

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Phlyogenetic analysis of the PR and RT sequences showed that 79 out of 80 sequences were HIV subtype C (Figures 4.2 and 4.3). One virus (08BBCR06ZA) from Bela Bela clustered with pure A1 subtypes on the PR gene and pure C subtypes on the RT gene. Further analysis of the partial *pol* of 08BBCR06ZA (comprising the complete PR and 900 nucleotides of the RT) with recombination identification programs showed that the *pol* gene has a mosaic structure consisting of A1 sequences alternating with C sequences confirming that the virus is a recombinant on the gene analyzed (Figures 4.4A and B). Analyzing the first 300 amino acids in the RT gene, sequence alignment showed that the consensus of the test sequences was identical to the global subtype C consensus; except at four positions (A36E, G23N, Q174K and R277K). It differed from the global subtype B consensus at 17 positions (V35T, T39E, S48T, K122E, D123G, K173A, D177E, T200A, Q207E, R211K, V245Q, A272P, K277R, T286A, E291D, V292I, and I293V). Alignment

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on the PR gene showed that the consensus was identical to the global subtype C consensus; except at position L63P. It differed from the global subtype B consensus at 9 positions (T12S, I15V, L19I, M36I, R41K, L63P, H69K, L89M, and I93L) (figures 4.5 and 4.6). Mean genetic distance for the PR sequences ranged from 0.0272 to 0.1311; and 0.0978 to 0.1014 for the RT sequences. This indicates that there is very little genetic difference between the test viruses.

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Table 4.1: Characteristics of a cohort of patients entering antiretroval treatment programme in Bela and Mankweng, Limpopo Province, South Africa.

Characteristics	Patients (n= 112) (%)
Sex	
Males	34 (30.3)
Females	78 (69.7)
Marital status	
Single	70 (62.5)
Married	26 (23.2)
Widow	03 (2.7)
Divorced	04 (3.6)
Data not available	09 (8.0)
Probable place of infection	
South Africa	99 (88.4)
Other countries	01 (0.9)
Data not available	12 (10.7)
Risk factor for transmission	
Unprotected Sex	110 (98.2)
Data not available	02 (1.8)
Probable Year of infection	
Before 2000	14 (12.5)
2000 – 2007	73 (65.2)
2008	24 (21.4)
Data not available	01 (0.9)
TB Treatment	
Yes	26 (23.2)
No	77 (68.8)
Data not available	09 (8.0)
WHO AIDS classification	
Stage I	01(0.9)
Stage II	11(9.8)
Stage III	48 (42.9)
Stage IV	52 (46.4)

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Table 4.2: Drug resistance related mutations, HIV-1 subtypes, CD4+ counts and viral load measurements in a cohort of patients entering an antiretroviral treatment programme in 2008 in Bela Bela, Northeastern South Africa

	Primary or major drug resistance mutations		Secondary or minor drug resistance mutations		HIV-1 subtype	CD4+ + count (cells/mm³)	Viral Load (log 10 copies/ml)
Sample ID	RTI	PI	RTI	PI			
08BBCR01ZA			E118I, E138S	L10I,M36I, L89M, I93L	C	85	5.69897
08BBCR04ZA				L63P,L89M ,I93L	C	26	4.634779
08BBCR05ZA			V90I	M36I,I93L	C	26	4.634779
08BBCR06ZA				M36I, L89M	A1/C*	116	5.391292
08BBCR07ZA				M36I, L63P,L89M,I93L	C	38	4.779863
08BBCR13ZA				M36I, L63P,L89M,I93L	C	109	5.49578
08BBCR14ZA				K20R,L89M, L63P,I93L	C	57	5.235031
08BBCR15ZA			V118I, E138A, V117D	K20R,M36I, L63P,L89M,I93L	C	128	5.099201
08BBCR16ZA				K20R,M36I, L63P,L89M,I93L	C	31	5.016444
08BBCR17ZA				K20R,M36I,L89M,I93L	C	51	5.153406
08BBCR19ZA				M36I, L63P,I93L	C	119	4.539001
08BBCR23ZA				M36I,I93L	C	182	3.827563
08BBCR24ZA			N348S,E138A	K20R,L24F, L63P,L89M,I93L	C	146	3.76812
08BBCR25ZA			V118I,E138A	M36I,L89M,I93L	C	187	5.045659
08BBCR26ZA				K20R,M36I, L63P,L89M,I93L	C	17	5.126807
08BBCR28ZA				M36I, L63P,L89M,I93L	C	1	4.934907
08BBCR29ZA	M41L		E138A,P255A	K20R,M36I,L89M,I93L	C	88	6.875061
08BBCR30ZA				M36I, L63P,L89M,I93L	C	7	5.25654
08BBCR31ZA			V118I, E138A	L23F, L63P,L89M,I93L	C	76	4.248268
08BBCR34ZA				L89M, L63P,I93L	C	472	5.623278
08BBCR36ZA				M36I, L63P,L89M,I93L	C	38	5.622409
08BBCR38ZA				K20R,M36I, I93L	C	46	4.742434
08BBCR39ZA				L89M, L63P,I93L	C	46	4.742434
08BBCR40ZA			M41V	M36I, L63P,L89M,I93L	C	139	4.676876
08BBCR41ZA			E138A	I93L	C	223	3.914237
08BBCR42ZA				M36I,L89M,I93L	C	123	5.106065
08BBCR43ZA				M36I,L89M,I93L	C	328	3.493737
08BBCR45ZA			E138A	M36I, L63P,L89M,I93L	C	106	3.697229
08BBCR47ZA				K20R,M36I,L89M,I93L	C	144	4.929112
08BBCR48ZA				K20R,M36I, L63P,T74S,I93L	C	316	3.671728
08BBCR50ZA				K20R,M36I, L63P,I93L	C	50	5.69897
08BBCR51ZA			E138A	L63P,I93L	C	168	4.776955
08BBCR53ZA				M36I, L63P,L89M,I93L	C	248	3.957559
08BBCR54ZA				M36I,L89M,I93L	C	50	4.800813
08BBCR55ZA				K20R,M36I,L89M,I93L	C	102	4.800813
08BBCR56ZA				M36I, L63P,L89M,T74S,I93L	C	108	5.69897
08BBCR57ZA				K20R,M36I,T74S,L89M,I93L	C	192	5.785693
08BBCR58ZA			V118I,E138A	L63P,L89M,I93L	C	113	5.181878
08BBCR59ZA				K20R,M36I,I93L	C	216	5.181878
08BBCR60ZA				K20R,M36I,L89M,I93L	C	211	4.197418
08BBCR61ZA				M36I, L63P,L89M,I93L	C	44	4.753177

* = *protease/reverse transcriptase* recombinant

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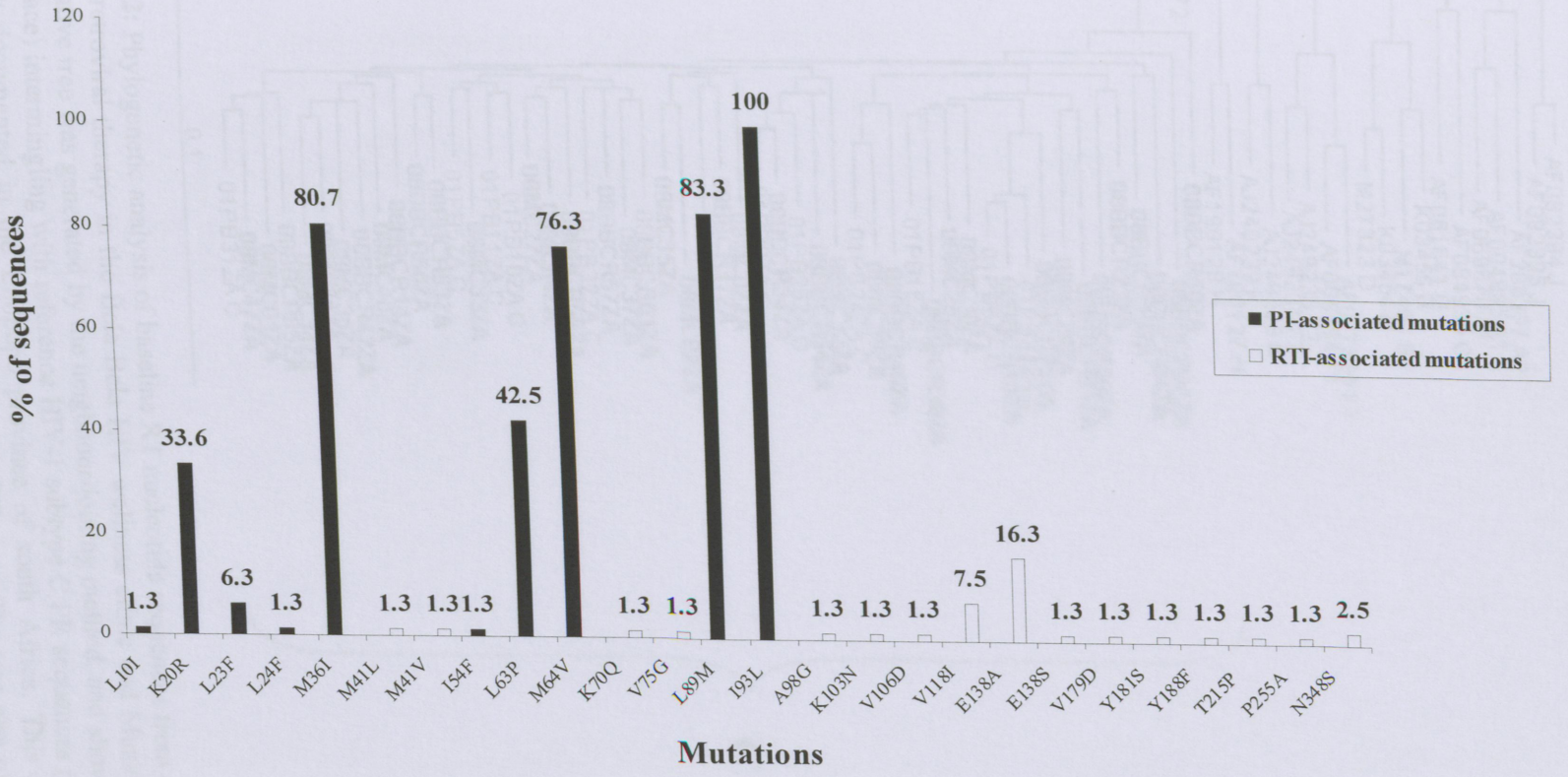

Figure 4.1: Distribution of resistance associated mutations on the *protease* and *reverse transcriptase* genes among HIV-1 sequences from two treatment sites in South Africa.

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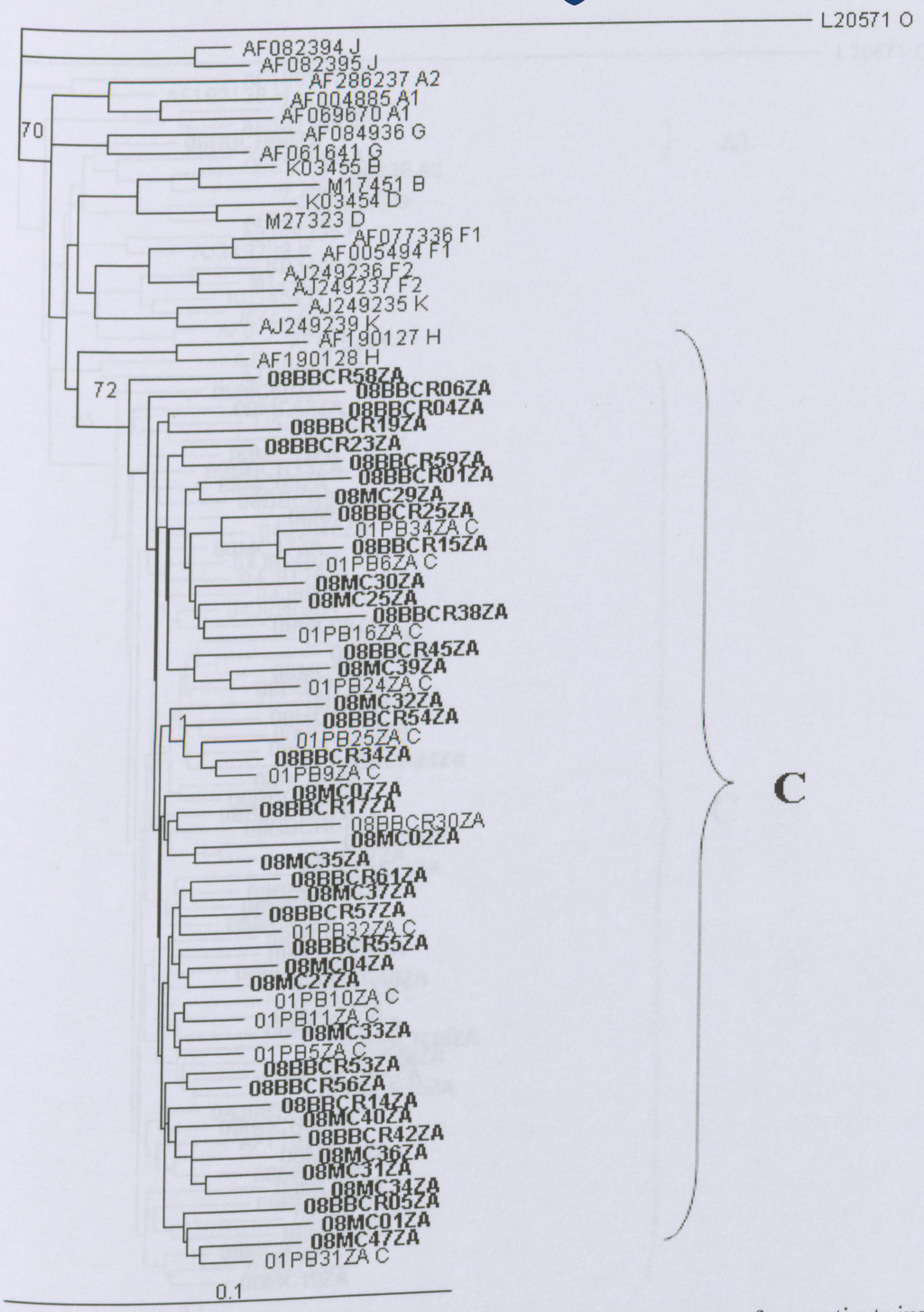

