4

AN INVESTIGATION OF HIV-1 DIVERSITY IN SOUTHERN AFRICANS, AND CHARACTERISATION OF VIRAL POPULATIONS IN RECENTLY INFECTED WOMEN

A dissertation submitted to the University of Cape Town for the Degree of Masters of Science

By

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Table of Contents

Abstract v
Acknowledgements vii

Chapter 1: INTRODUCTION 4

1.1 Historical perspective on HIV 4

1.1.1 The discovery of HIV 4
1.1.2 Current status of the epidemic 5
1.1.3 HIV-1 Subtypes 6
1.1.4 Origin of HIV 8

1.2 HIV-1: the virus 12

1.2.1 Taxonomic classification 12
1.2.2 Virus morphology, genomic organization and life-cycle 13
1.2.3 *Env* gp120 20
1.2.4 HIV-1 Genetic diversity 25
1.2.5 HIV-1 Variability 29

1.3 Heterosexual Transmission 34

1.3.1 Early events in HIV transmission and infection 35
1.3.2 Co-receptors and phenotype 38
1.3.3 Viral load 39
1.3.4 Viral population restriction during transmission 40

University of Cape Town

1.3.5 Gender influences on heterosexual transmission 43
1.3.6 Subtype influences on HIV transmission 46

1.3 Project motivation **48**

Chapter 2: *ENV* SEQUENCE DIVERSITY OF THE HIV EPIDEMIC IN SOUTHERN AFRICANS **50**

2.1 Introduction **50**

2.2 **Materials and Methods** **51**

2.2.1 Patient data 51
2.2.2 RNA extraction and cDNA synthesis 57
2.2.3 Polymerase chain reaction (PCR) and agarose gel electrophoresis 58
2.2.4 Sequencing 61
2.2.5 Sequence analysis 62

2.3 **Results** **65**

2.3.1 PCR and Gel electrophoresis 65
2.3.2 Phylogenetic analysis 66
2.3.3 Genetic Distances 69
2.3.4 V3 Loop 71
2.3.5 Phenotype predictions 73
2.3.6 Global geographic clustering within subtype C 73
2.3.7 Entropy plots 75

2.4 Discussion **77**

University of Cape Town

Chapter 3: AN INVETSIGATION OF HIV-1 DIVERSITY FOLLOWING HETEROSEXUAL TRANMISSION IN RECENTLY INFECTED WOMEN FROM A SOUTH AFRICAN SEX WORKER COHORT 82

3.1 Introduction 82

3.2. Methods and Materials 86

3.2.1 Patient data and sample selection 86
3.2.2 RNA quantification and sero-status confirmation 87
3.2.3 RNA Extraction 88
3.2.4 cDNA Synthesis 88
3.2.5 Polymerase Chain reaction and agarose gel electrophoresis 88
3.2.6 Quantitation of PCR target molecules from PCR-based limiting dilution assay 92
3.2.7 Cloning and Colony PCR 92
3.2.8 Heteroduplex Tracking Assay 94
3.2.9 Sequencing 99
3.2.10 Sequences analysis 100

3.3 Results 101

3.3.1 RNA quantification and sero-status confirmation 101
3.3.2 Total population PCR 104
3.3.3 Quantitation of target molecules from PCR-based limiting dilution assay 104
3.3.4 Cloning into pMOS*Blue* and Colony PCR 105
3.3.4 Heteroduplex Tracking Assay 106
3.3.5 Phylogenetic analysis 108
3.3.6 Genetic distances 111
3.3.7 Amino-Acid translation and Phenotype prediction 113

University of Cape Town

3.4 Discussion **117**

Chapter 4: CONCLUSIONS **122**

Appendices **125**

Appendix A: DNA Techniques 126
Appendix B: Reagents and recipes 130
Appendix C: Vector maps and Molecular Weight Markers 132
Appendix D: Amino Acids 134

References **135**

University of Cape Town

Abstract

The aim of this study was to firstly define HIV-1 *env* genetic diversity in southern Africa, and secondly to investigate transmission by characterising populations in HIV infected pre-seroconversion female sex workers from a cohort in Kwazulu/Natal.

Genetic diversity in the envelope region of 39 southern African individuals originating from Malawi, South Africa, Zambia and Zimbabwe was assessed. For 19 individuals sequence analysis was performed on gp120 (one individual each from Zimbabwe and Malawi, 6 from Zambia and 11 from South Africa), and for an additional 20 individuals only V3V5 was analysed (3 individuals from Malawi, 2 from Zimbabwe, 4 from Zambia, 5 from Durban, South Africa and 6 from Johannesburg, South Africa).

All gp120 and V3V5 sequences grouped within subtype C except one highly divergent V3V5 sequence originating from a Zambian individual. This outlier sequence did not group significantly with any sequence from other subtypes. This study provides the first complete gp120 sequences originating from Zimbabwe and Malawi, and significantly extends the database of sequences from South Africa. High intrasubtype diversity was observed among sequences (an average of 10,6% across gp120 and 14,1% across V3V5), however, there was some evidence for a founder type effect as indicated by subclusters within subtype C from sequences originating from Durban South Africa.

In a parallel study, we identified and characterised HIV-1 populations in a cohort of five heterosexual, recently infected South African sex workers by assessing C2V3 and p17 diversity through heteroduplex tracking assay (HTA). Samples were obtained before seroconversion and should thus best represent the virus population that was transmitted. The majority of women harboured HIV-1 populations which were highly homogeneous, with intraperson genetic diversity ranging from 0% to 1,8% in C2V3 and 0% to 1,2% in p17. This is in contrast to a published study from Kenya which showed high diversity following transmission.

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Two of the five women had lower diversity in *env* compared to *gag*, suggesting selection associated with transmission and subsequent establishment of infection.

This study is of particular relevance as it identified and characterised both viruses in recently infected individuals representing the currently circulating viral population as well as the viral populations that was transmitted in southern African individuals. The outcomes of this study could assist in the planning and design of vaccines formulated for this region.

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University of Cape Town

Chapter 1: INTRODUCTION

1.1 **Historical perspective on HIV** 4

1.1.1 The discovery of HIV 4
1.1.2 Current status of the epidemic 5
Sub-Saharan Africa 5
South Africa 6
1.1.3 HIV-1 Subtypes 6
Group M 7
Group O 8
Group N 8
1.1.4 Origin of HIV 8
Oldest possible HIV infection cases 8
Two Theories for the Origin of HIV 9
SIV 9
HIV Endemic to Africa 10
Spread of HIV into the Western world 12

1.2 **HIV-1: the virus** 12

1.2.1 Taxonomic classification 12
Family *Retroviridae* 12
Genus Lentiviruses 13
1.2.2 Virus morphology, genomic organization and life-cycle 13
Morphology 13
Genomic organization 14
Structural proteins and viral enzymes 15
Regulatory proteins 17
Accessory/auxiliary proteins 17
Life-cycle 19

University of Cape Town

1.2.3 *Env* gp120 20
Genomic organization 20
Host cell entry determinants 20
CD4+ cell receptor and other main receptors 20
Co-receptors 21
Neutralisation determinants 22
Definition of neutralisation 23
Mechanism of neutralisation 23
Cross-neutralisation between subtypes 23
Monoclonal antibodies 24
Neutralisation epitopes 24
1.2.4 HIV-1 Genetic diversity 25
Geographic distribution of HIV-1 subtypes 25
Global 25
Africa 26
South Africa 27
1.2.5 HIV-1 Variability 29
Variability in perspective 29
Forces driving genetic variation 30
Reverse transcription and rapid viral turnover 30
Immune responses 31
Antiretroviral therapy 32
Availability of cells 32
Genetic recombination 32

1.3 Heterosexual Transmission 34

1.3.1 Early events in HIV transmission and infection 35
First cells encountered by HIV 35
First cells infected by HIV 37
1.3.2 Co-receptors and phenotype 38
Co-receptors in transmission 39
1.3.3 Viral load 39

University of Cape Town

1.3.3 Viral population restriction during transmission 40
Selective transmission 41
Selective amplification 42
Compartmentalisation 42
1.3.5 Gender influences on heterosexual transmission 43
Female multiple variant transmission 44
Male multiple variant transmission 45
Explanation for female multiple variant transmission 45
Gender influences on HIV pathogenesis 45
1.3.6 Subtype influences on HIV transmission 46
Differential co-receptor use by subtypes 47

1.3 Project motivation 48

University of Cape Town

CHAPTER 1: Introduction

1.1 Historical perspective on HIV:

1.1.1 The discovery of HIV

Acquired Immunodeficiency Syndrome (AIDS) was first described in North America in patients with an unusual form of pneumonia, *Pneumocyctis carinii* (PCP) (Gottlieb *et al.*, 1981), an infection previously found in patients with severe immune suppression (Hymes *et al.*, 1981; Masur *et al.*, 1981). At the same time, an infection with a retrovirus called human T-lymphotropic virus type I (HTLV-I), was described in patients exhibiting similar symptoms (Poisez *et al.*, 1980). This observation was particularly important as it pointed to a retrovirus as a possible cause of AIDS (Gallo, 1987). The virus associated with AIDS was first isolated in 1983 by Barre-Sinoussi and colleagues who termed this novel retrovirus AIDS lymphadenopathy-associated virus (LAV) (Barre-Sinoussi *et al.*, 1983). Similarly Gallo and colleagues isolated a retrovirus which they called T-lymphotropic virus type III or HTLV-III (Gallo *et al.*, 1984). Around the same time, a third group named their retrovirus AIDS-associated retrovirus or ARV (Levy *et al.*, 1984). Confirmation that these retroviruses were indeed isolates of the same virus came in 1985 (Ratner *et al.*, 1985) and in 1986 the Human Retrovirus Subcommittee of the ICTV (International Committee for Taxonomy of Viruses) recommended that the virus should be named human immunodeficiency virus or HIV (Coffin *et al.*, 1986).

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In the mid-1980's a second AIDS virus, closely related to but genetically distinct from HIV, was discovered. This new virus was seemingly restricted to West Africa. The first AIDS virus, HIV, was renamed human immunodeficiency virus type 1 (HIV-1), and the second virus HIV-2 (Clavel *et al.*, 1986; Franchini *et al.*, 1987). This review will focus only on HIV-1.

1.1.2 Current Status of the Epidemic

The AIDS crisis now surpasses the worst-case scenarios predicted a decade ago. More-than-20 million people have died globally of AIDS since HIV/AIDS was first reported in 1981. In the year 2001 an estimated number of 5 million people became HIV infected of which 800 000 were children (Figure 1.1).

Sub-Saharan Africa.

Sub-saharan Africa is the worst affected region in the world. However, in certain countries there are hopeful signs that the epidemic might be controlled. Uganda for example has seen reduction in prevalence rates and Senegal has controlled its epidemic to below 2%. Positive results have also been observed in Zambia with its focus on HIV prevention among, farmers, schools and religious groups proving successful (Agha, 2002; UNAIDS, 2002).

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Figure 1.1: Global estimates of HIV/AIDS epidemic at the end of 2001. (Report on the global HIV/AIDS epidemic, UNAIDS, 2002)

However, in some countries in southern Africa the epidemic continues to increase. Prevalence rates in Botswana from pregnant women in urban areas rose from 38,5% in 1997 to 44,9% in 2001. Similar patterns are visible for Zimbabwe (29% in 1997 to 35%

in 2000), Namibia (26% in 1998 to 29,6% in 2000) and Swaziland (30,3% in 1998 to 32,3% in 2000). Startling as these figures are, they still do not reflect the actual risk of acquiring HIV, which are even higher in specific age groups. In 2001, 55 % of 25-29 year-old Botswana women attending urban antenatal care centres, are reported to be living with HIV. In 2000 the corresponding prevalence rate was 33,9% in Swaziland and 40,1% in Zimbabwe (UNAIDS, 2002). In sub-Saharan Africa it is estimated that only 30 000 people will have had the benefit of antiretroviral drugs by the end of 2001.

South Africa.

An estimated 3,5 million new HIV infections occurred in 2001 resulting in a total of 28,5 million HIV infected people living in sub-Saharan Africa. Of these 28,5 million, 5 million lives in South Africa, thus contributing nearly 20% to the African burden. Of this 5 million, 2,7 million are women and 250,000 are children (UNAIDS, 2002). Figures from antenatal clinics report that prevalence rates reached 24,8% in 2001, on par with the 24,5% level of the previous year. A slight drop in prevalence rates among adolescents was reported between 1998 and 2001, possibly due to large-scale information campaigns and condom distribution. However it is difficult to monitor the impact of prevention strategies without knowing incidence data. In recent surveys, 55% of sexually active teenage girls are reported to use condoms during sex. Older age groups (20-34) have less favorable outcomes, and there is a need to extend prevention education to older age groups. The human and socioeconomic toll will remain significant for many generations.

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1.1.3 HIV-1 Subtypes

HIV-1 exhibits extraordinary genetic variability and in 1989, strains in the global epidemic were subdivided into European/North American and African according to their distinct separate clusters in a phylogenetic tree. Since then classification has changed from being based on geographic occurrence to a phylogenetic based classification system (Gao *et al.*, 1996; Louwagie *et al.*, 1993). HIV-1 is divided into groups M (major group), O (outlier) and N (non-M and non-O). These groups are genetically divergent but share the HIV-like genome organization (Berger *et al.*, 1998; Braaten *et al.*, 1996;

Charneau *et al.*, 1994; Gurtler *et al.*, 1996; Simon *et al.*, 1998; Vanden Haesevelde *et al.*, 1994).

Group M. HIV-1 group M is responsible for more than 99% of infections. This is the only group with significant geographic spread both within and outside of Africa (Anderson *et al.*, 1996; De Cock, 1996; Hu *et al.*, 1996; Janssens *et al.*, 1997; Peeters *et al.*, 1997; Quinn, 1996). By the early 1990s several genetic subtypes of HIV-1 had been recognized within group M, defined on the basis of genetic distance and analysis of phylogenetically informative sites (Louwagie *et al.*, 1993; Louwagie *et al.*, 1995; McCutchan, 2000; Robertson *et al.*, 2000).

The following criteria are used to define a subtype (Alaeus, 2000; Robertson *et al.*, 2000):

1. At least 3 full-length sequences from unlinked samples are required to define a new subtype.
2. They should resemble each other but not other existing subtypes throughout the genome. Consistent clustering in all parts of the genome with both high bootstrap support and no 'jumping' between clusters indicative of recombination.
3. In order to designate a sequence as a new subtype, it should be equidistant from all other subtypes in all the regions of the genome.

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HIV-1 group M viruses are divided into nine subtypes (A, B, C, D, F, G, H, J, K), which are further subdivided into 4 sub-subtypes (A1, A2, F1, F2,) and at least nine different circulating recombinant forms (CRF01_AE, CRF02_AG, CRF03_AB, CRF04_cpx, CRF05_FD, CRF06_cpx, CRF07_BC, CRF08_BC, CRF09_cpx) (McCutchan, 2000). A recombinant lineage is designated a CRF when related forms are found in more than 3 epidemiologically unlinked individuals. CRFs have a designation that includes the letters of the parent genetic subtypes (e.g. CRF01_AE), although in CRFs derived by recombination of more than three subtypes, the letters are replaced by cpx (complex), e.g. CRF04_cpx (Robertson *et al.*, 2000). Prototype viruses representing the genetic subtypes E and I have not yet been found. The viruses originally identified as subtype E (the predominant group of viruses involved in heterosexual transmission in Thailand) (Carr *et*

al., 1996; Cornelissen *et al.*, 1996; Gao *et al.*, 1996) and I (a small group of viruses from the Mediterranean) (Kostrikis *et al.*, 1995), are now considered intersubtype recombinants and have been termed CRF01_AE and CRF04_cpx, respectively. CRFs and recombination are discussed in more detail in section 1.2.5.

Group O. HIV-1 group O is highly divergent from group M and is largely restricted to west and central Africa (Mauclere *et al.*, 1997; Peeters *et al.* 1997; Zekeng *et al.*, 1994), sporadic reports of group O identified in Europe and the United States have been reported (Loussert-Ajaka *et al.*, 1995; Soriano *et al.*, 1996). In Cameroon, they constitute less that 10% of HIV-1 infections (Gurtler *et al.*, 1996; Peeters *et al.* 1997). Within group O, a subtype classification has been difficult to establish, but may become apparent as more samples accumulate (Workshop Report from the European Commission (DG XII, INCO-DC) and the Joint United Nations Programme on HIV/AIDS, 1997) and the Joint UNITED Nations programme, 1997). Group O viruses were discovered much later than group M, due to their apparent low prevalence and highly circumscribed geographic distribution. (Janssens *et al.*, 1994; Nkengasong *et al.*, 1994; Peeters *et al.* 1997; Simon *et al.*, 1998; Zekeng *et al.*, 1994).

Group N. HIV-1 group N is also highly divergent from group M and have been identified in a very small number of individuals in Cameroon, and only two group N strains have been genetically characterized (Simon *et al.*, 1998).

1.1.4 Origin of HIV

Oldest possible HIV infection cases.

After the discovery of HIV, a few HIV infection cases were proposed in retrospect. Firstly that of a British sailor from Manchester who died of an AIDS–like illness in 1959 (Williams *et al.*, 1983). However, despite convincing serological evidence (Corbitt *et al.*, 1990) the authenticity of the sample could not be confirmed due to contamination of the source material (Zhu and Ho, 1995). More convincing evidence came from members of a Norwegian family infected earlier than 1971 who were shown to be infected with an HIV-1 outlier group (Simon *et al.*, 1998), a variant form that is mainly restricted to West Africa. The oldest verified case of HIV-1 was a sample taken in 1959 from an adult man

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from the Democratic Republic of Congo. This sequence is placed near the ancestral node of subtypes B and D in the major group viruses and may well have evolved from a single introduction into the African population not long before 1959 (Zhu *et al.*, 1998).

Two Theories for the Origin of HIV.

SIV origin. Simian Immunodeficiency viruses (SIVs) have been isolated from a number of diverse species of wild African primates. SIVs have the same complex genomic organization as HIV's but differ from HIV-1 in the absence or presence of certain accessory genes (Barre-Sinoussi, 1996). Six distinct groups of SIV have been detected in African primates (Table 1.1). SIVs are not naturally found in wild Asian macaques, (*Macaca mulatta)* but SIVsm have since been artificially introduced from infected sooty mangabeys to Asian macaques and are now named SIVmac (Kestler *et al.* 1988; Lowenstine *et al.*, 1986). This provides evidence that African simians and not Asian macaques are the natural hosts of SIV.

Table 1.1: African and Asian monkeys harbouring Simian Immunodeficiency viruses.

	SIV	Monkey		Reference
African primates	SIVagm	African green monkeys	*Cercopithecus aethiops*	Allan *et al.*, 1991
	SIVmnd	Mandrill	*Papio sphinx*	Tsujimoto *et al.*, 1988
	SIVsyk	Sykes's monkey	*Cerophiticus mitis*	Emau *et al.*, 1991
	SIVsm	Sooty mangabeys	*Cercocebus atys*	Marx *et al.*, 1991
	SIVcpz	Chimpanzees	*Pan troglodytes*	Peeters *et al.*, 1989
	SIVL'hoest	L'Hoest monkeys	*Cerophiticus l'hoesti*	Hirsch *et al.*, 1999
Asian macaques	SIVmac	Macaques	*Macaca mulatta*	Lowenstine *et al.*, 1986

HIV-1 and HIV-2 both originated from two separate zoonotic (cross-species or animal-to-human) infections by two different African primate SIVs (Gao *et al.*, 1992; Hirsch *et al.*, 1989; Huet *et al.*, 1990; Sharp *et al.*, 1995;). SIV as the origin of HIV-2 was substantiated by the following criteria: similarities in genome organization of SIV and

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HIV-2; phylogenetic relatedness; prevalence in natural host; geographic coincidence and finally plausible routes of transmission (Hahn *et al.*, 2000; Sharp *et al.*, 1995). SIVsm from wild-living sooty mangabeys was found to be genomically indistinguishable and phylogenetically closely related to HIV-2. Two further lines of evidence suggest that SIVsm is the origin of HIV-2. SIVsm's natural habitat coincides with the epicenter of the HIV-2 epidemic (Chen *et al.*, 1995; Hirsch *et al.*, 1989; Marx *et al.*, 1991) and contact between sooty mangabeys and humans are common as they are hunted for food and kept as pets (Chen *et al.*, 1996; Chen *et al.*, 1997). No fewer than six subtypes have been identified in HIV-2 which each represent a separate cross-species transmission (Figure 1.2) (Chen *et al.*, 1996; Chen *et al.*, 1997; Gao *et al.*, 1992).

The origin of **HIV-1** is much less certain (Sharp *et al.*, 1995). HIV-1 is most similar in sequence and genomic organization to viruses found in chimpanzees, *Pan trogolodytes* (SIVcpz) and have been proposed as a natural primate host for HIV (Gao *et al.*, 1999; Huet *et al.*, 1990). But until recently, only three SIVcpz infections in chimpanzees have been documented, one that involved a virus so divergent it might represent a different primate lentiviral lineage (Vanden Haesevelde *et al.*, 1996). This fact together with high diversity between HIV-1 and SIVcpz, a low prevalence of chimpanzees in geographic regions of Africa where AIDS was not initially recognized have cast doubt in chimpanzees as a natural host and reservoir for HIV-1. It has been suggested that another, as yet unidentified, primate species could be the natural host for both SIVcpz and HIV-1 (Huet *et al.*, 1990; Vanden Haesevelde *et al.*, 1996). However, in 1999 a chimpanzee (*Pan troglodytes troglodytes*) was identified with a natural SIVcpz infection. From sequence analysis results, as well as the observation that the natural range of *P. t. troglodytes* coincides uniquely with areas of HIV-1 group M, N and O endemnicity, it was suggested that *P. t. troglodytes* is the primary reservoir for HIV-1 and has been the source of at least three independent introductions of SIVcpz into the human population resulting in HIV-1 group M, N and O (Gao *et al.*, 1999).

HIV endemic to Africa. A second possibility for the origin of HIV is that AIDS could have been an old African endemic disease silent or unrecognised until now (Gallo *et al.*, 1987; Gallo and Montagnier 1988; Montagnier 1988). In isolated groups HIV would have had little opportunity to spread and could possibly have been contained for

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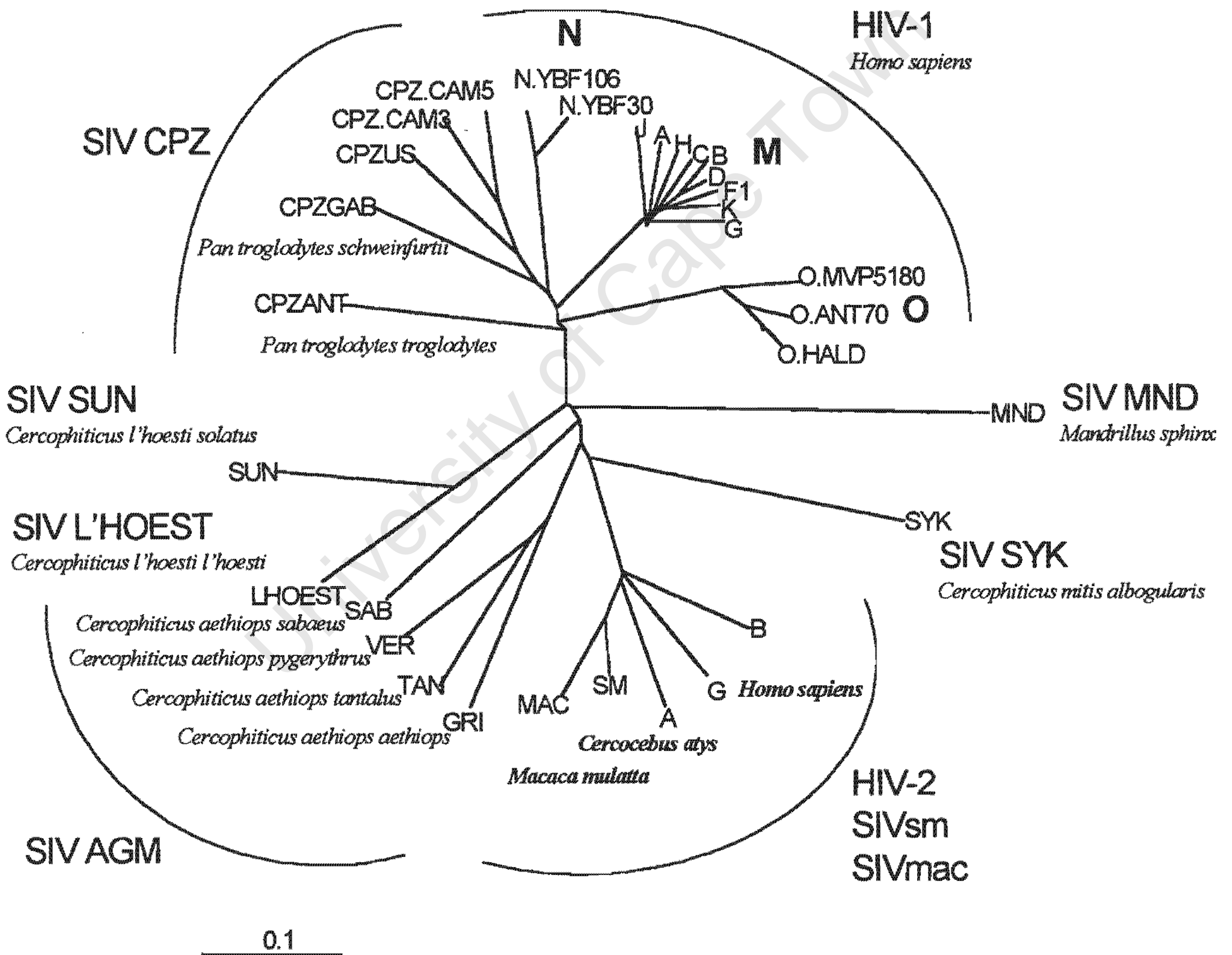
decades. Political developments in Africa, as well as regional and international travel revolution, may have broadened the ecological niche of the virus and could be one of the factors responsible for introducing HIV into the Western world, where the spread was facilitated by the sexual revolution and intravenous drug use (Mann *et al.*, 1988).

Figure 1.2: Phylogenetic relationships among primate lentiviruses. The tree is based on viral *gag* genome alignment. The scale bar indicates an approximate genetic distance of 10%



Both these hypotheses point to the African continent for the geographical origin of the virus and are probably not mutually exclusive. A recent HIV-1 genetic diversity study in the Democratic Republic of Congo (DRC) suggested this region to be the epicenter of HIV-1 group M. The DRC harbors an old and mature epidemic with all HIV-1 group M subtypes circulating in this area. This high intrasubtype diversity (from 15,7% for subtype D to 17,3% for subtype H), high numbers of recombinant viruses as well as additional unclassified strains would suggest that the epidemic has been evolving for a long period of time compared to regions with low diversity such as USA. More evidence to support the DRC as geographic origin of HIV-1 group M is the fact that the first HIV-1 group M sequence originated from the DRC in 1959, giving concrete evidence indicating that group M viruses had been present in humans for at least forty years (Vidal *et al.*, 2000).

Spread of HIV into the Western world.

HIV was probably introduced from Africa into USA where the first epidemic was associated with homosexual transmission of HIV subtype B (Auerbach *et al.*, 1984). From the USA, the virus presumably spread from one Western country to the next. However, the epidemic did not gather momentum in Europe until several years after it appeared in the US. In 1982, the prevalence of HIV among homosexuals was 42,6% in San Fransisco and 7,5% in Amsterdam (Van Griensven *et al.*, 1993).

1.2 HIV-1: the virus:

1.2.1 Taxonomic Classification

Family Retroviridae. HIV-1 and HIV-2 are members of the family, *Retroviridae* (Retroviruses). These RNA containing viruses are so named because they reverse what seemed to be the normal flow of genetic information (Latin, retro: backward). Retroviruses contain reverse transcriptase (RT) a RNA-dependant DNA polymerase that makes DNA from viral RNA (Levy *et al.*, 1994).

The retrovirus differs from all other RNA viruses in that their RNA genome, transcribed to DNA, can become incorporated into the host's cell genome. Depending on the genetic

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structure of a given virus, this event can lead to genetic transformation of the host cells. Retroviridae is the only virus family known able to transform host cells and produce virus remaining present in cells without any signs of infection. Retroviruses have frequently been isolated from birds and mice as well as from other species, including humans. (Levy *et al.*, 1994).

Genus Lentiviruses. The International Committee for Taxonomy of Viruses (ICTV) has sub-grouped the retroviruses into seven genuses. HIV belongs to the genus lentiviruses. Lentiviruses (Latin *lenti*: slow) obtained their name from the long incubation period from infection to disease. They are typically characterized by the cone-shaped morphology of the virus core and distinctive morphology at the budding stage. Human lentiviruses can be genomically distinguished from other retroviruses in that they contain five accessory genes in addition to *gag, env* and *pol* genes that are common in all retroviruses (Levy, 1994).

1.2.2 Virus morphology, genomic organization and life-cycle

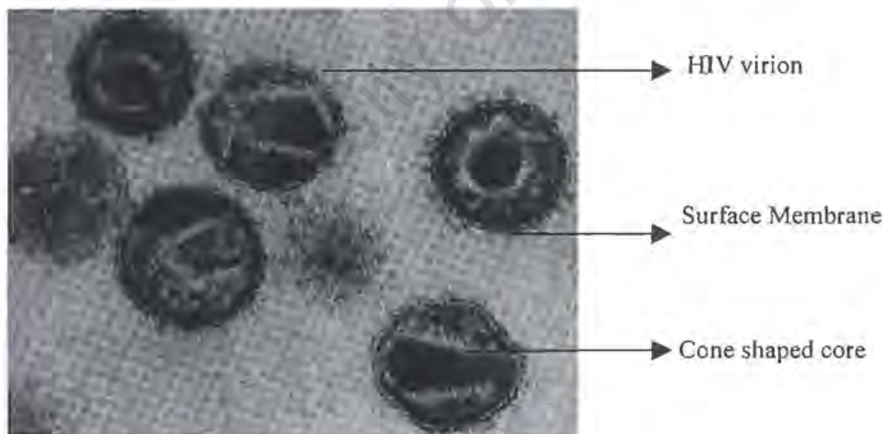

Figure 1.3: Electron micrograph of the HIV sphere (± 1,000 angstrom units, one tenth-thousand of a millimeter across), enclosed in a membrane (Adapted from Gallo and Montagnier, 1988).

Morphology.

HIV is approximately 100-150nm in diameter. It is roughly circular in shape, although may vary from oval to a somewhat irregular outline (Levy, 1994) (Figure 1.3). Encasing the virion is the envelope, a lipid bilayer that is acquired from the host cell. Protruding through the lipid membrane are numerous viral glycoprotein spikes. These envelope

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spikes consists of two combined glycoproteins, the surface glycoprotein (SU), and the transmembrane glycoprotein (TM). The matrix protein (MA) membrane composed of additional structural proteins, is located underneath the envelope. The inner capsid (CA) of the virus is characteristically cone-shaped and is the main distinguishing feature of the virus under the electron microscope. The capsid contains two coiled strands of RNA (the viral genome) together with the viral enzymes; reverse transcriptase (RT), protease, RNAse and integrase (Barre-Sinoussi, 1996) (Figure 1.5).

Genomic organization.

The ssRNA is reverse transcribed into dsDNA which, when integrated in the host genome, is referred to as the provius. The HIV proviral DNA is ±10 000 nucleotide in length and is flanked by two identical long terminal repeats (LTR) promoter regions (Figure 1.4). The HIV genome has three genes in common with all other retroviruses *gag*, *env* (coding for structural proteins) and *pol* (coding for viral enzymes). In addition the HIV has a clustered area in the 3' half of the genome encoding a number of accessory or auxiliary proteins which are virion or non-virion associated including Vif, Vpr, Vpu, Vpx and Nef. HIV also contains regulatory proteins coded for by *tat* and *rev*. On the basis of messenger RNA (mRNA), three classes of can be distinguished, (i) genome length mRNA, (ii) singly sliced RNAs and (iii) multiply spliced RNAs.

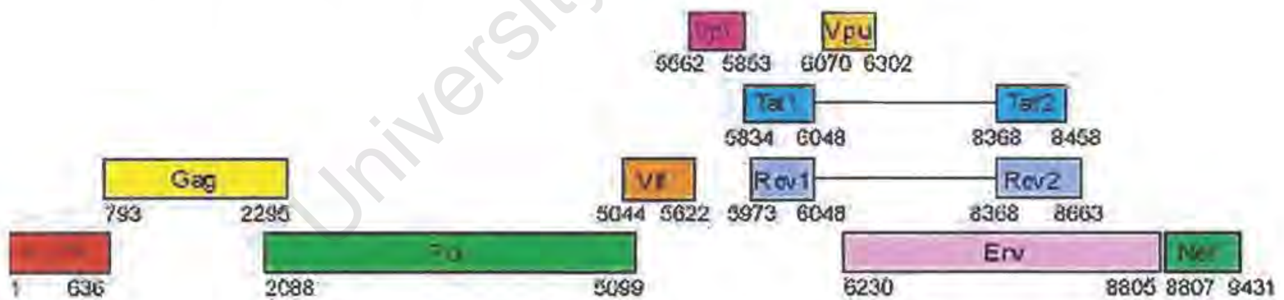

Figure 1.4: HIV-1 genomic organization (Adapted from http://hiv-web.lanl.gov)

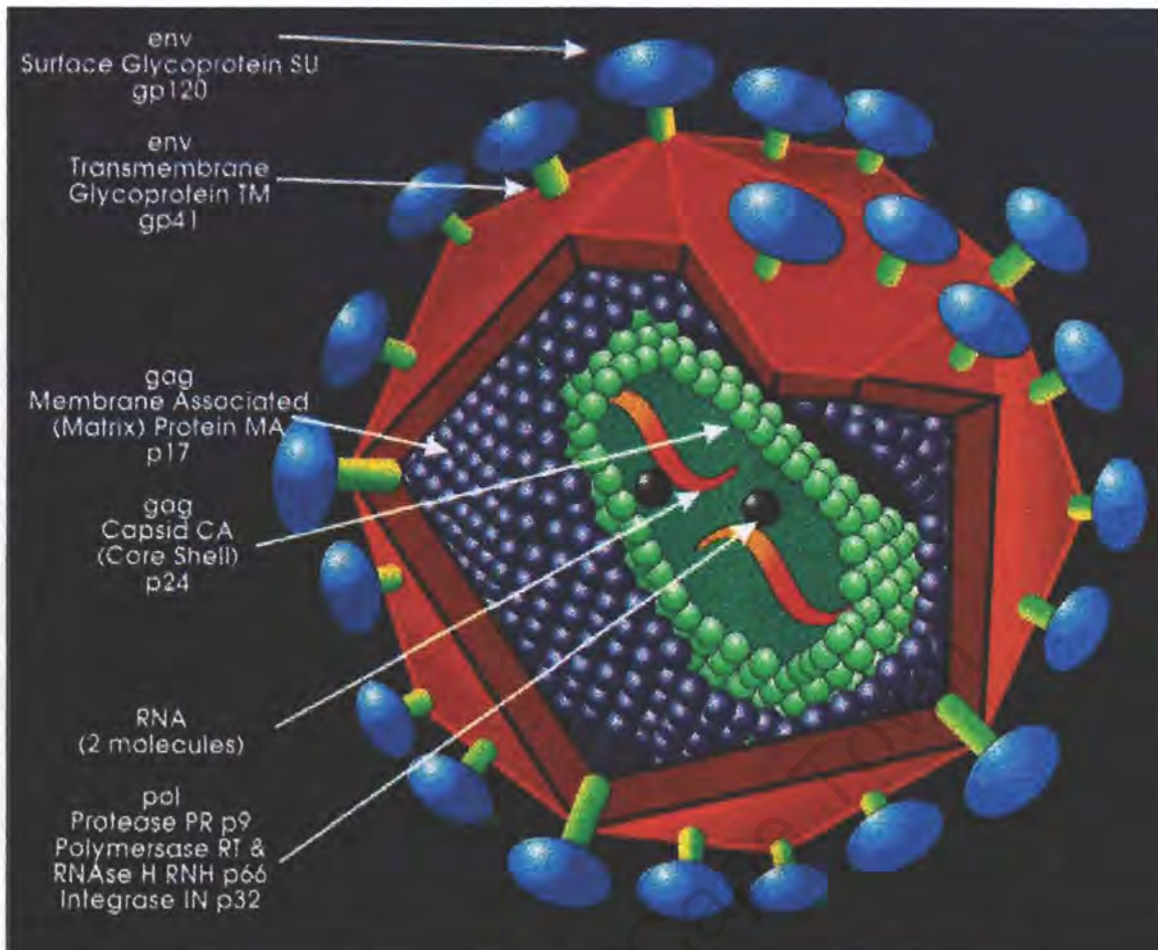

Figure 1.5: HIV structure (http://www.critpath.org/aric/library/img002.htm).

Structural proteins and viral enzymes.

Gag. The *gag* gene is located at 5' end of the genome and the p55 Gag polyprotein (55kD) is translated from genome length RNA transcripts. P55 is cleaved during or shortly after virus budding from the cell by HIV-1 protease (PR) mediated by recognition of a myristylated N terminus (Gottlinger *et al.*, 1989; Spearman *et al.*, 1997; Zhou and Resh, 1996). Proteolytic cleavage yields mature Gag proteins, p17 matrix (MA), p24 capsid (CA), p7 nucleocapsid (NC), p6 and two small spacer peptides p1 and p2 (Farmerie *et al.*, 1987; Kramer *et al.*, 1986; Krausslich *et al.*, 1995; Pettit *et al.*, 1994; Wiegers *et al.*, 1998). The p17 matrix protein plays a role in nuclear localization of the pre-integration complex (PIC) (Kiernan *et al.*, 1998), RNA binding, transport of viral RNA to the plasma membrane, antigen, particle assembly, regulation of lymphocyte and HIV-1 replication (De Francesco *et al.*, 1998). During later stages of the HIV life cycle p17 is involved in Env incorporation into virions, transportation of precursor polyproteins

to the plasma membrane for assembly and budding (Cannon *et al.*, 1997; Yu *et al.*, 1992). P24 capsid protein (CA) plays an important role in virus assembly and maturation by forming a shell surrounding the viral RNA genome and core-associated proteins (Huang and Martin, 1997). CA is also involved in early postentry steps (Gross *et al.*, 1998). The p7 nucleocapsid (NC) plays a role in RNA binding and encapsidation (Schwartz *et al.*, 1997; Zhang and Barklis, 1995), RNA dimerisation (Poon *et al.*, 1996), Gag-Gag interactions (Lee and Yu, 1998), stability of the preintegration complex, membrane binding (Sandefur *et al.*, 1998) and reverse transcription (Guo *et al.*, 1997). The function for p1 and p2 is unclear, they may regulate cleavage rates at cleavage sites and so influence the ordering of virion morphogenesis (Krausslich *et al.*, 1995; Pettit *et al.*, 1994; Wiegers *et al.*, 1998).