Figure 4.2: Phylogenetic analysis of baseline RT nucleotide sequences from patients just about to enter antiretroviral therapy in the Bela Bela HIV wellness clinic and Mankweng Hospital. This representative tree was generated by the neigbhour-joining method, and shows the test sequences (in bold face) intermingling with reference HIV-1 subtype C PR sequences (with 01PB prefixes) previously documented in the Limpopo province of south Africa. This shows that the test sequences are HIV-1 subtype C sequences on the RT gene. The tree was rooted with an HIV-1 group O sequence.

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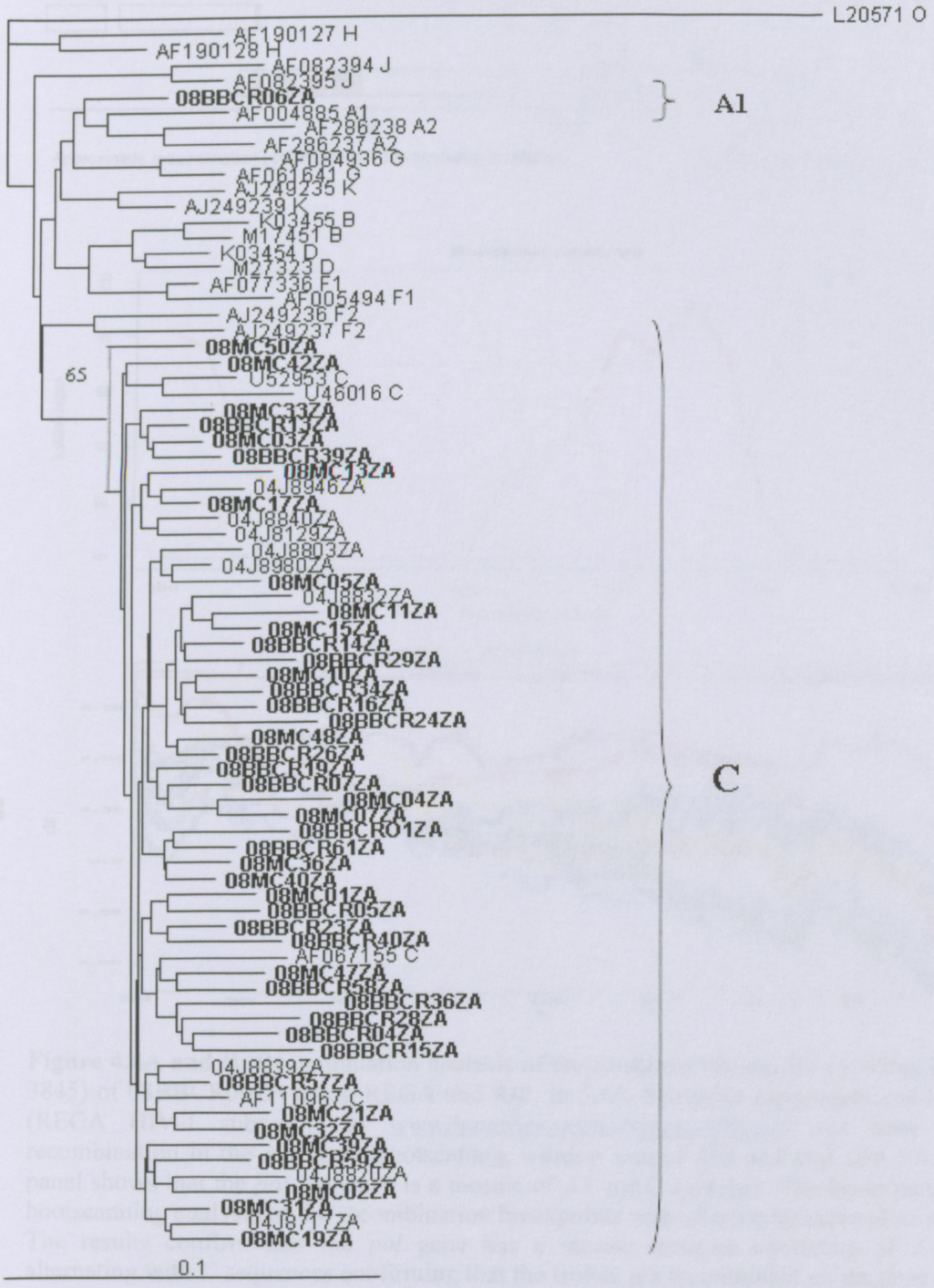

Figure 4.3: Phylogenetic analysis of baseline PR nucleotide sequences from patients just about to enter antiretroviral therapy in the Bela Bela HIV wellness clinic and Mankweng Hospital. This representative tree was generated by the neigbhour-joining method, and shows the test sequences (in bold face) intermingling with reference HIV-1 subtype C PR sequences (with 04J prefixes) previously documented. This shows that the test sequences are HIV-1 subtype C sequences at least on the PR gene; except one (08BBCR06ZA) which clusters with subtype A1 reference sequences. The tree was rooted with an HIV-1 group O sequence.

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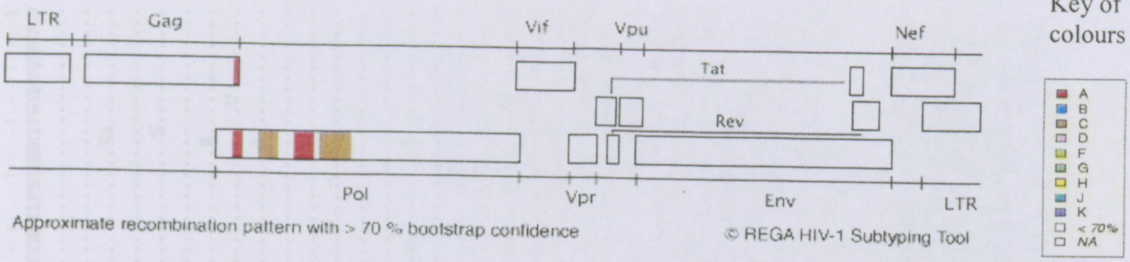
A



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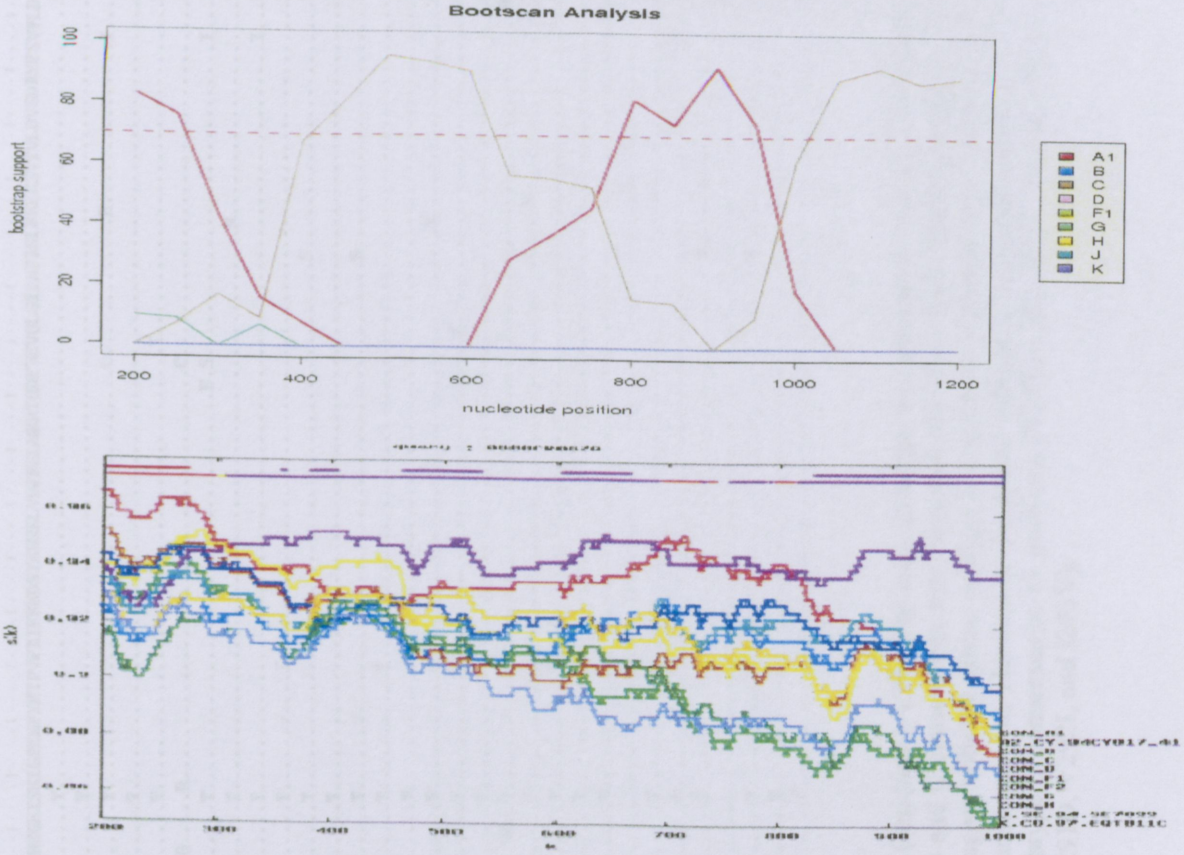

Figure 4.4A and B: Recombination analysis of the combined PR and RT (1197bp, HXB2:2253-3845) of 08BBCR06ZA using REGA and RIP. In 5.4A, Sequence assignment and bootscanning (REGA HIV-1 subtyping at www.bioafrica.net/subtypetool/html/) was done to confirm recombination in the *pol* gene (bootscaning, window size of 400 and step size 50). The upper panel shows that the *pol* sequence is a mosaic of A1 and C subtypes. The lower panel shows the bootscanning analysis of the recombination breakpoints with a bootstrap value of more than 70%. The results confirm that the *pol* gene has a mosaic structure consisting of A1 sequences alternating with C sequences confirming that the isolate is a recombinant on the gene analyzed. In 5.4B, the recombination identification program (RIP) (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html) was then used to confirm recombination. Results show a similar mosaic structure (similarity plot, window size 400, confidence threshold 0.9). This confirms that the isolate is a recombinant comprising subtypes A1 and C in the *pol* gene.