Pol. Occasionally, due to a frame shift, a large polyprotein is translated comprising of Gag-Pol products (Mervis *et al.*, 1988; Ratner and Haseltine, 1985). Proteolytic cleavage of *pol* yields viral enzymes protease (PR), reverse transcriptase (RT) and integrase (IN). Protease is active only as a dimer (Nutt *et al.*, 1988). Reverse transcriptase is responsible for generating dsDNA from viral RNA. Integrase, that forms part of the PIC, is involved in the incorporation of viral dsDNA into the host genome (Barre-sinoussi, 1996).

Envelope. Envelope is a type I integral membrane protein. The envelope precursor gp160 (160kDa) is transcribed from singly spliced mRNA transcripts. Processing and synthesis of gp160 occur via the endoplasmic reticilum (ER) pathway. The Golgi body is the site of further post-translational processing of gp160 through glycosylation, influencing both immunological and functional properties. Cleavage of gp160 by host cellular proteases produces the major external envelope glycoprotein SU gp120 (120kDa) and the transmembrane protein gp41 TM (41kDa). The envelope is partly derived from the cell membrane of the host cell as the virus buds through the cell membrane during the final stages of replication. The gp120 protein is visible protruding on the outside of the envelope as spikes and is anchored by non-covalent interactions to the outer surface of the envelope by gp41 protein. The mature gp120-gp41 proteins are associated as a trimer. Envelope gp120 is discussed in more detail in section 1.2.3.

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Regulatory proteins.

Regulatory proteins coded for by *tat* and *rev* are produced from overlapping reading frames by multiply spliced mRNAs. They control viral gene expression at the transcriptional and post transcriptional level respectively and are essential for virus propagation.

Tat. The *tat* gene consists of two genomically distinct regions, after transcription these exons are spliced together to produce a monocistronic mRNA. This codes for the 14kDa Tat protein. Tat has been localized primarily in the nucleus but can also exist extracellularly in large quantities (Barre-sinoussi, 1996; Kuiken *et al*., 2000). Tat plays a major role in regulation of viral gene expression. Tat acts by binding to the TAR (trans-activating response) RNA element within the long terminal repeats (LTR) transactivating transcription and elongation (Cullen, 1990; Haseltine, 1988). Tat is essential for HIV-1 viral replication (Cullen, 1993; Jones and Peterlin, 1994).

Rev. This 19 kDa phosphoprotein is also coded for by two exons that are spliced to produce a monocistronic transcript. Rev is localized primarily in the nucleus and regulates viral gene expression at post transcriptional level. They act by binding to RRE (rev-responsive element) on all unspliced or singly spliced HIV mRNAs (encoding structural proteins, *env*, *gag* and *pol*), regulating the ratio of spliced to unspliced viral mRNAs. They also promote nuclear export, stabilization and utilization of such transcripts. Rev is crucial for the generation of structural proteins (Barre-Sinoussi, 1996; Kuiken *et al*., 2000).

Accessory/auxiliary proteins.

All complex primate lentiviruses contain a series of accessory/auxillary proteins, *nef, vif, vpr, vpx* and *vpu*. Although these proteins appear to be dispensable for viral propagation *in vitro* they seems to be more important *in vivo*. Several accessory proteins are multifunctional with distinct roles at different stages in virus replication. Most accessory proteins are transcribed from multiply sliced mRNA transcripts and coded for by a single exon. They all are relatively small proteins of 80 to 210 amino acids (Miller and Sarver, 1997) All primate lentiviruses contain the *vif* and *nef* genes; *vpu* is restricted to HIV-1

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and SIVcpz; *vpx* is restricted to HIV-2, SIVsm, SIVmac and SIVagm; and all primate lentiviruses with the exception of SIVagm contain *vpr* (Barre-Sinnoussi, 1996).

Nef. Nef is a multifunctional 27kD myristylated protein produced by an ORF located at the 3' end of the HIV genome. Tat, Rev and Nef are not incorporated into virion particles but are the first viral components produced from multiply spliced viral mRNA (Barre-Sinoussi, 1996; Kuiken *et al.*, 2000). Nef is predominantly located in the plasma membrane via a myristyl residue, but has also been found in the nucleus. Nef was originally thought to be a down regulator of viral gene expression, hence the origin of the misnomer "negative factor". Nef is now known to down regulate CD4 (Aiken *et al.*, 1994) and MHC class I molecules, stimulating virion infectivity and to alter the activation state of cells.

Vif. Vif or viral infectivity factor is a basic protein of 23kD that is able to exists in both a soluble cytosolic form and a membrane associated form. Vif functions in transporting incoming virus to the nucleus (Karczewski and Strebel, 1996) and may play a role in HIV provirus formation (Simon and Malin 1996).

Vpu. Vpu is a 16kDa type I integral membrane protein with a 20 residue long hydrophobic helix acting as a membrane anchor region. Vpu functions firstly in degradation of CD4/gp160 complexes releasing gp160 for further processing (Bour *et al.*, 1995). Secondly oligomerised Vpu forms cell membrane ion channels enhancing virion release (Ewart *et al.*, 1996; Gottlinger *et al.*, 1993). Thirdly, Vpu is responsible for the decrease in cell surface expression of major histocompatibility complex (MHC) class I in HIV-1 infected cells (Kerkau *et al.*, 1997). It has been suggested that this decrease in antigen presentation is one reason for the inability of CD8+ T cells to eliminate HIV infection.

Vpr, viral protein R is a small 15kD protein (96 amino acid) translated from a single-spliced RNA, is dependent on Rev and is intracellularly localized in the nucleus (Kondo and Gottlinger, 1996; Lu *et al.*, 1995). Vpr functions by targeting the viral preintegration complex (PIC) to the nucleus (Heinzinger *et al.*, 1994) as well as arrests

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dividing cells in G2 increasing virus production (Bartz *et al.*, 1996; Di Marzio *et al.*, 1995; He *et al.*, 1995; Jowett *et al.*, 1995).

Life-Cycle.

Both viral and cellular proteins regulate HIV-1 replication. The precise ordering of steps in the lifecycle has not been definitely determined and multiple steps are likely to occur in a concerted, simultaneous fashion (Figure 1.6).

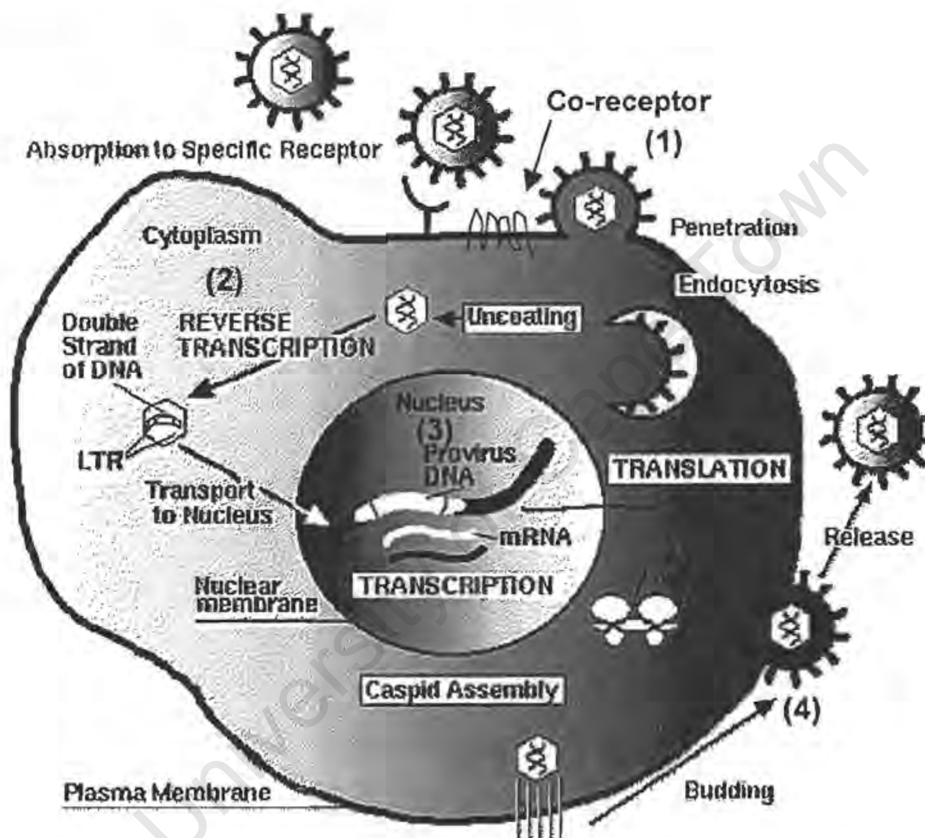

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Figure 1.6: The HIV-1 replication cycle. (1) **Adsorption and penetration.** This is through attachment of virus gp120 to the host cell CD4 receptor takes place. Binding to gp120 triggers a gp120 conformational change exposing a co-receptor binding site and allowing gp120 to bind to a co-receptor. This in turn causes an additional conformational change within gp120/gp41 allowing insertion of gp41 three coiled N terminal fusion peptide into the host cell membrane. Membrane fusion between the lipid bilayer of the virion and the host cell plasma membrane takes place. Now the viral core or nucleoprotein complex is released into the cell cytoplasm. (2) **Uncoating and reverse transcription.** Inside the cell, the viral core releases its viral RNA and enzymes where reverse transcription of the vRNA takes place through RT. This generates a double-stranded DNA copy (cDNA) termed the preintegration cDNA complex (PIC) which is transported to the nucleus. (3) **Integration.** cDNA is integrated into the host cell chromosome by IN, this is termed the provirus. The provirus may remain latent for years but produces viral mRNA transcripts through host

cellular enzymes upon activation. (4) **Translation, Assembly and Budding.** Viral protein synthesis and post-translational modifications takes place in the cytoplasm facilitated by host cellular machinery. Viral proteins are transported to the plasma membrane where maturation of viral particles takes place and budding occurs. During this process Gag p55 recruits two copies of sRNA and lines the inner face of the plasma membrane. This induces membrane curvature and budding while Env glycoproteins are incorporated into the host cell lipid bilayer. During budding protease cleaves the Gag-pol polyprotein to the mature functional capsid, nucleocapsid, matrix and enzymatic proteins forming a mature virus particle. (Adapted from Barre-Sinoussi, 1996; Freed, 1998; http://www.critpath.org).

1.2.3 *Env* Gp120

Env gp120 is essential for HIV-1 transmission as it contains determinants for entry into host cells as well as neutralisation determinants. As this study aims to characterise viral populations following transmission through determination of envelope diversity, a detailed description of envelope is provided.

Genomic organisation.

Gp120 consists of 5 genetically conserved regions C1 to C5 interspersed with 5 variable loops, V1 to V5. The first four variable regions form surface-exposed loops through disulphide bonds at the cystine residues at the base of the loops (Leonard *et al.*, 1990). The V3 loop protrudes through the surface of the gp120. It consists of 35 amino acids and has a relatively conserved tetrapeptide crown of 4 amino acids that allow only amino acid changes with similar chemical properties. The V3 loop has particular significance, as it is responsible for viral attachment to the CD4 cellular receptor, contains the principle neutralizing domain, determines cell tropism, cytopathicity and co-receptor usage of the virus.

Host cell entry determinants.

CD4+ cellular receptor and other main receptors. HIV-1 entry into host cells requires the sequential interaction of gp120 with the CD4 glycoprotein primary cellular receptor followed by interaction with a second cellular co-receptor. Gp120 binds to the most amino-terminal of the four immunoglobulin-like domains of CD4. CD4 binds to gp120 in a carbohydrate-devoid depression at the interface of the outer domain with the

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inner domain and the bridging sheet of gp120. Direct interaction only occurs between 26 gp120 amino acid residues and 22 CD4 residues (Kwong *et al.*, 1998).

Alternative cellular receptors can be utilised by HIV. The most striking of these are DC-SIGN, playing a particularly important role in mucosal transmission. This type II membrane protein with a mannose binding, C-type lectin domain is located on some dendritic cells (DC's) (Geijtenbeek *et al.*, 2000 a, b). A site-specific glycosylation site at the base of the V2 loop seems to be involved in the binding of DC-SIGN to gp120 envelope (Lue *et al.*, 2002). DC-SIGN is discussed in more detail in section 1.3.1.

Co-receptors. Binding to CD4 alone is not enough to result in viral fusion to the host cell (Chesebro *et al.*, 1990). CD4 binding induces conformational changes in the gp120 glycoprotein exposing a co-receptor binding site in the V3 loop that, until now, has been partially occluded by the V2 loop (Kong *et al.*, 1998). Other more conserved gp120 structures exposed upon CD4 engagement also seem to be involved in co-receptor binding. Co-receptor binding in turn triggers an additional conformational change that leads to exposure of the gp41 ectodomain. It is this step that finally mediates fusion (Trkola *et al.*, 1996; Wu *et al.*, 1996).

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Co-receptors belong to the 7 transmembrane G-protein coupled chemokine receptor family. The two most well defined cellular co-receptors are CXCR4 and CCR5, members of the CXC (a) and CC (b) chemokine receptors subfamilies, respectively. CXCR4 was the first HIV-1 co-receptor to be characterized but CCR5 was subsequently determined to be the principal co-receptor associated with sexually transmitted HIVs (Michael, 1999). During disease progression highly cytopathic X4 viruses either in addition to, or instead of, R5 viruses can evolve (Connor *et al.,* 1997; Scarlatti et al., 1997). In some the R5 to X4 shift may be concomitant with, or occurs shortly prior to, disease emergence (Xiao *et al.*, 1998). Other occurrences accompanying this switch is accelerated loss of CD4+ T cells (Koot *et al.*, 1993), reduced survival time after AIDS diagnosis (Tersmette *et al.*, 1989) and a broadened HIV-1 co-receptor profile (Bjorndal *et al.*, 1997; Connor and Ho, 1994; Dittmar *et al.*, 1997; Koot *et al.*, 1993; Liao *et al.*, 1997; Rucker *et al.*, 1997; Scarlatti *et al.*, 1997). This co-receptor switch seems to be a trait more associated with subtype B, where X4 viruses appear in up to 50% of subtype B

infected patients in late stage disease. Incidence of X4 in subtype C infections appears to be much lower, even though this subtype appears equally pathogenic (Abebe *et al.*, 1999; Ping *et al.*, 1999).

Other co-receptors have been implicated in HIV or SIV entry *in vitro*, such as CCR2b, CCR3, CCR8, gpr15 (BOB), STRL33 (Bonzo), APJ, V28 and MDC, but their relevance *in vivo* has not been well documented, and all HIVs at a minimum use CCR5 or CXCR4 (Doranz *et al.*, 1996; Dittmar *et al.*, 1997; He *et al.*, 1997; Liao *et al.*, Rucker *et al.*, 1997;). CCR3's role in the central nervous system infection through microglial cells, that express CCR3 in addition to CCR5, has been shown (He *et al.*, 1997). Virtually no primary HIV-1 isolates have been shown to utilize CCR2b (Chen *et al.*, 1998). Co-receptors have natural cellular ligands and HIV-1 competes with these. The natural ligands for the CCR5 receptor is RANTES, MIP1α and MIP1β and for the CXCR4 receptor, SDF-1α (Berger *et al.*, 1998). Determinants in CCR5 facilitating gp120 binding, is a cluster of residues in the amino terminal domain of CCR5 allowing fusion and entry for both R5 and R5X4 isolates (Farzan *et al.*, 1998). For CXCR4, residues dispersed throughout the extracellular domain of CXCR4 facilitate gp120 fusion and entry (Kajumo *et al.*, 2000). The determinants of co-receptor usage on gp120 (Hoffman *et al.*, 1998; Trkola *et al.*, 1996; Wu *et al.*, 1996) lie within the 35 amino acid stretch of the V3 region and to a lesser extent V1 and V2. The occurrence of positively charges amino acids at either one or two fixed positions in the V3 loop as well as an increase in V2 charge can result in a R5 to X4 switch (Fouchier *et al.*, 1992; Hoffman *et al.*, 2002; Kolchinsky *et al.*, 2001; Labrosse *et al.*, 2001; Ogert *et al.*, 2001;).

Neutralisation determinants.

The *env* gene contains neutralisation determinants for host neutralising antibodies (Palker *et al.*, 1988). Neutralising antibodies target the surface gp120 and transmembrane gp41 envelope glycoproteins of the virus (Parren *et al.*, 1999; Poignard *et al.*, 1996). Most of the envelope surface is hidden from humoral immune recognition by extensive glycosylation and oligomeric occlusion (Wyatt and Sodroski, 1998). The majority of broadly neutralising antibodies access only two surfaces, one that overlaps the CD4 binding site (shielded by the V1V2 loop) and another that overlaps the co-receptor site (shielded by V2V3). Thereby either blocking attachment of gp120 to its cellular

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receptor, and co-receptors or by preventing gp41 from mediating fusion with the target cell membrane (Alkhatib *et al.*, 1996; Trkola *et al.*, 1996; Wu *et al.*, 1996). CD4-induced exposure results in enhanced antibody binding (Kwong *et al.*, 1998).

Definition of neutralisation. Antibody-mediated neutralisation is defined as "the loss of infectivity which ensues when antibody molecule(s) bind to a virus particle, and usually occurs without the involvement of any other agency. As such this is an unusual activity of antibody paralleled only by the inhibition of toxins and enzymes" (Dimmock, 1995). Neutralising antibodies can act against virions and against infected cells. Activity to virions is most often considered as "neutralization" and is the most important for antibody-mediated protection in vivo as antibody is more effective against free virions than against infected cells (Pantaleo and Fauci, 1995).

Mechanism of neutralisation. The exact mechanism of neutralisation is still under debate, but is most likely a simple occupancy model. According to this model, neutralisation occurs when a fairly large proportion of available sites on the virion are occupied by antibody, leading to the inhibition of virus attachment or interference with the cellular entry process. This model is supported by an observed linear relationship between the surface area of a virus and the number of antibody molecules that are required to bind to the virus for neutralisation. This number is approximately that predicted to efficiently coat the virion particle, given the size of the antibody molecule. (Burton, 2002; Parren and Burton, 2001).

Cross neutralisation between subtypes. The uncertain relevance of genetic subtype to HIV-1 vaccine is owed in part to a poor understanding of the immunotype diversity of the virus as it relates both to cellular and humoral immunity. The fact that genetic subtypes tend to cluster geographically raises the possibility that distinct immunotypes of the virus have evolved along similar lines. Relatively little is known about the neutralisation capacity of subtype C isolates and serum from SA. A recent study examined South African (Du-cohort), subtype C sera neutralisation activity. Extensive cross-neutralisation (70%) of a large number of heterologous subtype C isolates from SA, obtained very early in infection, (Bures *et al.*, 2002), was observed. This suggests that SA serum and isolates have an unusual degree of shared

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neutralisation determinants at regional level. Poor cross neutralisation was observed between subtype C sera and subtype B isolates as was observed before (Smith *et al.*, 1998), although a few subtype C sera were able to cross neutralise a wide spectrum of subtype B and C isolates. This seems unusual as SA subtype C was shown to have been the result of multiple introductions of the virus into the country, with greater diversity as can be observed in clonal epidemics such as India and Thailand (Bredell *et al.*, 1998; van Harmelen *et al.*, 1997). It seems from these results that each subtype consists of multiple serotypes of varying subtype specificity, posing great obstacle in the way of vaccine design. However, shared neutralisation determinants in subtype C shows that neutralising antibodies induced by vaccines will have less epitope diversity to overcome at a regional level.

Monoclonal antibodies. Although HIV elicits weak cross-neutralisation responses, a small number of human monoclonal antibodies (Mabs) with broad neutralising activities have been isolated from infected individuals. Only a few monoclonal antibodies, b12, 2G12, 2F5, Z13, X5 and 4E10 are capable of neutralising primary isolates of HIV-1, this is due to HIV evolving various protective mechanisms to enable it to resist the binding of antibodies to its envelope glycoprotein complex (Parren and Burton, 2001; Parren *et al.*, 1999).

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In a study examining neutralisation through MAb's of subtype C isolates (Bures *et al.*, 2002), it was noted that subtype C isolates resemble subtype B in their sensitivity to neutralisation by G1b12 and to a lesser extent 2F5. However, unlike subtype B, all subtype C isolates in this study showed resistance against 2G12 neutralisation. This MAb, 2G12, was previously shown to have the ability to neutralise a broad spectrum of HIV-1 isolates (Bures *et al.*, 2000; Trkola *et al.*, 1995). In studies that did incorporate very small numbers of subtype C's, it was shown that high doses of 2G12 was required for neutralisation to occur (Trkola et la, 1995).

Neutralisation epitopes. Epitopes responsible for primary isolate neutralisation by serum samples from HIV-infected individuals remain largely unknown although some has been identified. These include the ELDKWA epitope of Mab 2F5 and as well as the CD4 binding site epitope of Mab b12 (Muster *et al.*, 1993; McInerney et al., 1997).

Neutralising and non-neutralising epitopes do not exist as distinct entities on the viral surface. These epitopes generally represent antibody accessible structures on the virion surface that are present in a valence high enough that a critical antibody density can be achieved (Parren and Burton, 2001).

A unique epitope for the binding of 2G12 have recently been identified. This relatively conserved epitope in an otherwise variable gp120 surface was shown to be associated with mannose sugars on N-linked glycosylation sites in the C2, C3, C4 and V4 domains and has probably no involvement with the gp120 peptide backbone (Sanders *et al.*, 2002; Scanlan *et al.*, 2002). This is indeed the only conserved, exposed surface on the gp120 trimer that does not interact with known cellular receptors and chemokine receptor (Kwong *et al.*, 2000). At least 6 different high-mannose and/or hybrid glycans of residues 295, 332, 392, 386, 448 and 339 have been identified in this epitope. There might be similarities between this epitope and the mannose-dependent binding sites on gp120 for DC-SIGN. Not all these positions are essential for 2G12 binding but analysis of subtype C isolates showing resistance to neutralisation by 2G12 (Bures *et al.*, 2002) may cast light on which of these N-linked mannose residues are essential for 2G12 binding. Position 295 seems crucial for the binding of 2G12 indicated by the fact that subtype C isolates from South Africa all lacked glycan 295 and were resistant to 2G12 neutralisation. This glycan is poorly conserved amongst subtype C isolates (Sanders *et al.*, 2002).

1.2.4 HIV–1 Genetic diversity

Geographical distribution of HIV-1 subtypes.

Global. Most subtypes have been detected in or are associated with Africa (Figure 1.7), with the greatest genetic variation in HIV-1 detected in Central Africa. Globally subtypes A and C account for most current infections followed by subtype B and the intersubtype recombinants CRF01_AE and CRF02_AG. Subtype C currently infects more people worldwide than any other subtype; it is common in southern Africa and India, accounting for >50% of all HIV infections (Essex, 1999; McCutchan, 2000) (Table 1.3).

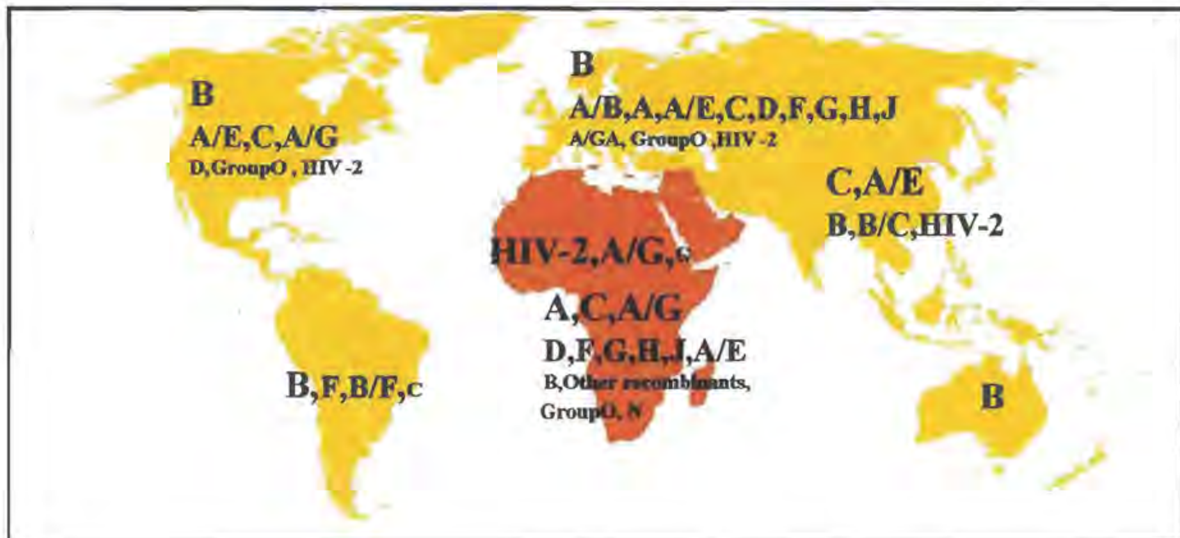

Figure 1.7: Geographic distribution of HIV-1 types, groups, subtypes and CRFs. The estimated distribution of HIV strains in sub-Saharan Africa, West Africa, Asia, Europe, North and South America, and Australia are shown. Size of type relates to relative abundance. A/E, CRF01_AE; A/G, CRF02_AG; A/G/I, CRF04_cpx; A/B, CRF03-kal153; B/C, two different CRFs (potentially CRF07_BC and CRF08_BC) (Adapted from McCutchan, 2000)

Africa. The most common subtypes circulating within Africa are A, C, D and CRF02_AG (Carr *et al*., 1999) (Table 1.2). Substantial prevalence of subtypes F, G, H, J, and CRF01_AE have also been noted. In contrast to Europe and for most of America, subtype B is considered a relatively rare strain in Africa. Other rare subtypes in Africa include a variety of other recombinants as well as groups O and N. All group M viruses have been found in central Africa. Indeed Central Africa harbours the greatest variety of subtypes, lending evidence for central Africa as the epicentre for the origin of HIV. West Africa harbours mostly CRF02_AG and G (McCutchan *et al*., 1999), and in some countries such as Cameroon group O viruses are found at low frequency. HIV-2 is more locally spread in the northwestern part of Africa, i.e. the Ivory Coast and Guinea-Bissau (Kanki *et al*., 1994). In Southern African countries such as Zambia, Zimbabwe, South Africa and Malawi, as well as Ethiopia, Eritrea and Somalia, subtype C dominates (Figure 1.8). Recent studies have indicated that recombinants are much more prevalent than originally thought in Africa, constituting up to 40% of infections in countries such as Tanzania and Kenya (Hoelscher *et al*., 2002).

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South Africa. Subtype C was the first HIV genotype from South Africa to be identified (Dietrich *et al.*, 1993) and was subsequently shown to be the major HIV-1 subtype in the heterosexual population of this country (Williamson *et al.*, 1995). More than 95% of isolates screened in South Africa were subtype C (Bredell *et al.*, 1998; Engelbrecht *et al.*, 1998, 1999; Moodley *et al.*, 1998; van Harmelen *et al.* 1997, 1999), with less than 5% of isolates screened belonging to subtype B, A, G and D (Bredell *et al.*, 2002).

Table 1.2: Published African full-length HIV-1 genomes, noting their subtype and country of origin (Adapted from McCutchan, 2000).

	Category†	Geographic origin*
Subtypes	A	Uganda, Tanzania, Kenya, Somalia
	B	Throughout Africa
	C	East, Southern Africa
	D	Uganda, DRC, Kenya
	F and F2	DRC, Kenya, Congo, Cameroon
	G	Nigeria, Kenya, Congo, DRC
	H	CAR
	J	DRC
	K	Cameroon
Circulating Recombinant forms	CRF01_AE CM 240	CAR
	CRF02_AG CM 240	Djibouti, Ivory Coast, Cameroon
	CRF05_FD VI 1310	DRC
	CRF06_cpx BFP90	Burkina Faso, Mali
	CRF09_cpx p291	Senegal
ISR – intersubtype recombinants	AC recombinant	Tanzania, Zambia Rwanda, Ethiopia, Uganda
	AD recombinant	Uganda, Kenya
Others	Unclassified	Cameroon, DRC, Gabon

† CRFs are numbered in the order discovered. Cpx refer to complex recombinants combining three or more subtypes. Intersubtype recombinants forms or IRS, are forms so far identified only in a single individual and without current evidence of epidemic spread. Unclassified strains fail to cluster with known subtypes in all genome regions examined, * CAR, Central African Republic; DRC, Democratic republic of Congo, formerly Zaire.

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The initial South African epidemic was associated mainly with homosexual subtype B transmission (Van Harmelen *et al.*, 1997; Williamson *et al.*, 1995). Evidence suggests subtype B may have been introduced into South Africa from other countries (Sher, 1989; van Harmelen *et al.*, 1997). There is preliminary evidence to suggest that subtype B in South Africa, is in the process of spreading from the homosexual community to establish an epidemic in the heterosexual community (Van Rensburg, EJ, unpublished data).

The heterosexual epidemic in South Africa is a relatively recent epidemic, since the first cases were reported in the late 1980s (Maartens *et al.*, 1997). Analysis of phylogenetic relationships between all published subtype C sequences from the southern African region, showed that subtype C viruses do not cluster according to geographical origin of infection (Bredell *et al.*, 1998; Van Harmelen *et al.*, 1999), supporting a multiple introduction scenario in South Africa. The origin of this epidemic is probably due to regional spread from other countries due to close socio-economic ties between SA and its neighbouring countries (Bredell *et al.*, 1998; van Harmelen *et al.*, 1997; Williamson *et al.*, 1995). As HIV-1 continues to spread globally, geographical restrictions are increasingly breaking down; many European countries, for example, have residents infected with multiple genetic subtypes (Clewley *et al.*, 1996; Courturier *et al.*, 2000; Jonassen *et al.*, 2000; Op de Coul *et al.*, 2000).

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Table 1.3: Table depicting geographic distribution of HIV-1 and HIV-2 subtypes outside of Africa.

Geographic region	Subtype	Reference
India and south east Asia	C, AE, B BC	Su *et al.*, 2000
Philippines	B, E, A, C, D	Paladin *et al.*, 1998
Thailand	B, E, AE	Subbarao *et al.*, 1998
Europe	B, all other subtypes and prevalent CRFs, O and HIV-2	Morgado *et al.*, 2002
North America	B, 1% of infections due to all other subtypes and prevalent CRFs	McCutchan, 2000
South America	B, 25% F and/or BF, C	Morgado *et al.*, 2002
Australia	B	Oelrichs *et al.*, 2000

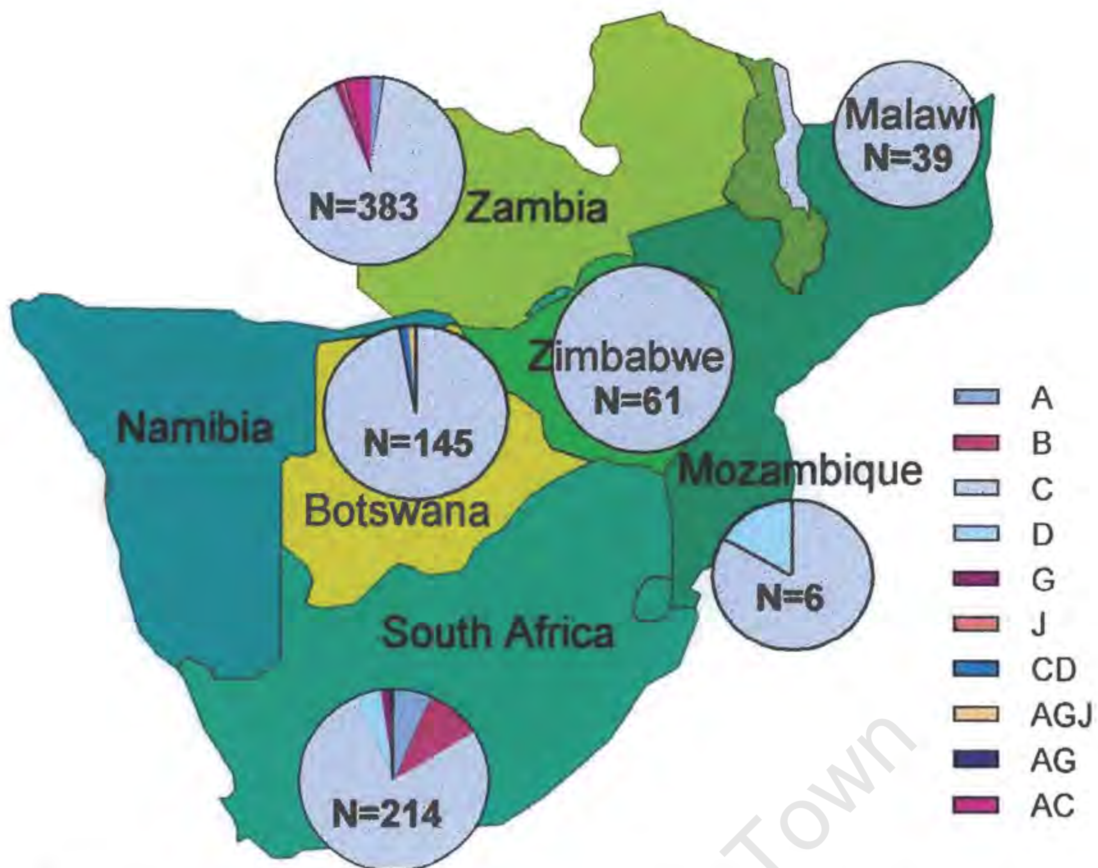

Figure 1.8: HIV-1 subtype distribution and abundance in Southern Africa as determined from the number of published HIV-1 genomic >300bp fragments in *gag* and *env*.

1.2.5 HIV-1 Variability

Variability in perspective.

The HIV-1 genetic diversity is greater than anything described for other human viral infections studied. Currently, strains within a single individual (intraperson) can differ as much as 10% in sequence (Kuiken *et al.*, 1996; Shankarappa *et al.*, 1999), while between subtypes (intersubtype) distances can be as much as 35%. A few amino acid changes in the envelope glycoprotein of influenza virus can be sufficient to trigger a new epidemic (Beirnaert *et al.*, 2000; Berman, 1998); re-assortants of influenza virus envelope genes can lead to devastating pandemics (Dowdle, 1999; Raymond *et al.*, 1986; Taubenberger *et al.*, 2000). A less than 2% amino-acid change can cause a failure in the cross-reactivity of the polyclonal response to the influenza vaccine and necessitates changing the vaccine strain (Gaschen *et al.*, 2002). Therefore, even within a subtype (intrasubtype), the extent of HIV-1 genetic diversity that varies up to 20% in envelope

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proteins is very high compared to the diversity found in viruses for which effective vaccines have been developed (Moore *et al.*, 2001). The capacity of the virus to mutate at any amino acid residue is constrained, however, by the functional or structural value of the residue to virus survival (Wrobel *et al.*, 1998).

Forces driving genetic variation.

Viral characteristics (error-prone reverse transcriptase, high viral turnover rate and genetic recombination), host specific influences (cellular and humoral immune responses and availability of cells to be infected) and external influences (antiretroviral therapy) drive genetic variation in HIV.

Reverse transcription and rapid viral turnover. The high error rate of reverse transcriptase was identified as the main source of HIV-1 diversity and is an identifying characteristic of all lentiviruses (Bebenek *et al.*, 1989; Boyer *et al.*, 1992). This high rate of incorrect nucleotide substitutions by reverse transcriptase (10^{-4} per nucleotide resulting in approximately one nucleotide miss-incorporation per replication cycle of each 10Kb genome) is due to its lack of proof reading activity (Pathak and Temin, 1990; Preston *et al.*, 1988). This effect is aggravated by the rapid viral turnover (10^{10} viral particles/day) and cell turnover within an HIV-infected individual (Ho *et al.*, 1995; Wei *et al.*, 1995). As a result individuals harbour a swarm of closely related viruses (quasispecies), each with their own unique viral genome (Nowak, 1992).

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Immune responses. Ongoing host immune defences (cellular and humoral immune responses), directed at HIV is a further driving force for genetic variation. Positive immune selection allows the genomic region under immune pressure to escape host defence immune recognition.

HLA-restricted CTL recognition plays a role in HIV-1 evolution *in vivo* (Moore *et al.*, 2002). Circulating CD8+ T cells recognize viral and self-peptides presented by cell surface molecules MHC class I. Both MHC class I and II are coded for by the human leukocyte antigen (HLA) loci. Allelic variants of the HLA molecule on the MHC can bind and display various antigenic peptides with differing affinities. Different ethnic

groups have differing HLA distributions and affect on CTL recognition and escape (Moore *et al.*, 2002; Novitsky *et al.*, 2001; Nowak *et al.*, 1995; Phillips *et al.*, 1991; Wolinsky *et al.*, 1996). Immune surveillance may be evaded at two levels, peptide binding in the HLA molecule and T cell response recognition (TCR) (Gotch *et al.*, 1996). CTL escape is accomplished by altering the genome to successfully interfere with epitope-HLA binding and so reduce T cell receptor recognition (McMichael and Rowland-Jones, 2001).

It has been difficult to document cellular immune selection directly, but recently cytotoxic T-lymphocytes (CTL) studies illustrated HIV evolution driven by host immune pressures more clearly. It was illustrated that cytotoxic T-lymphocyte responses (CTL) are directed at epitopes in several HIV-1 proteins and exert positive selection pressure on these genomic regions. CTL escape has been described in acute and chronically HIV-1 infected humans (McMichael and Rowland-Jones, 2001), SIV infected macaques (Evans *et al.*, 1999; Allen *et al.*, 2000) and SHIV (simian-human immunodeficiency virus) challenged rhesus monkeys after vaccination (Barouch *et al.*, 2002; Goulder *et al.*, 1997).

Selection can be quantified by measuring the extent to which nonsynonymous nucleotide substitutions (resulting in a changed amino acid) compared to synonymous nucleotide substitutions occur more frequently than would be expected by chance. This is called the Dn/Ds ratio (Nei and Gojobori, 1986).

In humoral immunity, antigenic variation and evasion of autologous neutralizing antibodies drive diversification of HIV (Arendrup *et al.*, 1992; Bongertz *et al.*, 1997; Fenyo *et al.*, 1996; Watkins *et al.*, 1996). Multiple rounds of neutralization escape diversify B cell responses to critical neutralization epitopes resulting in the broadening of the neutralization antibody response over time. This has been established as one of the factors involved in protection in LTNP (long term non progressors) (Bradney *et al*, 1999). Genetic studies of antibody-driven selection are complicated by the complex, discontinuous nature of many B-cell epitopes.

Antiretroviral therapy. Antiretroviral therapy (ARV) is also a driving force in HIV-1 genetic variability. The majority of drug-resistant mutations arise in immunogenic

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regions of the RT gene. ARV selective pressure exerted on a population of viruses results in the emergence of drug-resistant mutants and consequently lead to treatment failure (Coffin, 1995; Kijak *et al.*, 2002; Larder and Kemp, 1989). The emergence of such viral variants has been extensively described and is found even in the setting of highly active ARV (HAART) therapies (Deeks, 2001; Loveday, 2001; Miller, 2001). When ARV selective pressure is removed, drug-sensitive quasispecies with a higher fitness in a drug-free environment emerge. This rationale led to the many structured treatment interruption (STI) therapies carried out on patients with treatment failure and multi-drug resistant viruses (Deeks, 2001; Miller, 2001).

Availability of cells. The number of type of cells available for HIV to infect and replicate in also determines genetic variation. In early infection R5 viruses are almost exclusively found indicating that this phenotype is preferentially transmitted. As HIV infection progress preferential viral destruction and exhaustion of CCR5 expressing cell populations take place. This in part is thought to drive the switch from the R5 to the X4 phenotype, enabling HIV now to infect a whole new source of cells expressing CXCR4. As the switch for the R5 to X4 is genetically determined this is indirect evidence for genetic selection and increased genetic variation (Overbaugh and Bangham, 2001).

Genetic Recombination. Another source of variation is genetic recombination. Recombination between two genetically distinct isolates of the same retrovirus species was first described in the 1970s in Avian tumor viruses (Kawai and Hanafusa, 1972; Vogt, 1971) and Rous sarcoma viruses (Linial and Brown, 1979). The first HIV-1 to be identified as a recombinant was MAL, an A/D/? strain from the Democratic Republic of Congo (Alizon *et al.*, 1986).

Extent of recombination. Recombination of HIV has proved to be a more frequent event than originally recognized serving a central role in viral evolution (Robertson *et al.*, 1995). The recombination rate of HIV-1 has been established at approximately three events per genome during every replication round, with a range of one to seven crossovers (Jertzt *et al.*, 2000). A recent paper showed that 75-80% of cells harbour two or more proviruses and that a single splenocyte cell may contain up to eight different proviruses. This evidence indicates that recombination must be a frequent

University of Cape Town

event, readily detected only when recombination occurs between different subtypes (Jung *et al.*, 2002).

Method of recombination. A cell can be infected by two genetically distinct viruses, either of the same subtype (producing intrasubtype recombination) or different subtypes (producing intersubtype recombination). This multiple infection may occur as a result of the simultaneous passage of multiple viruses or through sequential passage from multiple transmission events (Blackard *et al.*, 2002). The resultant co-packaging of RNA genomes from the two genetically distinct viruses into a single viral particle makes recombination possible during the next cycle of infection (Coffin, 1979; Goodrich and Duesberg, 1990; Hu and Temin, 1990; Junghans *et al.*, 1982; Kartz *et al.*, 1990). Through strand displacement and template switching during dsDNA synthesis, reverse transcriptase creates a mosaic genome. Once integrated into the cellular genome, this mosaic DNA becomes the source of the mosaic RNA genomes of the progeny virions.

CRFs and ISRs. A recombinant lineage is designated a CRF (Circulating Recombinant Form) if they can be grouped phylogenetically with distinct subtypes in different genomic regions and when related forms are found in more than 3 epidemiological unlinked individuals (Table 1.4). Until this can be established, these recombinants are termed intersubtype recombinants (ISRs). ISRs are either less stable in the population or not had an opportunity to spread. They have ill defined crossover sites, and have been generated from more recent dual infections than CRFs (Cornelissen *et al.*, 1996; Cornelissen *et al.*, 1997; Jetzt *et al.*, 2000; Neilson *et al.*, 1999). Recombination can be distinguished from an accumulation of minor genotypic changes, by a distinct breakpoint or crossover site. Sequences on either side of the breakpoint in a recombinant, will group with different subtypes (Robertson *et al.*, 1995; Robertson *et al.*, 2000). A recent *in vitro* study reported a "hot spot" for recombination in the C2 *env* domain (Quinones-Matau *et al.*, 2002).

Recombination between group M and O has also been reported (Peeters *et al.*, 1999; Takehisa *et al.*, 1999). To date, no recombination between HIV-1 and HIV-2 has been reported, despite the possibility of such an event as they coexist in several countries in West Africa.

University of Cape Town

Table 1.4: Geographic spread of eight established CRFs (Adapted from McCutchan, 2000)

CRF	Geographic distribution	Number of full length sequences	Reference	Comments
CRF01_AE	Thailand, China, CAR, USA	9	McCutchan *et al.*, 1996	
CRF02_AG	Djibouti, Ivory Coast, Cameroon, Senegal, Guinea Bissau, Nigeria, Congo, Gabon, USA	11	Carr *et al.*, 1998 McCuthan, 2000	Commonly known as IbNG
CRF03_AB	Russia	1	Liitsola *et al.*, 1998	Circulates among IDU
CRF04_cpx	Cyprus, Greece	3	Gao *et al.*, 1998; Nasioulas *et al.*, 1999	Initially subtype I
CRF05_FD	DRC	2	Laukkanen *et al.*, 2000	
CRF06_cpx	Burkina Faso, Mali and several west African countries	??	Montavon *et al.*, 1999, Oelrichs *et al.*, 1998	Initially designated A/G/J/?
CRF07_BC	China	2	Piyasirisilp *et al.*, 2000	Circulates among IDU
CRF08_BC	China	3	Piyasirisilp *et al.*, 2000	Circulates among IDU

Being exposed to two or more subtypes must occur at a much higher frequency than the actual corresponding infections. This is predicted by the prevalence of co-circulating strains, incidence of new HIV infections and limited prevention measures (Quinones-Mateu *et al.*, 2002). ISRs are undoubtedly contributing to HIV-1 evolution and may ultimately result in a complete dissolution of defined HIV-1 subtypes (Quinones-Mateu *et al.*, 2002).

1.3 Heterosexual Transmission:

Transmission of HIV-1 occurs via a number of routes; in a vertical fashion, from mother to child; and in various horizontal ways, including heterosexually, homosexually and through contact with contaminated blood products (intravenous drug users with

University of Cape Town

contaminated needles and blood transfusions). By far the most persistent contributor to the HIV-1 epidemic is heterosexual contact, and this review will focus on male to female heterosexual transmission.

1.3.1 Early events in HIV transmission and infection

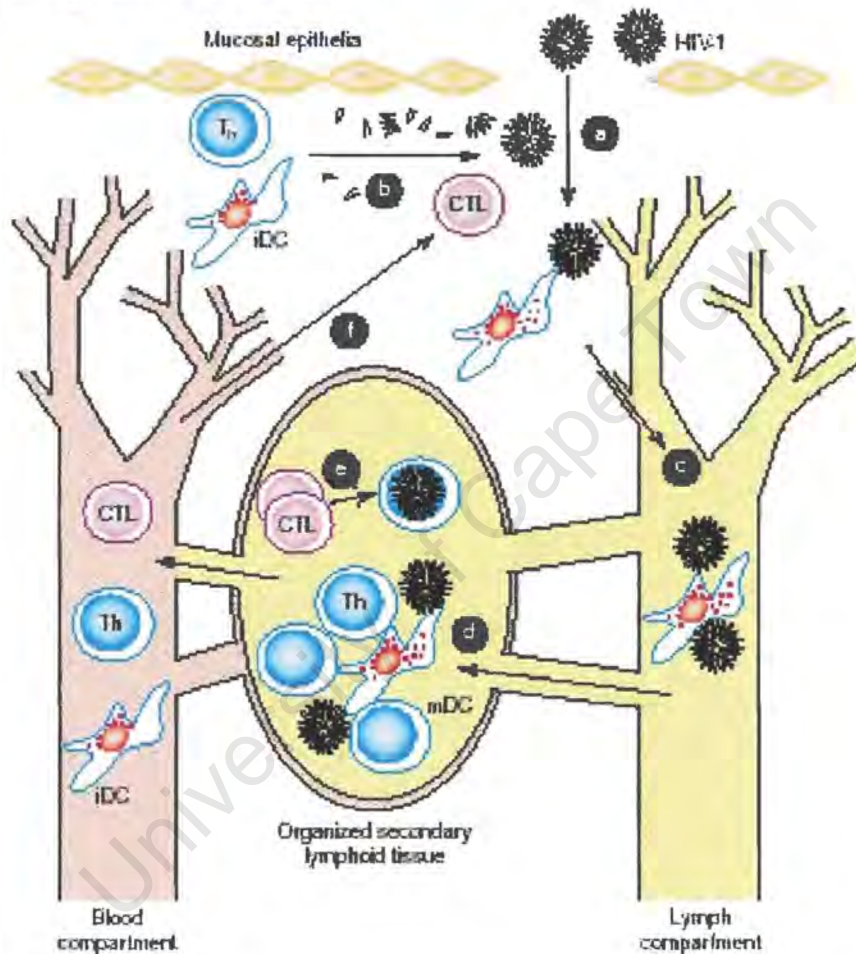
First cells encountered by HIV-1. Before infection can be established the virus needs to cross the mucosal layer through transport by epithelial cells in order to access cells permissive for viral infection like CD4+ T cells in lymphoid tissues. It has been shown that dendritic cells (DC) present in epithelial (Langerhans cells) and sub epithelial (dermal DCs) layers of the genital tract can transport HIV-1 to lymph nodes and CD4+ permissive T cells (Piguet and Blauvelt, 2002).

University of Cape Town

Dendritic cells play an important role in the generation and regulation of adaptive immune responses (Banchereau and Steinman, 1998) (Figure 1.9). Immature dendritic cells (iDCs) are widely distributed throughout the body and constantly sample the environment for antigens through phagocytosis, macropinocytosis and receptor-mediated pinocytosis. Upon stimulation by pathogenic antigen, activation of iDC takes place resulting in maturation. This involves upregulation of a variety of molecules important in activating T cells for example, cell surface MHCs to ensure efficient presentation of optimally processed antigens to cells. Maturation also coincides with migration of DCs to secondary lymphoid organs.

HIV-1 **capture** by DCs is mediated through one of a variety of mannose C-type lectin receptors (MCLRs) present on the surface of DCs in various tissues and scenario seems more complicated than first anticipated (Turville *et al.*, 2001, 2002). One recently characterized MCLR, DC-SIGN (DC-specific, ICAM-3, grabbing, nonintegrin) a type II membrane protein with an external domain is abundantly expressed in the surface of iDCs and to a lesser extent on mDCs (Geijtenbeek *et al.*, 2000a). The natural ligand for DC-SIGN is intracellular adhesion molecule 3 (ICAM-3) but HIV-1 also binds to DC-SIGN and uses it as its predominant receptor on DCs rather than CD4 through HIV-1s glycan rich envelope (Geijtenbeek *et al.*, 2000b). In particular a site-specific glycosylation site has been indicated at the base of the V2 loop to be involved in the

binding of DC-SIGN to gp120 envelope (Lue *et al.*, 2002). DC-SIGN is not exclusively required for virus capture by dendritic cells (Turville *et al.*, 2001). This function of DCs has an advantage in transporting whole pathogen to where the immune system can best see them, but becomes less advantageous to the host when the pathogen, like HIV, can infect and destroy lymphocytes. In this fashion HIV exploits the migratory nature iDC and T cell recruitment of mDC by being captured by DC-SIGN onto DCs at the mucosa, traveling to the lymph nodes to release infectious particles in to the midst of activated T cells, establishing infection in the host.



University of Cape Town

Figure 1.9: HIV-1 transmission and uptake by DCs. (a) Sexual transmission through mucosal epithelia (b) Productive infection of iDCs and CCR5+Th cells by M-tropic virions as well as HIV capture through DC-SIGN (c) Activated DCs carry HIV-1 to lymphoid tissues (d) Interactions between mDCs and CD4+CCR5+ cells leads to efficient propagation of infection within lymphoid tissues (e) Generation of HIV-1 specific CTLs. Destruction of HIV-1 infected Th cells and DCs impairs generation of CTLs specific for HIV-1 facilitating viral persistence (f) Migration of HIV-1 specific CTLs to sites of infection (Adapted from Sewell *et al.*, 2001)

It must be noted however that the most studies have been done on DCs from blood despite transmission involving mucosal DCs. Additional mucosal DC studies seem warranted. Nevertheless, DCs play a pivotal role in HIV transmission and disease establishment and this could have important implications in preventions and treatment of AIDS (Piguet and Blauvelt, 2002)

First cells infected by HIV-1. There is no direct *in vivo* data to indicate what the cell types are first infected in the reproductive tract of women. It has been shown however that chronic HIV-1 infected women harbour infected macrophages, Langerhans and T cells in cervical tissue, but these may not be the same cells infected during or directly after transmission (Pomerantz *et al.*, 1988; Pudney and Anderson, 1991).

SIV infected rhesus monkey models investigating male-to-female transmission since 1989 have produced some *in vitro* data (Miller *et al* 1989). However here have been conflicting findings. According to one group Langerhans cells present in the Lamina propria (submucosal layer of cells in the vagina) are the first cells in the female genital tract to become infected (Spira *et al.*, 1996). Another showed that activated and resting CD4+ T cells were the first to be infected after 3 days of intravaginal (IV) inoculation of SIV in monkeys (Zhang *et al.*, 1999). Hu and colleagues (2000) found that dendritic cells were the first infected, detecting infection within 60 minutes of intravaginal (IVAG) exposure to SIV-1. These discrepancies are due in part to the lack of a suitable *in vitro* model (Shattock *et al.*, 2000).

The latest evidence was proposed by Gupta and colleagues (2002). They developed a novel cervical tissue derived organ culture model mimicking *in vivo* transmission. This model showed that memory CD4+ T cells were the first to be infected 6 hours after mucosal HIV-1 (cell-free and cell-associated virus) exposure. At 96 hours after exposure Langerhans cells as well as macrophages were also detected in confirmation of previous reports (Gupta *et al.*, 2002).

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1.3.2 Co-receptor and phenotype

HIV entry in order to establish infection requires attachment firstly to CD4 and secondly to a co-receptor, either CCR5 or CXCR4 (Alkhatib *et al.*, 1996; Doranz *et al.*, 1996). Receptors and in particular co-receptors are important because they determine HIV phenotype and are used to classify HIVs.

Three systems that were not based on co-receptor use have been used until recently to classify and determine HIV phenotype. Such systems defined HIV phenotype by the cells in which they replicate *in vitro,* defining them either as macrophage (M)-tropic or T-cell-line (T)-tropic. The second system categorized viral isolates by their ability or inability to induce syncytia (multinucleated giant cells) in the MT-2 T-cell line, distinguishing them as syncytium inducing (SI) or non-syncytium-inducing (NSI) (Schuitemaker *et al.*, 1992; Tersmette *et al.*, 1988). The third system defined viruses as either slow/low (SL) or rapid/high (RH), depending on their replication kinetics in PBMCs and T-cell lines (Fenyo *et al.*, 1988). Roughly, SI and NSI correspond to RH and SL phenotypes respectively. These three classification systems were often used interchangeably, but are not synonymous (Table 1.5).

Table 1.5: Classification of HIV-1 biological phenotypes (Adapted from WHO-UNAIDS Guidelines for standard HIV isolation and characterization procedures, 2002)

Co – receptor Usage	**New Classification**	**Historic classification**		
		Cytopathology in MT2-cells	**Replication permissive cells**	**Replication rate in PBMCs and T-cell lines**
CXCR4	X4	SI	T cells tropic	Rapid/high RH
CCR5 CCR3/CCR2b	R5 R3 R2b	NSI	Macrophage tropic	Slow/low SL
CXCR4 and CCR5 and/or CCR3	R5X4 R3R5X4 or R3X4	SI	T cells or macrophage tropic	Rapid/high RH

In 1998 a new classification system was adopted where HIVs were classified according to their ability to bind to the two main co-receptors, CXCR4 and CCR5. Isolates that use CCR5 but not CXCR4 as co-receptor are termed R5 viruses, isolates using CXCR4 but

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not CCR5 are designated X4 viruses, and isolates capable of using both co-receptors, with comparable efficiencies are termed R5X4. This classification system can also be expanded to include other co-receptors if their use by an isolate proves to be a major determinant of tropism; for example, whether isolates use CCR3 and/or CCR5 to enter microglia could define then as R3, R5 or R3-R5 isolates (Berger *et al.*, 1998).

Co-receptors in transmission. CCR5 co-receptor usage is an important determinant for HIV-1 transmission *in vivo*. This is illustrated by the fact that some individuals with a deletion of 32 base pairs in both alleles of the CCR5 gene remain uninfected despite multiple HIV-1 exposures (Liu *et al.*, 1996a; Samson *et al.*, 1996). Primary mononucleated cells from individuals with the CCR5$\triangle$32 genotype were shown *in vitro* to be resistant to infection by R5 viruses, but not X4 viruses. This homozygous deletion is present in approximately 1% of Caucasians. Protection is not absolute and several studies have indicated a delayed HIV-1 disease progression in individuals homozygous for the $\triangle$32 deletion (Dean *et al.*, 1996; Huang *et al.*, 1996; Samson *et al.*, 1996). It has also been proposed that individuals heterozygous for the $\triangle$32 genotype have delayed disease progression (Dean *et al.*, 1996). An additional CCR5 mutation (CCR5m303) has been showed to assist in resistance of infection in conjunction with the CCR5$\triangle$32 genotype (Quillent *et al.*, 1998). CXCR4 is more highly conserved than CCR5 although polymorphisms in CXCR4 exist but have not been associated with resistance to infection (Martin *et al.*, 1998; Wegner *et al.*, 1998).

1.3.3 Viral load

Viral load plays an important role in transmission as the major predictor of HIV-1 heterosexual transmission risk. It has been shown that in individuals with HIV-1 RNA levels below 1500 copies per millilitre, transmission is rare (Mellors *et al.*, 1997; Quinn *et al.*, 2000). Thus reductions in viral load brought about by the use of antiretroviral drugs reduce the rate of transmission. Such reductions have been documented in studies of perinatal transmission (Mofenson *et al.*, 1999; Shaffer *et al.*, 1999; Sperling *et al.*, 1996), but not in studies of sexual transmission (Quinn *et al.*, 2000).

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Women progress to AIDS at lower viral loads compared to men although both progress at a similar time course to AIDS (Farzadegan *et al.*, 1998; Ghandi *et al.*, 2002; Quinn *et al.*, 2000; Sterling *et al.*, 1999). Women has been shown to have 2- to 6-fold lower levels of HIV RNA than do men, even when controlling for time since seroconversion (Farzadegan *et al.*, 1998; Gandhi *et al.*, 2002; Sterling *et al.*, 1999). There have been some studies contradicting this (Bush *et al*, 1996; Moore and Burton, 1999).

Several studies have shown a good correlation between PBMC viral load and viral load in seminal plasma, semen (Gupta *et al.*, 1997) and cervical secretions (Hart *et al.*, 1999; Iversen *et al.*, 1998). Viral loads in genital secretions appear to fall in concert with the declines in PBMC viral load after combination therapy (Gupta *et al.*, 1997; Musicco *et al.*, 1994; Vernazza *et al.*, 1997). This is significant, as the rate of transmission has been remarkably reduced in persons with very low serum viral loads (Quinn *et al.*, 2000).

Factors that influence viral load especially in genital secretions are important factors in determining risk of HIV-1 transmission. It was also shown that whereas cervical inflammation and ulceration can increase HIV-1 RNA in vaginal secretions (Lawn *et al.*, 2000), hormonal changes during the menstrual cycle do not have a significant effect of HIV-1 RNA levels (Villanueva *et al.*, 2002),

1.3.4 Viral population restriction during transmission

Both simian models and human studies have indicated that restriction of viral populations during male-to-female transmission across mucosa takes place. A marked higher genetic diversity was observed in rhesus macaques inoculated intravenously when compared to intra-vaginally inoculated animals, indicating that restriction of viral populations occurred especially during mucosal vaginal transmission (Enose *et al.*, 1997; Greenier *et al.*, 2001; Miller *et al.*, 1989). The same trend was seen in studies on newly infected individuals which harboured viruses that were largely R5 even though those from the corresponding donor were both X4 and R5 (Asjo *et al.*, 1986; Bjorndal *et al.*, 1997; Connor *et al.*, 1994; Cornelissen *et al.*, 1995; Scarlatti *et al.*, 1997; Tschering *et al.*, 1998;Zaitseva *et al.*, 1997; Zhu *et al.*, 1993). Additional evidence in concordant couple studies for restrictive mucosal transmission showed that the transmitted variant represents

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only a minor population in the semen of the corresponding transmitter (van't Wout *et al.*, 1994; Zhu *et al.*, 1993, 1996).

A few models have been proposed to explain the observed restricted viral populations directly after transmission including; selective transmission, selective amplification and compartmentalisation (Zhu *et al.*, 1993).

Selective transmission. Of all these possible explanations convincing evidence exists only for selective transmission. Selective transmission states that a viral variant possesses a selective advantage in penetrating the mucosal barrier of a new host (Zhang *et al.*, 1993; Zhu *et al.*, 1993). Epithelial cells in the intestines have been shown to selectively transport R5 viruses across the epithelial layer (without getting infected themselves) bringing them into contact with sub-mucosal CD4+ T cells that they are able to infect (Meng *et al.*, 2002). Intestinal epithelial cells selectively take up R5 viruses by expressing co-receptor CCR5 and glycoshingolipid galactosyl ceramide (GalCer - an alternative receptor in epithelial cell lines) but not CD4, DC-SIGN/LFA-1 (two alternate receptors) or the CXCR4 co-receptor (Fantini *et al.*, 1993; Meng *et al.*, 2002). This mechanism for R5 selective transmission could possibly be true for all other epithelial cells and in particular genital epithelial cells at the site of sexual transmission.

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Dendritic cells have also been implicated in participating in the selective restriction of R5 viruses. Productive infection of iDCs by HIV-1 has been described but this is controversial. iDCs express higher levels of CCR5 and little or no CXCR4 and only replicate M-tropic viruses. Replication though, has been shown to occur only at very low levels (Canque *et al.*, 1996; Granelli-Piperno *et al.*, 1998; Reece *et al.*, 1998; Zaitseva *et al.*, 1997). Mature DCs express less CCR5 and higher levels of CXCR4 (Kawamura *et al.*, 2001).

Although mucosal epithelial cells may be involved in the selective transfer of mucosal acquired HIV-1, other mechanisms of R5 virus selection may exist, particularly for viruses transmitted intravenously This is illustrated by observed transmission of mostly R5 viruses by intravenous drug users (Van't Wout *et al.*, 1994) and the occasional sexual transmission of X4 viruses in acute infection (Pratt *et al.*, 1995).

Selective amplification. Selective amplification is another proposed model in explanation for the observed restricted viral populations after transmission. This states that it is possible that multiple HIV variants from the transmitter penetrate the new host, but only a minority is selectively amplified to become the dominant population. Selective amplification has been described in blood where SI viruses before seroconversion were replaced by NSI viruses at seroconversion. This suggests that HIV-1 selective amplification occurred after transmission (Cornelissen *et al.*, 1995). This model can be further explained by compartmentalization. It seems probable that selective amplification and selective transmission are together responsible for selective infection of HIV.

Compartmentalization. In heterosexual transmission it is virus present in the donor genital secretions (either seminal fluid in the case of male to female transmission or cervicovaginal secretions in the case of a female to male transmission) that is directly responsible for establishing infection. In trying to explain viral population restriction during transmission, compartmentalization states the possibility that selected viruses are preferably found in the transmitting genital compartment such as semen in the case of male-to-female transmission.

HIV-1 has different abilities to persist and replicate in different compartments in the host, dependant on host and viral factors. Compartmentalization for both proviruses and cell free viruses detected in PBMCs and plasma has been described. Distinctly different proviral sequences in PBMCs have been detected in various host compartments such as brain tissue (Hughes *et al.*, 1997), cerebrospinal fluid (Kuiken *et al.*, 1995), spleen (Epstein *et al.*, 1991), lung (Itescu *et al.*, 1994), semen (Zhu *et al.*, 1996), and cervicovaginal secretions (Ellerbrock *et al.*, 2001). It is also possible that in the same compartment, proviral and plasma RNA can be distinctly different (Zhu *et al.*, 1996).

In female genital secretions, proviral and cell-free HIV in blood were found to be phylogenetically and phenotypically distinct. This was first determined for proviral populations (Panther *et al.*, 2000; Poss *et al.*, 1995, 1998) and recently also determined for cell-free virions. It was also indicated that the principal source of HIV in the genital

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tract was due to local viral replication (Ellerbrock *et al.*, 2001). This suggests that natural and therapeutic induced selection pressures in the female genital tract occur independent of those in blood. It has indeed been determined that local cytokine, chemokine and HIV-1 specific immunoglobulin production in female genital tract secretions are different than those in blood (Artenstein *et al.*, 1997; Belec *et al.*, 1995).

In male genital secretions proviral HIV as well as cell-free virions have been detected in the non spermatozoa mononuclear cell fraction of semen and seminal fluid respectively (Gupta *et al.*, 1997; Hamed *et al.*, 1993). Drug resistance mutations were showed to be unequally distributed in blood and semen and serve as evidence for compartmentalization in semen (Kroodsma *et al.*, 1994). In addition other genetic and phenotypic differences between proviral variants in blood and semen were described although this does not seem to be a trend (Delwart *et al.*, 1998; Zhu *et al.*, 1996). More macrophages and lymphocytes are present in semen relative to blood (Wolff and Anderson, 1988) and anti-immunoglobulin G in semen including antibodies to HIV-1 proteins p55, p24 and p17 are less prevalent in blood than semen (Wolff *et al.*, 1992). This raises the possibility that viral evolution in semen may be different from that in the blood because of differences in immune pressures.

It was thought that since R5 viruses are primarily transmitted they might preferably be found in genital secretions (Cheng-Mayer *et al.*, 1989; Wolff and Anderson, 1988). However, both X4 and R5 viruses have been found present in semen and thus it is unlikely that compartmentalization can account for this observed restriction (Zhang *et al.*, 1993; Zhu *et al.*, 1993)

1.3.5 Gender influences on heterosexual transmission

Much of our understanding of the virological determinants of sexual transmission of HIV is derived from examining recently subtype B infected American and European men. These studies showed transmission of a genetically homogeneous viral population (Zhang *et al.*, 1993; Zhu *et al.*, 1993) that over time diversifies and becomes more heterogeneous, evolving in a multitude of closely related, but still different variants, 'quasispecies'. During later stage of disease the population again becomes more homogeneous

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(McNearney *et al.*, 1992). Transmission of genetically homogeneous viral populations as observed in recently infected subtype B American and European men has been noted despite the donors harbouring genetically complex viral populations and was attributed to either selective amplification or selective transmission (Zhang *et al.*, 1993; Zhu *et al.*, 1993). However most infecting individuals or donors harbour a complex viral population due to the viral dynamics within the individual during the course of HIV infection.

Transmission data obtained from men have until recently been extrapolated to women. Almost half of HIV infections occur in women worldwide and in South Africa in particular, it is estimated that 2,7 million women are infected in an epidemic of 5 million (UNAIDS, 2002). Despite women being one of the most substantial groups affected by the epidemic they have been a relatively understudied group and little is known about virological determinants involved in transmission in women, and in particular, African women.

Female multiple variant transmission. Recent studies have shown that there may be gender differences in transmission and that women are unlike men with regard to HIV transmission. These studies showed that the pronounced genetic restriction seen in heterosexually infected men during HIV transmission, is often not observed in heterosexually infected African women (Kampinga *et al.*, 1997; Long *et al.*, 2000; Poss *et al.*, 1995). This was detected specifically in a Kenyan cohort of female sex workers by two independent studies comparing viral diversity in acutely infected males and female sex workers infected with subtype A and D (Long *et al.*, 2000; Poss *et al.*, 1995). The first study showed five out of six acutely infected women from this Kenyan sex worker cohort, harboured heterogeneous viral populations (Poss *et al.*, 1995). In the second study transmission of multiple variants of HIV-1 took place in twenty out of 32 women, resulting in individuals harbouring a heterogeneous viral population soon after transmission (Long *et al.*, 2000). Multiple variant HIV transmission has also been indicated in studies on other cohorts such as a Rwandan pregnant women cohort (Kampinga *et al.*, 1997). This indicates that infection by multiple variants is not unique to the sex worker cohorts. The viruses that were found in these women early in infection were genetically diverse, with up to 6,4% difference in the envelope region encompassing

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variable region 1 (V1) through to V3 (Long *et al.*, 2002). It is possible that the level of viral diversity in early infection may influence disease progression.

Male multiple variant transmission. It has however been shown in a few instances that transmission of multiple variants can take place in subtype B infected men, despite overwhelming evidence of the opposite. The first study indicated co-infection in a homosexual male (Zhu *et al.*, 1995) and the second study, showed transmission of a heterogeneous virus mixture in one male out of seven individuals (Zhu *et al.*, 2002). This study indicated that the viral population became homogeneous in *env* but not in *gag* soon after transmission to another male. Another study found heterogeneous HIV populations in eight homosexual men and also found homogenisation in *env* but not in *gag* over time (Learn *et al.*, 2002). This indicates that early selective processes focus on viral properties within Env but not Gag.

Proposed explanations for female multiple variant transmission. It may be possible that viruses transmitted to women differ phenotypically from those transmitted to men. It was thought that the mechanism restricting viral diversity selects for R5 viruses, and that in women a failure of phenotypic restriction for R5 viruses may result in heterogeneous viral population transmission (Zhang *et al.*, 1993; Zhu *et al.*, 1993). If this were true more frequent transmission of X4 viruses would be observed in women. This was not the case in a recent study showing R5 viruses present in all African female study subjects regardless of viral complexity (Long *et al.*, 2002). Thus is seems unsupported that phenotypic restriction absent in women could explain HIV multiple-variant transmission. A number of reasons have been proposed to explain why women may be more susceptible to infection with multiple quasispecies when compared to men. These include an increased muscosal area of exposure, increased susceptibility through greater mucosal trauma, the presence of undiagnosed sexually transmitted infections, prolonged exposure to infected male secretions and immunological differences between men and women (Ray and Quinn, 2000).

University of Cape Town

Gender influences on HIV pathogenesis. Studies have also reported gender differences in HIV pathogenesis, with progression to AIDS in women being associated with lower viral loads when compared to men although progression to AIDS in men and

women occurs at a similar time course (Farzadegan *et al.*, 1998; Quinn *et al.*, 2000; Sterling *et al.*, 2001). Women have been shown to have two to six fold lower levels of HIV RNA than men at a similar stage of HIV infection. This was found even when controlling for possible confounding factors such as, age, race, mode of transmission and antiretroviral use (Gandhi *et al.*, 2002; Sterling *et al.*, 1999). Some studies contradicting this have also been reported (Bush *et al*, 1996; Moore and Burton, 1999).

These observed gender differences in viral levels could be explained by hormone fluctuations in women. During ovulation oestrogen and progesterone levels fluctuate and some studies have shown that HIV viral levels follow suit (Greenblatt *et al.*, 2000; Reichelderfer *et al.*, 2000). A mechanism proposed for this observed hormone and viral level correlation is oestrogen-mediated down-regulation of TNF-α directly influencing HIV-1 expression (Mellors *et al.*, 1991; Shanker *et al.*, 1994). Progesterone that has been implicated in increased susceptibility to a range of pathogens including to SIV through hormone-related impairment in cell-mediated immunity (Marx *et al.*, 1996). In addition, human lymphocytes have a distinct progesterone binding domain and it is possible that during ovulation with fluctuating levels of progesterone this may exert a dose dependant inhibitory effect on CCR5 expression on activated T cells (Vassiliadou *et al.*, 1999). Contradicting evidence has however been published, indicating that hormonal levels during the menstrual cycle do not have a significant effect on HIV-1 RNA levels in vaginal secretions (Mostad *et al.*, 1998; Villaneuva *et al.*, 2002).

1.3.6 Subtype influences on HIV transmission

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It is possible that subtype could be one factor that influences the pattern of viral transmission that not all subtypes transmit in the same manner (Essex, 1995; Soto-Ramirez *et al.*, 1996). A viral diversity study on women found that only subtype A and D infected females, but not subtype C infected individuals, harboured a heterogeneous viral population soon after transmission (Long *et al.*, 2000). Heterogeneous viral transmission is not restricted to subtype A and D infected African women alone however. A study on Subtype B infected American individuals showed that one in four women were infected with a heterogeneous viral population soon after HIV transmission. All men in this study were infected with homogeneous populations (Delwart *et al.*, 2002). There is currently

not sufficient information as to whether subtype could be a factor that influences the pattern of viral transmission or whether heterogeneous virus populations can be transmitted in subtype C infections.

There is also evidence suggesting that subtypes may play no role in the efficiency of transmission. Maternal-infant HIV transmission studies showed similar rates of vertical transmission between different HIV-1 subtypes. In addition HIV pathogenic data such as CD4 T-cell decline (Alaeus *et al.*, 1999; Amornkul *et al.*, 1999; Galai *et al.*, 1997), clinical progression and plasma viral levels (Alaeus *et al.*, 1999) among different subtypes have also showed no subtype-specific differences. The only evidence for differences in disease progression with regards to subtypes was illustrated in a small Senegalese study showing subtype specific differences in virulence (Kanki *et al.*, 1999).

Differential co-receptor use by subtypes. There has been evidence to suggest that not all subtypes behave the same with regards co-receptor requirements. Potential subtype specific differences in co-receptor usage among different subtypes was first suggested by the observation that subtype C X4 variants were rare in a group of 16 people under care in Sweden independent of clinical status, CD4 counts, or treatment. This study investigated whether co-receptor usage differed between all different genetic subtypes of HIV-1 and showed that CXCR4 usage is perfectly correlated to biological phenotype for all subtypes (Tschering *et al.*, 1998). Subtype C X4 variants were rare among a group of 22 French military personnel infected during overseas deployment (Peeters *et al.*, 1999), as well as in a group of Malawian men (Ping *et al.*, 1999). It has been reported that subtype-C viruses have an extra NF-κ site in the LTR-region, which could lead to more efficient transcription of mRNA without displaying the SI phenotype resulting in increased pathogenesis (Johansson *et al.*, 1995). Additional studies have confirmed this (Adebe *et al.*, 1999).

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1.4 Project motivation:

> ***First Aim.*** *This project aims firstly to define env sequence diversity of the HIV epidemic within the southern African region including countries such as Malawi, Zambia, Zimbabwe and two sites in South Africa, Johannesburg and Durban.*

Viral diversity is especially relevant in vaccine design and it is thus important to monitor HIV-1 genetic diversity and evolution, particularly in the countries targeted for vaccine trials where limited HIV-1 sequence data exists. This study characterises *env* viral diversity in Malawi, Zambia, Zimbabwe and South Africa with a focus on the Durban sex-worker seroconversion cohort, which will be used for subsequent transmission studies.

Characterisation of the gp120 region was performed as this region contains important information for HIV-1 transmission and pathogenesis. The gp120 include determinants for attachment through receptors and co-receptors mediating cellular fusion during host invasion. The gp120 also includes neutralization determinants.

> ***Second Aim.*** *Secondly, we aim to characterise viral populations in recently infected South African women to investigate transmission with regard to gender, subtype and predicted co-receptor usage.*

Recent evidence indicates that women may be unlike men with regards to genetic diversity after transmission, with women harbouring a genetically more complex viral population (Zhang *et al.*, 1993; Zhu *et al.*, 1993; Kampinga *et al.*, 1997; Long *et al.*, 2000; Poss *et al.*, 1995). Although almost half of HIV infections occur in women, they have been a relatively understudied group. Very little is known about virological determinants involved in HIV transmission in women, and in particular, in African women.

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Chapter 2: *ENV* SEQUENCE DIVERSITY OF THE HIV EPIDEMIC IN SOUTHERN AFRICANS

2.1 Introduction **50**

2.2 Materials and Methods **51**

2.2.1 Patient data 51
2.2.2 RNA extraction and cDNA synthesis 57
2.2.3 Polymerase chain reaction (PCR) and agarose gel electrophoresis 58
2.2.4 Sequencing 61
2.2.5 Sequence analysis 62

2.3 Results **65**

2.3.1 PCR and Gel electrophoresis 65
2.3.2 Phylogenetic analysis 66
2.3.3 Genetic Distances 69
2.3.4 V3 Loop 71
2.3.5 Phenotype predictions 73
2.3.6 Global geographic clustering within subtype C 73
2.3.7 Entropy plots 75

2.4 Discussion **77**

University of Cape Town

CHAPTER 2: *Env* sequence diversity of the HIV-1 epidemic in southern Africans

2.1 Introduction:

It is important to monitor HIV-1 genetic diversity and evolution as certain regions of the genome exhibit extraordinary variability that poses an obstacle to HIV-1 vaccine design. For example, vaccines that include *env* may differ up to 30%. Defining viral diversity is of particular relevance for countries targeted for vaccine trials such as countries in southern Africa. Southern African countries with a predominantly subtype C epidemic (McCormack *et al.*, 2002; Novitsky *et al.*, 2001, Ping *et al.*, 1999; Trask *et al.*, 2002; UNAIDS, 2002; van Harmelen *et al.*, 2001, 1999) exhibit high intrasubtype C diversity which further complicates vaccine development (Novitsky *et al.*, 2001; Van Harmelen *et al.*, 2001). Subtype C is also responsible for most current infections globally (including HIV-1 epidemics in countries outside of Africa, such as India and China) representing at least 56% of all circulating group M infections (Esparza and Bhamarapravati, 2000; Essex, 1999; McCutchan, 2000). This study focuses on viruses from South Africa, Zambia, Zimbabwe and Malawi. Although partial *env* sequences from these countries are available, limited full-length sequences exist (http://hiv-web.lanl.gov/) (Table 2.1). The same is true for most other southern Africa countries with the exception of recent sequence publications from Botswana (Novitsky *et al.*, 2002).

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Table 2.1: Full-length, gp120 and partial sequences available for HIV-1 from South Africa, Zambia, Zimbabwe and Malawi (http://hiv-web.lanl.gov/).

County of Origin	Number of full-length genomes	Number of gp120 sequences	Number of partial genomes >300bp
South Africa	8	31	275
Zambia	2	4	98
Zimbabwe	0	0	67
Malawi	0	0	5

It is also of interest to monitor HIV-1 diversity as it may impact on HIV pathogenesis. Some evidence indicates possible differences in pathogenesis between different subtypes (Kanki *et al.*, 1999). This is controversial, as other studies have shown no association between HIV subtype and disease progression. Diversity monitoring can also be used to track the epidemic. In South Africa the epidemic changed from a predominantly subtype B epidemic, in the early 1980s, associated with homosexual transmission to a subtype C epidemic predominantly infecting the heterosexual population (van Harmelen *et al.*, 1997). This chapter focuses on the *env* region as it contains determinants that impact on HIV-1 transmission and host cell entry, pathogenesis and neutralisation. This region is responsible for virus attachment through receptors, co-receptors and cellular fusion during host invasion and cell entry. Phenotype is determined by amino acids in certain positions in the V3 loop and the resulting change in charge they have. SI viruses have a higher positive charge than NSI viruses in this region (Fouchier *et al.*, 1992).

In this chapter we aim to characterise sequence diversity of the complete gp120, with a subset of samples analysed only in the V3V5 region. Samples were collected from Malawi, Zambia, Zimbabwe and two sites in South Africa (Durban and Johannesburg) with a focus on the Durban sex worker seroconversion cohort (Du-cohort) that will be used for subsequent transmission studies (see chapter 3). This research forms part of the larger HIVNET 028 study (#N01-AI-45202), investigating virological and immunological characteristics of subtype C. These samples were from individuals with recent infections and thus represent both the current circulating virus as well as the virus that is transmitted.