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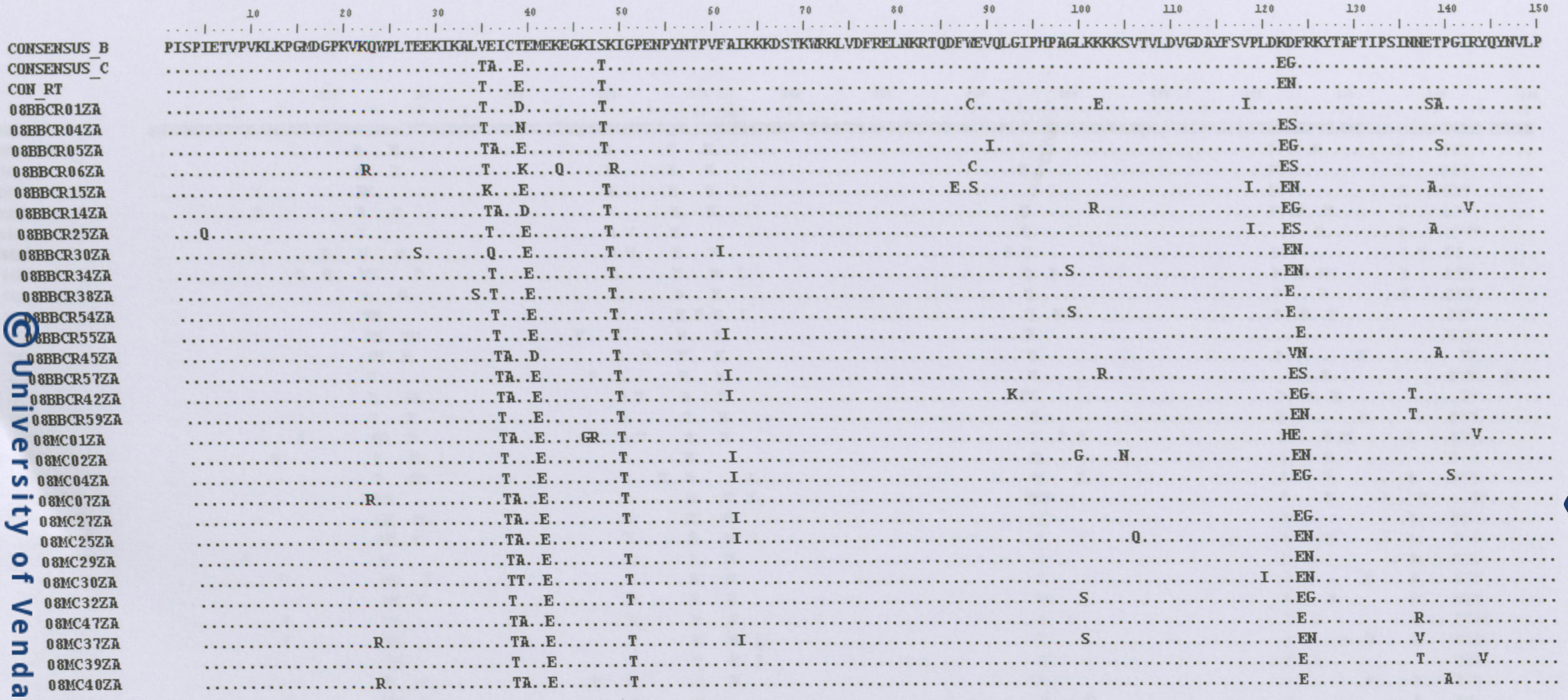

Figure 4.5: Amino acid alignment of RT sequences (residues 1-150). The test consensus was generated from the HIV database (GenBank) using some 29 sequences from this study. The global subtype B and C consensuses were obtained also from HIV database program. The entire consensuses were aligned using BioEdith software. All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid (if any) is denoted by an X. The test consensus was identical to the global subtype C consensus; except at four positions (A36E, G23N, Q174K and R277K). It differed from the global subtype B consensus at 17 positions (V35T, T39E, S48T, K122E, D123G, K173A, D177E, T200A, Q207E, R211K, V245Q, A272P, K277R, T286A, E291D, V292I, and I293V).

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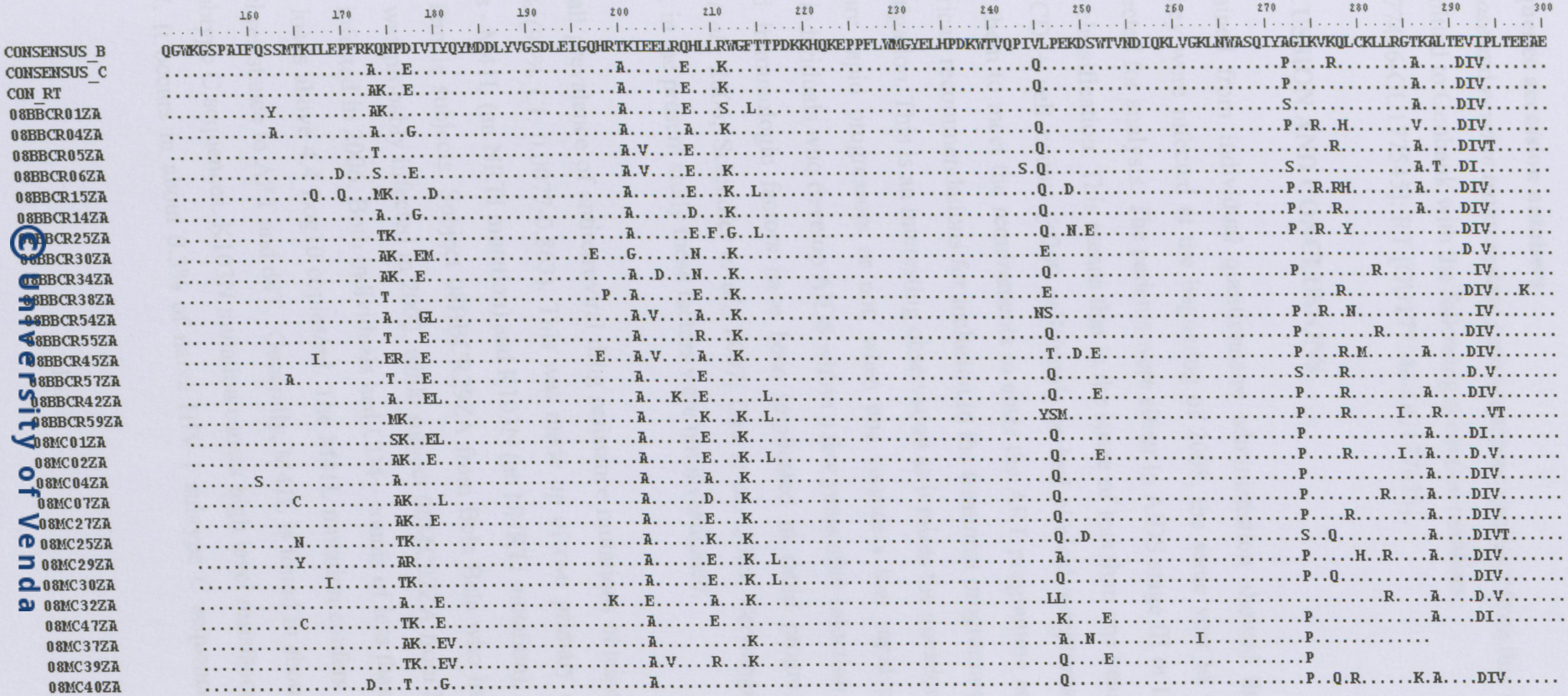

Figure 4.6: Amino acid alignment of RT sequences (residues 151-300). The test consensus was generated from the HIV database (GenBank) using some 43 sequences from this study. The global subtype B and C consensuses were obtained also from HIV database program. The entire consensuses were aligned using BioEdith software. All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid (if any) is denoted by an X.

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4.3.4. Sequence accession numbers

The *protease* and *reverse transcriptase* gene sequences ontained from this work have been submitted to Genbank with the following accession numbers:
PR [GU177466-GU177545]; RT [GU177546-GU177625].

4.4. DISCUSSION AND CONCLUSIONS

Data obtained from individual questionnaire administration showed that 21 patients thought they were infected at the beginning of 2008, the same year in which samples were collected for analysis. The patients were either in AIDS stage III or IV according to the WHO classification. This means that in the space of less than one year these patients had low CD4+ cell counts (<200 cells/mm^3) or had clinical symptoms suggestive of AIDS for them to meet the requirements to enter the ART programme according to the South African recommendations for inclusion in the treatment programme at the time of this investigation. This is an interesting observation as it raises the question whether these patients are rapid progressors or not, when one considers that rapid progressors are normally individuals who develop AIDS within a few years after infection. Host genetics, viral, and immunologic factors have been implicated in rapid progression to AIDS (Choge *et al*., 2006; Schindler *et al*., 2007; Cao *et al*., 2009; Serwanga *et al*., 2009). However, in the present study these factors were not investigated.

The overall prevalence of antiretroviral drug resistance mutations obtained in this study was 2.5% (95% CI: 0.0077-0.863). This was made up of two primary drug resistance mutations - M41L (an NRTI mutation) and K103N (an NNRTI mutation), detected in two different female subjects. Subject 08BBCR29ZA from Bela Bela who harboured M41L mutation was probably infected in 2002, while subject 08MC02ZA from Mankweng was probably infected in 2006. Both individuals had CD4+ counts of less than 200 cells/mm^3 and viral loads above 4.4 Log10 copies/ml. The M41L mutation confers intermediate to high level resistance to AZT and d4T. Generally, M41L is found in about 0.1% of naive HIV-1 subtype C sequences. K103N mutation causes high level resistance to NVP, DLV and EFV. It occurs in about 0.3% of naive HIV-1 subtype C sequences. K103N was

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observed in a 28 year old unmarried female. It could not be confirmed whether or not this patient had previously received NVP in a mother-to-child transmission prevention programme. In addition, it could not be ascertained whether or not these individuals are epidemiologically linked to individuals on ART. No major PR resistance mutation was found among the analyzed sequences. The frequencies of the observed PR and RT resistance associated mutations for the entire study population are depicted in Figure 1. Some amino acid substitutions at resistance associated sites and polymorphisms were also observed particularly in the PR gene. The potential impact of these changes observed frequently in non HIV-1 subtype B viruses especially subtype C viruses on the evolution of resistance and on the sensitivity of resistance testing protocols have been discussed (Bessong, 2008; Martinez-Cajas *et al.*, 2008). The low level of antiretroviral drug resistance recorded in this study suggests that despite the rapid ART scale-up, transmission of HIV drug resistance is still relatively uncommon in these two communities of Northern South Africa. An important consequence of this low level of resistance is that the current first line treatment may be working well and not much built-up resistance is being transmitted in new infections within the population. This low level is most lokely an indication of the success of the ART programme in minimizing the emergence of resistance in these communities. However, an expanded HIV drug resistance surveillance is needed to inform the ART program as it continues to scale-up.

In a previous cross-sectional study of viruses obtained in 2001 from Bela Bela, no important resistant mutations were reported (Bessong *et al.*, 2005; 2006). A low genetic resistance prevalence of 2.3% was recently reported in a cross-sectional study in the Free State Province of South Africa (Haung *et al.*, 2009). And where transmitted drug resistance has been surveyed, low levels of resistance have also been reported among adults, for example in the Gauteng and Western Cape Provinces (Pillay *et al.*, 2008; Orrell *et al.*, 2009); and in children born of drug-experienced mothers (van Zyl *et al.*, 2010). Recent reports suggest that the level of transmitted drug resistance may vary across regions in Southern and Eastern Africa such as Cape Town (20%), Entebbe (19%), Kigali 8% and Lusaka 3% (Price *et al.*, 2011), and below 5% in Botswana (Bussman *et al.*, 2011).

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Limitations of the present study include the use of population-based sequencing and investigating on chronic infections. Bulk sequencing has reduced sensitivity in detecting minority viral populations, while resistant viruses may revert to wild type over time. These together may lead to underestimation of resistance levels. However, the data obtained is similar to what has been observed in other parts of South Africa, and Southern Africa where access to ART is expanding; and complements resistance data in a region of South Africa with a relatively high HIV prevalence but with very little data on resistance mutational patterns. Regular studies are needed to assess the transmission of resistant viruses as access to ART expands. Such studies are required to inform policy on resistance testing.

As expected, most of the viruses (79/80) were shown to be HIV-1 subtype C by phylogenetic analysis of the polymerase gene sequences (Figures 2). However, one virus (08BBCR06ZA) obtained from Bela Bela was assigned A1 and C on the *protease* and *reverse transcriptase* genes respectively. The detailed mosaic nature of 08BBCR06ZA as a unique recombinant form derived from HIV-1 sub-subtype A1 and subtype C parental genomes has been described (Iweriebor *et al.*, 2011).

The present study indicates that the prevalence of drug resistance mutations in a cohort of patients about to enter ART programmes in 2008 in Bela Bela and Mankweng, two early treatment sites in northeastern South Africa is low. Overall, the patients in the study cohort would likely achieve therapeutic benefit from the first line ART regimen, and there may be no need for resistance testing prior to ART initiation in the study community at least at the population level.

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CHAPTER FIVE

ANALYSIS OF HIV-1 *INTEGRASE* SEQUENCES FROM TREATMENT NAIVE INDIVIDUALS IN NORTH-EASTERN SOUTH AFRICA

ABSTRACT

HIV-1 *integrase* (IN) is a key viral enzyme in the infection of susceptible cells and offers an important target for chemotherapy. Raltegravir is the most recent drug in the *integrase* inhibitor (INI) class of drugs to be licensed for use in treatment. The use of Raltegravir has not begun in South Africa but with the rise in treatment failure, it could be introduced in South Africa in the near future as part of salvage therapy. However, information on the mutations and their pattern is still lacking. The aim of this study therefore was to analyze the complete HIV-1 IN gene from two communities in North-eastern South Africa, for the presence of Raltegravir associated drug resistant mutations; and document HIV subtypes based on the IN gene. Gathering of this baseline data is in anticipation of the eventual use of *integrase* inhibitors in South Africa.

A total of 127 sequentially HIV positive individuals with no prior history of antiretroviral therapy were recruited between February 2008 and December 2008 from the Bela Bela HIV Wellness Clinic and Mankweng Hospital – two major treatment sites in the Limpopo province of South Africa. The complete *integrase* gene was amplified by a one tube RT-PCR followed by a nested PCR. Population based sequencing was done by the dideoxynucleotide chain termination method. Genetic drug resistance mutations were determined using the Stanford HIV Drug Resistance Interpretation programme and the International AIDS society-USA guidelines. Viral subtyping was done by phylogenetic analysis; while recombination analysis was carried out using REGA and RIP recombination tools.

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Viral DNA was obtained for 120/127 (94.5%) samples; while reliable nucleotide sequences were obtained for 89 IN genes. No major drug resistance mutations were detected in any of the subjects. However, 6/89 (7%) minor mutations and polymorphisms (L74M, Q95K, Q95P, E157K, I203M, and R263S) were detected. Phlyogenetic analysis showed that 88/89 (99%) of the viruses were HIV-1 subtype C on the IN gene. One isolate (08BBVCT28ZA) did not cluster with any reference subtype. The IN (864bp, HXB2 location: 4230-5093) and a partial *pol* fragment of sample 08BBVCT28ZA (1127bp, HXB2 location: 2253-3379) was further analysed for recombination using REGA and RIP recombination tools.

The results revealed that the sequence is a mosaic of A1, C, F1, and F2 subtypes on the IN gene and HIV-1 subtype C on the partial *pol* fragment (PR and RT genes). This suggest that the isolate is a recombinant on the IN gene and HIV-1 C on the partial *pol* gene region.

The identification of a mosaic virus indicates the introduction of hitherto undocumented variants in a region where HIV-1 subtype C viruses are known to overwhelmingly predominate. The lack of known major mutations to Raltegravir indicates that infecting viruses would be sensitive to IN when introduced. However, the presence and significance of unknown amino acid substitutions at resistance related sites need further investigation.

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5.1. INTRODUCTION

In all currently targeted HIV enzymes, inhibition with antiretroviral (ARV) drugs has led to the emergence of resistance in treated patients, leading to failure in treatment and necessitating changes in highly active antiretroviral therapy (HAART) regimens (Goethals *et al.,* 2008). The emergence of new drug classes such as the INI has made possible, the use of new drug combinations, thereby offering more treatment options to HIV-infected patients (Goethals *et al.,* 2008).

The IN gene is one of the three important genes of HIV-1, consisting of 288 amino acids encoded by the 3' end of the HIV-1 *pol* gene (Lataillade *et al.,* 2007). It catalyzes three reactions – 3' processing, strand transfer and integration (Malet *et al.,* 2008). During 3'-end processing, the terminal guanine-thymine dinucleotide (GpT) is cleaved from the 3' end of each long terminal repeat, producing cytosine-adenosine dinucleotide (CpA) 3'-hydroxyl ends. The reaction occurs in the cytoplasm, within a nucleoprotein complex known as the preintegration complex (PIC) (Malet *et al.,* 2008). The PIC is then transported through the nuclear pore to the nucleus, where strand transfer occurs. During strand transfer and integration, IN transfers both newly 3' extremities of the viral DNA into the target DNA via a single step transesterification. Thereafter, the viral genome is inserted and covalently linked into the host genome to form a provirus (Malet *et al.,* 2008). HIV-1 IN is composed of three functional domains: the N-terminal domain (NTD), which encompasses amino acids 1-49 and contains a histidine-histidine-cysteine-cysteine (HHCC) motif that coordinates zinc binding; the catalytic core domain (CCD) which encompasses amino acids 50-212 and contains the catalytic triad D64, D116, and E152, known as the DDE motif; and the C-terminal domain (CTD), which encompasses amino acids 213-288 and is involved in host DNA binding (McKee *et al.,* 2008). There is no human homolog of the IN enzyme and so HIV IN represents a rational and important target for treating HIV infection and preventing AIDS (Ceccherini-Silberstein *et al.,* 2009).

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South Africa has one of the highest HIV/AIDS prevalence in the world, and according to the South African National HIV and Syphilis survey conducted in 2007, the estimated prevalence is 28% (Department of Health, 2008). Limpopo Province has a prevalence rate of 20.7% from the antenatal survey. Prior to 2004, ARVs were available only through private health establishments in South Africa; and due to the scarcity and prohibitive high cost majority of patients could not afford them. In early 2004, a national antiretroviral delivery programme was initiated to provide drugs to those who had a CD4+ count less than 200 cells/mm^3 or with a clinically defined AIDS condition (Department of Health, 2004). Other psychosocial considerations for eligibility required patients to demonstrate reliability, no active alcohol or other substance abuse, no untreated active depression, disclosure of HIV status to at least one friend or family member or have joined a support group, accepted their HIV positive status, have insight into the consequences of HIV infection, understood the purpose of antiretroviral therapy, and be able to visit the treatment centre on a regular basis.

Recently, the food and drug administration (FDA) of the USA approved the first INI (Raltegravir – a strand transfer inhibitor) for inclusion in treatment regimens of HIV-1 patients failing their current regimens with multi-drug resistance strains. The decision was based upon the efficacy by which Raltegravir in combination with an optimized background regimen could improve the viral load and CD4+ count at week 24 in phase III clinical trials (Cooper *et al.,* 2008). Its use is still limited to treatment-experienced patients and HIV positive subjects with ARV drug resistant virus. Apart from its activity in the setting of resistance and treatment failure, it seems to be good for first-line therapy and as a switch option for subjects with intolerance to other ARVs (Stellbrink, 2009). It further has a great advantage because of its independence from 'boosting' of drug levels with Ritonavir and this is very suitable for many patients suffering from Ritonavir-associated side effects.

The South African national drug regimen guideline until 2009 recommended the use of Stavudine, Lamivudine and Efavirenz as the first-line therapy for adults who have never been exposed to antiretrovirals, with nevirapine replacing efavirenz for women of child

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bearing age. The second-line therapy comprises Zidovudine, Didansone and Lopinavir/Ritonavir. The 2010 ART guidelines have introduced the use of Tenofovir for those with poor tolerance of Stavudine in the first line regimen. It also recommends eligibility for treatment of pregnant women and patients with HIV/TB co-infection with a CD4+ count of less than 350 cells/mm^3 (Department of Health, 2004; Department of Health, 2010). Since the introduction of ART in 2004 in South Africa, the development of resistance and treatment failure has begun to emerge (Marconi *et al.,* 2008). Raltegarvir may be introduced in South Africa in the near future as part of salvage therapy. Despite this, only a few studies in South Africa have focused on the IN gene in terms of viral genetic variability and resistance mutational patterns (Marconi *et al.,* 2008; Fish *et al.,* 2010). Futhermore, no data is available from the north-eastern South Africa, where HIV prevalence is relatively higher. It is equally important to provide data sequence on drug targets in the evolving dynamic HIV landscape of treatment options and genetic variability.

5.1.1. Objectives of the study

The main objective of this study was to determine using the *integrase* gene, the genetic characteristics of HIV-1 sequences from treatment naive individuals in North-eastern South Africa. The specific objectives were:

1. To set up a PCR protocol for amplification of the *integrase* gene.
2. To genetically classify HIV sequences from Limpopo using the *integrase* gene.
3. To determine the intragenetic diversity of the *integrase* gene in HIV-1 infected individuals in this region of South Africa.
4. To examine *integrase* gene from drug-naive individuals for known mutations that confer resistance to *integrase*.
5. To analyze the functional domain of HIV-1 *integrase* gene sequences.

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5.2. MATERIALS AND METHODS

5.2.1. Ethical approval

This was obtained as described in sections 2.2.1 and 3.2.1

5.2.2. Study sites

Study subjects were recruited from two study sites in the Limpopo province of South Africa - the Phela O. Phedishe (POP) HIV clinic in Mankweng Hospital and the Bela Bela HIV/AIDS Wellness Clinic in Bela Bela. Details about these sites have been described in sections 2.2.2 and 3.2.2.

5.2.3. Study population, sample collection and plasma preparation

A total of 127 HIV positive individuals without prior exposure to ARVs visiting the voluntary counseling and testing facility of the Bela Bela Wellness Clinic and Mankweng Hospital were recruited consecutively between February 2008 and December 2008 and used for this study. Blood and demographic data collection, plasma preparation and storage were carried out as described in section 2.2.3. Data on viral load and CD4+ count were not available.

5.2.4. Viral RNA isolation, RT-PCR and nested PCR

Viral RNA was isolated using the viral mini RNA kit (Qiagen), stored at -80°C until used. A one-tube *reverse transcriptase* PCR was performed with AMV RT (Roche) and Taq DNA polymerase (Invitrogen), followed by a nested PCR. The first round primers were INFORI (5′-GGA ATC ATT CAA GCA CAA CCA GA-3′; HXB2 location 4059→4081) and INREV-I (5′-TCT CCT GTA TGC AGA CCC CAA *TAT*-3′; HXB2 location 5244←5267) and the nested primers HIV+4141(5′-TCT ACC TGG CAT GGG TAC CA-3′; HXB2 location 4141→4160) and INREVII (5′-CCT AGT GGG ATG TGT ACT TCT GA-3′; HXB2 location 5197←5219). This generated approximately a 1078 bp product (HXB2 location 4230-5093) of the entire IN gene. The PCR cycling conditions for the first round were: 1 cycle of 42°C for 60 minutes, 1 cycle of 95°C for 3

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minutes. This step will be followed 35 cycles of 95°C for 1 minute, 54°C for 1 minute, and 72°C for 2 minutes. A final extension at 72°C for 10 minutes ended the RT-PCR, with a 4°C hold. 5µl of the first round reaction served as template for the second round amplification reaction and the conditions were: 1 cycle of 95°C for 3 minutes, 35 cycles of 95°C for 1 minute, 54°C for 1 minute, and 72°C for 2 minutes, followed by a final extension cycle at 72°C for 10 minutes and a hold at 4°C. This protocol was established and optimised to enable the amplification of samples even with a low viral load (<1000copies/ml).

5.2.5. Detection of amplified products (agarose gel electrophoresis)

This was performed as described in section 2.2.5.

5.2.6. Purification of PCR product, sequencing and editing

This was performed as described in section 2.2.6. However, the sequencing primers used were different and are shown in table 5.1

Table 5.1: Sequencing primers for the *integrase* gene

Primer name and HXB2 location	Primer sequence
HIV+4141 (4141→4160)	5′-TCTACCTGGCATGGGTACCA-3′
INREV II (5197←5219)	5′-CCTAGTGGGATGTGTACTTCTGA-3′
INFORSEQ (4743→4767)	5′-CTTAAGACAGCAGTACAAATGGCAG-3′
INREVSEQ (4750←4772)	5′-GAATACTGCCATTTGTACTGCTG-3′

5.2.7. Viral genetic subtyping and recombination analysis

HIV subtyping was performed as described in section 2.2.7. Putative recombinant sequences were analyzed as described in section 4.2.7.

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5.2.8. Genetic drug resistance determination

This was performed as described in section 2.2.8.

5.2.9. Amino acid alignment determination and analysis of the functional domains of HIV-1 IN gene

Predicted amino acids for the IN gene were aligned using the BioEdit program (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Briefly, consensus amino acid sequences for the IN gene region was created and then compared to the global subtype C and the global subtype B consensus sequences obtained from GenBank (year 2000). The three functional domains (F1-A49, M50- E112 and L213 – D288) on the test consensus were then compared with those of the global C and global B, in order to determine conserved and substituted amino acid residues. The mean genetic distances among the IN sequences were determined by the Kimura 2- parameter model.