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2.2 Materials and Methods:

2.2.1 Patient data

This study constitutes part of the HIVNET 028 study in which the overall aim was to evaluate immune responses to recent HIV-1 subtype C infection in five geographically distinct southern African populations, Johannesburg and Durban South Africa, Malawi, Zambia and Zimbabwe that will facilitate and/or optimise the design of a relevant vaccine

candidate. HIVNET028 (Principal Investigators HW Sheppard, Viral and Rickettsial Disease Laboratory, 2151 Berkley Way, Berkley, CA 94704, USA; C. Gray, National Institutes for Communicable Diseases, Sandringham, Johannesburg, SA) was initiated in 1999 (#N01-AI-45202). Participants were recruited and samples collected by co-investigators at the individual sites including E. Vardas, S. Abdool Karim (Durban); G. Gray, J. McIntyre (Soweto, Johannesburg); L. Zjenah, D. Katzenstein (Zimbabwe); Rosemary Masunda and colleagues (Zambia); Newton Kumwenda (Malawi).

Samples from individuals with recent HIV-1 infection were included from five existing southern African HIVNET sites. Thirty-nine samples were selected, 16 from Durban (615- prefix), 6 from Johannesburg (616-prefix), 10 from Zambia (541- prefix), 4 from Malawi (525- prefix) and 3 from Zimbabwe (536- prefix) (Figure 2.1). Patient samples were recruited as follows:

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Durban, SA: Participants were recruited from five truck stops in the Kwazulu-Natal Midlands area that served as a source of recent seroconvertors. This cohort was established as part of an UNAIDS-funded Phase III vaginal microbicide trial, Col-1492 (Van Damme *et al.*, 2002). Sex workers of this cohort were screened for seroconversion on a monthly basis. To date 452 sex workers have been screened and 20 recent seroconverters were recruited for the HIVNET study.

Johannesburg, SA: The Chris Hani Baragwanath hospital in Johannesburg was used as a site for identifying seroconvertors among pregnant women attending the antenatal clinic. Recent infections were identified using the standard testing algorithm for recent HIV seroconversion (STARHS) (Gouws *et al.*, 2002).

Zimbabwe: There existed several study populations in Zimbabwe. Study participants forming part of the microbicide CDC N-9 study were screened for HIV infection and seroconvertors identified for inclusion in the HIVNET study. Pregnant women in Harare as well as women screened for perinatal transmission studies in Chitungwiza were also identified as a source for recent seroconvertors. In addition,

serum samples pre- and post-seroconversion with estimated dates of seroconversion were collected from 150 seroconvertors from a factory workers cohort.

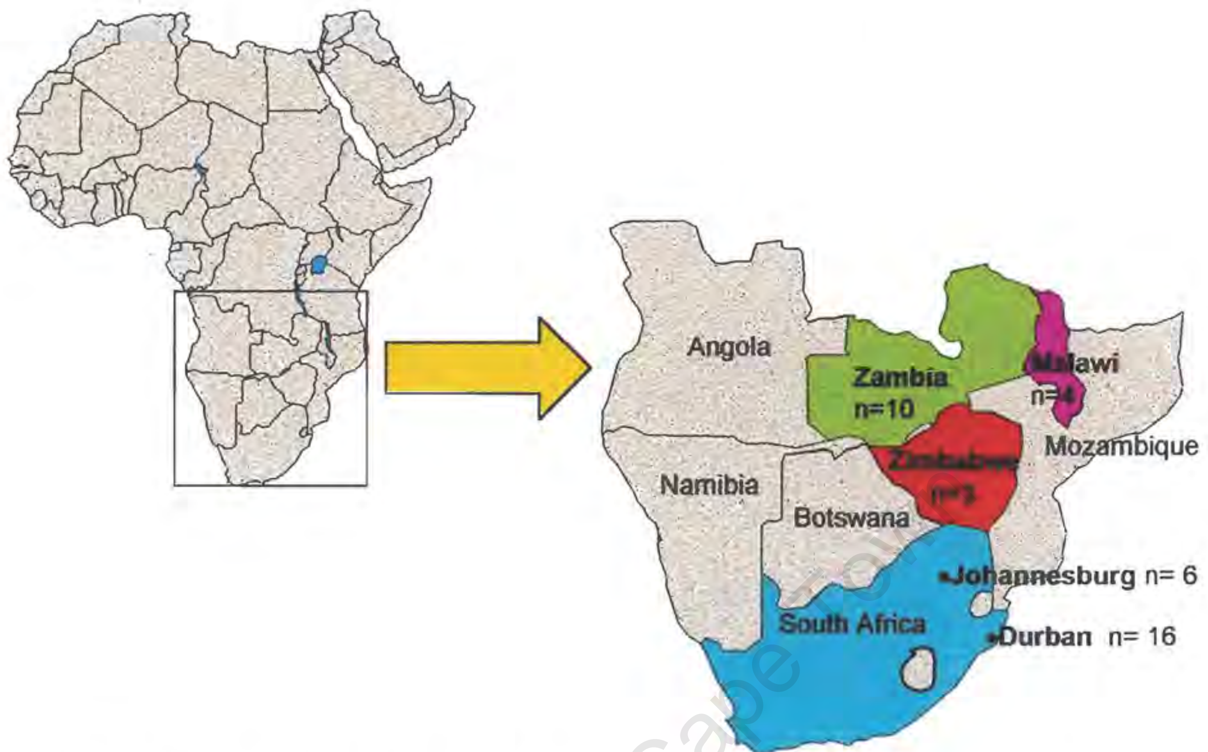

Figure 2.1: Map of southern Africa indicating geographical origin of 39 samples employed in this study

Malawi: Samples from Malawi were taken from a follow-up study conducted by the Johns-Hopkins Project in Blantyre at the Sugar Corporation of Malawi (SUCOMA) estate. HIV incidence in men from this cohort is approximately 21%. A cohort exists of 1 600 men who have follow-up visits every six months.

Zambia: An NIH-funded, observational discordant heterosexual couple cohort in Lusaka was recruited. This study consists of 600-700 enrolled couples.

Patient demographic data and clinical information was generated as part of the HIVNET 028 study by co-investigators. Demographic data collected, included, age, sex, date and origin of sample collection (Table 2.2). Ethical approval for all samples was obtained. Almost 80% of individuals (34/39) from this study were female. Heterosexual contact

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was determined to be the mode of transmission as no other possible risk factors could be determined. Contaminated needles were excluded as possible mode of transmission as no reported use of non-medical injections existed. For the Du-cohort, condom use data was not available and almost all women had evidence for STDs as was evident from the high incidence of genital and rectal discharges and ulcerations. Clinical information obtained, included CD4+ lymphocyte, viral load, estimated date of transmission or infection, and months post infection (Table 2.3). The estimated date of infection or transmission was taken as the midpoint between the last seronegative date and first seropositive date. Months post infection was calculated from the midpoint. CD4+ lymphocyte counts were available in the majority of individuals.

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Table 2.2: Demographic data of 39 HIV-1 infected subjects evaluated in this study originating from the five HIVNET sites.

Origin	Subject	Sample Date	Age (yr)	Sex
Malawi (525-)	525-00003-4	23-08-2000	45	M
	525-00016-0	15-03-2001	44	M
	525-00030-1	25-10-2001	18	F
	525-00034-3	05-12-2001	33	F
Zimbabwe (536-)	536-00008-0	19-01-2001	18-45	F
	536-00010-7	13-10-2000	18-45	F
	536-00016-2	11-05-2001	18-45	F
Zambia (541-)	541-00005-4	09-08-2000	18-45	M
	541-00006-2	26-07-2000	18-45	F
	541-00008-5	24-01-2001	18-45	F
	541-00009-8	14-02-2001	18-45	M
	541-00011-5	12-07-2000	18-45	F
	541-00012-6	21-06-2000	18-45	F
	541-00014-4	02-08-2000	18-45	M
	541-00017-8	17-01-2001	18-45	F
	541-00019-3	10-01-2001	18-45	F
	541-00022-8	12-07-2000	18-45	F
South Africa, Durban (615-)	615 00011-0	24-08-2000	ND	ND
	615-00002-6	17-08-2000	18-45	F
	615-00004-4	01-06-2000	36	F
	615-00005-9	17-08-2000	18-45	F
	615-00007-8	17-08-2000	18-45	F
	615-00008-0	24-08-2000	18-45	F
	615-00009-3	24-08-2000	18-45	F
	615-00010-7	24-08-2000	18-45	F
	615-00012-1	05-10-2000	25	F
	615-00013-6	25-01-2001	18-45	F
	615-00014-9	16-11-2000	34	F
	615-00016-2	31-08-2000	18-45	F
	615-00018-5	31-08-2000	18-45	F
	615-00037-7	12-12-2001	ND	F
	615-00038-1	12-12-2001	44	F
	615-00032-5	12-12-2001	ND	F
South Africa, Johannesburg (616-)	616-00001-7	10-04-2001	18-45	F
	616-00009-1	06-12-2001	ND	F
	616-00043-7	07-12-2001	ND	F
	616-00046-1	06-12-2001	ND	ND
	616-00048-6	29-11-2001	ND	F
	616-00050-3	05-10-2001	ND	F

ND, not done; N, No; Y, Yes.

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Table 2.3: Clinical data for all 39 subjects participating in this study (HIVNET028, #N01-AI-45202).

	Subject	Sample Date	Estimated date of Transmission* (month-year)	Months post Transmission	CD4+Ts (cells/μl)	Viral Load (copies/ml)
Malawi	525-00003-4	23-08-00	6-99	14	242	33812
	525-00016-0	15-03-01	12-99	15	340	53140
	525-00030-1	25-10-01	ND	ND	608	8583
	525-00034-3	05-12-01	ND	ND	254	12766
Zimbabwe	536-00008-0	19-01-01	1-00	12	455	20439
	536-00010-7	13-10-00	ND	ND	373	1250
	536-00016-2	11-05-01	ND	ND	424	4160
Zambia	541-00005-4	09-08-00	12-98	20	387	ND
	541-00006-2	26-07-00	2-99	17	321	3989
	541-00008-5	24-01-01	1-99	24	136	115968
	541-00009-8	14-02-01	1-99	25	318	5668
	541-00011-5	12-07-00	1-99	18	412	22706
	541-00012-6	21-06-00	1-99	17	ND	24093
	541-00014-4	02-08-00	10-98	22	357	843
	541-00017-8	17-01-01	4-99	21	443	55466
	541-00019-3	10-01-01	3-99	22	476	93697
	541-00022-8	12-07-00	2-99	17	409	50295
SA, Durban	615 00011-0	24-08-00	7-98	25	186	2204
	615-00002-6	17-08-00	11-99	9	215	21763
	615-00004-4	01-06-00	8-99	10	527	4046
	615-00005-9	17-08-00	7-99	13	408	2136
	615-00007-8	17-08-00	3-99	17	355	15916
	615-00008-0	24-08-00	1-99	19	10	1367
	615-00009-3	24-08-00	12-98	20	521	655
	615-00010-7	24-08-00	7-99	13	523	1707
	615-00012-1	05-10-00	12-98	22	299	1486
	615-00013-6	25-01-01	12-98	25	734	2062
	615-00014-9	16-11-00	1-99	22	855	108
	615-00016-2	31-08-00	ND	ND	365	1520
	615-00018-5	31-08-00	6-98	26	343	3955
	615-00037-7	12-12-01	12-99	24	32	177259
	615-00038-1	12-12-01	10-00	14	592	2349
	615-00032-5	12-12-01	10-00	14	255	16952
SA, Johannesburg	616-00001-7	10-04-01	12-99	16	1183	2061
	616-00009-1	06-12-01	ND	ND	371	15565
	616-00043-7	07-12-01	ND	ND	ND	105
	616-00046-1	06-12-01	ND	ND	45	202735
	616-00048-6	29-11-01	ND	ND	153	ND
	616-00050-3	05-10-01	ND	ND	348	159

*Estimated date of infection or transmission was taken as the midpoint between the last seronegative and first seropositive sample collection dates; ND, not done.

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2.2.2 RNA extraction and cDNA synthesis

Manual RNA Extraction. RNA was extracted using the QIAamp® Viral RNA Mini Kit for purification of viral RNA from plasma, serum, sell-free body fluids and cell-culture supernatants (Qiagen, Valencia, CA, USA). This method is used to extract viral RNA of more than 200bp from 140 to 280μl sample (depending on sample volume availability) through the selective binding properties of a silica gel based membrane. According to manufacturers instructions, between 280μl plasma sample were used to extract RNA and subsequently eluted in 30μl RNase-free buffer (Appendix A1).

cDNA Synthesis. Complementary DNA (cDNA) was reverse transcribed from viral RNA by the Invitrogen Thermoscript ™ RT-PCR System (Invitrogen, GmbH, Karlsruhe, Germany). The Thermoscript ™ RT-PCR System uses Thermoscript RT, an avian RNase H-minus reverse transcriptase engineered to have high thermal stability and to produce high yields of cDNA. cDNA was generated from a total of 8μl HIV-1 RNA primed with 2μl of 10pmol/μl (2μM) gene specific primer denatured at 65°C for 5 minutes. Env F-r was used as gene specific primer for gp120 sequences and Env A-r for V3V5 sequences. Ten microlitres of a master mixture was added to primed RNA to give a total of 20μl. The master mixture was prepared as follows:

Reagent	**Amount**
5X cDNA buffer (250mM Tris acetate (pH 8,4), 375mM potassium acetate, 40mM magnesium acetate, stabilizer)	1 X
DTT	10mM
dNTP mixture (dATP, dTTP, dCTP, dGTP)	2mM
RNaseOUT™	40U
Thermoscript RT	15U
Total	**10μl**

This reaction was incubated at 50°C for 1 hour and terminated by heating to 85°C for 5 minutes. RNA template was then removed with 2U *E. coli* RNase H supplied with the kit and incubated at 37°C for 20 minutes.

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2.2.3 Polymerase Chain Reaction (PCR) and Agarose Gel Electrophoresis

PCR. For 19 samples the complete envelope gp120 was amplified by nested PCR (nPCR) (Table 2.5). The gp120 outer reaction was performed with gene specific primers Env G-f and Env F-r producing a 1,968Kb fragment incorporating the complete gp120. Nested inner reactions were done resulting in three overlapping fragments using Env A-f and Env E-r (702bp) for the first fragment, Env B-f and Env B-r (512bp) for the second fragment and Env D-f and Env A-r (594bp) for the third fragment. An additional 20 samples were amplified by PCR generating a region spanning the variable region 3 to 5 (V3V5) within gp120 PCR. The outer fragment for V3V5 was generated using Env A-f and Env A-r producing a 1,599kb fragment. Nested inner reactions were done using DR-7 and DR-8 (674bp) (Table 2.4).

Quality control. A water negative control was included in parallel in all PCR runs as well as during the initial cDNA step in order to detect possible contamination. Steps taken to avoid contamination included, setting up cDNA synthesis and PCR master mixtures in a cubicle in a tightly controlled separate PCR-only area where no patient products or other DNA was allowed. Adding patient RNA to cDNA master mixture was done in a separate area where only cDNA synthesis was performed. Subsequent carry-over of cDNA to the outer PCR reaction was performed in a cubicle in a third separate area designated as a PCR nested area only. PCR master mixtures were never taken into the general laboratory area were possible contamination could occur.

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Table 2.4: Primers for cDNA synthesis and PCR for amplification of gp120 and V3V5

Primer	Position*	Sequence
Env F-r	7932-7949	5' CTG CTT AAT GCC CCA GAC 3'
Env G-f	5981-6000	5' AAG CGG AGA CAG CGA CGA AG 3'
Env B-f	6826-6847	5' TAA CAC AAG CCT GTC CAA AGG T 3'
Env B-r	7317-7337	5' AAT TTC TAG GTC CCC TCC TGA 3'
Env D-f	7208-7226	5' AGC ACA TTG TAA CAT TAG T 3'
Env A-r	7783-7802	5' TGC TGC TCC CAA GAA CCC AA 3'
Env A-f	6203-6223	5' GAA AGA GCA GAA GAC AGT GGC 3'
Env E-r	6886-6903	5' TTA GAA TCG CAT AAC CAG 3'
DR7	6990-7021	5' TCA ACT CAA CTG CTG TTA AAT GGC AGT CTA GC 3'
DR8	7638-7668	5' CAC TTC TCC AAT TGT CCC TCA TAT CTC CTC C 3'

* Sequence positions were determined relative to HXB2 (Genbank accession number K03455)

Table 2.5: Twenty samples were used to produce V3V5 sequences and 19 samples to produce gp120.

19 Gp120 Sequences	20 V3V5 Sequences
525-00003-4	525-00016-0
536-00010-7	525-00030-1
541-00005-4	525-00034-3
541-00006-2	536-00008-0
541-00011-5	536-00016-2
541-00012-6	541-00008-5
541-00014-4	541-00009-8
541-00022-8	541-00017-8
615 00011-0	541-00019-3
615-00002-6	615-00013-6
615-00004-4	615-00014-9
615-00005-9	615-00037-7
615-00007-8	615-00032-5
615-00008-0	615-00038-1
615-00009-3	616-00001-7
615-00010-7	616-00009-1
615-00012-1	616-00043-7
615-00016-2	616-00046-1
615-00018-5	616-00048-6
	616-00050-3

The master mixtures contained:

Outer reaction of 50µl contained

Reagent	**Working Concentration**
10X PCR buffer (Super-therm DNA polymerase, JMR Holdings, Sevenoaks, UK)	1X
PCR grade dNTP (Roche, GmbH, Mannheim, Germany)	200µM
Primer Env G-f and Env F-r or Env A-f and Env A-r	0,1µM
$MgCl_2$ for Env G-f + F-r (gp120) or Env A-f + Env A-r (V3V5) respectively	3mM or 2mM
Super-therm DNA polymerase (Super-therm DNA polymerase, JMR Holdings, Sevenoaks, UK)	0,625U
Patient cDNA	5µl
Total	**50µl**

PCR cycling reaction was carried out using a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, Norwalk CT, USA) according to the following parameters:

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Initial denaturing step at 94°C for 2 min.

94°C for 1 min - denaturing
50°C for 1 min – annealing → 3 cycles
72°C for 1 min – elongation

94°C for 15 sec - denaturing
50°C for 45 sec – annealing → 32 cycles
72°C for 1 min - elongation

Final elongation step at 72°C for 7 min

Inner reactions of 50μl contained

Reagent	**Working concentration**
10X PCR buffer (Super-therm DNA polymerase, JMR Holdings, Sevenoaks, UK)	1X
PCR grade dNTP (Roche, GmbH, Mannheim, Germany)	200μM
Primers for gp120 Env A-f and Env E-r, Env B-f and Env B-r, Env D-f and Env A-r and primers for V3V5, DR7 and DR8.	0,1μM
$MgCl_2$ for Env pairs, Bf+Br, DR7+DR8 Af+Er and Df +Ar respectively	2mM and 4,48mM
Super-therm DNA polymerase (Super-therm DNA polymerase, JMR Holdings, Sevenoaks, UK)	0,625U
Outer reaction PCR product	5μl
Total	**50μl**

PCR cycling reaction was carried out using a PCR express thermocycler (Hybaid, Middlesex, UK) according to the following parameters:

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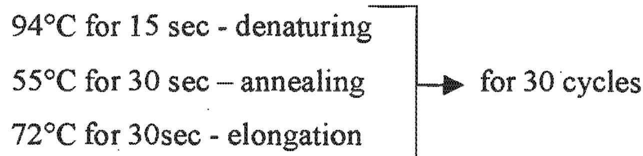
Initial denaturing step at 94°C for 2 minutes



Final elongation step at 72°C for 7 min

Agarose gel electrophoresis and PCR Amplicon purification. Amplicons were visualized by 2% agarose gel electrophoresis (Appendix A4). Second round PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Valencia CA, USA) (Appendix A2).

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2.2.4 Sequencing

Fragments constituting the gp120 as well as the V3V5 region were sequenced in both directions with forward and reverse primers for each of the three fragments. This was performed by standard dideoxy chain termination method (Sanger *et al.*, 1977). This method makes use of 2', 3' ddNTPS, which upon incorporation, into the growing DNA strand by polymerases, results in chain termination. ddNTP's lack a 3'-hydroxyl residue that is needed for phosphodiester bond formation between successive dNTPs. These ddNTPs are added to PCR reaction together with an abundance of dNTPs, resulting in competitive chain-extension and termination. Oligonucleotide chains are produced with varying length, differing by single nucleotides. These are then fluorescently labeled, allowing for detection upon electrophoresis.

The sequencing reaction was performed through the ABI PRISM® BigDye ™ terminator V3.0 Cycle sequencing Ready reaction Kit with AmpliTaq® DNA Polymerase FS (Applied Biosystems, Warrington, UK). According to manufacturers recommendations we performed half reaction cycle sequencing, adding a total of approximately 50ng

amplicon DNA per reaction plus 3,2pmol primer, either the forward or the reverse. To this was added 4μl of the enzyme ready reaction mixture containing AmpliTaq® DNA Polymerases, FS with thermostable pyrophosphatase from the BigDye ™ terminator V3.0 Cycle sequencing Ready reaction Kit plus 4μl 2,5X sequencing buffer (Tris-HCL, pH 9 and $MgCl_2$).

This was then thermocycled in a GeneAmp PCR system 2400 thermocycler (Perkin Elmer Corporation, Norwalk, CT, USA) using the following cycle conditions.

96°C for 30 sec – initial denaturing step

96°C for 30 sec - denaturing
50°C for 15 sec - annealing → for 25 cycles
60°C for 4 min - elongation

Sequencing was done on an ABI PRISM ™ 3100 Genetic Analyser (Applied Biosystems, Warrington, UK). All sequences were subjected to quality control measures to ensure that there were no sample mix-ups or contamination from other sources (Learn *et al.*, 1996).

2.2.5 Sequence analysis

Quality Control. For each sample both forward and reverse sequences were generated and samples were sequenced until no ambiguities could be detected. Screening for contamination was done before the analysis of the sequences, and periodically during the course of large sequencing studies, so problems could be detected and corrected early. Recent sequences generated in the laboratory was also compared to study sequences through phylogenetic analysis to identify possible sample cross contamination from the surrounding laboratory. Phylogenetic trees clarify relations between sequences and our trees were scrutinized for signs of extreme interpatient similarity and mixed clusters indicative of contamination. In addition, BLAST (Basic Logical Alignment Search Tool, http://ncbi.nlm.nih.gov/ BLAST) searches of sequences (Altschul *et al.*, 1990) were

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performed in order to identify a best match of the sequences to the already published HIV sequences.

Phylogenetic trees. Phylogenetic trees were generated from sequences in both the V3V5 and gp120 genomic regions. This was done to determine subtype and investigate sequence relatedness. A total of 39 V3V5 sequences (19 V3V5 sequences cut from the gp120 sequences and 20 sequences for which only the V3V5 were generated) and 19 gp120 sequences were analysed. The 39 V3V5 sequences consisted of 16 from Durban South Africa, 6 from Johannesburg South Africa, 4 from Malawi, 3 from Zimbabwe and 10 from Zambia. The 19 gp120 sequences consisted of 11 sequences from Durban South Africa, 1 each from Malawi and Zimbabwe and 6 from Zambia.

For both V3V5 and gp120 the forward and reverse sequences were corrected using Auto Assembler (Applied Biosystems, Warrington, UK). The resulting consensus sequences were then aligned in CLUSTAL X (Thompson *et al.*, 1997) and manually edited using both BioEdit Sequence Alignment Editor v5.0.2 (Hall, 1999) and DNAMAN (version 4.0, Lynnon BioSoft, Vaudreuil-Dorion, Quebec, Canada).

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A total of 34 reference sequences obtained from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov/Align_current /SUBTYPE-REF/subtype.html) were included in the phylogenetic tree. These reference sequences included 5 from subtype C, 4 from subtype A, 4 from subtype B, 4 from subtype D, 4 from subtype F1, 2 from subtype F2, 2 from subtype K, 4 from subtype G, 2 from subtype J and 3 from subtype H, SIV_{CPZGAB} was included as an outlier. Phylogenetic trees (Figure 2.4 and Figure 2.5) were generated based upon the distance matrix neighbor-joining method in TREECONW 1.0 (van de Peer and de Wachter, 1994). Tree topology was inferred using the Kimura two-parameter correction for multiple substitutions, not taking into account insertions and deletions (Kimura, 1980). The bootstrap method for determination of reliability of tree topology was performed using 100 bootstrap replicates with SIV_{CPZGAB} sequence as outgroup. Trees were drawn in Treeview (version 1.5.2; R.D.M. Page, http://taxonomy.zooogy.gla.ac.uk/rod/rod.html).

Genetic distances. MEGA (Molecular Evolutionary Genetic Analysis, version 2,1) analysis of both gp120 and V3V5 sequences were completed using the Kimura 2-parametre model and taking into account transitions and transversions (Kumar *et al.* 2001). Percentage nucleotide divergence was compared both within and between samples, subtype reference alignments and all subtype C sequences available from Africa in the HIV sequence database (http://hiv-web.lanl.gov/content/hiv-db). This was performed to assess intrasubtype, intersubtype and intersample genetic diversity.

The same 34 reference sequences used in the phylogenetic trees (Figure 2.4 and 2.5) were included for *intersubtype* diversity assessment. *Intrasubtype* C diversity was determined in both the gp120 and V3V5 genomic regions. For V3V5 sequences, *intrasubtype* C diversity calculations between subtype C sequences from South Africa (25 published sequences and 22 sequences from this study), Zambia (4 published and 10 from this study), Zimbabwe (3 from this study alone), Malawi (4 published and 4 from this study) and Botswana (52 published sequences) were included. *Intrasubtype* diversity in the gp120 region was calculated between sequences from South Africa (11 published sequences and 19 samples sequenced from this study), Zambia (6 published sequences and 4 samples sequences from this study), and Botswana (53 published sequences).

Amino Acid translation and Phenotype Prediction. Nucleotide sequences were translated into amino-acid sequences using BioEdit version 5.0.2 and phenotype prediction was performed through two different methods. The so-called 11/25 rule has been used widely for phenotype prediction. This rule designates sequences with basic residues (K or R) at V3 amino acid position 306 and 322 relative to the CDS start of HXB2, as X4 or SI and those with no basic residues at these positions as R5 or NSI (Connor *et al.*, 1997; Fouchier *et al* 1992; Hoffman *et al.*, 2002) (Figure 2.10). A method by Briggs and colleagues 2000, takes into account the possibility of dual tropism (R5X4) and has a higher accuracy at 91% in predicting the true phenotype. Four variables have been identified as predictors of viral phenotype, the number of positive (K or R) and negatively (D or E) charged amino acids in the V3 loop, the V3 loop net charge and the presence of an isoleucine (I) at HXB2 amino acid position 327. The actual phenotype is assigned a numerical value according to an equation:

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Predicted phenotype = 0.94 + [1.68 X (V3 net charge)]
- [1.37 X (total positive charges, (K or R))]
+ [1.54 X (total negative charge (D or E))]
- [1.19, if amino acid 327 = I]

A predicted phenotype output value of 0,5 to 1,4 is assigned a number 1, which means the virus is R5, 1,5 to 2,4 is assigned a number 2 predicting the virus to be X4 alone or in combination with R5, thus dual tropic and 2,5 to 3,4 is number 3 and predicts X4 virus.

Entropy Plots. Entropy plots were generated in BioEdit version 5.0.2. This refers to a measure of the lack of predictability for an alignment position. Entropy is at maximum variability in nucleotide positions with high variation but low in positions with low variation (Schneider and Stephens, 1990).

2.3 Results:

2.3.1 PCR and Gel electrophoresis

The V3V5 region was amplified to yield a band of approximately 674 bp (Figure 2.2). Gp120 was amplified as three overlapping fragments yielding fragments of 702bp (Env A-f and Env E-r), 512bp (Env B-f and Env B-r) and 594bp (Env D-f and Env D-r). PCR was optimized for increased sensitivity and specificity such that only clear single bands were visualized, with no evidence of non-specific PCR. Amplicons that were visualized as discrete bands on agarose gels, were selected for sequencing. PCR was routinely monitored for contamination by including a positive control and water negative control for each reaction set of PCR. An RNA positive control consisted of a pool of RNA samples from different patients. This resulted in generation of amplicons of different lengths after amplification of certain regions of the genome (Figure 2.3 lane 19).

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Figure 2.2: V3V5 nested amplicons, amplified by DR-7 and DR-8 (674bp) and visualized after 2% agarose electrophoresis 1h at 120 V. Molecular weight marker 6 (Roche, GmbH, Mannheim, Germany) is in lane 1. Lane 2, 615-00037-7; lane 3, 615-00032-5; lane 4, 616-0001-7; lane 5, 616-00009-1; lane 6, 616-00043-7; lane 7, 615-00013-6; lane 8, negative water control and lane 9, positive control.

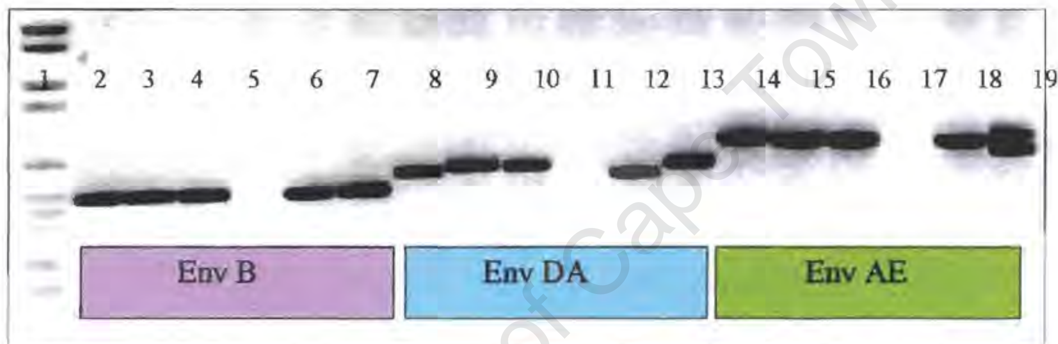

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Figure 2.3: Gp120 nested amplicons, amplified by Env AE (702bp), Env B (521bp) and Env DA (594bp) visualised by 2% agarose gel electrophoresis for 1h at 120 V. Molecular weight marker 6 (Roche, GmbH, Mannheim, Germany) is in lane 1. Lane 2, 8 and 14 contains 541-00011-5; Lane 3, 9 and 15, 541-00012-6; Lane 4,10 and 16, 541-00014-4; Lane 5, 11 and 17, the water negative control; Lane 6 and 7, 12 and 13, 18 and 19 are positive controls, the second positive control in each fragment (lane 7, 13 and 19) were performed on a mixed population of HIVs explaining the double bands observed in some cases.

2.3.2 Phylogenetic Analysis

Phylogenetic analysis of 19 gp120 and a total of 39 V3V5 sequences were performed. All gp120 sequences grouped with subtype C reference sequences (Figure 2.4) with similar results for V3V5 (Figure 2.5). One Zambian V3V5 sequence, 541-00017-8 was

an exception grouping outside of subtype C with a bootstrap value of 90% (Figure 2.5). We could detect clusters of sequences that grouped together according to geographical origin in samples from South Africa in both trees. However, these clusters were not supported by significant bootstrap values. There was no evidence of contamination, as in both phylogenetic trees generated from V3V5 and gp120 sequences, we found no interpatient sequences to be identical and no tight clustering of patient sequences with reference sequences.

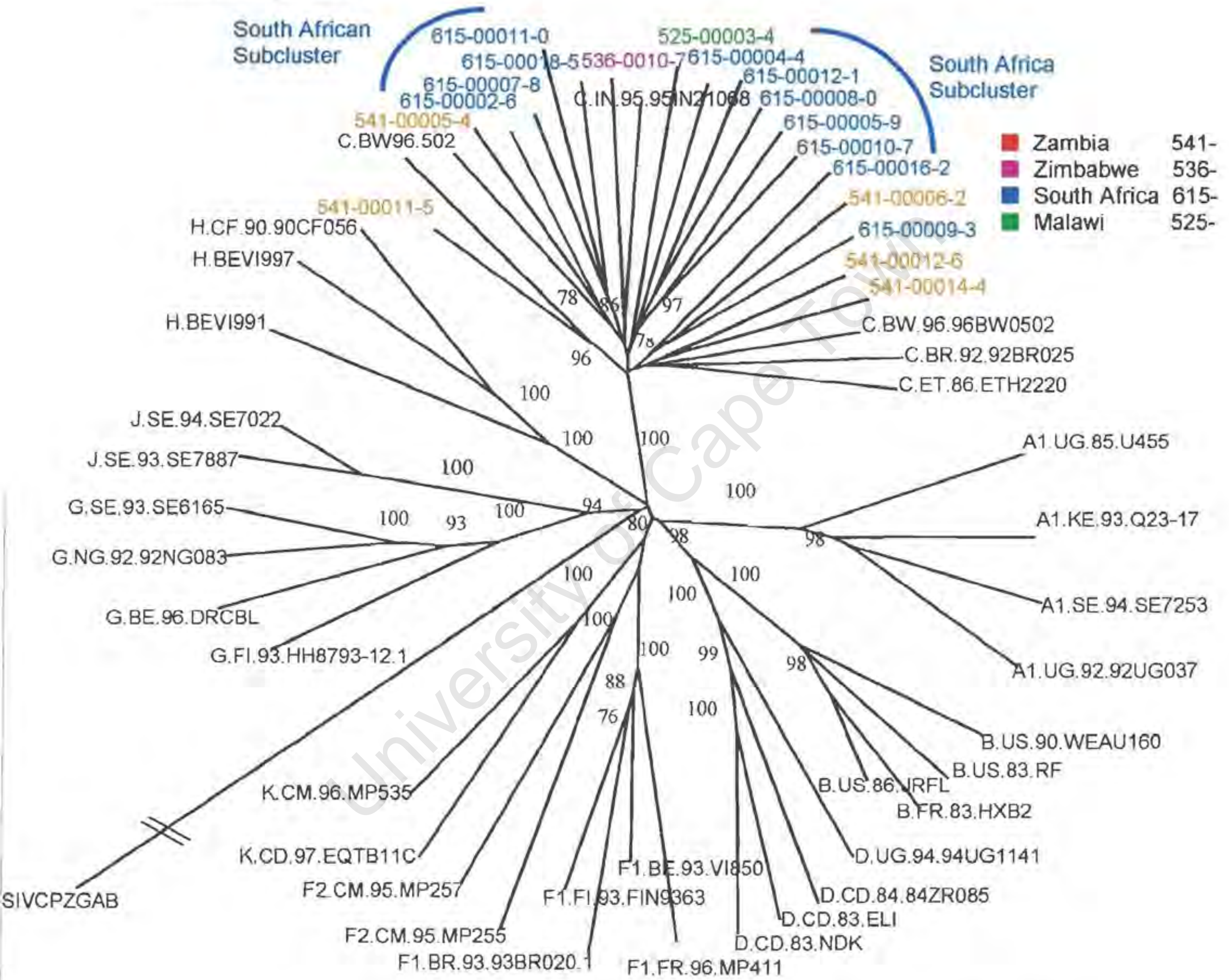

Figure 2.4: Phylogenetic tree analysis of 54 **gp120** sequences, 19 from this study including 11 from SA (prefix 615-), 1 from Malawi (prefix 525-), 1 from Zimbabwe (prefix 536) and 6 from Zambia (prefix 541-) and 34 reference sequences. Neighbor-joining trees were constructed using SIVcpz as an outlier; bootstrap values >75% out of 100 replications was represented on the branches.

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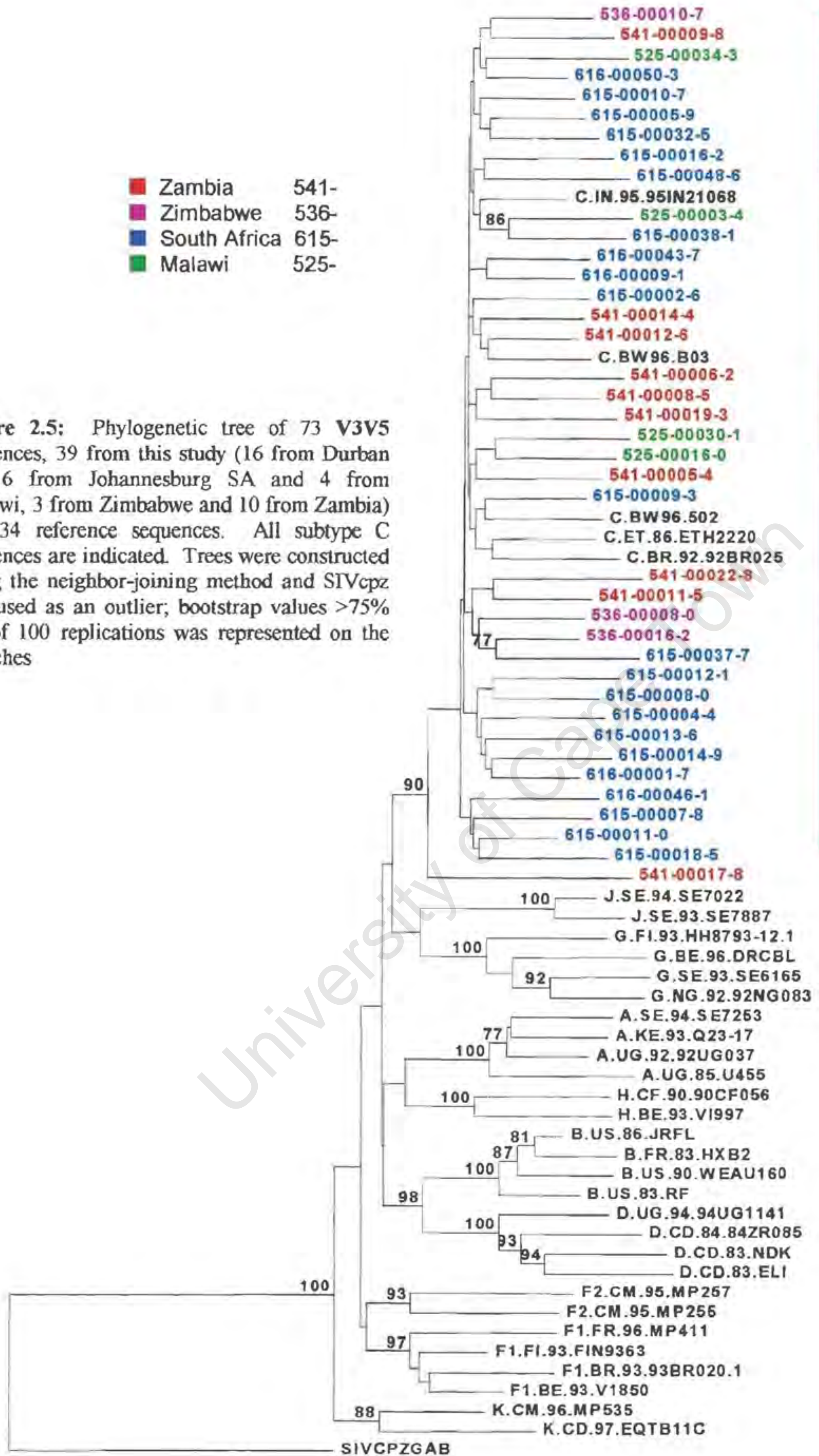

Figure 2.5: Phylogenetic tree of 73 **V3V5** sequences, 39 from this study (16 from Durban SA, 6 from Johannesburg SA and 4 from Malawi, 3 from Zimbabwe and 10 from Zambia) and 34 reference sequences. All subtype C sequences are indicated. Trees were constructed using the neighbor-joining method and SIVcpz was used as an outlier; bootstrap values >75% out of 100 replications was represented on the branches

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Gp120.