5.3. RESULTS

5.3.1. Study subject demographics

A total of 127 HIV positive individuals were sequentially recruited for this study. The mean age was 43.5 years (18 – 69 years). The proportion of women and men was 64.6% and 33.1% respectively. Eighty one percent of the study subjects were single and 13.4% were married. The most important risk factor for HIV transmission was sexual intercourse (94.5%). However, our questionnaire was not designed to determine whether it was heterosexual or homosexual. In 88.2% of the participants, the most probable place of HIV infection was South Africa. Thirty-three percent of the study subjects reported to have been infected in 2008, while 56.7% estimated that they were infected between 2000 and 2007. The highest level of education for most of them was grade 6 and above (78.7%), and 4.0% were university students. A monthly income range of less than R1000 (125 USD) was common to most (48.8%) of the subjects, while households with 1-5 persons were reported by 77.2% of the respondents. Details of the demographics of the study population are shown in Table 5.2.

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5.3.2. Viral DNA amplification and sequencing

Viral DNA was obtained for 120 out of 127 (94.5%) individuals (Figure 5.1). Reliable nucleotide sequences were obtained for 89 IN genes. Genetic subtyping and resistance analysis were done on these sequences.

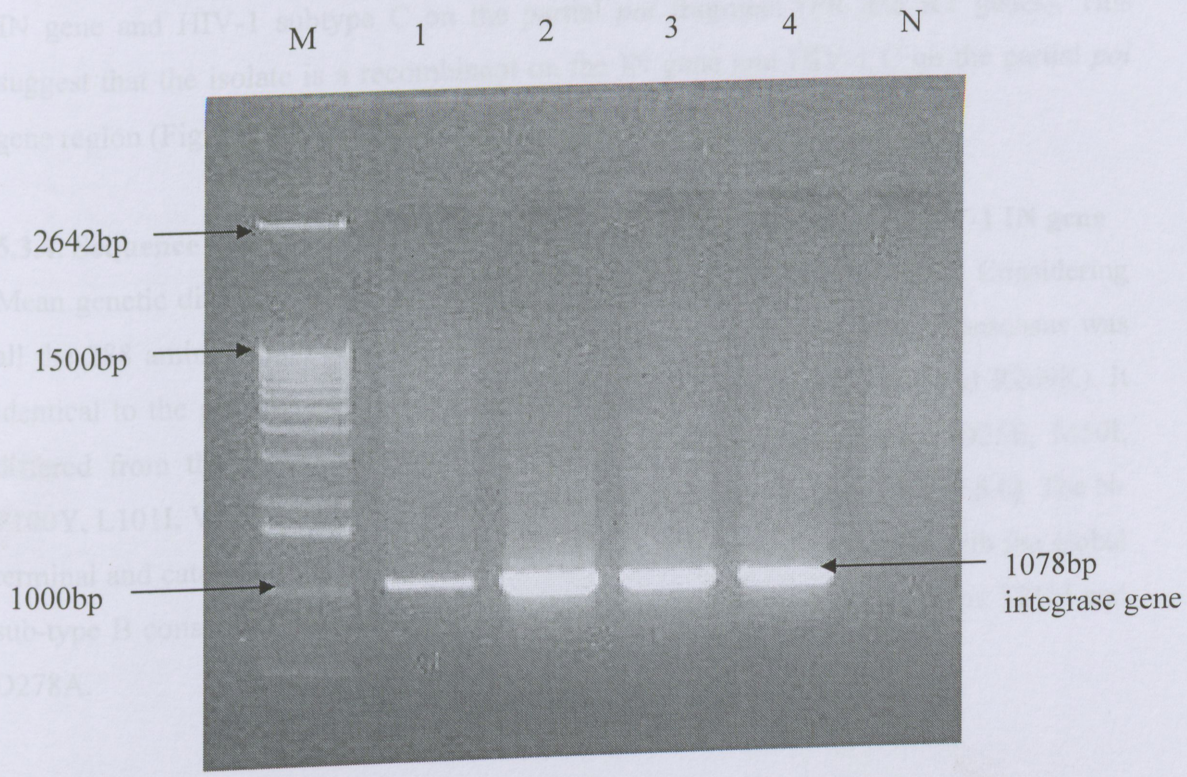

Figure 5.1: A representative gel electrophoresis photo of amplified IN gene. Lane M: Molecular weight marker XIV (1.5kbp); lanes 1-4: test samples and lane N: Negative control.

5.3.3. Drug resistance mutations and HIV subtypes

No major drug resistance mutations were detected in any of the subjects. However, minor mutations and polymorhisms (L74M, Q95K, Q95P, E157K, I203M, and R263S) were detected in 6 different subjects (08MB73ZA, 08BBVCT27ZA, 08MB33ZA, 08BBVCT49ZA, 08BBVCT38ZA, and 08MB34ZA). The detected mutations, nucleotide substitutions, and their potential significance are shown in Table 5.3.

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Phlyogenetic analysis of the IN gene showed that 88/89 (99%) of the isolates were HIV-1 subtype C. One virus (08BBVCT28ZA) did not show any assignment to any subtype (Figure 5.2). The IN (864bp, HXB2 location: 4230-5093) and a partial *pol* (complete PR and part of RT) fragment of sample 08BBVCT28ZA (1127bp, HXB2 location: 2253-3379) was further analysed for recombination using REGA and RIP recombination tools. The results revealed that the sequence is a mosaic of A1, C, F1, and F2 subtypes on the IN gene and HIV-1 subtype C on the partial *pol* fragment (PR and RT genes). This suggest that the isolate is a recombinant on the IN gene and HIV-1 C on the partial *pol* gene region (Figures 5.3 and 5.4).

5.3.4. Sequence alignment and analysis of the functional domains of HIV-1 IN gene

Mean genetic distance for the IN sequences ranged from 0.0124 to 0.1004. Considering all the 288 amino acids, sequence alignment showed that the test virus' consensus was identical to the global subtype C consensus except at 2 positions (V72I and R269K). It differed from the global subtype B consensus at 10 positions (R14K, D25E, M50I, F100Y, L101I, V113I, N134G, E167D, T218I, and D278A) (Figures 5.5 and 5.6). The N-terminal and catalytic core domains were very conserved when compared with the global sub-type B consensus. However, the C-terminal domain differed at positions T218I and D278A.

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Table 5.2: Characteristics of the study population of drug naive subjects from Bela Bela and Mankweng.

Characteristics	Patients (N= 127) Number (%)
Sex	
Males	42 (33.1)
Females	82 (64.6)
Data not available	01 (0.8)
Marital status	
Single	103 (81.8)
Married	17 (13.4)
Widow	02 (1.6)
Divorced	02 (1.6)
Data not available	03 (2.4)
Probable place of infection	
South Africa	112 (88.2)
Other countries	01 (0.8)
Unknown	04 (3.1)
Data not available	10 (7.9)
Risk factor for transmission	
Sex	120 (94.5)
Blood & blood product route	01 (0.8)
Unknown	01 (0.8)
Data not available	05 (4.0)
Probable Year of infection	
Before 2000	00 (0)
2000 – 2007	72 (56.7)
2008	42 (33.1)
Unknown	08 (10.1)
Data not available	05 (3.9)
Household income (SAR)	
<R1000	62 (48.8)
R1000 – R5000	31 (24.4)
>R5000	00 (0)
Data not available	34 (26.7)
Number of dependents	
1-5 persons	98 (77.2)
≥ 6 persons	15 (11.8)
Data not available	14 (11.0)
Level of education	
Not educated	02 (1.6)
≤ Grade 5	08 (6.3)
Grade 6 & above	100 (78.7)
University	05 (4.0)
Data not available	12 (9.4)

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Table 5.3: Drug resistance associated mutations, frequency, and coding nucleotides on IN gene sequences of drug naive subjects from Bela Bela and Mankweng.

Patient code	*Integrase* gene mutation	Frequency	Coding nucleotides		Comment
			Wild type	mutant	
08BBVCT73ZA	L74M	1/89 (1.1%)	CTG	ATG	Polymorphism (Stanford); minor mutation involved in the Q148H/K/R pathway to Raltegravir resistance (IAS).
08BBVCT27ZA	Q95K	1/89 (1.1%)	CAA	AAA	Minor mutation to Raltegravir (Stanford); undocumented (IAS).
08MB33ZA	Q95P	1/89 (1.1%)	CAA	CCA	Unusual mutation (Stanford); undocumented (IAS).
08BBVCT49ZA	E157K	1/89 (1.1%)	GAA	AAA	Unusual mutation (Stanford); undocumented (IAS).
08BBVCT38ZA	I203M	1/89 (1.1%)	ATA	ATG	Polymorphism (Stanford); undocumented (IAS).
08MB34ZA	R263S	1/89 (1.1%)	AGG	AGC	Unusual mutation (Stanford); undocumented (IAS).

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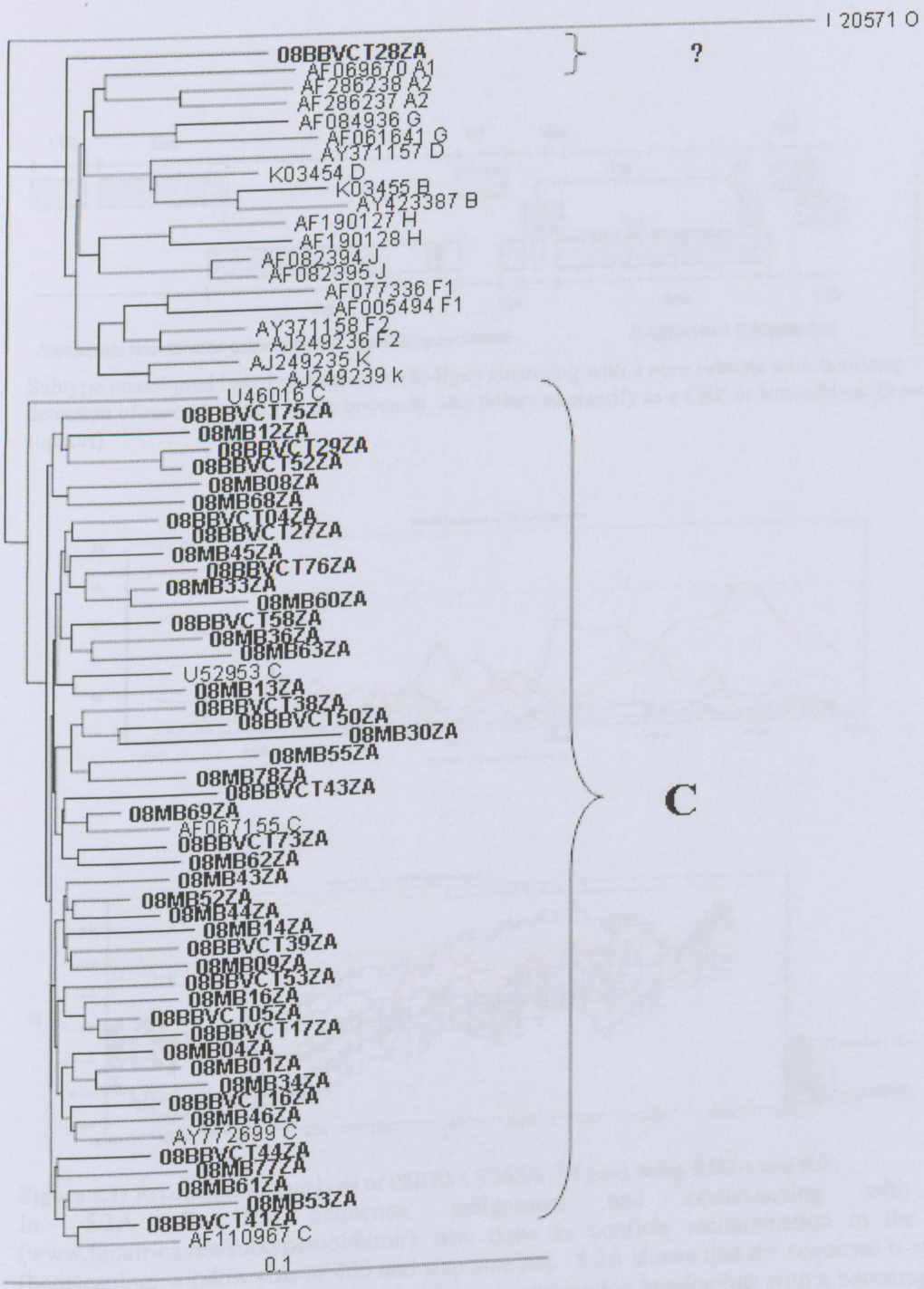

Figure 5.2: Phylogenetic analysis of IN nucleotide sequences from drug naive patients from Bela Bela HIV wellness clinic and Mankweng Hospital. This representative tree was generated by the neigbhour-joining method, and shows the test sequences (in bold face) clustering and intermingling with described reference HIV-1 subtype C IN sequences previously documented from Southern Africa and elsewhere. This shows that the test sequences are HIV-1 subtype C sequences on the IN gene; except one (08BBVCT28ZA) which did not cluster with any subtype reference sequences.