The *intersubtype* diversity calculated for 34 subtype reference sequences included in phylogenetic trees, ranged from 14,6%, (between subtype B and D), to 21,4 % (between subtype J and F). *Intrasubtype* C diversity of sequences from Africa, n=115 (including 20 gp120 sequences generated in this study) ranged from 2,9% to 14,2% (mean 9,9%). The pairwise genetic distance between the 39 southern African HIVNET sequences generated from this study ranged from 7,6% to 13,1% (mean 10,6%) (Table 2.6). Similar ranges for intrasubtype C sequences were found when including a complete analysis of all sequences available from South Africa as well as sequences from other southern African countries such as Zambia and Botswana (Figure. 2.6).

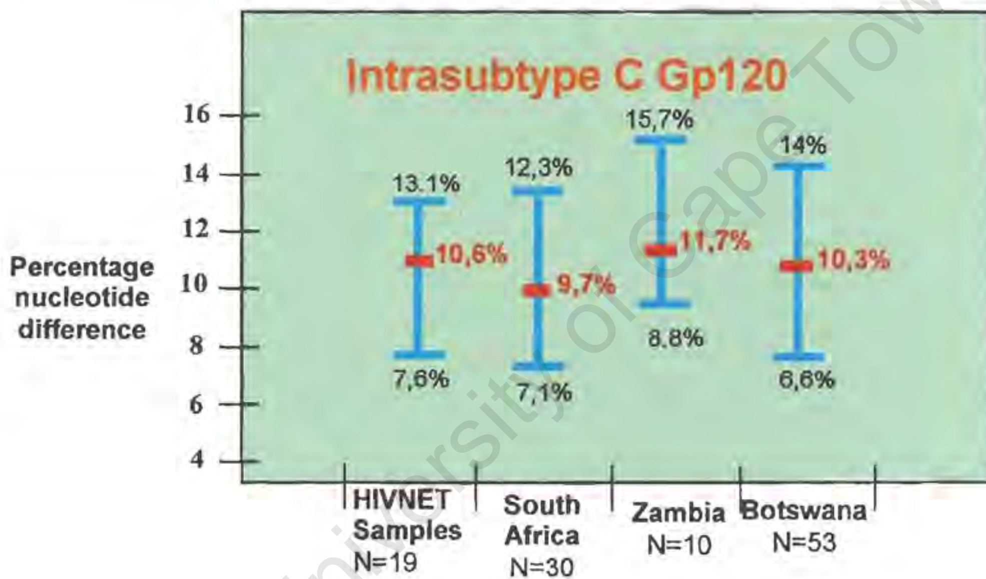

Figure 2.6: Intrasubtype C and intrasample nucleotide diversity across the gp120 region. Values are in percentage nucleotide diversity.

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V3V5

The mean *intersubtype* genetic distance between the 34 reference subtypes in V3V5 region ranged from 20,6% between subtype B and D to 30,2% between subtype D and J. Subtype C was the most similar to subtype A with a nucleotide distance of 23,3% and the least similar to subtype G with a nucleotide divergence of 26,5%. The overall mean for *intersubtype* genetic distance was 24,6%. The V3V5 pairwise genetic distance between

subtype C sequences from Africa including samples from this study (*intrasubtype*) were calculated and ranged from 4% to 20,4% (average 12,6%). (Table 2.6 and Figure 2.7). The genetic diversity among HIVNET southern African sequences ranged from 8,1% to 24,7% with an average of 14,5%. When excluding the Zambian outlier 541-00017-8 from our calculations the pairwise genetic distance range changed from 8,1% to 19,6% (average of 14,1%) (Figure 2.8).

		Subtype								
		A	B	C	D	F	G	H	J	K
	A									
	B	0.246								
	C	0.233	0.248							
	D	0.264	**0.206**	0.248						
Subtype	F	0.264	0.247	0.244	0.276					
	G	0.254	0.285	0.265	0.293	0.251				
	H	0.246	0.271	0.251	0.291	0.283	0.268			
	J	0.285	0.278	0.235	**0.303**	0.280	0.244	0.267		
	K	0.295	0.245	0.259	0.276	0.239	0.282	0.278	0.289	

Figure 2.7: Intersubtype genetic distances for the V3V5 region of the HIV-1 genome of 34 reference subtype samples from Los Alamos HIV Sequence database (http://hiv-web.lanl.gov/content/hiv-db).

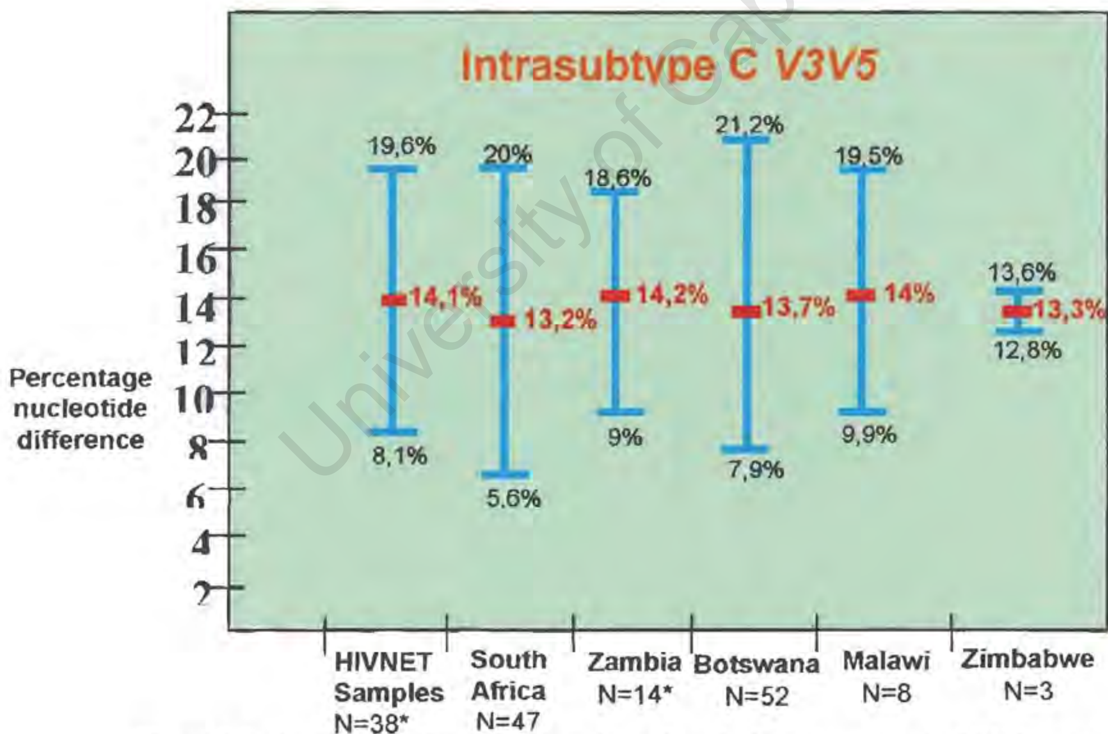

Figure 2.8: Intrasubtype C and intrasample nucleotide diversity across the V3V5 region for all samples in this study. Values are in percentage nucleotide diversity. * Excluding the Zambian outlier 542-00017-8.

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Table 2.6: Genetic diversity assessed for both the V3V5 and gp120 region between subtype reference sequences obtained from Los Alamos (*intersubtype*), within published African subtype C sequences (intrasubtype C) and within HIVNET subtype C sequences from this study (http://hiv-web.lanl.gov/content/hiv-db).

	% Nucleotide difference in *V3V5*	% Nucleotide difference in *Gp120*
Intersubtype	20,6 - 30,3 (24,6) n = 34	14,6 - 21,4 (18,4) n = 34
Intrasubtype C (Africa)*	4 - 20,4 (12,6) n = 143	2,9 - 14,2 (9,9) n = 115
Intrasample	8,1 - 19,6 (14,1) n = 39	7,6 - 13,1 (10,6) n = 19

Average % nucleotide difference in brackets, Zambian outlier (541-00017-8) were excluded from intrasample and intrasubtype C calculations. Intersubtype % nucleotide differences calculated using 34 reference sequences. "*" Including HIVNET sequences from this study.

2.3.4 V3 loop

The predicted V3 loop amino acid sequences consisted of 35 amino acids in 35 out of 39 HIVNET sequences. Four sequences, one from Zambia and three from South Africa had a single amino acid deletion each (Figure 2.9). The region in which these deletions occurred seemed to be clustered across an area of 4 amino acids (HXB2 numbering 319 to 322).

```
HXB2 numbering       300        310        320        330
                 ....|....| ....|....| ....|....| ....|..
ST C consensus   CTR NNNTRK SIRI**G GQ TFYATGDIIG DIRQAHC
541-00008-5      CTR SNNTRK GVRI**G GQ VFYA-NEIIG DIRQAHC
615-00004-4      CIR NNNTRR SVRI**G GQ TFY-TNDIIG DIRQAYC
615-00032-5      CTR GNNTRK SVRI**G GQ VFYA-NDIIG DIRQAHC
615-00048-6      CTR NNNTRK SIRI**G GQ VFYATN-IIG DIRQAYC
```

Figure 2.9: V3 loop amino acid alignment of four sequences that each had a deletion clustered in a 5 amino acid region spanning amino acid position 319 to 322, included is the subtype C consensus V3 loop amino acid sequence. "– " Indicates a deletion in the amino acid sequence, "**" represents two amino acids present in HXB2 but not in most subtype C sequences. HXB2 numbering was done relative to the CDS start of gp160.

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The N-linked glycosylation reportedly absent in most South African subtype C sequences site proximal to the first cystine of the V3 loop at position 295 was present in two individuals, one from South Africa and Zimbabwe each. Valine was the most common amino acid for our samples in this position (Sanders *et al.*, 2002) (Figure 2.10).

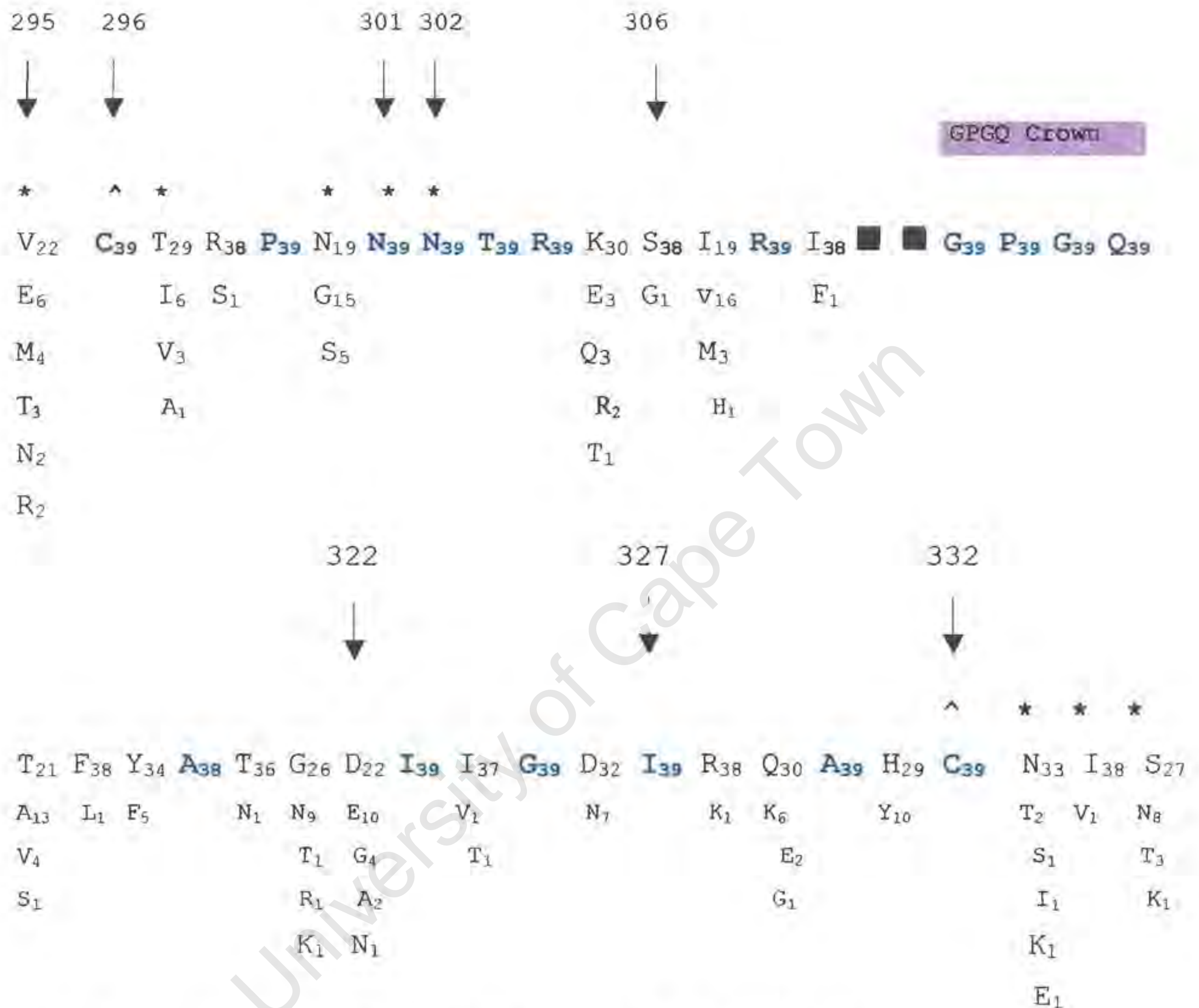

Figure 2.10: Consensus amino acid alignment of 39 subtype C V3 loop sequences. Boldface blue letters show conservation in all 39 sequences. Documented N-linked glycosylation sites are indicated with an asterisk (*), cystines at the start, position 296 and end, position 332, are indicated by a ^. The sequence is numbered relative to subtype B HXB2 CDS start of gp160 sequence. " " This symbol indicates two amino acids present in HXB2 but not in any of our sequences.

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2.3.5 Phenotype Predictions

According to the conventional phenotype prediction 11/25 rule, (Foucier *et al.*, 1995) no sequence from this study had a positive/basic residue (K or R) at V3 position 11 or 25 (HXB2 number 306 or 322) indicating all viruses were R5. The method by Briggs and colleagues (2000) was also applied to predict phenotype (Table 2.7). According to this method the numerical value calculated for all 39 samples analysed was 1, confirming that all samples were indeed R5.

2.3.6 Global Geographic Clustering within Subtype C

Global relatedness of gp120 sequences from this study to all other available subtype C gp120 sequences available from the HIV sequence database (http://hiv-web.lanl.gov/Align_current /SUBTYPE-REF/subtype.html) was assessed phylogenetically. The phylogenetic tree consisted of 175 subtype C sequences which included 19 gp120 sequences from this study, 4 reference sequences from Brazil, 8 from Europe, 11 from India, 2 from, the Middle East and 131 sequences from sub Saharan Africa (Figure 2.11).

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Table 2.7: Predicted phenotype calculated according to Briggs and colleagues (2000).

Sample	+ Charges	- Charges	Net charge	Ile @ aa 327	Output Value	Numerical Value	Predicted phenotype
525-00003-4	5	2	+3	YES	1.02	1	R5
525-00016-0	4	2	+2	YES	0.71	1	R5
525-00030-1	4	1	+3	YES	0.85	1	R5
525-00034-3	5	2	+3	YES	1.02	1	R5
536-00008-0	5	2	+3	YES	1.02	1	R5
536-00010-7	6	1	+5	YES	1.47	1	R5
536-00016-2	5	3	+2	YES	0.88	1	R5
541-00005-4	4	3	+1	YES	0.57	1	R5
541-00006-2	6	1	+5	YES	1.47	1	R5
541-00008-5	5	2	+3	YES	1.02	1	R5
541-00009-8	5	1	+4	YES	1.16	1	R5
541-00011-5	6	2	+4	YES	1.33	1	R5
541-00012-6	5	2	+3	YES	1.02	1	R5
541-00014-4	5	2	+3	YES	1.02	1	R5
541-00017-8	6	3	+3	YES	1.19	1	R5
541-00019-3	4	2	+2	YES	0.71	1	R5
541-00022-8	6	2	+4	YES	1.33	1	R5
615 00011-0	5	1	+4	YES	1.16	1	R5
615-00002-6	5	2	+3	YES	1.02	1	R5
615-00004-4	5	2	+3	YES	1.02	1	R5
615-00005-9	5	2	+3	YES	1.02	1	R5
615-00007-8	5	2	+3	YES	1.02	1	R5
615-00008-0	5	1	+4	YES	1.16	1	R5
615-00009-3	5	1	+4	YES	1.16	1	R5
615-00010-7	5	2	+3	YES	1.02	1	R5
615-00012-1	5	1	+4	YES	1.16	1	R5
615-00013-6	5	2	+3	YES	1.02	1	R5
615-00014-9	5	2	+3	YES	1.02	1	R5
615-00016-2	5	1	+4	YES	1.16	1	R5
615-00018-5	5	2	+3	YES	1.02	1	R5
615-00032-5	5	2	+3	YES	1.02	1	R5
615-00037-7	5	1	+4	YES	1.16	1	R5
615-00038-1	5	3	+2	YES	1.02	1	R5
615-00048-6	5	1	+4	YES	1.16	1	R5
616-00001-7	5	1	+4	YES	1.16	1	R5
616-00009-1	5	1	+4	YES	1.16	1	R5
616-00043-7	5	2	+3	YES	1.02	1	R5
616-00046-1	5	2	+3	YES	1.02	1	R5
616-00050-3	5	2	+3	YES	1.02	1	R5

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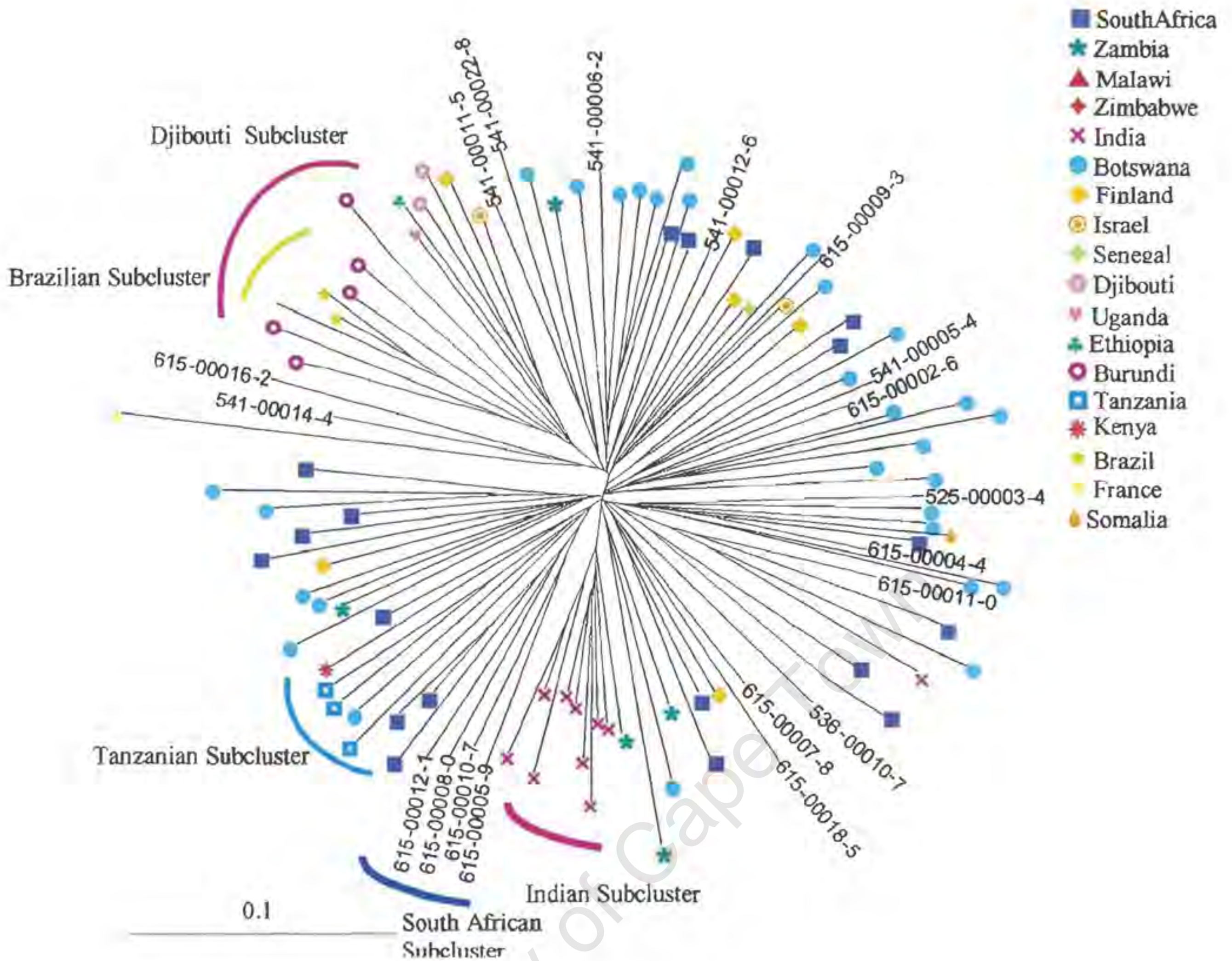

Figure 2.11: Global phylogenetic relationship among subtype C HIV-1 gp120 sequences including 19 samples from this study. Possible subclusters are indicated.

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2.3.1 Entropy Plots

Entropy plots for alignments of V3V5 and gp120 sequences show a large amount of variability across the HIV-1 *env* genome. Entropy is at maximum variability in positions with high nucleotide variability such as in the variable regions V1 to V5 and low in positions with low variation such as the constant regions C1 to C5 (Figure 2.12 and 2.13). Of the variable regions, V3 had the lowest entropy while V1 and V5 had the highest entropy.

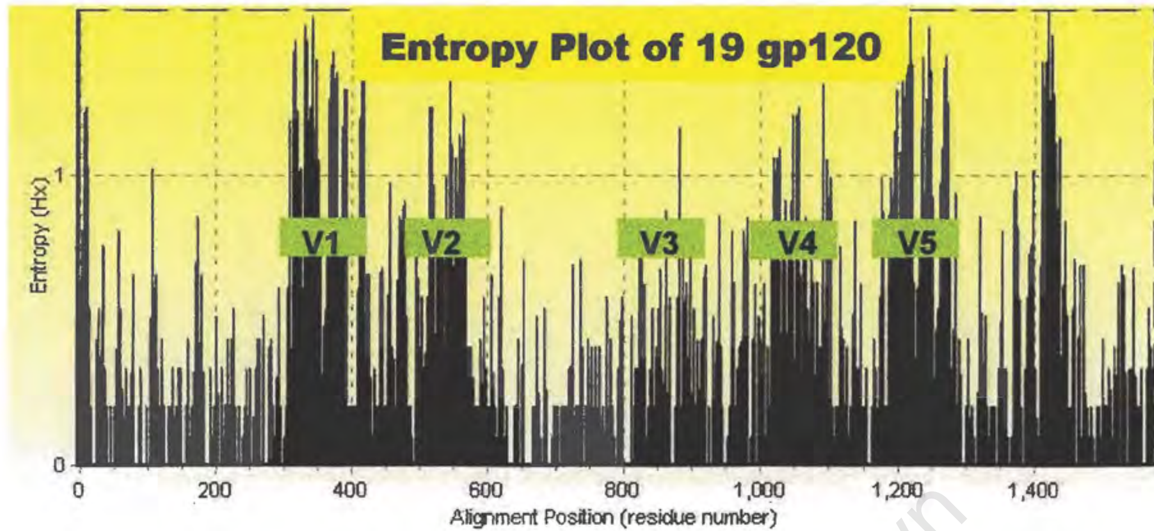

Figure 2.12: Entropy plots of sequences from this study across the gp120 region, variable regions 1 to 5 are indicated.

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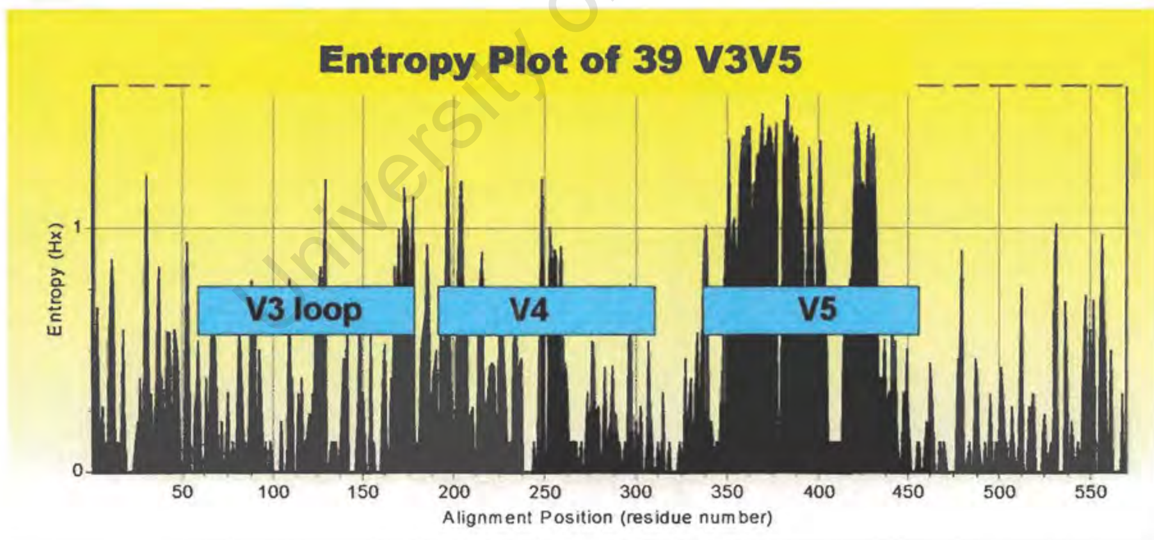

Figure 2.13: Entropy plots of sequences from this study across the V3V5 region.

2.4 Discussion:

This study confirms the dominance of *env* subtype C in southern Africa in that all 19 gp120 sequences and all but one of the 39 V3V5 sequence grouped with subtype C. One sequence from Zambia (541-00017-8) was highly divergent and did not group significantly with other subtype C sequences. The 19 full-length gp120 sequences provided the first full-length gp120s sequences from Zimbabwe and Malawi and extends the database of sequences from South Africa. This study is unique in that the majority of these sequences came from individuals within 2 years of infection and therefore reflects viruses currently circulating in the region.

However, subtype analysis based on only partial sequencing as in our case, may allow recombinants to go undetected. Additional analysis such as full-length sequencing and bootscanning would be required to confirm the true subtype. The outlier Zambian sequences may be a recombinant and sequences from this virus clustered similarly in trees generated from RT, *nef* and *gag*. However the fact that this sequence is always an outlier in all four genomic regions suggests that it may not be a recombinant but rather a highly divergent subtype C virus (H. Bredell, personal communication).

The subtype C epidemic in the heterosexual South African population has in previous studies been demonstrated to have a high level of intrasubtype diversity (van Harmelen *et al.*, 2001). These studies reported percentage nucleotide diversity in V3V5 to be 10% (Bredell *et al.*, 1998) and in V3, 14% (van Harmelen *et al.*, 1999). The high diversity and lack of phylogenetic relationships observed in the South African HIV-1 epidemic was thought to be due to multiple introductions of subtype C into South Africa (Shankarappa *et al.*, 2001; Engelbrecht *et al.*, 2001; Bredell *et al.*, 1998; van Harmelen *et al.*, 1997). We found overall V3V5 DNA distance of 13.2 %, which was similar when compared to the 10% observed in South African subtype C V3V5 sequences in 1998 (Bredell *et al.*, 1998) (Figure 2.8). This increase in DNA distance from 10% to 13,2% in South African subtype C V3V5 sequences is consistent with a 1% increase in diversity described for HIV-1 per year. Multiple introductions of HIV into South Africa are facilitated by a good transport infrastructure used by highly mobile populations such as migrant workers

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and truck drivers, allowing for rapid movement of the virus into new communities (Gilbert and Walker, 2002; Ramjee and Gouws, 2002).

In contrast to the high diversity observed in the South African subtype C epidemic the subtype C diversity in regions such as Thailand and India is much less. Indian subtype C sequences has a nucleotide divergence of 4,2% in the V3V5 region and 7,2% across gp120, much lower when compared to South African V3V5 at 10% (Bredell *et al.*, 1998) and 14% in the V3 (van Harmelen *et al.*, 1999). A close similarity between subtype C strains from these countries is the result of a recent spread from a single ancestor and represents a founder type effect (Delwart *et al.*, 1993; Grez *et al.*, 1994). Indeed it has been shown that the Indian epidemic may have originated from southern Africa (Dietrich *et al.*, 1993). Our analysis showed a close relationship between sequences from India and South African sequences generated in this study in that gp120 subtype C sequences from both countries grouped close together in a monophyletic lineage confirming results from previous studies (Shankarappa *et al.*, 2001) (Figure 2.11).

Global phylogenetic relationship of subtype C gp120 sequences resulted in a radial tree with strains from Djibouti, Brazil, Burundi, India and Tanzania grouping together according to geographical origin (Figure 2.11). In contrast, most gp120 sequences from South Africa exhibited a starburst phylogeny with sequences scattered throughout the tree with limited internal structure, typical of an epidemic that resulted due to multiple introductions of HIV. A South African subcluster could be identified, which contained four of the eleven South African sequences from this study as well as several subtype C from the sequence database. These subclusters were not supported with high bootstrap values.

The predicted V3 loop amino acid sequence exhibited the characteristic tetrapeptide sequence, GPGQ (HXB2 nucleotide position 312 to 315), at the tip of the V3 loop typical of all subtype C sequences (Figure 2.10). A N-linked glycosylation site proximal to the first cystine of the V3 loop at position 295 is reportedly absent in subtype C (Sanders *et al.*, 2002). This N-linked mannose seems to be crucial for the binding of monoclonal antibody 2G12 as in the absence of this glycan Mab 2G12 is unable to bind (Sanders *et*

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al., 2002; Scanlan *et al.*, 2002). Analysis of amino acid sequences generated in our study showed that this N-linked glycosylation site was present in only one South African (615-00048-6) and one Zimbabwean (535-00008-0).

CCR5 is the major co-receptor used by HIV during heterosexual transmission and acute infection (Deng *et al.*, 1996). In subtype B viruses it has been shown that R5 viruses switch to using CXCR4 as co-receptor in later stages of disease progression. The R5 to X4 co-receptor switch has rarely been observed in subtype C although subtype C X4 variants have been observed (Bjorndal *et al.*, 1999; Morris *et al.*, 2001; Tien *et al.*, 1999). This subtype specific differences in co-receptor usage was first suggested by the observation that subtype C X4 variants were rare in a group of 16 people under care in Sweden (Tschering *et al.*, 1998), and among a group of 22 French military personnel infected during overseas deployment (Peeters *et al.*, 1999), as well as in a group of Malawian men (Ping *et al.*, 1999), since then it has been confirmed in subtype C infected Ethiopian individuals (Bjorndal *et al.*, 1999) and 10 South African HIV isolates (Morris *et al.*, 2001).

According to two methods for prediction of HIV phenotype, including the so-called 11/25 rule (Connor *et al.*, 1997; Fouchier *et al* 1992; Hoffman *et al.*, 2002) and the method by Briggs (Briggs *et al.*, 2000) it was determined that all sequences were R5. These results confirm previous findings that CCR5 is the major co-receptor used by HIV-1 subtype C isolates predicted by two independent methods.

Findings from our study support previous reports that subtype C is the major contributor to the southern African HIV-1 epidemic, including countries such as South Africa, Zambia, Zimbabwe and Malawi (Bredell *et al.*, 1998; Moodley *et al.*, 1998). Our results showed that subtype C sequences from southern Africa, exhibited a starburst phylogeny with limited geographic clustering typical of an epidemic resulting from multiple introductions of HIV. We also determined that sequences circulating in recently infected individuals from this study were all infected with HIV viruses utilising CXCR5 as co-receptor. In addition the reported absence of the N-linked glycosylation site as the reason for no 2G12 Mab neutralisation in subtype C viruses was confirmed by our study.

University of Cape Town

Chapter 3: AN INVESTIGATION OF HIV-1 DIVERSITY FOLLOWING HETEROSEXUAL TRANSMISSION IN RECENTLY INFECTED WOMEN FROM A SOUTH AFRICAN SEX WORKER COHORT

3.1 Introduction..........82

3.2. Methods and Materials..........86

3.2.1 Patient data and sample selection 86
3.2.2 RNA quantification and sero-status confirmation 87
3.2.3 RNA Extraction 88
3.2.4 cDNA Synthesis 88
3.2.5 Polymerase Chain reaction and agarose gel electrophoresis 88
3.2.6 Quantitation of PCR target molecules from PCR-based limiting Dilution assay 92
3.2.7 Cloning and Colony PCR 92
3.2.8 Heteroduplex Tracking Assay 94
3.2.9 Sequencing 99
3.2.10 Sequences analysis 100

3.3 Results..........101

3.3.1 RNA quantification and sero-status confirmation 101
3.3.2 Total population PCR 104
3.3.3 Quantitation of target molecules from PCR-based limiting dilution assay 104
3.3.4 Cloning into pMOS*Blue* and Colony PCR 105
3.3.4 Heteroduplex Tracking Assay 106
3.3.5 Phylogenetic analysis 108
3.3.6 Genetic distances 111
3.3.7 Amino-Acid translation and Phenotype prediction 113

University of Cape Town

3.4 Discussion......117

University of Cape Town

CHAPTER 3: An Investigation of HIV-1 Diversity following heterosexual transmission in recently infected women from a South African female sex worker cohort

3.1 Introduction:

Over 90% of HIV-1 infections in South Africa are thought to occur heterosexually. There are more women than men infected with approximately 2,7 million HIV positive women compared with only 2,3 million infected men (UNAIDS 2002). Women have been reported to be infected with a genetically more complex HIV-1 population soon after transmission (Kampinga *et al.*, 1997; Long *et al.*, 2000; Poss *et al.*, 1995) compared to men who are thought to harbor a homologous viral population (Zhang *et al.*, 1993; Zhu *et al.*, 1993). It has been proposed that women may be more susceptible to infection with multiple quasispecies due to: an increased muscosal area of exposure, increased susceptibility through greater mucosal trauma, the presence of undiagnosed sexually transmitted infections, prolonged exposure to infected male secretions and immunological differences between men and women (Ray and Quinn, 2000). HIV-1 populations following transmission may reflect the virus transmitted and be the consequence of selective transmission / selective penetration (differential ability to traverse the mucosa), or may occur after transmission as a result of selective amplification (outgrowth of a particular quasispecies) (Zhang *et al.*, 1993; Zhu *et al.*, 1993). Additional gender differences HIV-1 pathogenesis have been noted in that women have been reported to progress to AIDS at lower viral loads compared to men (Farzadegan *et al.*, 1998; Sterling *et al.*, 2001). To understand the mechanisms of transmission and to develop interventions such as vaccines, identification and characterization of recently transmitted HIV-1 variants are crucial.

This study aims to characterize HIV populations present early in infection in five South African women belonging to the Durban sex worker cohort (Du-cohort). The women were being monitored monthly for seroconversion as part of an UNAIDS-funded Phase

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III microbicide trial (Van Damme *et al.*, 2002). In order to more accurately investigate viruses that were transmitted, the last HIV antibody negative sample and the first HIV antibody positive sample were obtained. Genetic characterization of viruses was performed in two distinct HIV-1 genomic regions p17 and C2V3. These two regions were investigated, as they would be under different selective pressures, with the *env* region being important for cell tropism. In addition, characterization of both regions ensured that we would be able to assess both the upper and lower margin of viral population diversity within an individual. This is possible as *gag* is a well-conserved region in the genome compared to the *env* gene.

Mass cloning and sequencing is the most conventional method for viral population studies yielding detailed data, but are labor and cost intensive. An alternative, simple, cost and labor effective technique is the Heteroduplex tracking assay (HTA), which is a highly specific and sensitive assay to define different populations (Delwart *et al.*, 1993, 1994). This method and subsequent sequence analysis was used to identify and characterize viral populations based on C2V3 and p17.

HTA uses a double stranded DNA probe, radioactively labeled on a single strand, that after denaturing and re-annealing together with fully complementary ddDNA/PCR product (template derived from the total viral population or from colony PCR) generate probe-PCR product consisting of homoduplexes and heteroduplexes (Figure 3.1). On a HTA autoradiograph a number of bands are visible: single stranded probe bands, homoduplexes (HO) and heteroduplexes (HE). The duplexes can be separated and visualized through polyacrylamide gel electrophoresis (PAGE) and subsequent autoradiography. The migration rate of DNA through polyacrylamide gel is dependant on the flexibility of the particular DNA fragment. DNA homoduplexes have a rigid, rod-like structure that migrates fastest in a polyacrylamide gel. When the rigid structure is disrupted by unpaired bases, a "kink" will form, similarly a cluster of mismatches form a "bubble" increasing the flexibility of the DNA structure. Both these structural changes lead to reduced mobility of heteroduplexes in the polyacrylamide gel relative to the probe which represents a particular population against which all other population clones for an individual can be compared (the probe is also called the reference population). Evidence

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for a population genetically diverse from the reference probe is seen by the observation of heteroduplex bands. HTA has been used to investigate the highly variable envelope region of HIV and other viruses such as Hepatitis C virus (Delwart *et al.*, 1994; Nelson *et al.*, 1997; Ping *et al.*, 1999). This study used the HTA to investigate different viral populations within individuals characterizing both the p17 and the C2V3 region with some modification of the method to improve resolution of the less variable p17 genomic region.