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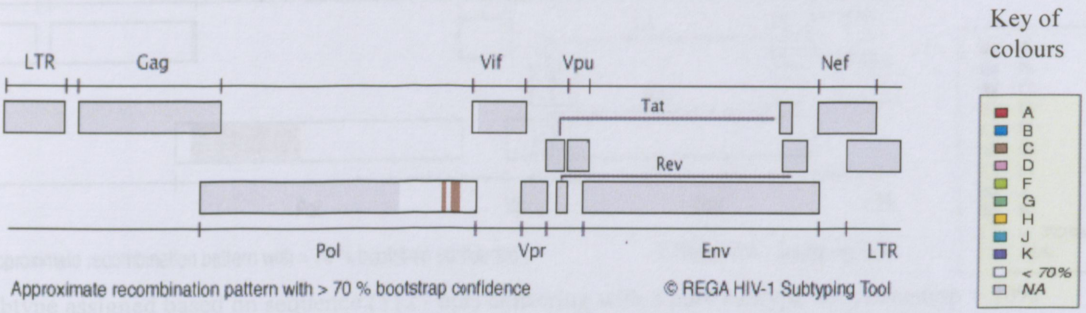
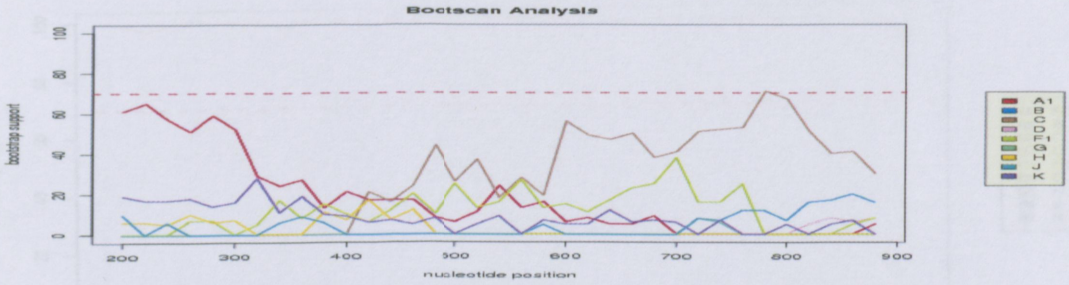
A



Subtype unassigned based on sequence (864bps) clustering with a pure subtype with bootstrap > 70% with detection of recombination in the bootscan, and failure to classify as a CRF or sub-subtype (bootstrap support).

B



C

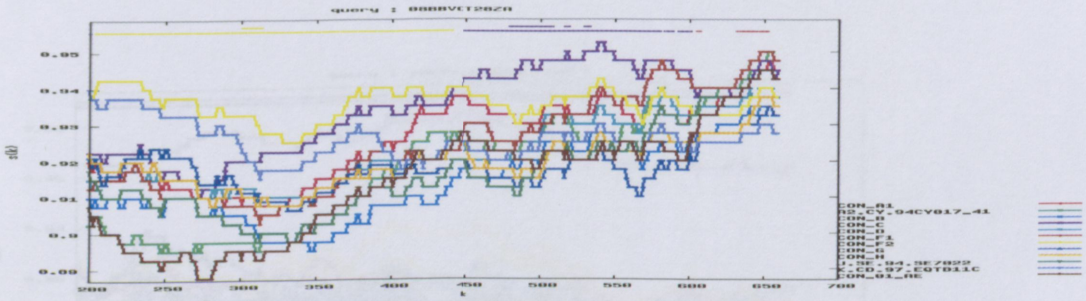


Figure 5.1: Recombination analysis of 08BBVCT28ZA IN gene using REGA and RIP.
In 5.3A & B, Sequence assignment and bootscanning with REGA (www.bioafrica.net/subtypetool/html/) was done to confirm recombination in the IN gene (bootscaning, window size of 400 and step size 20). 5.3A shows that the sequence is subtype C. 5.3B shows the bootscanning analysis of the recombination breakpoints with a bootstrap value of more than 70%. The results show that the IN gene has a mosaic structure consisting of A1 sequences alternating with C sequences, suggesting that the isolate is a recombinant on the IN gene analyzed. In 5.3C, the recombination identification program (RIP) (http://www.hiv.lanl.gov/content/sequence/RIP/RIP.html) was then used to confirm recombination. Results show a different mosaic structure (similarity plot, window size 400, confidence threshold 0.9) of C, F1 and F2 subtypes. This further suggests that the isolate is a recombinant in the IN gene.

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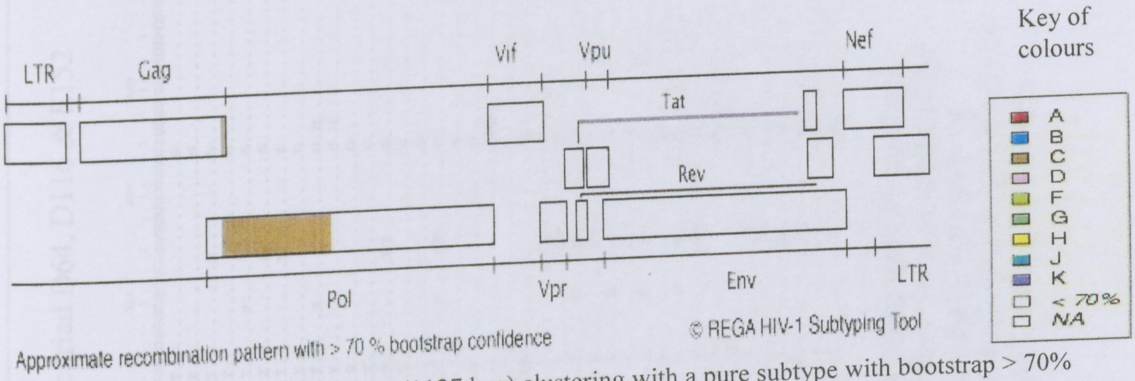
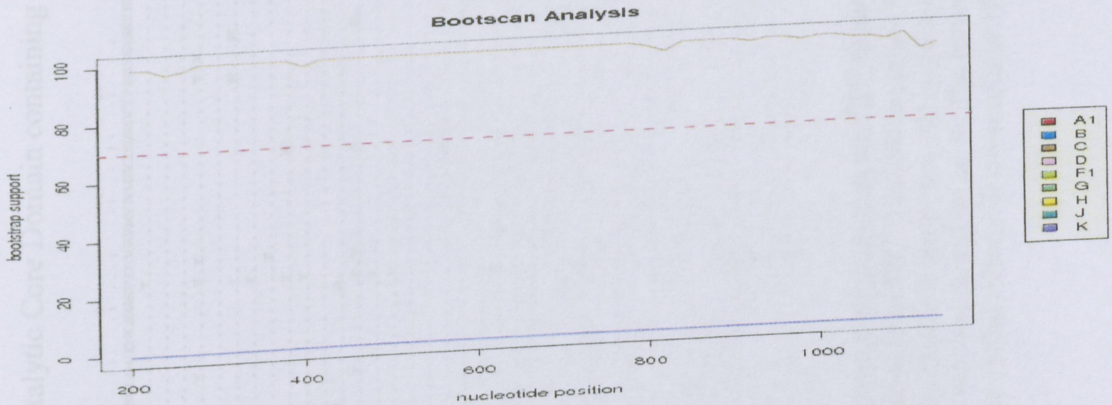
A



Subtype assigned based on sequence (1127 bps) clustering with a pure subtype with bootstrap > 70% without recombination in the bootscan.

B



C

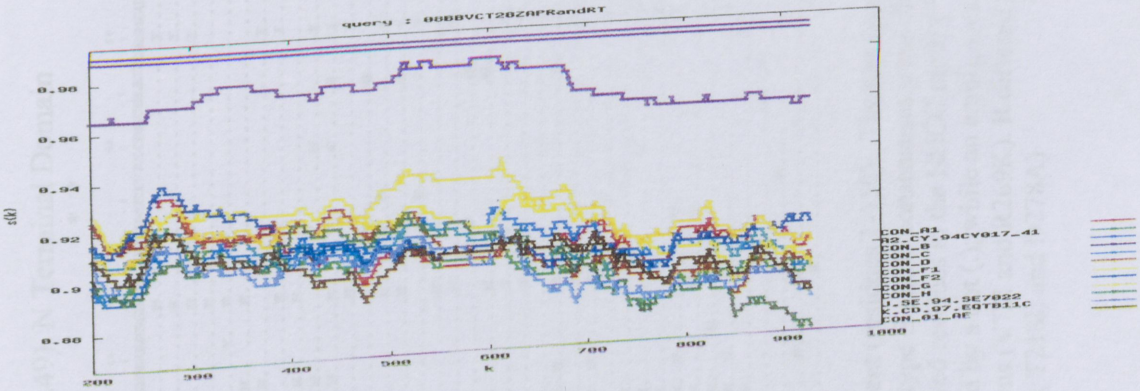


Figure 5.4: Recombination analysis of 08BBVCT28ZA partial *pol* gene using REGA and RIP. In 5.4A & B, sequence assignment and bootscanning of the partial *pol* (1127bp combined PR and RT, HXB2:2253-3379) using REGA, shows that the sequence is HIV-1 C on this gene region. In 5.4C, the recombination identification program was further used to check for recombination. Results also show that the virus is HIV-1 C on the PR and RT genes.

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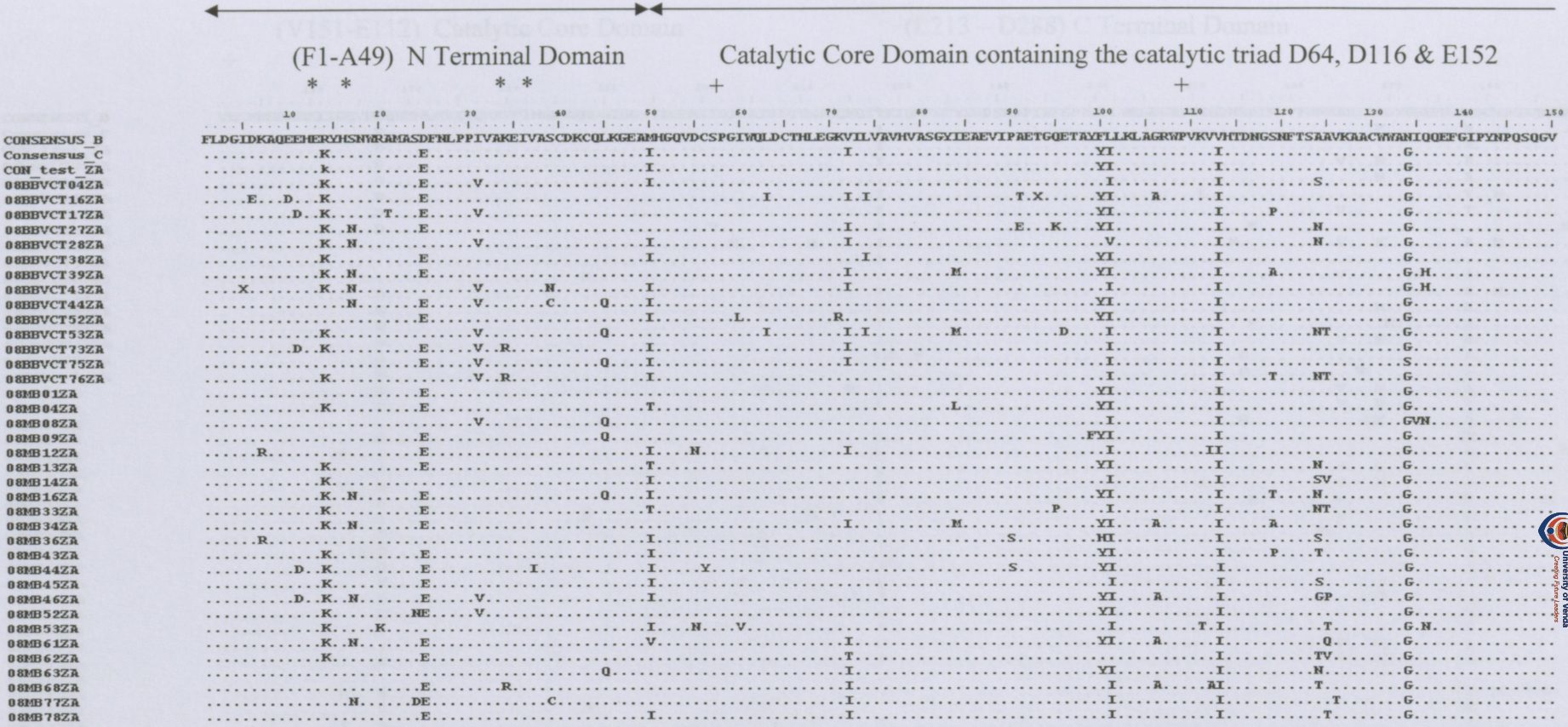