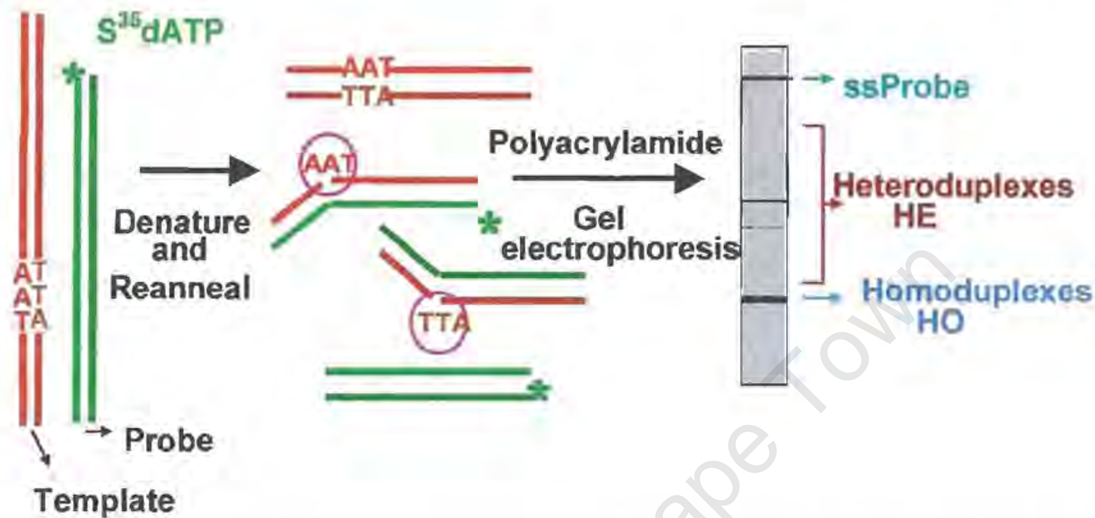

Figure 3.1: The Heteroduplex Tracking Assay. Complementary double stranded template and double stranded radio labelled probe (probe are labelled on one strand only) are allowed to denature and re-anneal. Upon re-annealing, homoduplexes and heteroduplexes form in addition to some single stranded probe (ssProbe). Single stranded probe bands are probe that has not re-annealed after denaturing, and homoduplex bands represent double stranded re-annealed sequences that are identical to the probe, either between two probe strands or between a probe strand and a homologous template strand. Any population of sequences that differ from the population represented by the probe will result in heteroduplex bands, the result of annealed sequences that differ either by mismatches or by insertions and deletions (indels). On a polyacrylamide gel these duplexes exhibit different migration rates, with fully complementary homoduplexes migrating the fastest at the bottom of the gel and heteroduplexes migrating slower, in the middle of the gel. The single stranded probe migrates the slowest at the top of the gel.

It was necessary to ensure that all or at least the greatest majority of HIV populations in the pre-seroconversion samples were detected, so that the extent of the transmitted population diversity could be determined accurately. In addition the likelihood of re-sampling the same RNA molecule had to be avoided, as this would distort the measure of sequence diversity (Liu *et al.*, 1996b). Amplifiable copy number was thus determined;

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this is an estimation of the number of target molecules in an aliquot of sample that could be amplified for further analysis. Below this number of molecules, amplification was not possible.

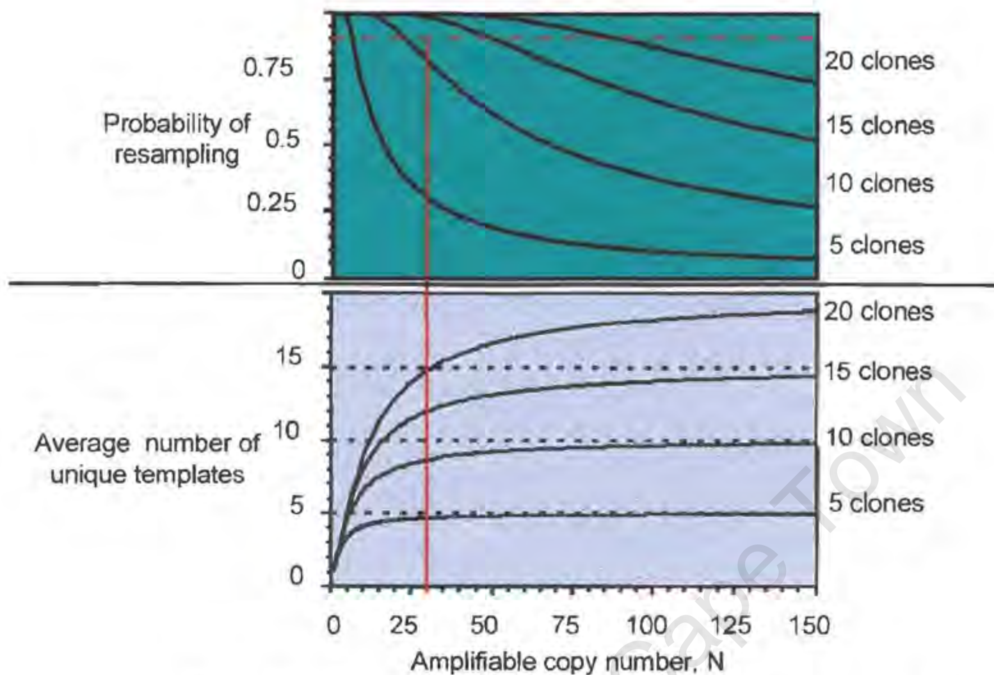

Figure 3.2: The relationship between the amplifiable copy number (N), the number of clones selected for screening, the average number of unique templates and the probability of re-sampling (Adapted from Jim Mullins).

A number of factors determine the relationship been RNA copy number and amplification product including: the efficiency of the primers depending on their design, PCR conditions specified, and specific binding affinity of primers for the different populations present in the sample. This makes some primers more efficient in amplifying certain populations than others. It is thus crucial to accurately quantify the number of HIV RNA copies that each primer pair is able to amplify for each sample to ensure accurate representation of the total population. We determined the amplifiable copy number per primer pair per sample by a method based on an end-point dilution followed by the detection of viral nucleic acid (PCR-based limiting dilution assay - PDLA) (Mulder *et al.*, 1994). An end point dilution of cDNA would represent populations present in the sample, as the RT reaction is not an amplification step, but merely a method to copy the existing viral RNA to cDNA. According to the number of positive

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PCR amplifications (more than one PCR-based limiting dilution can be performed) per dilution an estimate of the number of HIV molecules that were amplified can be made by inputting the results into a program, QUALITY, (Quantitation Using A LImiting diluTion assaY) (Rodrigo *et al.*, 1997) (http://ukib.microbiol.washington.edu/cbg/jquality.html). This program estimates the amplifiable copy number or the number target molecules in an aliquot of sample that could be amplified. There is a relationship between the amplifiable copy number and the number of clones to be selected for sequencing (Figure 3.2). According to this relationship an amplifiable copy number of at least 25 and screening of at least 20 clones is required to sample the majority of unique templates and ensure that no re-sampling takes place.

3.2. Methods and Materials:

3.2.1 Patient data and sample selection

Samples were obtained from the Du sex worker cohort (Du being the UNAIDS assigned abbreviation for Durban). This cohort was established in 1992 as part of an UNAIDS-funded Phase III vaginal microbicide trial, Col-1492 (Abdool Karim *et al.*, 1995; Van Damme *et al.*, 2002). Participants recruited for this cohort originated from five truck stops on the main transport route between Durban and Johannesburg in the Kwazulu/Natal Midlands.

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Women were screened for seroconversion on a monthly basis. To date, 452 sex workers have been screened. On enrollment, the mean period as sex worker per individual was 2,5 years (range one month to 31 years). Sex worker clients originated mainly from South Africa but also from surrounding countries including Mozambique, Zimbabwe, Zambia, Namibia and Kenya. The clients had an estimated HIV prevalence of 56,3%. On average these women had four partners per day and were involved in 28 sexual acts per week. Most women from this cohort used no contraceptive method, and despite extensive education and counseling, only one fifth of the women used condoms in just over 50% of coital acts. Forty-one percent engaged in anal sex (Karim and Ramjee,

1998), while 24% said that they had oral sex with their clients. There is no reported use of anti-retroviral drugs in this cohort.

The last HIV antibody negative sample (preseroconversion), and first antibody positive sample was obtained. To confirm that samples collected a month before documented seroconversion were indeed preseroconversion samples and to determine viral load, RNA quantification was performed and the sample was tested for possible seroconversion.

3.2.2 RNA quantification and Seroconversion detection

RNA Quantification. Plasma samples collected a month before documented seroconversion (preseroconversion) were screened HIV RNA using the Nucleic Acid Sequence Based Amplification (NASBA)/NucliSens assay developed by Organon Teknika, Boxtel, The Netherlands. Viral loads were determination was performed by the Medical Virology Diagnostic Laboratory (UCT). Samples that had detectable viral RNA without a measurable antibody response were then selected for inclusion into the study.

Seroconversion determination. HIV antibody status was confirmed with the Trinity Biotech Capillus™ assay (Trinity Biotech USA, Jamestown). The Capillus™ assay is a rapid qualitative assay for the detection of antibodies to HIV-1 and HIV-2 in human whole blood, serum or plasma. HIV-1 and HIV-2 antibodies are detected by a direct latex aggregation between antibodies and HIV-1 and HIV-2 envelope proteins bound to polystyrene latex beads supplied in the kit. In this assay 10μl plasma of each sample equilibrated to room temperature was added to 120μl latex reagent on a capillary slide, mixed and allowed to flow via capillary action to the so-called viewing window. This window shows a smooth milky liquid in a negative result and aggregation or flocculation in a positive result.

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3.2.3 RNA Extraction

Due to low volumes of sample available, HIV-1 viral RNA extracted as part of the NASBA procedure was also used for p17 and C2V3 PCR amplification. Although this RNA contained the kit calibrators, the primers used to co-amplify the calibrators and sample RNA in the viral load quantification procedure were in a different genomic region than the primers used in the study for both genomic regions p17 and C2V3 (Van Gemen *et al.*, 1994), thus there was no possibility of amplifying and analysing calibrator RNA.

Where necessary, additional RNA was extracted using the QIAamp® Viral RNA Mini Kit for purification of viral RNA from plasma, Serum, Cell-free body fluids and Cell-culture supernatants (Qiagen, Valencia, CA). According to manufacturers specifications RNA was extracted from 140 - 280μl plasma and eluted in 60μl buffer. This was then divided into 10μl aliquots and either used directly in complementary DNA (cDNA) synthesis or stored at –70°C. (Appendix A1).

3.2.4 cDNA Synthesis

Complementary DNA was generated in the same manner as detailed in section 2.2.2. As two distinct genomic regions in the HIV-1 genome was under investigation two separate cDNA reactions were performed, one for copying the C2V3 region and another for the p17 region. Gene specific primers used was either Env A-r for C2V3, or Gag D-r for p17 (Table 3.1).

3.2.5 Polymerase chain reaction and Agarose gel electrophoresis

Quality control. Quality control was performed in the same manner as described in detail in section 2.2.3.

PCR. Both the C2V3 and p17 regions were amplified by nested PCR (nPCR) for all samples. Each nPCR consisted of an outer reaction and a subsequent single inner reaction with primers nested within the outer fragment (Figure 3.3). For the C2V3 region

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the outer reaction was performed using primer pairs Env A-f and Env A-r producing a 1,599Kb fragment and the inner reaction was performed using primer pair Env B-f and Env B-r yielding a 512bp fragment. The p17 outer reaction was done using Gag D-f and Gag D-r producing a fragment of 1,776Kb and the inner reaction was performed using primer pair Gag A-f and Gag A-r yielding a 621bp fragment (Table 3.1)

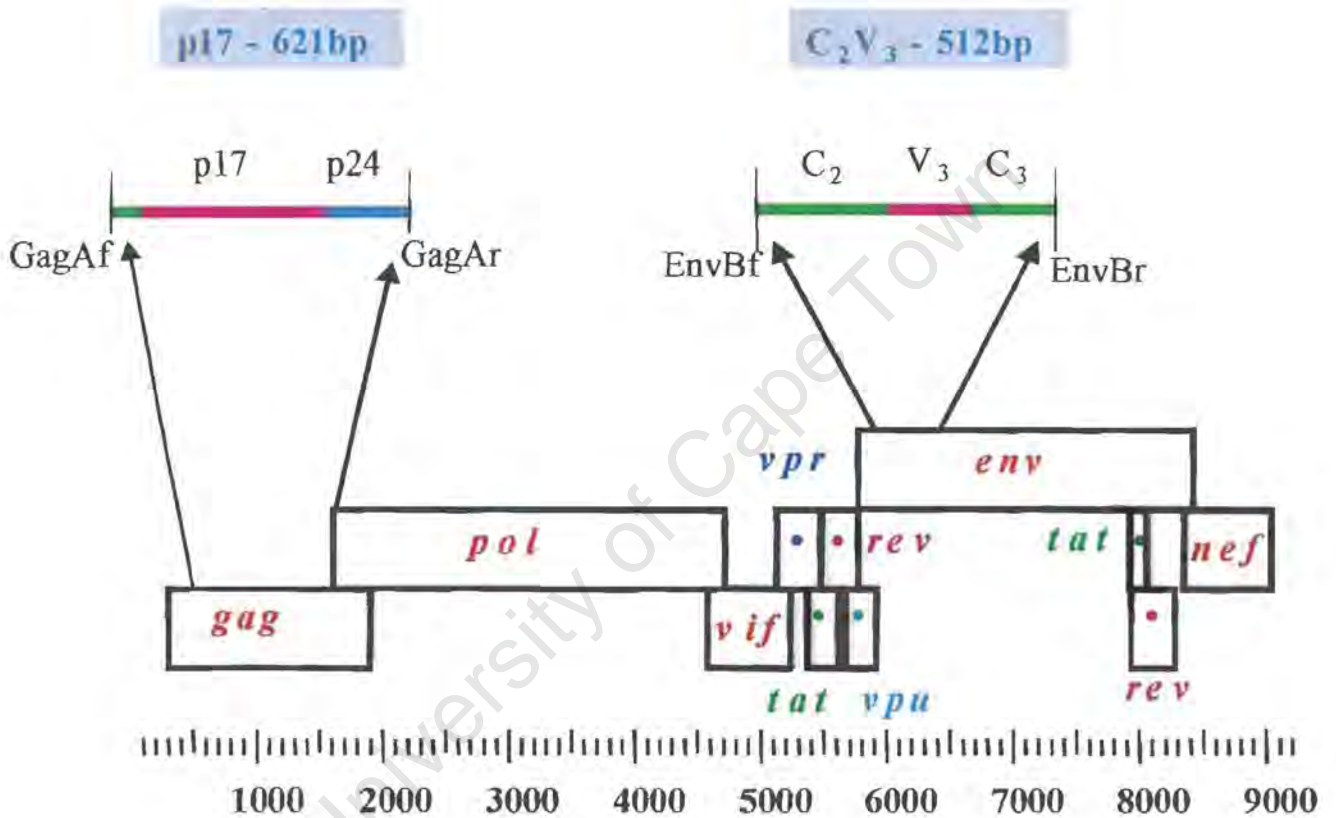

Figure 3.3: Genomic organization of HIV-1 showing C2V3 and *P17* regions under investigation in this study.

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Table 3.1: Primers for cDNA synthesis and PCR for amplification of C2V3 and p17

Primer	Position*	Sequence
Env A-r	7783-7802	5' TGC TGC TCC CAA GAA CCC AA 3'
Env A-f	6203-6223	5' GAA AGA GCA GAA GAC AGT GGC 3'
Env B-f	6826-6847	5' TAA CAC AAG CCT GTC CAA AGG T 3'
Env B-r	7317-7337	5' AAT TTC TAG GTC CCC TCC TGA 3'
Gag D-f	626-643	5' TCT CTA GCA GTG GCG CC G 3'
Gag D-r	2382-2402	5' AAT TCC TCC TAT CAT TTT TGG 3'
Gag A-f	683-704	5' CTC TCG ACG CAG GAC TCG GCT T 3'
Gag A-r	1282-1303	5' ACA TGG GTA TCA CTT CTG GGC T 3'

* Sequence positions were calculated relative to HXB2. Primers produced at the DNA synthesis laboratory of UCTs Biochemistry Department were individually designed and optimized.

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The PCR master mixes contained:

Outer nested PCR (50μl)

Reagent	Amount
10X PCR buffer (Supplied with Super-therm DNA polymerase)	1X
PCR grade dNTP (Roche Diagnostics, GmbH, Mannheim, Germany)	200μM
Primer Env A-f + Env A-r or Gag D-f + Gag D-r	0,1μM
$MgCl_2$ for Env A-f + A-r (C2V3) $MgCl_2$ for Gag D-f + Gag D-r (p17)	2mM 1,5mM
Super-therm DNA polymerase (Super-therm DNA polymerase, JMR Holdings, Sevenoaks, UK)	0,625U
Patient cDNA	5μl
Total	**50μl**

PCR cycling was carried out using a GeneAmp PCR System 2400 thermocycler (Perkin Elmer, Norwalk, CT) according to the following parameters:

Initial denaturing step at 94°C for 2 min.

94°C for 1 min
50°C for 1 min → for 3 cycles
72°C for 1 min

94°C for 15 sec
50°C for 45 sec → for 32 cycles
72°C for 1 min

Final elongation step at 72°C for seven minutes.

Inner nested PCR (50µl)

Reagent	**Amount**
10X PCR buffer (Supplied with Super-therm DNA polymerase)	1X
PCR grade dNTP (Roche Diagnostics, GmbH, Mannheim, Germany)	200µM
Primer Env Bf+Br (C2V3) and Gag A-f+Ar (p17)	0,1µM
$MgCl_2$ for Env B-f+B-r $MgCl_2$ for Gag A-f +A-r	2mM 1mM
Super-therm DNA polymerase (Super-therm DNA polymerase, JMR Holdings, Sevenoaks, UK)	0,625U
Outer reaction PCR product	5µl
Total	**50µl**

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PCR cycling reaction was carried out using a PCR Express thermocycler (Hybaid limited, Middlesex, UK) according to the following parameters:

Initial denaturing step at 94°C for 2 min

94°C for 15 sec
55°C for 30 sec → for 30 cycles
72°C for 30sec

Final elongation step completed at 72°C for seven minutes.

Agarose gel electrophoresis and PCR purification. C2V3 and p17 amplicons were visualized by 2% agarose gel electrophoresis (Appendix A4). Second round PCR C2V3 and p17 amplicons were purified using the QIAquick PCR purification protocol (Qiagen, Valencia, CA) (Appendix A2).

3.2.6 Quantitation of PCR target molecules from PCR-based limiting dilution assay

In this study we determined the amplifiable copy number by performing a PCR-based limiting dilution assay (PDLA). We performed a one in ten serial dilution of cDNA for each of the five samples and amplified each dilution by nPCR as described above in section 3.2.5. The number of positive PCR amplifications (in some cases more than one PCR-based limiting dilution was performed per sample) per dilution was inputted into QUALITY, (Quantitation Using A LImiting diluTion assaY) (Rodrigo *et al.*, 1997) (http://ukib.microbiol.washington.edu/cbg/jquality.html) and an estimate of the number of HIV molecules that were amplified were calculated, ie the amplifiable copy number. For our purposes we chose to add enough RNA per RT-nPCR reaction to ensure that at least 30 copies were amplified (Figure 3.2).

3.2.6 Cloning and Colony PCR

To generate probes for HTAs both the C2V3 (512bp) and the p17 (621bp) fragments need to be cloned into separate pMOS*Blue* vectors for all five samples included in this study (Appendix C1). The MOS*Blue* vector is supplied in a kit, linearised at *EcoRV* in the multiple cloning site (pMOS*Blue* Blunt ended cloning kit, RPN5110, Amersham Pharmacia Biotech, Buckinghamshire, UK). Cloning was performed according to manufacturers recommendations.

pk Reaction. In this first step, both fragments are converted into blunt, phosphorylated products in a single step:

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Reagent/ Stock solution	Amount/Final Concentration
10X pk buffer	1X
100mM DTT	10mM
pk enzyme mix	1μl
Either amplicons C2V3 or p17	Xμl
Water	Yμl
Total	**10μl**

The amount of amplicon DNA added to the pk reaction was calculated on a vector:insert ratio of 1:2,5 for both the C2V3 fragment of 512bp and the p17 fragment of 621bp. The optimal vector:insert ratio of 1:2,5 for a 214bp fragment was determined by the manufacturers. The pk reaction was incubated at 22°C for 40 minutes where after the reaction was terminated at 75°C for 10 minutes and then cooled on ice for two minutes.

Ligation reaction. In this step blunted fragments are ligated into the blunt de-phosphorylated linearised pMOS*Blue* vector during a brief heat incubation step:

Reagent/ Stock solution	Amount/Final Concentration
Product form pk reaction	10μl
pMOSBlue vector (50ng/μl)	50ng
T4 ligase (4 units/μl)	4 U
Total	**12μl**

Transformation. Highly efficient MOS*Blue* competent cells provided with the kit was determined by the kit manufacturers to result in 75% of recombinant colonies containing an insert. The competent cells were allowed to thaw on ice for no more than five minutes before use. Cells were mixed gently to suspend evenly. A total of 20μl of competent cells were added to pre-chilled 1,5ml eppendorf tubes. Of the ligation mix 1μl was added directly to the cells and stirred gently to mix. The tubes were left on ice for 30 minutes and subsequently heat shocked in a 42°C water bath for 40 seconds without shaking. Tubes were then incubated on ice for 2 minutes and 80μl SOC medium supplied with the kit (2g Tryptone, 0,55g Yeast extract, 10mM NaCl, 10mM KCl, 2mM Mg^{++} ($MgCl_2$ and $MgSO_4$), 2mM glucose up to 1 litre), equilibrated to room temperature, was added to each tube. Tubes were then shaken at 200 - 250 rpm at 37°C for 1 hour.

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After one hour, 2 X 25μl and 1 X 50μl of each transformation mixture was plated on selective indicator LB plates containing tetracycline (15μg/ml), ampicillin (100μg/ml), X-gal (80μg/ml) and IPTG (0,5mM) for blue white screening (Appendix B3). Plates were incubated inverted overnight at 37°C and checked for positive white colonies the following day. These resulting pMOS*Blue* clones were used both for constructing HTA probes as well as for screening HIV population complexity.

Ligation and Transformation controls. To test the efficiency of both the ligation reaction and the transformation reaction, positive and negative controls were included in the cloning procedure. Ligation efficiency was determined through use of a ligation positive control of 9ng of a 214bp supplied by kit manufacturers. They determined an optimum vector:insert ratio for ligation at 1:2,5 with this 214bp control insert. In order to determine the efficiency of transformation 0,2ng of pUC18 was transformed into the supplied pMOS*Blue* competent cells. A 5μl plating of cells transformed with this plasmid onto X-gal and IPTG LB plates should give an efficiency of >4×10^7 blue cfu/μg test plasmid or approximately 400 colonies per plate according to kit manufacturers.

3.2.7 Heteroduplex Tracking Assay

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The HTA consists of a number of steps including first of all, probe construction. Probes were constructed by growing up a selected clone for subsequent plasmid DNA maxipreparation. One clone each per genomic region was selected for radioactive labeling. After an *EcoRI* restriction enzyme digestion the overhang was filled in with α^{35}S-dATP and released from the vector by *NdeI* digestion and purified. Secondly, denaturation and annealing of probes in the presence of both C2V3 and p17 amplicons generated by colony PCR resulted in the formation of homoduplexes and heteroduplexes. Thirdly, homoduplexes and heteroduplexes are separated by polyacrylamide gel electophoresis and visualized by gel drying and autoradiography. Heteroduplexes indicated which clones represented divergent populations. Where available two examples of each such divergent heteroduplex population were selected for sequencing and genomic characterization. For each sample the HTA was performed in duplicate to confirm findings.

Probe construction

Plasmid DNA maxipreparation. Two selected plasmid clones, one pMOS*Blue*-C2V3 clone and one pMOS*Blue*-p17 clone per sample, were each inoculated into separate aliquots of 100ml LB broth (Appendix B7) each containing ampicillin (100μg/ml) and grown up overnight at 37°C with shaking. A Nucleobond® AX 100 maxi-preparation of plasmid DNA from this 100ml culture was then performed according to manufacturers instructions (Machery-Nagel, Duren, Germany) (Appendix A3). The resulting plasmid DNA was redisolved in TE (pH 7,8). In order to determine the concentration of the re-suspended plasmid DNA, a one in hundred diluted aliquot in TE was scanned using the Beckman DU-40 spectrophotometer (Beckman Industries, California, USA) from 310nm to 220nm wavelengths. The spectrophotometer was blanked using TE after which the samples were read. The DNA concentration was determined by multiplying the OD reading at A_{260} by 100 to correct for dilution and then by 50, which is the factor for double stranded DNA.

Probe labeling. Intraperson C2V3 and p17 α^{35}S-dATP isotope labeled probes were constructed by first digesting 10μg pMOS*Blue* plasmid with *EcoRI* (Roche Diagnostics GmbH, Mannheim Germany) at 37°C for one hour:

Reagent/ Stock solution	**Amount/Final Concentration**
pMOSBlue C2V3 or p17 clone	10μg
10 X Buffer H (Roche Diagnostics, GbmH, Mannheim, Germany)	1X
EcoRI (10U/μl) (Roche Diagnostics, GbmH, Mannheim, Germany)	60U
Made up with dH_2O to a total of	60μl

The reaction was terminated for 10 minutes at 70°C. In the next step only one strand with a 3' end overhang due to the *EcoRI* digestion was labeled with the α^{35}S-dATP isotope according to the following method:

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Reagent/ Stock solution	Amount/Final Concentration
EcoRI restriction mixture	60μl
370 MBq/ml α^{35}S-dATP (10mCi/ml) (Amersham Pharmacia Biotech)	0,05mCi
Klenow fragment (4U/μl) (Takara Biomedicals, Japan)	10U
Made up with dH_2O to a total of	67,5μl

The reaction was allowed to complete for 15 minutes at room temperature and stopped at 70°C for 10 minutes. A second *NdeI* restriction enzyme (Roche Diagnostics GmbH, Mannheim Germany) digest was performed to cut the labeled insert from the plasmid:

Reagent/ Stock solution	Amount/Final Concentration
Isotope reaction mixture	67,5μl
10X buffer H (Roche Diagnostics GmbH, Mannheim Germany)	1X
NdeI (10U/μl) (Roche Diagnostics GmbH, Mannheim Germany)	60U
Made up with dH_2O to a total of	100μl

The probes were purified by QIAquick PCR purification protocol (Qiagen, Germany) following the manufacturers protocol (Appendix A2). Purified probe DNA was eluted in 100μl of 10mM Tris-Cl, pH8,5.

Heteroduplex formation.

Colony PCR. Twenty clones were randomly selected for screening of HIV-1 population complexity through colony PCR for each genomic region analyzed. Colony PCRs were performed in the same manner as inner nested PCRs described in section 3.2.5.

Denaturing and re-annealing. Denaturing and re-annealing of double stranded probe in the presence of double stranded template (amplicons of colony PCR) results in the formation of heteroduplexes as well as homoduplexes. From 20 clones per sample, an 8μl aliquot of the colony PCR amplicon (either C2V3 or p17) was added to a 1,5ml eppendorf tube, this represents the template DNA. An additional two tubes were prepared per sample per genomic region analyzed, one with 8μl sterile distilled water in

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the place of 8μl colony PCR amplicon, serving as the probe homoduplex positive control and another containing 8μl of the PCR product originating from PCR reaction of total population serving as the total population control. To each of these tubes the following was added and mixed well by pipette action:

Reagent/Stock solution	Amount/Final concentration
Probe	0,5μl
10X HTA annealing buffer (Appendix B6)	1X
Template	8μl
Made up with dH_2O to a total of	10μl

This reaction mixture was next subjected to heat denaturation for two minutes at 95°C on a Dri-block-DB 2A heating block (Techne® Inc., Princeton, N.J.). The reaction mixture was then removed from the heating block and allowed to re-anneal at room temperature for five minutes. Tube contents were collected by a short centrifugation step.

Polyacrylamide gel electrophoresis.

The resulting homoduplexes and heteroduplexes were separated by PAGE. For separation of both the C2V3 and p17, a 5% polyacrylamide gel was prepared. The p17 genomic region is less variable and fewer mismatches can be expected between different intraperson populations when compared to the C2V3 region. Thus, 20% urea was added to pronounce the effect of mismatches and indels in the less variable p17 region.

5% Polyacrylamide gel for C2V3 heteroduplex separation was made up as follows:

Reagent/Stock Concentration	Amount/Final Concentration
40% acrylamide stock (1% bis-acrylamide)	5%
10 X TBE	1X
Made up with dH_2O to a total of	30ml

5% Polyacrylamide gel for p17 heteroduplex separation was made up as follows:

Reagent/Stock Concentration	Amount/Final Concentration
40% acrylamide stock (1% bis-acrylamide)	5%
10 X TBE	1X
Urea	20%
Made up with dH_2O to a total of	30ml

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Gels were polymerized by addition of 125μl 25% ammonium persulphate together with 19,8μl TEMED. This was then mixed and poured into 16.5 x 17.5cm, 1,5mm thick glass plates positioned in a SCIE-Plas vertical 2-Gel Cam Casting tray (SCIE-PLAS, Southam, UK) and allowed to polymerise at room temperature. The plates were separated by spacers that allowed 1mm thick gels to be poured. The polymerized polyacrylamide gel was next transferred to a SCIE-Plas Maxi V20-CDC electrophoresis apparatus (SCIE-PLAS, Southam, UK) containing 1,5 liter of 1X TBE (Appendix B4) for electrophoresis of the samples. Gels used for separation of p17 heteroduplexes were pre-run at 250V for 15 minutes and the wells rinsed with a syringe to remove residual urea to prevent distortion of DNA bands. After denaturing and annealing, duplexes were prepared for separation through PAGE by adding 2μl 6X PAGE loading dye (Appendix B5) to each tube. Samples were then loaded onto the respective gels. C2V3 heteroduplexes were separated at constant 17mA per gel for 6 hours. P17 heteroduplexes were separated through electrophoresis at constant 250V for 4,5 hours.

Gel drying and autoradiography. After electrophoresis the gels were removed from the electrophoresis apparatus. Glass plates were carefully pried open with a thin blade and a nick with the aid of a scalpel was made in the right top hand corner of the gel to track the orientation of gels. Two pieces of Whatmann 3MM chromatography paper were cut to the size of the gel, laid on top and smoothed over to ensure adequate adsorption of the gel to the paper. The gel was next lifted from the glass plate by careful removal of the paper and covered in Gladwrap®. Gel drying was performed under vacuum on a DrygelSr.Slab-gel dryer model SE1160 (Hoefer Scientific instruments, San Fransisco) connected to a refrigerated condensation trap (Savant RT100, USA) for 1 hour at 80°C. Dried gels were exposed to 18X24cm Hyperfilm MP high performance autoradiography film (Amersham Pharmacia biotech, UK) overnight at –80°C. The film was developed by submerging for 3 minutes in developer (1 part Novolith TM liquid A to 3 parts mixed water with an equal volume of 1 part Novolith TM Liquid B to 3 parts water) [May and Baker, Essex, UK], followed by one minute in stop solution (2% acetic acid) and then 3 minutes in fixative (Agfa G334C [Bayer, RSA] 1 part solution A for four parts water, mixed with 1 part solution B to 20 parts water). After a water rinse the developed film was air dried.

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3.2.8 Sequencing

Clones that differed from the reference probe population as identified by heteroduplex bands were selected for sequencing. At least two clones per divergent population were sequenced where possible. A total of 37 C2V3 sequences were generated, 9 for Du145, 5 for Du204, 5 for Du114, 9 for Du421 and 9 for Du23. A total of 31 p17 sequences were generated, 8 from Du204, 5 from Du114, 5 from Du421 and 8 from Du23. The clone representing the probe was also sequenced in addition to at least two other clones representing the homoduplex population.

Fragments constituting the C2V3 region and the p17 fragment were sequenced in both directions with forward and reverse gene specific primers, Gag A-f and A-r as well as Env B-f and B-r. The basic principles used in sequencing are explained in more detail in section 2.2.3. Cycle sequencing was performed using the ABI PRISM® BigDye ™ terminator V3.0 Cycle sequencing Ready reaction Kit with AmpliTaq® DNA Polymerase FS (Applied Biosystems, Warrington, UK). According to manufacturers recommendations we performed half reaction cycle sequencing, adding a total of more or less 50ng amplicon DNA per reaction plus 3,2pmol of either primer. To this was added 4μl of the enzyme mix from the BigDye ™ terminator V3.0 Cycle sequencing Ready reaction Kit plus 4μl 2,5X sequencing buffer (Tris-HCL, pH 9 and $MgCl_2$) supplied with the kit.

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Thermocycling was performed in a GeneAmp PCR system 2400 thermocycler (Perkin Elmer Corporation, Norwalk, CT).

96°C for 30 sec – initial denaturing step

96°C for 30 sec - denaturing
50°C for 15 sec - annealing → for 25 cycles
60°C for 4 min - elongation

Sequencing was done on an ABI PRISM ™ 310 Genetic Analyser (Applied Biosystems). All sequences were subjected to quality control measures to ensure that there were no sample mix-ups or contamination from other sources (Learn *et al.*, 1996).

3.2.11 Sequence analysis

Phylogenetic trees. Quality Control and phylogenetic trees generated from sequences in both the C2V3 and p17 genomic regions was performed according to the same methods described in chapter 2, section 2.2.4.

Trees were constructed for C2V3 sequences including a total of 32 reference sequences ranging from subtype A through K (5 from subtype C, 2 from subtype J, 3 from subtype B, 4 from subtype D, 2 from subtype F2, 4 from subtype F1, 2 from subtype F, 2 from subtype K, 3 from subtype H, 3 from subtype A and 4 from subtype G) obtained from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov/Align_current /SUBTYPE-REF/subtype.html). For p17 phylogenetic tree a total of 22 reference sequences were included, 4 from subtype C, 3 from subtype A, 2 from subtype G, 2 from subtype J, 2 from subtype H, 1 from subtype U, 2 from subtype F1, 2 from subtype D and 4 from subtype B.

Genetic distances. MEGA (Molecular Evolutionary Genetic Analysis, version 2,1) analysis between sequences generated from within one individual was completed using the Kimura 2-parametre model and taking into account transitions and transversions (Kumar *et al.* 2001). Percentage nucleotide divergence was determined to assess intraperson genetic diversity.

Amino Acid translation and Phenotype Prediction. Nucleotide sequences were translated into amino-acid sequences using BioEdit version 5.0.2 and phenotype prediction was performed by a method described by Briggs and colleagues (2001) employed in section 2.2.4.

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3.3. Results:

3.3.1 RNA quantification and sero-status confirmation

Five individuals, Du23, 114, 145, 204 and 421 had a plasma sample that were taken one month before documented seroconversion and were identified as RNA positive. As the exact date of infection for all samples was unknown an estimated date of infection was taken as the midpoint between the last HIV RNA negative sample and the first HIV RNA positive sample. According to the estimated date of infection, samples were collected 7 to 30 days (mean 14,8 days) after infection (Table 3.2). The viral loads for these preseroconversion samples ranged from 16 000 copies per millilitre (4,2 log) in Du23 to 5 100 000 copies per millilitre (6,7 log) for Du421. The viral loads for the first antibody positive (seroconversion) samples were not available.

Log viral load graphs measured from just after seroconversion to 30 months post seroconversion for four of the five study samples, Du23, Du204, Du114 and Du421 were available from previous studies (provided by C. Gray) (Figure 3.4). For Du145 we did not have enough viral load data to generate reliable plots, however. More detailed plots representing viral load fluctuations over a shorter time (up to 26 months post seroconversion) were produced for Du23, Du114, Du204 and Du421, but only two of these are shown for Du23 and Du204 (Figure 3.5 and Figure 3.6). Two trends were observed, in the case of Du204, Du114 and Du421 (Figure 3.5) the sample taken at preseroconversion had a higher viral load than the seroconversion sample, whereas for Du23 the inverse was true, with a higher viral load at seroconversion than at preseroconversion (Figure 3.6). Where viral loads are not available for drawing these plots, the mean between two known viral loads were calculated. These estimated readings are indicated in red.

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Table 3.2: Collection dates for samples selected for inclusion into study including, viral load, estimated date of infection, and estimated days post infection.

Sample Number	Collection Date	Sample Description	Viral Load (cp/ml plasma)*	Estimated date of infection	Estimated days post infection
Du23	2/10/98	Last RNA negative	<400		
	03/12/98	1st RNA Positive	$1,6 \times 10^4$ (log 4,2)	2/11/98	30
	27/1/99	1st Antibody positive	ND		
Du114	21/8/98	Last RNA negative	<400		
	9/9/98	1st RNA Positive	$5,6 \times 10^5$ (log 5,7)	30/8/98	8
	6/11/98	1st Antibody positive	ND		
Du145	15/3/99	Last RNA negative	<400		
	14/4/99	1st RNA Positive	$3,9 \times 10^4$ (log 4,5)	29/3/99	14
	10/5/99	1st Antibody positive	ND		
Du204	8/4/98	Last RNA negative	<400		
	24/4/98	1st RNA Positive	$9,3 \times 10^4$ (log 4,9)	16/4/98	7
	20/5/98	1st Antibody positive	ND		
Du421	10/5/99	Last RNA negative	<400		
	11/6/99	1st RNA Positive	$5,1 \times 10^6$ (log 6,7)	25/5/99	15
	26/7/99	1st Antibody positive	ND		

* <400, viral loads are below assay detection limit. ND, Not done; Preseroconversion samples indicated in bold; VL determined by NASBA.

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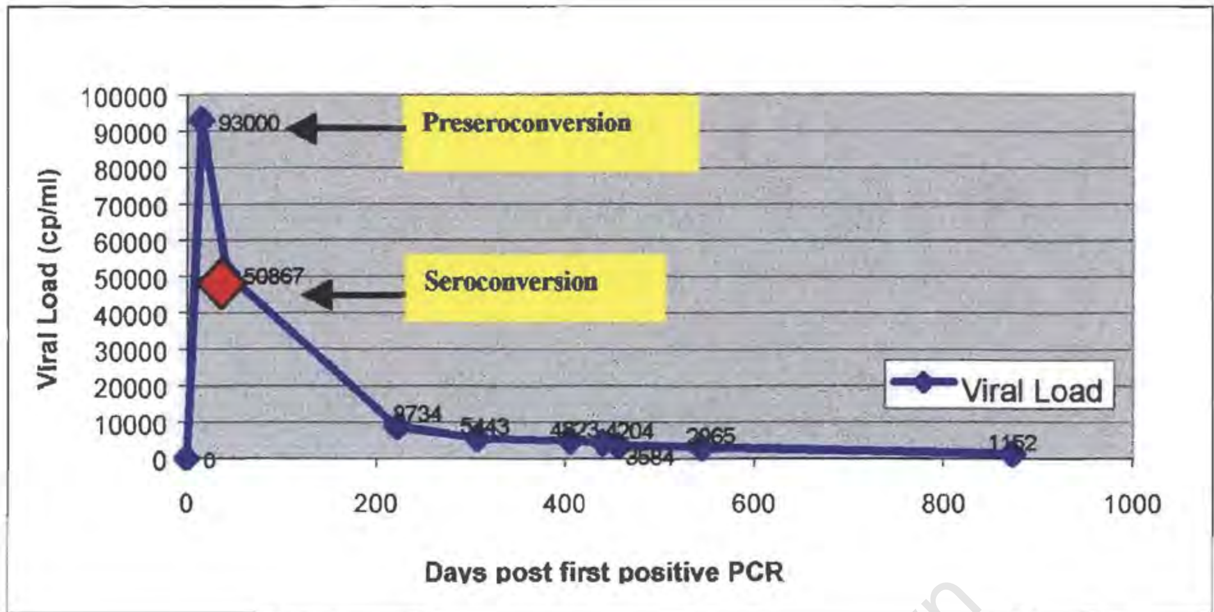

Figure 3.5: Du204 viral load fluctuations with time. For samples where viral loads were not available the mean between the two known viral loads were calculated, these are indicated in red diamonds (Adapted from Dr. Clive Gray, NICD).

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Sero-status confirmation. Seroconversion dates that were documented for the five samples were confirmed by an additional Cappilus test. One sample, Du145, was also seropositive in our assay.

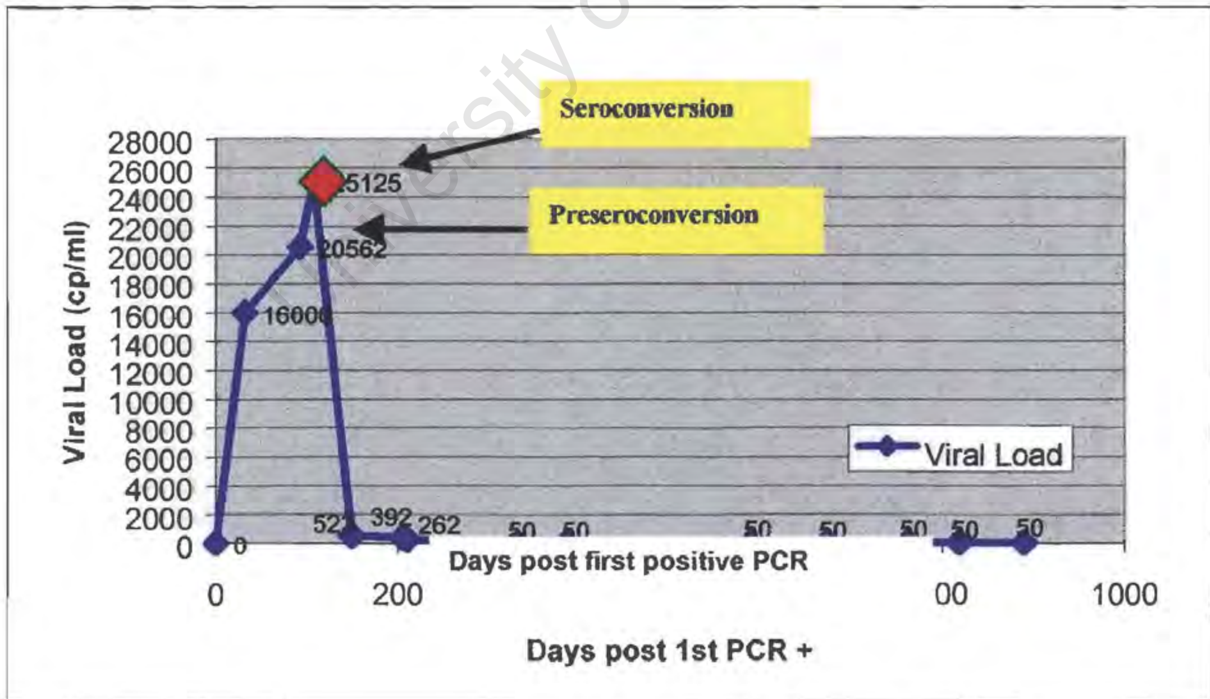

Figure 3.6: Du23 viral load fluctuations with time, For samples where viral loads were not available the mean between the two known viral loads were calculated, these are indicated in red diamonds (Adapted from Dr. Clive Gray, NICD

3.3.2 Total population PCR

To analyse representative populations present in the patient, total population PCR was performed. The C2V3 region was amplified to yield a band of approximately 512bp and the p17 region was amplified to yield a band of approximately 621bp (Figure 3.8). PCR was optimized for increased sensitivity and specificity such that only clear single bands were visualized, with no evidence of non-specific PCR. Amplicons that were visualized as discrete bands on agarose gels were selected for sequencing. PCR was routinely monitored for contamination by including a positive control and water negative control for each reaction set of PCR.

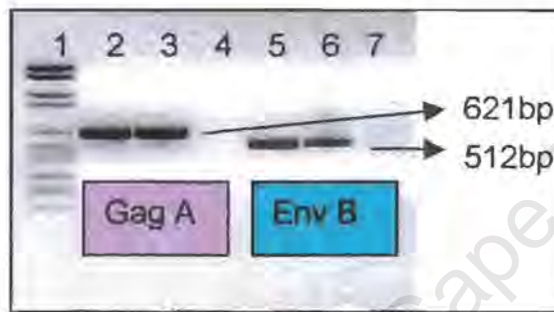

Figure 3.7: Du114 C2V3 and p17 amplicons visualized after 2% agarose gel electrophoresis for 30min at 120V. Lane 1 contains molecular marker 6 (Roche Diagnostics GmbH, Mannheim, Germany). The Gag A-f and Gag A-r primers amplified a 621bp fragment incorporating p17 visible in lane 2 and 3. The Env B-f and Env B-r primers amplified a 521bp fragment incorporating *env* C2V3 in lane 5 and 6. Lane 3 and 4, 6 and 7 each represents the positive and negative control respectively for p17 and C2V3.

3.3.3 Quantification of amplifiable copy number from PCR-based limiting dilution assay

To ensure that the generated amplified product represented all viral populations present in the particular sample, the amplifiable copy numbers per primer pair had to exceed 30 copies. Figure 3.8 shows the limiting dilution performed of a 10-fold serial dilution, in this figure amplicons were generated up to a dilution factor of one in 24 (Figure 3.8). In all instances the amplifiable copy number were more than 32 copies. Du23 with the lowest viral load at 16 000 copies/ml, had an input copy number of 731,4 copies and that was sufficient for envelope primers to generate an amplifiable copy number of 302 and

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32 for *gag* primers. Du421 with the highest viral load at 5100 000copies/ml, Du421, had an input copy number of 214 737copies and that was sufficient to generate an amplifiable copy number of 302 for both *env* and *gag* primers (Table 3.3).

Table 3.3: Table depicting viral load (copies per ml plasma), input copy number and amplifiable copy number per primer pair per sample.

Sample number	NASBA Viral load (cp/ml plasma)	Input copy number	Amplifiable copy number *Gag*	Amplifiable copy number *Env*
Du23	16 000	731,4	32	>302
Du114	560 000	14933	>302	>302
Du145	39 000	1560	>302	>302
Du204	93 000	762,5	>302	>302
Du421	5100 000	214737	>302	>302

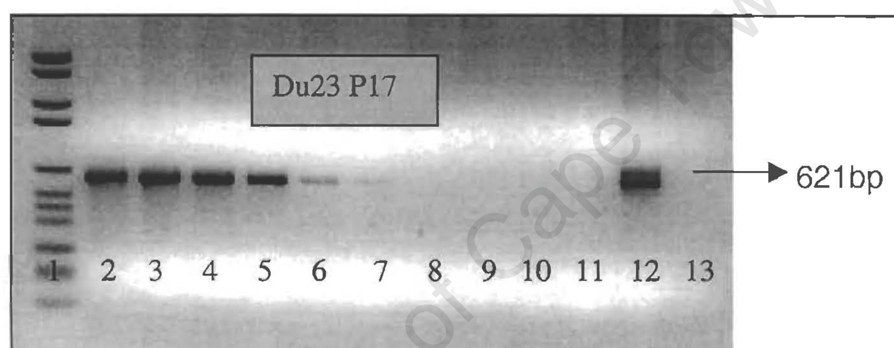

Figure 3.8: PCR based limiting dilution assay of Du23 p17 (621bp). This 2% agarose gel represents a nPCR of 10-fold serial dilution. Lane 2 to 11 contains dilution series of 1 to 1/152 of cDNA. Lane 1 contains molecular marker 6 (Roche Diagnostics, GmbH, Mannheim, Germany), positive control is in lane 12 and negative water control in lane 13.

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3.3.4 Cloning into pMOS*Blue* and Colony PCR

Viral populations within an individual were cloned for screening for diversity by HTA. In each case where clones were selected for downstream processing the transformation efficiency surpassed 4×10^7cfu per μg pUC18. The ligation positive control plates for most cloning steps had insert containing white colonies comprising >90% of total number of colonies present indicating a high efficiency of ligation. pMOS*Blue* clones were tested for inserts by amplifying the insert with gene specific primers, either C2V3 or p17 primer pairs, termed a colony PCR (Figure 3.9). In figure 3.9 a Du23 p17 colony PCR is

visualized and out of 20 picked colonies, 17 were positive, as expected for the p17 fragment.

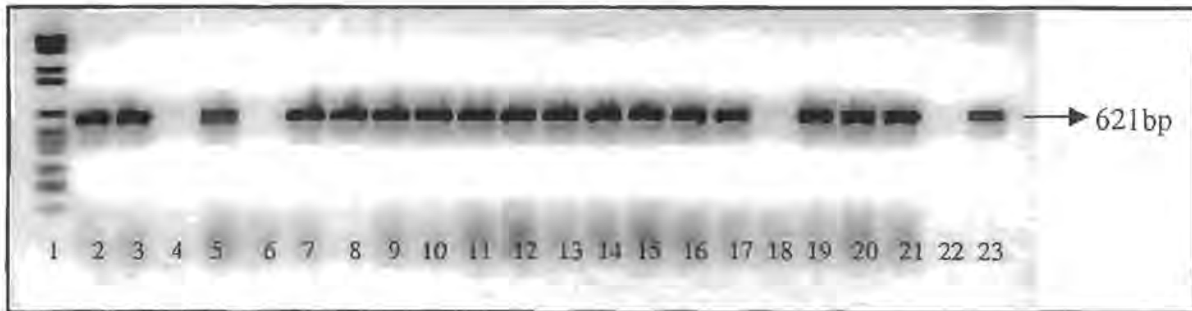

Figure 3.9: Du23 p17 colony PCR yielding fragments of 621bp in length. Lane 1 contains molecular marker 6 (Roche Diagnostics GmbH, Mannheim, Germany), lane 2 to 21 samples or clones, lane 22, the negative control and lane 23 the positive control.

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3.3.5 Heteroduplex Tracking Assay

HTA was used to identify clones divergent from the probe, which were generated from the same individual. A table is included that indicates the proportion of clones that were identical and different to the probe reference based on migration patterns, as well as the number of clones selected for sequencing (Table 3.3b). A detailed description of only two representative HTA autoradiographs is supplied; Du145 C2V3 and Du114 p17. Most HTA (p17 and C2V3) autoradiographs showed a majority of homoduplexes indicating that mainly one genomic population was present within an individual (Figure 3.10). Du114 is such an example, a single pattern of migration, with heteroduplexes in four of the 20 clones. Limited mobility shifts were detected in lanes containing clone 3,29 33 and 47 (Figure 3.10). In a single individual, Du145 two C2V3 heteroduplex populations with two different patterns of migration were detected. One population represented in lanes containing clones 19, 22 and 23 and another in the lane-containing clone 5. In this individual, the heteroduplex population exhibiting the largest shift was found in three of the twenty clones and the heteroduplex representing the lesser shift was found in only one clone (Figure 3.11).

Table 3.3b: HTA results indicating how many clones were identical to probe and how many were different including how many were selected for sequencing.

	Env			*Gag*		
	Number of identical clones	**Number of different clones**	**Number of sequenced clones ***	**Number of identical clones**	**Number of different clones**	**Number of sequenced clones***
Du23	20	0	8 identical	20	0	7 identical
Du114	20	0	4 identical	20	0	4 identical
Du145	16	4	8 – total 4 – different 4 - identical	20	0	4 identical
Du204	20	0	4 identical	12	8	7 total 5– different 2 - identical
Du421	20	0	8 identical	20	0	4 identical

*In addition to the number of clones sequenced the probe sequence were also obtained.

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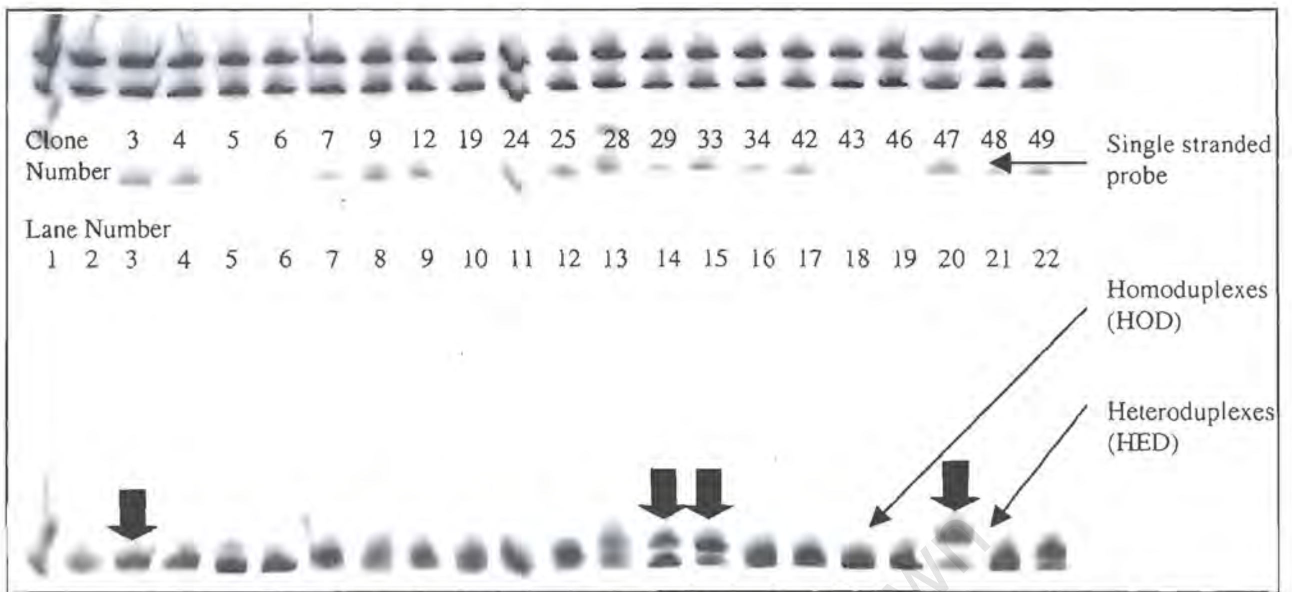

Figure 3.10: Autoradiograph of Du114 p17 HTA. Lane one contains the total population, lane 2, the probe or the reference by itself; the following lanes are numbered according to the clone it contains. Clones that formed heteroduplexes were selected for sequencing and are indicated by an arrow.

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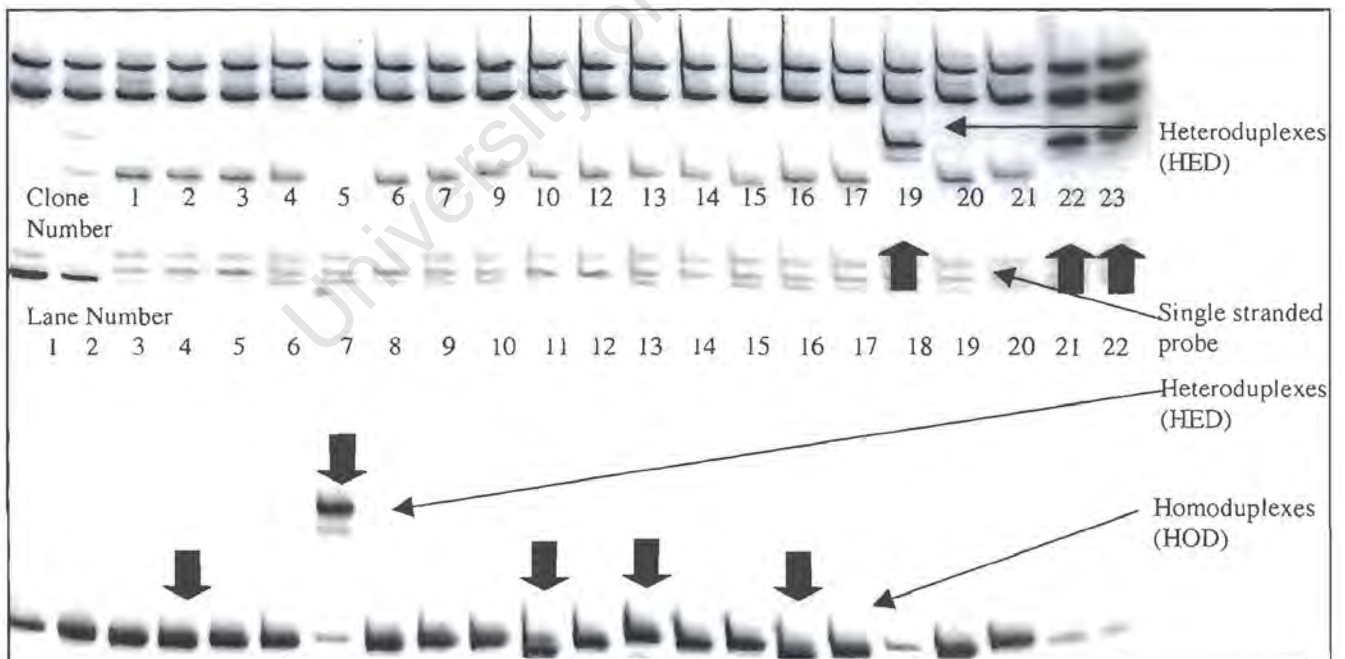

Figure 3.11: Autoradiograph of Du145 C2V3 HTA. Lane one contains the total population, lane 2, the probe or the reference; the following lanes are numbered according to the clone it contains. Clones that formed heteroduplexes were selected for sequencing and are indicated by an arrow.

3.3.6 Phylogenetic analysis

Phylogenetic analysis of a total of 37 C2V3 and 31 p17 sequences was performed (Figure 3.12 and Figure 3.13)

C2V3 phylogenetic tree. We could detect clusters of sequences that grouped together representing individual clone sequences generated from one individual supported with a bootstrap value of 100%. All sequences grouped within the subtype C reference sequences. There was no evidence of contamination as no sequences from different individuals grouped together and no close grouping was observed between sample sequences and reference sequences

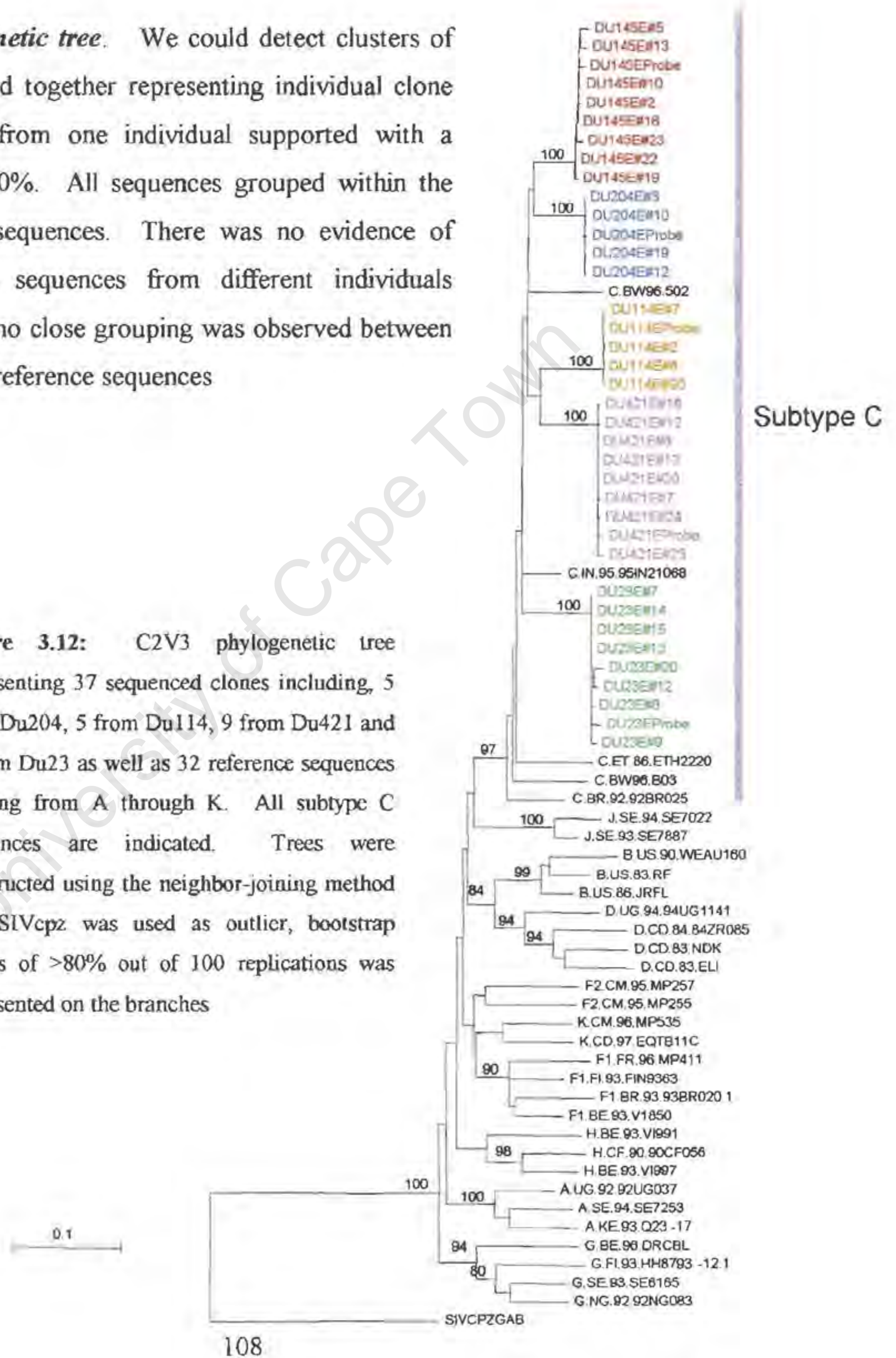

Figure 3.12: C2V3 phylogenetic tree representing 37 sequenced clones including, 5 from Du204, 5 from Du114, 9 from Du421 and 9 from Du23 as well as 32 reference sequences ranging from A through K. All subtype C sequences are indicated. Trees were constructed using the neighbor-joining method and SIVcpz was used as outlier, bootstrap values of >80% out of 100 replications was represented on the branches

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P17 phylogenetic tree. All sequences from this study grouped with subtype C supported with a bootstrap value of 89%. All sequences from a particular individual grouped together and were supported with a bootstrap value of 100%. No interpatient sequences grouped together and no tight clustering of patient sequences with reference sequences could be observed. Sequences from within a sample showed high conservation (Figure 3.13).

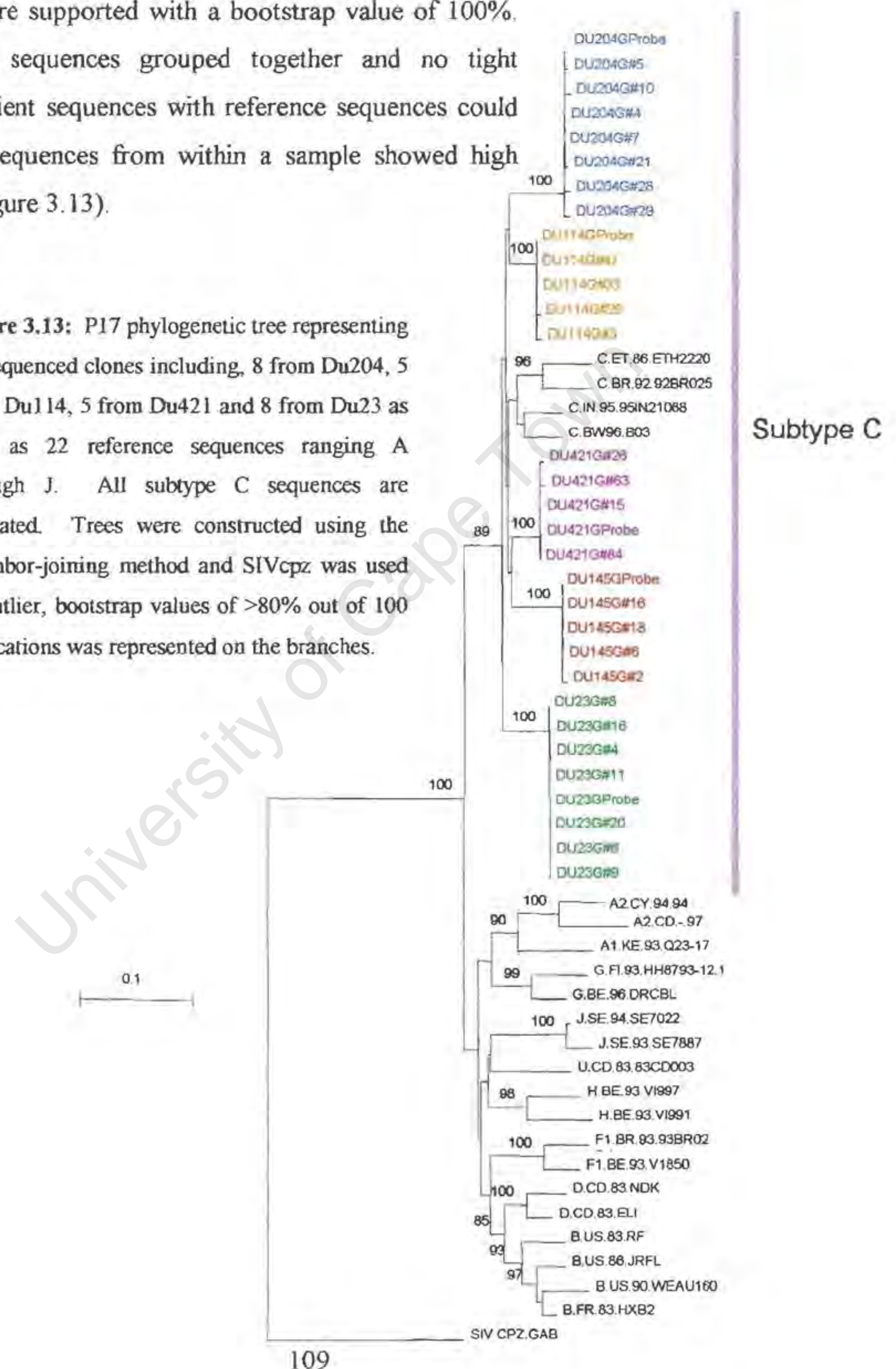

Figure 3.13: P17 phylogenetic tree representing 31 sequenced clones including, 8 from Du204, 5 from Du114, 5 from Du421 and 8 from Du23 as well as 22 reference sequences ranging A through J. All subtype C sequences are indicated. Trees were constructed using the neighbor-joining method and SIVcpz was used as outlier, bootstrap values of >80% out of 100 replications was represented on the branches.

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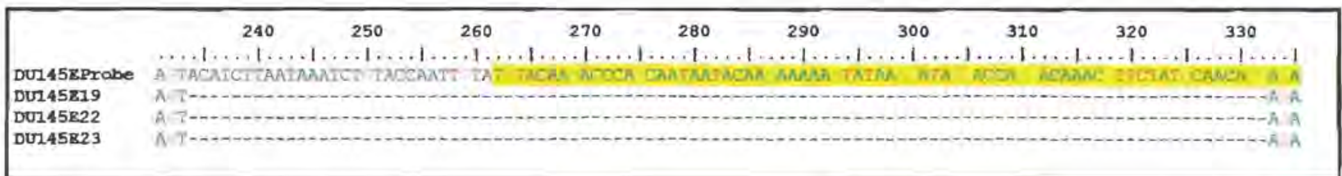

Figure 3.14: Nucleotide alignment of Du145 C2V3 probe reference sequence and clones number 19,22 and 23 under investigation. The V3 loop that lies partly within the 99bp deletion is blocked. Gaps are indicated by dashes "-".

In Du145, the C2V3 heteroduplexes observed (Figure 3.11) were confirmed to be due to a deletion. The three clones exhibiting a large shift each had a 99bp deletion spanning the second constant region (C2) and the first part of the V3 loop (positioning according to HXB2 *gag*, 858 to 962) (Figure 3.14). The heteroduplex with smaller shift detected in Du145 C2V3 clone number five was due to a small insertion of 6bp (Figure 3.15).

Figure 3.15: Nucleotide alignment of Du145 C2V3 probe reference sequence and clone number 5 under investigation showing the smaller insertion of 6bp. Positioning was determined according to HXB2 gp160, 1035 to 1040. Gaps are indicated by dashes "-".

In this study HTAs were able to distinguish between as little as a single nucleotide mismatch. In Du114 p17 autoradiograph (Figure 3.10), the shift observed in lane 15 was the result of a single A to G mismatch between clone number 33 and the reference probe (Figure 3.16).

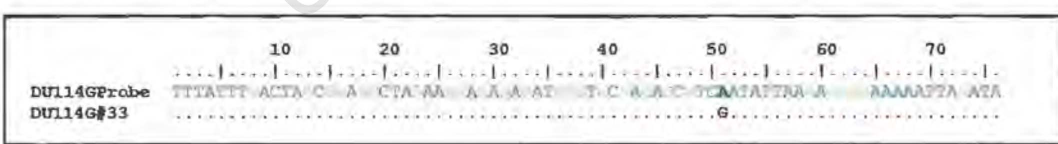

Figure 3.16: Nucleotide alignment of Du114 p17's probe reference sequence and clone number 33 under investigation. Homology is indicated by full stop"."

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3.3.7 Genetic distances

C2V3 Intrasubtype C diversity. In the previous study, chapter 2, it has already been determined that the intrasubtype C diversity for sequences from southern Africa ranged from 5,6% to 21,2% across the V3V5 region and 6,6% to 15,7% across the gp120 region (Figure 2.8 and Figure 2.6). When analyzing subtype C sequences from this study across the V3 region alone, the greatest diversity between these samples were 15%.

C2V3 Interperson diversity. The overall mean genetic distance for sequences from the five individuals from this study was 8,6%. When comparing the mean nucleotide diversity calculated per individual, Du204 and Du145 was the most similar, differing by 8,6%. Individual sequences between Du204 and Du145 differed from 7,8% to 9,2%. Sequences from Du114 and 23 were the least related with the mean nucleotide diversity measured between these individuals differing by 12,5% (Table 3.4). Individual population sequences between Du114 and 23 differed from 13,5% to 14,6%.

C2V3 Intraperson diversity. The intraperson nucleotide diversity was found to be low, ranging from sequences being identical at 0%, to as much as 1,8% for Du145. The overall mean intraperson diversity was 0,32%. Sequences within Du114 were most similar (0% to 0,2%, mean 0,02%) with sequences from Du145 being the least similar (0,3% to 1,8%, mean 0,6%). The mean intraperson diversity ranged from 0,02% for Du114, 0,1% for Du204, 0,3% for Du421, 0,6% for Du23 and 0,6% for Du145 (Figure 3.17).

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Table 3.4: C2V3 mean interperson diversity calculated between individuals in this study.

	Du114	Du145	Du204	Du23	Du421
Du114					
Du145	10,9%				
Du204	11,3%	8,6%			
Du23	12,5%	11,4%	9,6%		
Du421	9,4%	10,1%	9,4%	11,2%	

P17 Intrasubtype C diversity. Subtype C sequences from South Africa (n=131) excluding sequences from our study, differed across the p17 region with up to 11,6%. The diversity between samples measured in our study ranged from 5,5% to 10,3%. This was lower than C2V3.

P17 Interperson diversity. The mean genetic distance for sequences from all individuals were 7,1%. When comparing the genetic distances calculated for all populations within different individuals in the p17 region, Du114 and Du421 were the most similar, with a difference of 5,5%. Individual sequences from Du114 and Du421 differed from 5% to 5,9%. Du204 and Du145 differed by 10,3% when comparing mean genetic distances and individual population sequences differed from 9,7% to 10,9% (Table 3.5).

Table 3.5: p17 mean interperson diversity calculated between samples included in this study.

	Du23	Du114	Du421	Du145	Du204
Du23					
Du114	7,3%				
Du421	7,9%	5,5%			
Du145	10%	8%	7,2%		
Du204	10,2%	7,4%	9,3%	10,3%	

P17 Intraperson diversity. The intraperson nucleotide diversity was found to be low, ranging from sequences being identical at 0%, to as much as 1,2% for Du204. The overall mean intraperson diversity was 0,08%. The mean intraperson genetic distance was 0,1% for Du23, 0,08% for Du114, 0,06% for Du145, 0,2% for Du204, 0,17 for Du421 (mean 0,08%). Sequences within Du23 were the most similar to each other with divergence ranging from 0% to 0,4%, sequences from Du204 were the least similar with divergence ranging from 0,2% to 1,2% (Figure 3.17).

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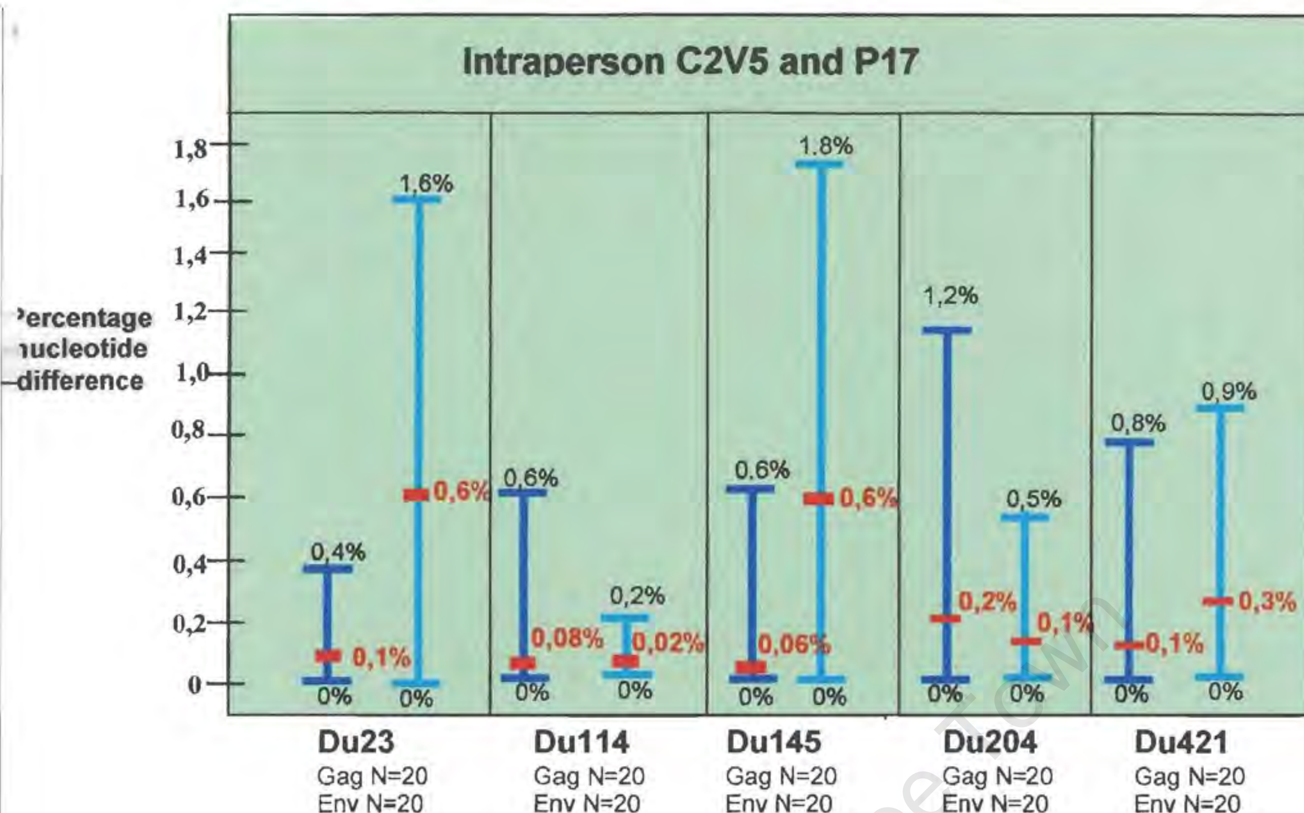

Figure 3.17: Intraperson p17 (dark blue) and C2V3 (light blue) percentage nucleotide diversity.

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3.3.8 Amino-Acid translation and Phenotype prediction

Complete C2V3 and p17 amino acid sequences generated are illustrated (Figure 3.18 and 3.19).

C2V3. The C2V3 amino acid sequence for Du114 was identical in all five clones sequenced. Du204 also had low variation with two amino acid changes of the total of 145 amino acids in three of five clone sequences. Amino acid sequence for Du23 differed at the most with six amino acid changes only in clone number 12 of the total of 9 clones. Du145 had a single deletion of 33 amino acids spanning the 3' end of C2 and more than half of the V3 loop in three clones, 19, 22 and 23 out of a total of nine. An additional insertion of three amino acids in C3 was also observed in clone number five (Figure 3.18).

p17. The p17 amino acid translation of Du145 clones was the most homogeneous of the five individuals analyzed, with only one amino acid change between all five clones in clone number 2. Du204 showed the most variation with up to five amino acid changes in clone number 29. No indels were observed (Figure 3.19).

V3 Loop. The V3 loop in particular was investigated for all five C2V3 probe sequences representing the major population present in the individual (Figure 3.20).

```
                             300       310       320       330
                           ....|....|....|....|....|....|....|..
HXB2                     N CTRPNNNTRKRIRIQRGPGRAF-VTIGKIGNMRQAHC NIS
Subtype C consensus      V CTRPNNNTRKSIRI**GPGQTFYATGDIIGDIRQAHC NIS

DU114EPROBE              E CTRPNNNTRKSVRI**GPGQTFYATNGIIGDIRQAYC NIS
DU204EPROBE              V CIRPNNNTRQSIRI**GPGQAFFATKDIIGDIRQAYC NIS
DU23EPROBE               V CARPNNNTRKSIRI**GPGQAFYATGAIIGNIREAHC NIS
                                Y - Clone #9
DU421EPROBE              V CTRPNNNTRKSVRI**GPGQTFFATGEIIGNIRQAHC NIS
                                  G - Clone #23
                                       M - Clone #12
DU145EPROBE              V CTRPSNNTRKSIRI**GPGQTFYATGDIIGDIRKAHC NIS
                         - ----------------------------
                         Deletion in clone # 19, 22 and 23
```

Figure 3.20: Probe reference C2V3 translated amino-acid sequence from samples investigated in this study. The probe reference sequence also served as the intraperson consensus sequence. Deviations are indicated; the deletion in Du145 is blocked in blue, amino acid changes in clones within DU23 and Du421 are indicated below the consensus sequence. Position relative to reference sequence HXB2 is indicated at the top. Two amino acids indicated by ** are present in HXB2 and influences position relative to HXB2.

The V3 loop was identical for all clones in Du114 and Du204. For Du23 a single amino acid change in one clone (number 9), an asparagine to tyrosine change (position 302) was observed. Du421 had two amino acid changes in two different clones, an arginine to glycine change (position 304) in clone number 23 and an isoleucine to methionine switch in clone number 12 (position 309). For Du145 two definite V3 amino acid populations could be observed. One population had a large 25bp deletion in the 5' start of the V3 loop, this deletion was seen in three of the nine clones sequenced, clones 19, 22 and 23. The predicted phenotype in all cases was R5 (Table 3.21).

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Figure 3. 18: Predicted amino acid translation of C2V3. Homology indicated by a full stop ".",deletions indicated by a dash "-". V3 loop highlighted. Number at the far right hand side indicates the number of amino acid mismatches.

Figure 3.19: Predicted amino acid translation for p17. Homology in all sequences indicated by a full stop ".", a stop codon is indicated by a "*". P17 matrix highlighted. Number at the far right hand side indicates the number of amino acid mismatches.