Figure 5.5: Amino acid alignment (residues 1-150). The test consensus was generated from the HIV database (GenBank) using some 37 sequences from this study. The global subtype B and C consensuses were obtained also from HIV database program. The entire consensuses were aligned using BioEdith software. The conserved residues in the HHCC motif (*) and the DDE (+) are noted. All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid is denoted by an X. The test consensus was identical to the global subtype C consensus except at 2 positions (V72I and R269K). It differed from the global subtype B consensus at 10 positions (R14K, D25E, M50I, F100Y, L101I, V113I, N134G, E167D, T218I, and D278A)

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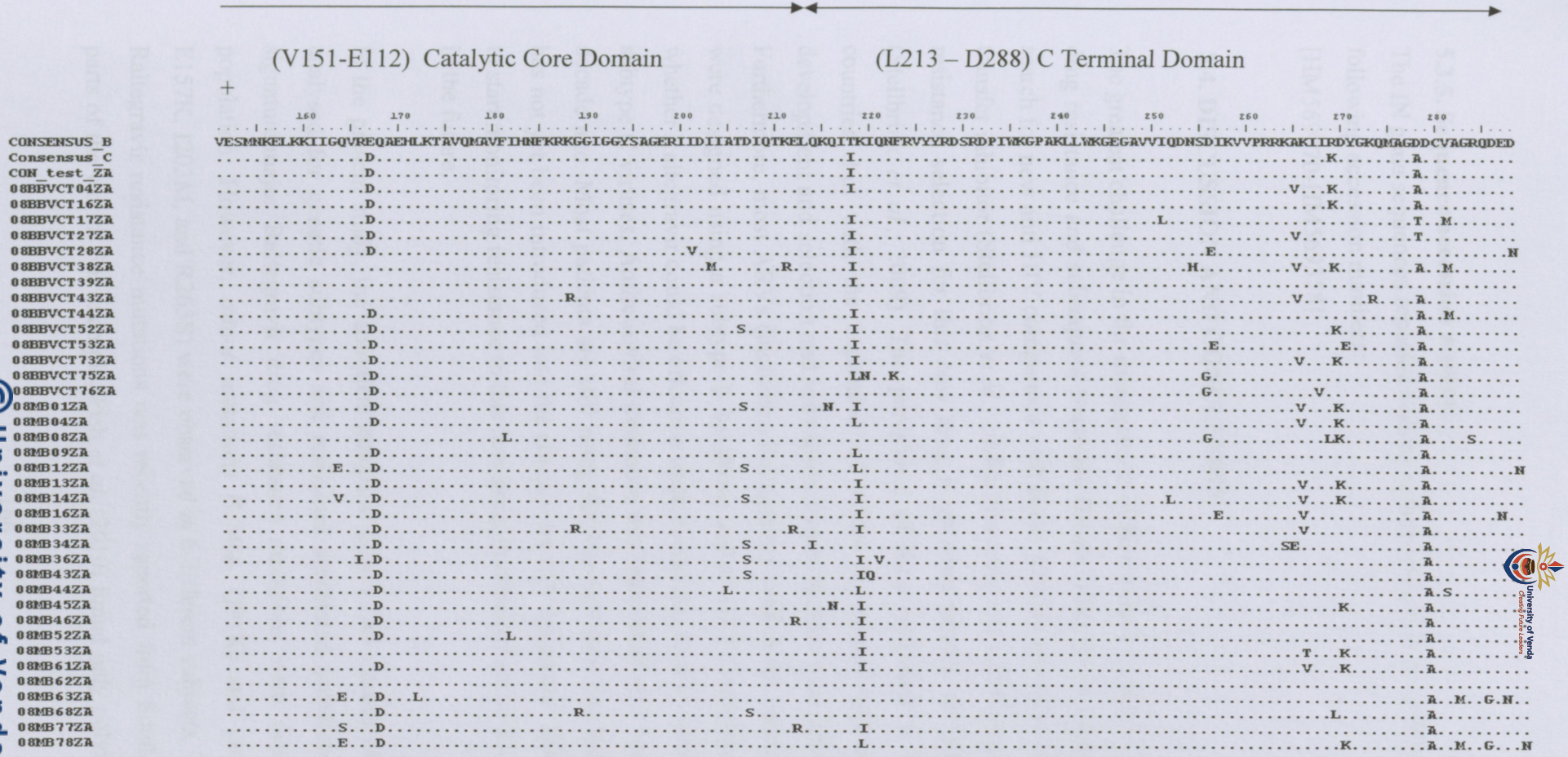

Figure 5.6: Amino acid alignment (residues 151-288). The test consensus was generated from the HIV database (GenBank) using some 37 sequences from this study. The global subtype B and C consensuses were obtained also from HIV database program. The entire consensuses were aligned using BioEdith software. The conserved residue E which is an integral part of the DDE motif is noted (+). All positions that agree with the global subtype B consensus sequence are denoted by a dot (.); while an ambiguous amino acid (if any) is denoted by an X.

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5.3.5. Sequence accession numbers

The IN gene sequences reported here have been submitted to GenBank with the following accession numbers:
[HM569270-HM569358].

5.4. DISCUSSION AND CONCLUSION

The greatest challenge in the management of HIV-infected patients is the development of drug resistance and subsequent treatment failure. Thus in the absence of a vaccine, the search for new anti HIV compounds with better efficacy continues. Raltegravir is a strand transfer inhibitor (Stellbrink *et al.,* 2009). Nevertheless, knowledge about the patterns of resistance selection for this new drug is growing rapidly but still poorly understood (Stellbrink *et al.,* 2009). The prevalence of drug resistance is low in most African countries, but with the expansion of access to therapy and over time, resistance development and selection and subsequent treatment failure are bound to be experienced. Furthermore, most ARVs (including Raltegravir) used in the treatment of HIV infection were designed using a subtype B backbone and it will be necessary to start finding out whether Raltegravir could be effective when eventually used in the future for treatment of subtype C viruses. Antiretroviral treatment was introduced into South Africa less than a decade ago. Most patients are still using the standard first line therapy and Raltegravir has not yet been introduced into the country for HIV treatment. However, it is important to start monitoring resistance to this new drug, in anticipation for its eventual introduction in the future.

In the present study, the complete *integrase* gene of 89 viruses obtained in 2008 were analysed for genetic subtypes and resistance associated mutations to Raltegravir. No signature/major Raltegravir drug resistance mutations were detected in the studied population. However, minor mutations (L74M, Q95K) and polymorphisms (Q95P, E157K, I203M, and R263S) were observed in 6 different subjects. The absence of major Raltegravir resistance mutations was recently reported from South Africa and in other parts of the world. For example, Fish *et al.* (2010) found only polymorphisms among 73

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samples collected from HIV-1 infected South African patients in 2010 (Fish *et al.,* 2010). Arruda *et al*. (2010) also reported the absence of INI resistance among 76 HIV-1 infected *integrase* inhibitor-naive patients in Sao Paulo, Brazil (Arruda *et al.,* 2010).

The potential impact that the polymorphisms observed in this study could have on treatment in the future is not known. The relevance of all IN mutations in clinical practice has yet to be defined; in light of the lack of long-term follow-up of treated patients, the limited data about the prevalence of *integrase* inhibitor-associated mutations in INI-naive patients (either untreated, or treated with ARVs not containing INIs), and the scattered information about conservation and variability of HIV IN in clean datasets (Ceccherini-Silberstein *et al.,* 2009).

On the whole, 11 out of the 288 (3.8%) amino acid residue positions had substitutions. The three functional domains of HIV are generally conserved. The NTD which encompasses amino acids 1-49 and contains a HHCC motif is conserved and the two substitutions observed are not within the HHCC motif. The HHCC motif coordinates zinc binding and helps in protein stability. The CCD which encompasses amino acids 51-212 and contains the catalytic DDE motif is also conserved and the six substitutions observed are not within the DDE motif. The CTD which encompasses amino acids 213-288 is generally conserved. However, two substitutions (T218I and D278A) occur in this region of the IN gene, representing a variability of 2.6% (2/76). These substitutions are conservative changes which have been shown in previous studies not to have any effect on IN activity (Van den Ent *et al.,* 1998; Engelman *et al.,* 1997). All the three domains are important in protein stability, multimerization, catalytic activity, DNA binding and in the binding with the human cellular co factor LEDGF/p75 (Ceccherini-Silberstein *et al.,* 2009).

The present study indicates that there are no major Raltegravir associated drug resistant HIV mutations among the treatment naive population in Bela-Bela and Mankweng, South Africa. The significance of the polymorphisms observed is not yet known; however, more studies need to be carried out in order to generate baseline data in anticipation of the

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eventual use of *integrase* inhibitors in South Africa. The identification of a recombinant virus indicates the introduction of hitherto undocumented variants in a region where C viruses are known to overwhelmingly predominate.



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CHAPTER SIX

GENERAL CONCLUSIONS AND RECOMMENDATIONS

The following general conclusions and recommendations could be drawn from the study:

1. The prevalence of antiretroviral genetic drug resistance in newly diagnosed persons in Bela Bela is low (3.5%; 95%CI = 0.019796-0.119077). This low prevalence was also observed in individuals about to enter therapy. Apparently, the testing of patients for drug resistant viruses before the initiation of therapy may not be required in this community. Despite this low prevalence, subsequent surveillance studies are needed to confirm the findings of this report and to generate data relevant to sentinel surveillance policy, and treatment initiation guidelines.

2. In Mankweng community, there is a difference in the prevalence rate of drug resistance mutations among newly diagnosed persons and chronic infected individuals about to start therapy. A moderately high prevalence (9.3%; 95%CI = 0-16.99) of drug resistance mutations was observed in newly diagnosed persons; while a low prevalence (2.5%; 95%CI = 0077-0.0863) was observed in chronically infected persons about to start treatment. The low prevalence observed in the latter group could be as a result of the progressive decay of drug resistance mutations. Overall, the prevalence of drug resistant mutations in Mankweng community could be high. Apparently, this is the first time that genetic drug resistance data is presented for Capricorn district. According to WHO recommendations, this moderate drug resistance prevalence may necessitate routine drug resistance testing for all persons newly diagnosed with HIV or all persons beginning HIV treatment in this community. This level also requires a review of the treatment program

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monitoring data for this community and the investigation of potential problems with regards to factors such as: continuous access to services, drug supply, drug quality, prescribing practice, compliance toxicity and/or adverse events, drug sharing and treatment failures. However, resistance in this population does not reflect current transmission of resistant HIV, because many newly diagnosed persons have been infected many years prior to diagnosis. Subsequent surveillance studies are needed to confirm the findings of this report and to generate data relevant to sentinel surveillance policy, and treatment initiation guidelines.

3. The present study also indicates that there are no major Raltegravir associated drug resistant HIV among the treatment naive population in Bela Bela and Mankweng, South Africa. The lack of known major mutations to Raltegravir indicates that infecting viruses would be sensitive to Raltegravir when introduced. The significance of the polymorphisms observed is not yet known. However, more studies need to be carried out in order to confirm the current finding in anticipation of the eventual use of this drug in South Africa.

4. Even though previous studies have reported HIV-1 subtype C as the predominant subtype prevalent in the Limpopo province, this study shows that other genetic variants have been introduced over time and the genetic landscape may be evolving. The identification of HIV-1 A1/C, HIV-1 subtype B, HIV-1 subtype J-like viruses, and mosaic viruses with components of A1, C, F1, and F2 subtypes may be an indication of the introduction of complex viruses in northern South Africa. This change in the landscape could impact diagnosis, treatment, and HIV-1 subtype C vaccine research in South Africa.

Overall, this study has elaborated on the data on genetic drug resistance in northern South Africa, providing new information on drug resistance levels among drug inexperienced patients, and previously undocumented genetic variants. In conclusion, it is

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recommended that genetic studies are continued on a regular basis to update data on trends relevant for research and patient management.

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APPENDIX

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Prevalence of drug-resistant mutations in newly diagnosed drug-naïve HIV-1-infected individuals in a treatment site in the Waterberg district, Limpopo province

Julius Nwobegahay, Pascal Bessong, Tracy Masebe, Lufuno Mavhandu, Cecile Manhaeve, Norbert Ndjeka, Gloria Selabe

Aim. We studied the prevalence of resistance mutations in drug-naïve HIV-infected individuals at the Bela-Bela treatment site to gather information on the presence of antiretroviral (ARV) drug-resistant viruses in drug-naïve populations, so as to improve treatment guidance.

Subjects and methods. Drug-naïve HIV-1-infected individuals were sequentially recruited between February 2008 and December 2008 from individuals visiting the voluntary counselling and testing (VCT) services of the Bela-Bela HIV/AIDS Wellness Clinic. Viral subtyping was done by phylogenetic analysis; drug-resistant mutations were determined according to the Stanford HIV Drug Resistance Interpretation and the International AIDS Society-USA Guidelines.

Results. A drug-resistant mutation prevalence of 3.5% (95% confidence interval 0.019796 - 0.119077) comprising Y181C and L33F was observed; 98% of the viruses were HIV-1 subtype C on the protease (PR) and reverse transcriptase (RT) gene regions.

Conclusion. The prevalence of drug-resistant mutations in drug-naïve persons may be low in Bela-Bela after 8 years of access to antiretroviral treatment (ART), and resistance testing before initiating treatment may not be needed.

S Afr Med J 2011;101:335-337.

Highly active antiretroviral therapy (HAART) has dramatically reduced morbidity and mortality among HIV/AIDS patients. However, a major drawback of HAART is the development of drug resistance.[1,2] South Africa has a high HIV prevalence, and access to treatment is expanding. A regimen based on the genetic resistance profiles of patients' viruses before initiating therapy generally correlates with a better outcome, compared with the absence of such resistance data.[2] The emergence of drug resistance in drug-naïve populations should be monitored, particularly in regions such as Limpopo Province, where such data are scarce. We aimed to determine the prevalence of ARV drug-resistance among drug-naïve HIV-infected individuals in a community where treatment has been provided for a considerable time.

Methods

Study subjects were recruited from the HIV/AIDS Wellness Clinic in Bela-Bela, Waterberg District, Limpopo Province. Treatment at the clinic began in 2001. HIV-positive individuals without prior exposure to ARV were recruited sequentially between February 2008 and December 2008. Five ml of venous blood was collected into EDTA vacutainer tubes from each consenting subject. Demographic data were obtained by questionnaire administration. Viral RNA was isolated using the viral mini RNA kit (Qiagen). Viral DNA was synthesised by a one-tube reverse transcription-polymerase chain reaction (RT-PCR), followed by a nested PCR.[3] The PCR products were verified for expected size by electrophoresis of 1% agarose gel. PCR amplicons were purified with a QIAquick PCR purification kit (Qiagen), and direct population-based sequencing was performed on both strands. HIV subtyping was done by phylogenetic analysis. Predicted amino acids for the PR and RT genes were aligned using the BioEdit programme (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Owing to the broad scope of viral mutations, drug-resistant mutations were identified using the Stanford HIV Drug Resistance Interpretation Algorithm (http://hivdb.stanford.edu/) and the International AIDS Society (IAS)–USA Guidelines.[4] Two algorithms were used to describe as broadly as possible the significance of observed amino acid substitutions. The Bayesian analysis was used to calculate the 95% confidence interval (CI), to estimate the confidence limits around the prevalence estimate. The PR and RT gene sequences reported here have been submitted to GenBank with the following respective accession numbers: PR [GU139577-GU139633] and RT [GU139634-GU139688]. Approval of the study protocol was obtained from the Health, Safety, and Research Ethics Committee of the University of Venda, Thohoyandou.

Results

A total of 79 HIV-infected individuals were sequentially recruited. The mean age was 43.5 (18 - 69) years. The most important risk factor for HIV transmission was sexual intercourse (88.6%), but the questionnaire was not designed to determine whether this was heterosexual or homosexual. In 90% of the cases the most probable place of HIV infection was South Africa. Viral DNA was obtained for 62 out of 79 (78%) individuals. Reliable nucleotide sequences were obtained for 57 PR genes and 55 RT genes. Using the Stanford and IAS guidelines, major drug-resistant mutations were detected in 2 out of 57 subjects (3.5%) (95% CI 0.019796 - 0.119077). One primary NNRTI mutation (Y181C) and one major PI mutation (L33F) were

AIDS Virus Research Laboratory, Department of Microbiology, University of Venda, Thohoyandou, Limpopo Province
J Nwobegahay, MSc
P Bessong, PhD
T Masebe, MSc
L Mavhandu, MSc

The HIV/AIDS Prevention Group, Bela-Bela, Limpopo Province
C Manhaeve, MA
N Ndjeka, MD

HIV/AIDS and Hepatitis Unit, Department of Medical Virology, University of Limpopo, MEDUNSA Campus, Garankuwa
G Selabe, PhD

Corresponding author: *P Bessong (bessong@univen.ac.za)*

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Prevalence of Antiretroviral Drug Resistance Mutations and HIV-1 Subtypes among Newly-diagnosed Drug-naïve Persons Visiting a Voluntary Testing and Counselling Centre in Northeastern South Africa

Julius M. Nwobegahay[1], Pascal O. Bessong[1], Tracy M. Masebe[1], Lufuno G. Mavhandu[1], Benson C. Iweriebor[1], and Gloria Selabe[2]

[1]AIDS Virus Research Laboratory, Department of Microbiology, University of Venda, PMB X5050, Thohoyandou 0950, South Africa and
[2]HIV/AIDS and Hepatitis Unit, Department of Medical Virology, University of Limpopo, MEDUNSA Campus, Pretoria, South Africa

ABSTRACT

Data on antiretroviral drug resistance among drug-naïve persons are important in developing sentinel surveillance policies. This study was conducted to determine the prevalence of antiretroviral drug resistance mutations among drug-naïve HIV-infected individuals attending a voluntary testing and counselling centre at the Mankweng Hospital in northeastern South Africa. In total, 79 drug-naïve HIV-positive individuals were sequentially recruited during February 2008–December 2008. Drug resistance mutations were determined using the calibrated population resistance tool available on the Stanford HIV drug resistance database. Viral DNA was obtained from 57 (72%) of the 79 individuals. Reliable nucleotide sequences were obtained for 54 reverse transcriptase (RT) and 54 protease (PR) gene regions from 54 individuals. Overall, five sequences (9.3%) harboured drug resistance mutations (95% confidence interval -1.53 to 16.99). Four (7.4%) of these were nucleoside RT inhibitor mutations (D67G, D67E, T69D, and T215Y), and one (1.9%) was a PR inhibitor mutation (M46I). No major non-nucleoside RT resistance mutation was detected. Several minor resistance mutations and polymorphisms common in subtype C viruses were observed in the PR and RT genes. Phylogenetic analysis of the partial *pol* sequences showed that 52 (96%) of the 54 isolates were HIV-1 subtype C. One isolate (08MB08ZA) was HIV-1 subtype B while another (08MB26ZA) was related to HIV-1 subtype J. HIV-1 subtype recombination analysis with REGA assigned the *pol* sequence to HIV subtype J (11_cpx) with a bootstrap value of 75%. The prevalence of drug resistance mutations observed in the population studied was relatively higher than previously reported from other parts of South Africa. In addition, this is apparently the first report of an HIV-1 subtype J-like virus from northeastern South Africa.

Key words: Antiretrovirals; Drug resistance; Drug-naïve persons; HIV-1; South Africa

INTRODUCTION

The health and quality of life of patients infected with HIV/AIDS have dramatically improved since the introduction of highly-active antiretroviral therapy (HAART) in 1996 (1,2). However, one of the major drawbacks of HAART is the development of drug resistance which usually accompanies the use of antiretrovirals (ARVs) (2,3). Drug-naïve individuals with ARV drug resistance have a relatively higher risk of virologic failure as they start antiretroviral treatment (ART) with a lower genetic barrier to resistance (4). In addition, harbouring resistance viruses before the initiation of treatment complicates alternative treatment algorithms.

In the developed world, HAART has been used with remarkable success, and treatment is accompanied with regular virologic monitoring, including measurements of viral load and testing of drug resistance to guide management of patients (5,6). On the other hand, the use of HAART in resource-poor settings still poses a lot of problems. Virological monitoring is less frequently done due to involvement of cost and dearth of qualified personnel.

Correspondence and reprint requests should be addressed to:
Dr. Pascal O. Bessong
AIDS Virus Research Laboratory
Department of Microbiology
University of Venda
Thohoyandou
South Africa
Email: bessong@univen.ac.za
Fax: +27 15 962 4749

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Low prevalence of genetic drug resistance in a cohort of HIV infected patients entering antiretroviral treatment programmes at two sites in Northern South Africa

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Keywords:	Genotypic drug resistance, Drug naïve patients, HIV-1 subtype C, northern South Africa

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Dr PO Bessong
Department of Microbiology
University of Venda
27 November 2006

Application for Ethical Clearance – Dr PO Bessong

The Health, Safety and Research Ethics Committee has at its meeting on the 27 November 2006, approved Dr Bessong's project entitled: *"Human Immunodeficiency Virus Drug Resistance in Limpopo Province, South Africa."*

Prof OS Fatoki
Director: Research and Development

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LIMPOPO

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REPUBLIC OF SOUTH AFRICA

DEPARTMENT OF HEALTH & SOCIAL DEVELOPMENT

Enquiries: Malomane EL

Ref: 4/2/2

7 August, 2007

Dr Pascal O. Bessong
University of Venda

Dear Dr Pascal O. Bessong

"Human Immunodeficiency virus Drug Resistance in Limpopo South Africa"

- Permission is hereby granted to Dr Pascal O. Bessong to conduct the study as mentioned above in selected sites in Limpopo Province
- The Department of Health and Social Development will expect a copy of the completed research for its own resource centre after completion of the study.
- The researcher is expected to avoid disrupting services in the course of his study
- The Researcher/s should be prepared to assist in interpretation and implementation of the recommendations where possible
- The Institution management where the study is being conducted should be made aware of this,
- A copy of the permission letter can be forwarded to Management of the Institutions concerned

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Department:
Health
REPUBLIC OF SOUTH AFRICA

UMNYANGO WEZEMPILO
LEFAPHA LA MAPHELO

Privaatsak X828
PRETORIA, 0001
Republiek van Suid-Afrika

Telephone: 012-312 0509
Fax: 012-312 0503

Enquiries: Dr LE Makubalo
Reference: H2/2/19

Dr P O Bessong
Department of Microbiology
University of Venda for Science and Technology
Private Bag X5050
THOHOYANDOU
0950

Dear Dr Bessong

Approval in Respect of Request to Conduct Research in Limpopo Province

This letter serves to indicate that approval is hereby granted to the above-mentioned researcher to proceed with research in respect of the study funded by the Department of Health titled **"HUMAN IMMUNODEFICIENCY VIRUS DRUG RESISTANCE IN LIMPOPO, SOUTH AFRICA"**. The approval to conduct this study is based on the conviction that the study will seek to evaluate and make recommendations regarding HIV resistance testing options, and anti-retroviral regimens in HIV-1 subtype C infected persons.

The onus rests with the researcher to negotiate appropriate and relevant time schedules with the research sites involved to conduct the research. A separate copy of this letter must be presented to the research unit and the Provincial Head of Department of Health confirming that permission has been granted for the research to be conducted.

Permission has been granted to proceed with the above study subject to the conditions listed below being met, and may be withdrawn should any of these conditions be flouted:

1. *The Provincial Head of Department of Health must be approached in writing, for permission to involve participants in the research study.*

2. *The patients concerned must be presented with copies of both this letter and the Provincial Department of Health letter that would indicate that the said researcher has been granted permission from the Departments of Health to conduct the research study.*

3. *A letter/document that outlines the purpose of the research and the anticipated outcomes of such research must be made available to the Provincial Department of Health Regional/District/Head Office Senior Manager/s concerned.*

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4. *The Researcher will make every effort to obtain the goodwill and co-operation of all the participants involved. Persons who offer their co-operation will not receive any form of remuneration for their participation in the study from the Department of Health while those that opt not to participate will not be penalised in any way.*

5. *The Researcher will make every effort to ensure that research may only be conducted in a way that the departmental normal official duties and programmes are not interrupted. The Senior Manager must be consulted about an appropriate time when the researcher may carry out his/her research at the site/s that they manage.*

6. *It is the researcher's responsibility to obtain written consent of all participants that are expected to participate in the study.*

7. *The researcher is responsible for supplying and utilising his/her own research resources, such as stationery, photocopies, transport, faxes and telephones and should not depend on the goodwill of the participating research sites for supplying such resources.*

8. *The names of the research participants in the study may not appear in the research report without the written consent of each of these individuals.*

9. *On completion of the study the researcher must supply the Department of Health and Provincial Department of Health Head Office with one Hard Cover bound copy of the final, approved research report. The researcher must also make available, on request to the Regional/District/Head Office Senior Managers, the final approved research report.*

The Department of Health wishes you well in this important undertaking and looks forward to examining the findings of your research study.

Kind regards

DR L E MAKUBALO
CLUSTER MANAGER: HEALTH INFORMATION, EVALUATION, EPIDEMIOLOGY AND RESEARCH
DATE: 2007 -05- 10

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