Table 3.21: Predicted phenotype calculated according to Briggs and colleagues (2000).

Sample	+ Charges	- Charges	Net charge	Ile @ aa 327	Output Value	Numerical Value	Predicted phenotype
Du23 probe	5	1	+4	YES	1.16	1	R5
Du114 probe	5	1	+4	YES	1.16	1	R5
Du145 probe	6	2	+4	YES	1.33	1	R5
Du204 probe	5	2	+3	YES	1.05	1	R5
Du421 probe	5	1	+4	YES	1.16	1	R5
Du421 clone 23*	4	1	+3	YES	0.85	1	R5

* This was the only clone that differed from the probe sequence in the V3 loop in all five individuals.

3.4 Discussion

This study characterised viral populations, in recently infected women of the Du sex worker cohort. Samples from five women, Du23, Du114, Du145, Du204 and Du421, obtained on average 15 days post transmission, were identified as being HIV seronegative but which still had a detectable HIV viral load. Viral populations were defined by analysing diversity in the C2V3 and p17 genomic region through heteroduplex tracking assays (HTAs), sequencing and phylogenetic analysis. All women were infected with subtype C based on C2V3 and p17 phylogenetic analysis.

To control for possible re-sampling of the same viral template, resulting in a bias estimation of diversity, we ensured sufficient amplifiable templates were added. It was also important to take the error rate of the taq polymerases into account, as this may contribute to diversity. The error rate of the Super-therm DNA polymerase (JMR Holdings, Sevenoaks, UK) used in this study was one miss-incorporation per 83 000bp for each PCR cycle completed. Calculated per fragment amplified, it indicates that in each RT-nPCR reaction amplifying the 621bp p17, 0,48bp will be miss-incorporated and for 512bp C2V3, 0,39bp. It is thus unlikely that the diversity seen in study samples was influenced markedly by the error prone nature of the taq polymerase used.

In HTAs it has been shown that there is a general relationship between migration and percentage DNA difference, but this relationship is only clear when analyzing HIV-1 populations exhibiting high genetic diversity. It is thus difficult to predict diversity of closely related populations within an individual based on migration patterns. A number

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of factors impact on the mobility of heteroduplexes. Firstly, the type of difference between two strands in a heteroduplex, with indels having the greatest effect compared to mispaired bases. Secondly, the type of mismatch also influence duplex mobility with pyrimidine-pyrimidine > purine-purine > purine-pyrimidine. Thirdly, a mismatch or indel towards the end of the duplex has a lesser effect compared to mismatches in the center or mismatches that are clustered (Nelson *et al.*, 1997; Upchurch *et al.*, 2000). Finally, the sequence flanking the region of difference also has an influence.

Various studies have detected heterogeneous viral populations in women recently infected, including women from sex worker cohorts, and in pregnant Rwandan women (Kampinga *et al.*, 1997; Long *et al.*, 2000; Poss *et al.*, 1995). However, the level of diversity found in our study, indicated that all five individuals harbored highly homogeneous viral populations.

In a study by Poss *et al* (1995) the proviral *env* V1, V2 and V3 regions of six recently seroconverted Kenyan sex worker women were analyzed. In five of the six infected females a heterogeneous viral population were detected. When comparing the V3 loop analyzed in common between the Kenyan study and our study, they found a maximum difference of 11 amino acid mismatches (ranging from 1,2,5 to 11 amino acid differences respectively) out of a total of 35 amino acids (31%) among the intraperson clones in the V3 loop, whereas in our study a maximum of two amino acid changes per 35 amino acid V3 loop stretch (5,7 %) was identified.

In a subsequent study, in the same Kenyan sex worker cohort an additional 15 out of a total of 26 women (42%) were found to harbor a heterogeneous viral population soon after infection. This study determined a mean intraperson difference across the V1, V2 and V3 regions of more than 5%. These results were not directly comparable to our study, however, as the V1 and V2 regions were not analyzed in our study. This study also included men, and interestingly all ten men were infected with homogeneous viral populations that differed by less than 1% in the V1, V2 or V3 regions, suggesting that women have a higher population diversity than men.

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A recent study (Learn et al., 2002), looking at diversity after transmission in eight homosexually infected men, showed that all individuals harbored viral populations with a median sequence diversity within individuals ranging from 0,4% to 3,02% (mean 1,8%) in *env* C2V5 and from 0% to 2,56% (mean 0,81 %) in p17. Median sequence diversity detected for samples from our study were 0% to 1,8% (mean 0,52%) in the C2V3 region and 0% to 1,2% (mean 0,42%) in the p17 regions. The diversity encountered in our samples was thus almost half of that observed in individuals from the Learn study, refuting evidence that soon after transmission, women have a higher population diversity than men. In addition this study showed that early selective forces focused on viral properties in *env* C2V5 but not *gag* p17, as diversity in *env* was lower than in *gag*. Similar results were found in our study, with two of the five women (Du114 and Du204) exhibiting lower diversity in *env* compared to *gag*. This suggests that selection associated with heterosexual transmission and subsequent establishment of infection do occur, resulting in the presence of homogeneous viral populations soon after transmission (Figure 3.17).

The differences observed between our study and what was found in these studies could be explained by a number of factors that may impact on transmission. These include: the type of sample analyzed (whether proviral DNA or plasma viral RNA), the frequency of exposure for the different cohorts used (high risk versus low risk), subtype of infecting virus and the sample collection time respective to the estimated time of infection.

The Kenyan cohort used proviral DNA, whereas this study used plasma viral RNA. Where RNA reflects the currently circulating virus, provirus could reflect archival virus, as provirus persists for the lifetime of the cell and RNA exists only in the order of several days due to the rapid turnover of plasma viral RNA (Long et al., 2000; Poss et al., 1995). However, studies have shown that viral divergence (from a founder stain) and viral diversity (breath of population at a given time point) were the same when assessed in the proviral DNA and plasma RNA of nine individuals, measured from seroconversion over nine to 12 years in the C2V3 region (Shankarappa et al., 1999). In addition, five out of six women from the Kenyan cohort harboring heterogeneous viral populations after seroconversion in proviral DNA also had heterogeneous viral populations in plasma RNA

before seroconversion (Long et al., 2000). This indicates that limited diversification occurred after the development of HIV-1 specific humoral immunity and also proves that HIV-1 population diversity is similar in both circulating plasma RNA and proviral DNA. Due to the availability of sample material we could only characterize circulating viral populations and not provirus.

The women from the Kenyan sex worker cohort had a much lower frequency of HIV-1 exposure with an average of two sexual encounters per week compared to the Du-cohort who had an average of 28 unprotected (<20% condom usage) sexual encounters per week. Increased exposure may result in increased ulcerative STI and thus an increased susceptibility to infections with multiple variants. In addition high frequency of exposure may also result in multiple infections.

Another possible explanation for the limited diversity observed in our study may be that not all subtypes transmit in the same way (Kanki *et al.,* 1999). Women from the Kenyan cohort were predominantly infected with viruses from subtype A. Of a total of 32 women, 24 were infected with subtype A, 4 with subtype D, 2 with viruses of unknown subtype and two with subtype C. Heterogeneous populations have also been detected in one American woman recently infected with subtype B (Delwart et al., 2002). In the Kenyan cohort, neither of the two individuals infected with subtype C harbored a heterogeneous viral population.

Overall samples for our study were taken much closer to infection compared to the Kenyan study. On average, samples for the 32 women in the Kenyan study were 66 days post estimated infection (range 17 to 163 days). On average women with homogeneous viral populations (n=12) were sampled within 54 days of infection much closer to infection when compared to women with heterogeneous populations (n=20) which were sampled on average 73 days post infection. In the study by Learn and colleagues (2002), samples were collected on average 43,3 days post onset of symptoms (range 26 to 63 days). The five women from our study were sampled on average within 14 days from estimated date of infection before seroconversion (range 7 to 30 days) (Table 3.2). Preseroconversion samples more accurately reflect transmitted variants, however our

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study still differs from the Kenyan study in this respect as they found high diversity in a subset of six individuals analyzed pre seroconversion (Long et al., 2000).

In conclusion, in our study group of five subtype C HIV-1 infected sex worker women we were able to detect highly homogeneous populations in the C2V3 region with diversity ranging from 0% to 1,8% (mean 0,52%). This low diversity in intraperson HIV-1 populations was confirmed in the p17 region, with nucleotide diversity ranging from 0% to 1,2% (mean 0,42%). This stands in contrast with HIV-1 diversity described in a large group of women from a Kenyan sex worker cohort infected with heterogeneous subtype A and D HIV-1 populations, with nucleotide diversity of more than 5% across the V1, V2 and V3 regions. Our results fit in with proposed models of selective transmission or selective amplification (Zhang *et al.*, 1993 and Zhu *et al.*, 1993). This is distinct from the Kenyan studies which showed transmission of multiple variants.

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CHAPTER 4: Conclusions

Subtype C is becoming more prevalent and is currently responsible for over 56% of global HIV infections (Esparza and Bhamarapravati, 2000; UNAIDS 2002). The results described in this dissertation contribute to our understanding of HIV-1 *env* genetic diversity in southern African countries such as Malawi (n=4), South Africa (n=27), Zambia (n=10), and Zimbabwe (n=3). In this study, phylogenetic analysis of HIVs infecting 44 individuals originating from these countries reinforces the fact that subtype C is still the major contributor to the southern African HIV-1 epidemic (van Harmelen *et al.*, 1997; Bredell *et al.*, 1998; Moodley *et al.*, 1998). Analysis of the phylogenetic relationships between published gp120 subtype C sequences originating from Africa, including the sequences generated from this study, showed that southern African subtype C viruses do not cluster according to geographical origin. The southern African epidemic is characterised by high diversity and is different to the more recent explosive, genetically restricted clonal epidemics that have been observed in other developing countries, such as India (Grez *et al.*, 1994; Lole *et al.*, 1999). However, in this study there was some evidence of a founder type effect, as gp120 sequences that originated from Durban South Africa formed a subcluster within subtype C.

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Subtype C viruses is reported to be resistant to neutralization by the monoclonal antibody, 2G12 (Bures et al., 2002; Sanders et al., 2002). Resistance to neutralisation has been attributed to a N-linked mannose residue at position 295, crucial for 2G12 binding that was 80% absent in a panel of 15 subtype C viruses (Sanders et al., 2002; Scanlan et al., 2002). In this study the N-linked mannose 295 was absent in 95% of sequences.

Much of our understanding of the virological determinants of sexual transmission of HIV is derived from examining recently subtype B infected American and European men. Transmission data obtained from men have until recently been extrapolated to women, however, recent studies have shown that there may be gender differences in virus transmission. Women have been shown to harbor heterogeneous virus populations directly after transmission compared to homologous viral populations reported to be

present in men (Zhang *et al.*, 1993; Zhu *et al.*, 1993; Kampinga *et al.*, 1997; Long *et al.*, 2000; Poss *et al.*, 1995). In addition, SIV transmission studies in female macaques indicated that a multitude of variants could be readily transmitted. These studies found greater restriction of viral diversity during vaginal transmission compared to intravenous transmission. This suggests that heterogeneous population transmission is possible and that selection of virus populations takes place during heterosexual transmission of HIV-1 to women (Enose *et al.*, 1997; Greenier *et al.*, 2001).

It has been proposed that women may be more susceptible to infection with multiple quasispecies due to: an increased muscosal area of exposure, increased susceptibility through greater mucosal trauma, the presence of undiagnosed sexually transmitted infections, prolonged exposure to infected male secretions and immunological differences between men and women (Ray *et al.*, 2000). Gender differences have also been reported in HIV pathogenesis, with progression to AIDS in women being associated with lower viral loads when compared to men (Farzadegan *et al.*, 1998; Sterling *et al.*, 2001).

This dissertation described and characterised HIV-1 variants in plasma samples collected from five recently infected South African women belonging to the Durban sex worker cohort, collected on average 15 days post infection before seroconversion. Our findings clearly showed that HIV-1 populations in these women were highly homogeneous when analysed in both C2V3 (genetic diversity ranging from 0% to 1,8%) and p17 (genetic diversity ranging from 0% to 1,2%). This differs from other studies investigating genetic diversity in a Kenyan female sex worker cohort where 20 women with heterogeneous viral populations in acute infection had genetic diversity of up to 5% across the V1, V2 and V3 genomic regions (Long *et al.*, 2000, Poss *et al.*, 1995). In addition, heterogeneous viral populations have also been found in women from Rwanda who became infected around the time of their pregnancy (Kampinga *et al.*, 1997) as well as one American woman, containing highly divergent variants differing by up to 6% in C2V5 (Delwart *et al.*, 2002).

A possible reason for the differences observed in HIV population diversity between our study and previous female studies may be due to our analysis of plasma viral RNA. Less

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fit viral variants may be quickly displaced in the rapidly turning over plasma compartment while they may remain archived as proviruses. It is also possible that subtype may have an influence on transmission as previous studies indicated heterogeneous viral populations only in HIV subtype A, D and B infections but not in subtype C infections (Delwart *et al.*, 2002; Long *et al.*, 2000, Poss *et al.*, 1995).

Although our sample number was small, our results support the fact that the majority of HIVs transmitted heterosexually are subjected to a selection process resulting in the presence of homogeneous HIV populations soon after transmission, confirming heterosexual transmission models of selective transmission and amplification.

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Appendices

Appendix A: DNA Techniques

A1. RNA Extraction.

A2. PCR Purification

A3. Nucleobond AX 100 Purification of Plasmids

A4. Agarose gel electrophoresis

Appendix B: Reagents and recipes

B1. 50X TAE

B2. 6X Agarose gel electrophoresis loading dye

B3. X-Gal, IPTG, Ampicillin LB plate

B4. 10X TBE

B.5 6X Polyacrylamide gel electrophoresis loading dye

B6. HTA Annealing Buffer

B7. LB Broth

Appendix C: Vector maps and Molecular Weight Markers

C1. pMOS*Blue* vector map

C2. Roche Molecular Weight Marker VI

Appendix D: Amino Acids

D1. Amino acid codes

D2. Codons

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Appendix A: DNA Techniques

A1. RNA Extraction

Manual Extraction. RNA was extracted using the QIAamp® Viral RNA Mini Kit for purification of viral RNA from plasma, serum, cell-free body fluids and cell-culture supernatants (Qiagen, Valencia, CA). This method is used to extract viral RNA of more than 200bp from 140 to 280µl sample through the selective binding properties of a silica-gel based membrane. Plasma sample is lysed under denaturing conditions inactivating harmful RNases ensuring intact RNA isolation. Buffering conditions are adjusted to ensure optimum binding of RNA to the QIAamp membrane after loading the sample to the spin column. Bound RNA to the silica membrane is subsequently washed in two steps to remove contaminants with two different wash buffers. This method yields RNA free of protein, nucleases and other contaminants and inhibitors without the use of phenol/chloroform or alcohol precipitation.

1. Plasma sample, 140-280µl is added to 560µl prepared Buffer AVL containing carrier RNA in a 1.5ml micro-centrifuge tube, mixed by pulse-vortexing for 15 seconds, allowed to incubate at room temperature (15 -25°C) for 10- minutes and briefly centrifuged to remove drops form the inside of the lid.
2. 96-100% 560µl ethanol is added and mixed by pulse vortexing for 15 seconds, and briefly centrifuged to remove drops form the inside of the lid
3. This solution is carefully applied to the QIAmp spin columns and centrifuged at 6000x g (8 000rpm) for 1 minute. The column is placed in a clean 2ml collection tube and the tube containing the filtrate is discarded.
4. Step 3 is repeated.
5. Next 500µl Buffer AW1 is added and centrifuged at 6000x g (8 000rpm) for 1 minute, the column is placed in a clean 2ml collection tube and the tube containing the filtrate is discarded.

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6. Next 500µl Buffer AW2 is added and centrifuged at 20 000x g (14 000rpm) for 3 minutes, the column is placed in a clean 2ml collection tube and the tube containing the filtrate is discarded.
7. The column placed in a clean 2ml collection tube is then centrifuged for an additional 1 minute at full speed.
8. The column is placed in a clean 2ml collection tube and the old tube containing the filtrate is discarded.
9. Buffer AVE equilibrated to room temperature, 60µl is added to the column and allowed to incubate for 1 minute.
10. It is then centrifuged for 1 minute at 6000x g (8 000rpm), aliquoted to 10µl and either used directly in the cDNA synthesis or stored at –80°C.

A2. PCR Purification

PCR products were purified by the QIAquick PCR Purification Kit (Qiagen, Valencia, CA). QIAquick PCR purification is based on a spin-column method each with silica-gel membranes to which DNA adsorbs in the presence of high salt while contaminants are allowed to pass through the column. In this way impurities are efficiently washed away.

1. To the 50µl PCR reaction five volumes of buffer PB were added and mixed.
2. A QIAquick spin column were placed in a 2ml supplied collection tube and the sample applied to this column.
3. The DNA was bound to the column by a quick centrifugation of 1 minute at 13 000 rpm in a standard table top centrifuge.
4. The flow through was discarded and the column replaced into the same collection tube.
5. To wash, 750µl buffer PE was added to the column and centrifuged for a minute at maximum speed.
6. The column was next placed into a clean 1,5ml eppendorf micro-centrifuge tube.

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7. The DNA is eluted with 30μl buffer EB (10mM Tris-Cl, pH8,5) placed on the centre of the membrane without disruption. The column is allowed to stand for one minute and then centrifuged for one minute.

A3. Nucleobond AX 100 Purification of Plasmids

The AX 100 column is a silica based, anion exchange column for purification of plasmid DNA form 10 to 100ml broth.

1. The culture was pelleted at 5 500rpm for 10 minutes at 4°C using the Beckmann J2-21 centrifuge.
2. The supernatant was removed and the resulting bacterial cell pellet resuspended for cell disruption in 4ml supplied buffer S1 (50mM Tric/HCl, 10mM EDTA, 100μg RNase A/ml, pH 8.0) equilibrated to 4°C.
3. Next 4ml of buffer S2 (200mM NaOH, 1% SDS) was added with immediate inversion of tube 6-8 times. The mixture was then incubated at room temperature for not longer than 5 minutes.
4. Chromosomal DNA was removed from precipitation with 4ml buffer S3 (2,8M KAc, pH 5,1), inverted 6-8 times and incubated on ice for 5 minutes.
5. The bacterial lysate was clarified by loading onto a moistened filter paper and allowed to flow-through. No precipitate was allowed to contaminate the clear lysate.
6. The AX 100 column was first equilibrated by 2ml buffer N2 (100mM Tris/H_3PO_4. 15% Ethanol, 900mM KCl, pH 6,3) and allowed to flow-through. Care was taken that the columns did not dry out.
7. Next the lysate was loaded and allowed to flow-through with subsequent two washes with 4ml buffer N3 (100mM Tris/H_3PO_4. 15% Ethanol, 1150mM KCl, pH 6,3).
8. Finally the plasmid DNA was eluted with 4ml buffer N5 (100mM Tris/H_3PO_4. 15% Ethanol, 1000mM KCl, pH 8,5).

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9. Eluted plasmid in 4ml buffer was next precipitated with 0,6 – 0,8 volumes RT equilibrated isopropanol and centrifuged immediately at 1 1500rpm for 30 minutes at 4°C.
10. The DNA was then washed with 70% ethanol at 4°C and centrifuged at 1500rpm for 10 minutes at 4°C. The plasmid DNA pellet was then briefly dried for 5 minutes at room temperature and redisolved in TE (pH 7,8).

A4. Agarose gel electrophoresis

Amplicons were visualized by 2% agarose gel electrophoresis. Agarose gel electrophoresis was performed using horizontal gel apparatus (Stratagene, La Jolla, USA). The 2% agarose gel was prepared by melting agarose powder (Agarose Di LE, Hispanagar, Burgos, Spain) in 1X TAE (diluted from 50X TAE in deionised water, Appendix B1). Once melted 10mg/ml ethidium bromide stock solution was added to a final concentration of 1µg/100ml agarose. The agarose was poured into gel setting trays and cooled down to room temperature to allow setting. The gel was prepared for electrophoresis by being placed in gel apparatus and submerged in 1X TAE. Before loading, 10µl PCR product was mixed with 2µl of 6X agarose gel electrophoresis loading dye (Appendix B2). In order to determine the size of the amplicons a DNA molecular weigh marker VI (Roche, GmbH, Mannheim, Germany, Appendix C2) was included in the first lane of all gels. The gel was electrophoresed at 120V for 30 minutes that were sufficient for separation of bands. The DNA fragments were then visualized on a UVP transilluminator (UVP, San Gabriel, California, USA) at 256nm wavelength and photographed with a Kodak ds 1D, Electrophoresis Documentation and Analysis System 120, V 2.0.3. computerized gel imager (Kodak ds 1D digital science, version 2.03).

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Appendix B: Reagents and recipes

B1. 50X TAE

424g Tris base

57,1 ml Glacial Acetic Acid

100ml of 0,5M EDTA pH 8,0

Made up to 1l with deionised water.

B2. 6X Agarose Gel Electrophoresis Loading Dye

0,25% bromophenol blue

0,25% xylene cyanol FF

30% glycerol

In deionised water

B3. X-Gal, IPTG, Ampicillin LB agar plates

10g tryptone

5g yeast extract

10g NaCl

15g agar

Made up to one litre with dH_2O

After agar has been sterilised by autoclavation and allowed to cool down to just prior to setting at 50°C the following selective regents were added: 15µg/ml tetracycline, 100µg/ml ampicillin, 80µg/ml X-gal, 0,5mM IPTG.

B4. 10X TBE

108g Tris-HCl

55g Boric acid

20ml 1,5M EDTA

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made up to one litre with dH_2O.

B.5 6X Polyacrylamide gel electrophoresis loading dye

0,25% Bromophenolblue

0,25% Xylene Xylanol

15% Ficoll

In deionised water

B6. HTA Annealing Buffer

100mM Tris/HCl

1M NaCl

20mM EDTA

B7. LB Broth

10g NaCl

5g Yeast extract

10g Tryptone

up to 1l with deionised water

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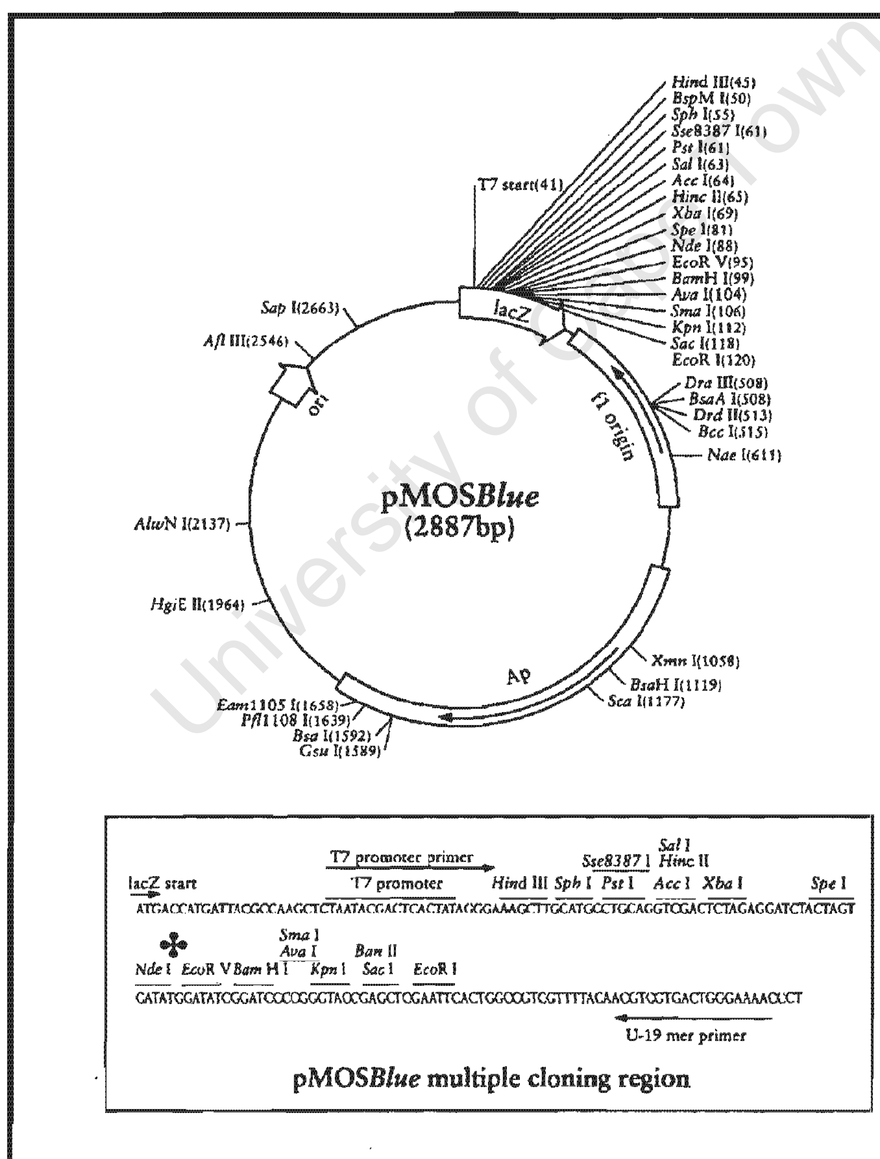
Appendix C: Vector maps and Molecular Markers

C1. pMOS*Blue* vector map

pMOS*Blue* vector map showing multiple cloning site and specific site of amplicon insertion indicated by a "✣ ". (Adapted from pMOS*Blue* blunt ended cloning kit, Amersham, Pharmacia Biotech)



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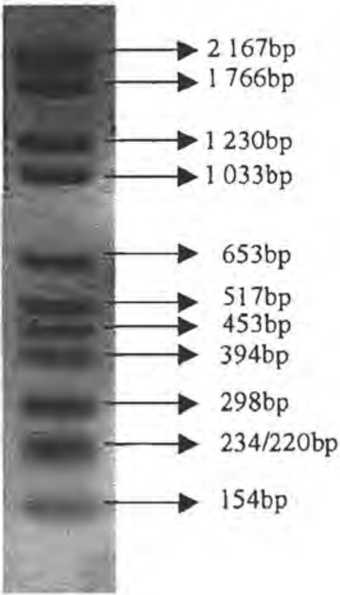
C2. Roche Molecular Weight Marker VI (Roche, GmbH, Mannheim, Germany)



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Appendix D: Amino Acids

D1. Amino acid codes

Alanine	Ala	A	Leucine	Leu	L
Arginine	Arg	R	Lycine	Lys	K
Asparagine	Asn	N	Methionine	Met	M
Aspartic acid	Asp	D	Phenylalanine	Phe	F
Cystine	Cys	C	Proline	Pro	P
Glutamine	Gln	Q	Serine	Ser	S
Glutamic acid	Glu	E	Threonine	Thr	T
Glycine	Gly	G	Tryptophan	Trp	W
Histidine	His	H	Tyrosine	Tyr	Y
Isoleucine	Ile	I	Valine	Val	V

D2. Codons

Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid	Codon	Amino Acid
AAA	K	CAA	Q	GAA	E	TAA	*
AAC	N	CAC	H	GAC	D	TAC	Y
AAG	K	CAG	Q	GAG	E	TAG	*
AAT	N	CAT	H	GAT	D	TAT	Y
ACA	T	CCA	P	GCA	A	TCA	S
ACC	T	CCC	P	GCC	A	TCC	S
ACG	T	CCG	P	GCG	A	TCG	S
ACT	T	CCT	P	GCT	A	TCT	S
AGA	R	CGA	R	GGA	G	TGA	*
AGC	S	CGC	R	GGC	G	TGC	C
AGG	R	CGG	R	GGG	G	TGG	W
AGT	S	CGT	R	GGT	G	TGT	C
ATA	I	CTA	L	GTA	V	TTA	L
ATC	I	CTC	L	GTC	V	TTC	F
ATG	M	CTG	L	GTG	V	TTG	L
ATT	I	CTT	L	GTT	V	TTT	F

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References

Abdool, K. S., K. Q. Abdool, M. Adhikari, S. Cassol, M. Chersich, P. Cooper, A. Coovadia, H. Coovadia, M. Cotton, A. Coutsoudis, W. Hide, G. Hussey, G. Maartens, S. Madhi, D. Martin, J. M. Pettifor, N. Rollins, G. Sherman, S. Thula, M. Urban, S. Velaphi, and C. Williamson. 2002. Vertical HIV transmission in South Africa: translating research into policy and practice. Lancet **359:**992-993.

Abebe, A., D. Demissie, J. Goudsmit, M. Brouwer, C. L. Kuiken, G. Pollakis, H. Schuitemaker, A. L. Fontanet, and T. F. Rinke de Wit. 1999. HIV-1 subtype C syncytium- and non-syncytium-inducing phenotypes and coreceptor usage among Ethiopian patients with AIDS. AIDS **13:**1305-1311.

Agha, S. 2002. Declines in casual sex in Lusaka, Zambia: 1996-1999. AIDS **16:**291-293.

Aiken, C., J. Konner, N. R. Landau, M. E. Lenburg, and D. Trono. 1994. Nef induces CD4 endocytosis: requirement for a critical dileucine motif in the membrane-proximal CD4 cytoplasmic domain. Cell **76:**853-864.

Alaeus, A., K. Lidman, A. Bjorkman, J. Giesecke, and J. Albert. 1999. Similar rate of disease progression among individuals infected with HIV-1 genetic subtypes A-D. AIDS **13:**901-907.

Alaeus, A. 2000. Significance of HIV-1 genetic subtypes. Scand. J. Infect. Dis. **32:**455-463.

Alizon, M., S. Wain-Hobson, L. Montagnier, and P. Sonigo. 1986. Genetic variability of the AIDS virus: nucleotide sequence analysis of two isolates from African patients. Cell **46:**63-74.

Alkhatib, G., C. Combadiere, C. C. Broder, Y. Feng, P. E. Kennedy, P. M. Murphy, and E. A. Berger. 1996. CC CKR5: a RANTES, MIP-1alpha, MIP-1beta receptor as a fusion cofactor for macrophage-tropic HIV-1. Science **272:**1955-1958.

Allan, J. S., M. Short, M. E. Taylor, S. Su, V. M. Hirsch, P. R. Johnson, G. M. Shaw, and B. H. Hahn. 1991. Species-specific diversity among simian immunodeficiency viruses from African green monkeys. J. Virol. **65:**2816-2828.

Allen, T. M., D. H. O'Connor, P. Jing, J. L. Dzuris, B. R. Mothe, T. U. Vogel, E. Dunphy, M. E. Liebl, C. Emerson, N. Wilson, K. J. Kunstman, X. Wang, D. B. Allison, A. L. Hughes, R. C. Desrosiers, J. D. Altman, S. M. Wolinsky, A. Sette, and D. I. Watkins. 2000. Tat-specific cytotoxic T lymphocytes select for SIV escape variants during resolution of primary viraemia. Nature **407:**386-390.

Altschul, S. F., W. Gish, W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. **215:**403-410.

University of Cape Town

Amornkul, P. N., S. Tansuphasawadikul, K. Limpakarnjanarat, S. Likanonsakul, N. Young, B. Eampokalap, J. Kaewkungwal, T. Naiwatanakul, J. Von Bargen, D. J. Hu, and T. D. Mastro. 1999. Clinical disease associated with HIV-1 subtype B' and E infection among 2104 patients in Thailand. AIDS **13:**1963-1969.

Anderson, R. M., B. Schwartlander, F. McCutchan, and D. Hu. 1996. Implications of genetic variability in HIV for epidemiology and public health. Lancet **347:**1778-1779.

Arendrup, M., C. Nielsen, J. E. Hansen, C. Pedersen, L. Mathiesen, and J. O. Nielsen. 1992. Autologous HIV-1 neutralizing antibodies: emergence of neutralization-resistant escape virus and subsequent development of escape virus neutralizing antibodies. J. Acquir. Immune. Defic. Syndr. **5:**303-307.

Artenstein, A. W., T. C. VanCott, K. V. Sitz, M. L. Robb, K. F. Wagner, S. C. Veit, A. F. Rogers, R. P. Garner, J. W. Byron, P. R. Burnett, and D. L. Birx. 1997. Mucosal immune responses in four distinct compartments of women infected with human immunodeficiency virus type 1: a comparison by site and correlation with clinical information. J. Infect. Dis. **175:**265-271.

Asjo, B., L. Morfeldt-Manson, J. Albert, G. Biberfeld, A. Karlsson, K. Lidman, and E. M. Fenyo. 1986. Replicative capacity of human immunodeficiency virus from patients with varying severity of HIV infection. Lancet **2:**660-662.

Auerbach, D. M., W. W. Darrow, H. W. Jaffe, and J. W. Curran. 1984. Cluster of cases of the acquired immune deficiency syndrome. Patients linked by sexual contact. Am. J. Med. **76:**487-492.

Banchereau, J. and R. M. Steinman. 1998. Dendritic cells and the control of immunity. Nature **392:**245-252.

Barouch, D. H., J. Kunstman, M. J. Kuroda, J. E. Schmitz, S. Santra, F. W. Peyerl, G. R. Krivulka, K. Beaudry, M. A. Lifton, D. A. Gorgone, D. C. Montefiori, M. G. Lewis, S. M. Wolinsky, and N. L. Letvin. 2002. Eventual AIDS vaccine failure in a rhesus monkey by viral escape from cytotoxic T lymphocytes. Nature **415:**335-339.

Barre-Sinoussi, F., J. C. Chermann, F. Rey, M. T. Nugeyre, S. Chamaret, J. Gruest, C. Dauguet, C. Axler-Blin, F. Vezinet-Brun, C. Rouzioux, W. Rozenbaum, and L. Montagnier. 1983. Isolation of a T-lymphotropic retrovirus from a patient at risk for acquired immune deficiency syndrome (AIDS). Science **220:**868-871.

Bartz, S. R., M. E. Rogel, and M. Emerman. 1996. Human immunodeficiency virus type 1 cell cycle control: Vpr is cytostatic and mediates G2 accumulation by a mechanism which differs from DNA damage checkpoint control. J. Virol. **70:**2324-2331.

University of Cape Town

Bebenek, K., J. Abbotts, J. D. Roberts, S. H. Wilson, and T. A. Kunkel. 1989. Specificity and mechanism of error-prone replication by human immunodeficiency virus-1 reverse transcriptase. J. Biol. Chem. **264:**16948-16956.

Beirnaert, E., P. Nyambi, B. Willems, L. Heyndrickx, R. Colebunders, W. Janssens, and G. G. van der. 2000. Identification and characterization of sera from HIV-infected individuals with broad cross-neutralizing activity against group M (env clade A-H) and group O primary HIV-1 isolates. J. Med. Virol. **62:**14-24.

Belec, L., T. Dupre, T. Prazuck, C. Tevi-Benissan, J. M. Kanga, O. Pathey, X. S. Lu, and J. Pillot. 1995. Cervicovaginal overproduction of specific IgG to human immunodeficiency virus (HIV) contrasts with normal or impaired IgA local response in HIV infection. J. Infect. Dis. **172:**691-697.

Berger, E. A., R. W. Doms, E. M. Fenyo, B. T. Korber, D. R. Littman, J. P. Moore, Q. J. Sattentau, H. Schuitemaker, J. Sodroski, and R. A. Weiss. 1998. A new classification for HIV-1. Nature **391:**240.

Berman, P. W. 1998. Development of bivalent rgp120 vaccines to prevent HIV type 1 infection. AIDS Res. Hum. Retroviruses **14 Suppl 3:**S277-S289.

Bjorndal, A., H. Deng, M. Jansson, J. R. Fiore, C. Colognesi, A. Karlsson, J. Albert, G. Scarlatti, D. R. Littman, and E. M. Fenyo. 1997. Coreceptor usage of primary human immunodeficiency virus type 1 isolates varies according to biological phenotype. J. Virol. **71:**7478-7487.

Bjorndal, A., A. Sonnerborg, C. Tscherning, J. Albert, and E. M. Fenyo. 1999. Phenotypic characteristics of human immunodeficiency virus type 1 subtype C isolates of Ethiopian AIDS patients. AIDS Res. Hum. Retroviruses **15:**647-653.

Blackard, J. T., D. E. Cohen, and K. H. Mayer. 2002. Human immunodeficiency virus superinfection and recombination: current state of knowledge and potential clinical consequences. Clin. Infect. Dis. **34:**1108-1114.

Bongertz, V., C. I. Costa, V. G. Santos, E. C. Joao Filho, B. Galvao-Castro, and M. G. Morgado. 1997. Correlation between susceptibility of primary HIV-1 isolates to autologous and heterologous neutralizing antibodies. Hospital Evandro Chagas AIDS Clinical Research Group. AIDS **11:**969-975.

Bour, S., U. Schubert, and K. Strebel. 1995. The human immunodeficiency virus type 1 Vpu protein specifically binds to the cytoplasmic domain of CD4: implications for the mechanism of degradation. J. Virol. **69:**1510-1520.

University of Cape Town

Boyer, J. C., K. Bebenek, and T. A. Kunkel. 1992. Unequal human immunodeficiency virus type 1 reverse transcriptase error rates with RNA and DNA templates. Proc. Natl. Acad. Sci. U. S. A **89:** 6919-6923.

Braaten, D., E. K. Franke, and J. Luban. 1996. Cyclophilin A is required for the replication of group M human immunodeficiency virus type 1 (HIV-1) and simian immunodeficiency virus SIV(CPZ)GAB but not group O HIV-1 or other primate immunodeficiency viruses. J. Virol. **70:**4220-4227.

Bradney, A. P., S. Scheer, J. M. Crawford, S. P. Buchbinder, and D. C. Montefiori. 1999. Neutralization escape in human immunodeficiency virus type 1-infected long-term nonprogressors. J. Infect. Dis. **179:**1264-1267.

Bredell, H., C. Williamson, P. Sonnenberg, D. J. Martin, and L. Morris. 1998. Genetic characterization of HIV type 1 from migrant workers in three South African gold mines. AIDS Res. Hum. Retroviruses **14:**677-684.

Bredell, H., G. Hunt, A. Casteling, T. Cilliers, C. Rademeyer, M. Coetzer, S. Miller, D. Johnson, C. T. Tiemessen, D. J. Martin, C. Williamson, and L. Morris. 2002. HIV-1 Subtype A, D, G, AG and unclassified sequences identified in South Africa. AIDS Res. Hum. Retroviruses **18:**681-683.

Briggs, D. R., D. L. Tuttle, J. W. Sleasman, and M. M. Goodenow. 2000. Envelope V3 amino acid sequence predicts HIV-1 phenotype (co-receptor usage and tropism for macrophages). AIDS **14:**2937-2939.

Bures, R., A. Gaitan, T. Zhu, C. Graziosi, K. M. McGrath, J. Tartaglia, P. Caudrelier, R. El Habib, M. Klein, A. Lazzarin, D. M. Stablein, M. Deers, L. Corey, M. L. Greenberg, D. H. Schwartz, and D. C. Montefiori. 2000. Immunization with recombinant canarypox vectors expressing membrane-anchored glycoprotein 120 followed by glycoprotein 160 boosting fails to generate antibodies that neutralize R5 primary isolates of human immunodeficiency virus type 1. AIDS Res. Hum. Retroviruses **16:**2019-2035.

Bures, R., L. Morris, C. Williamson, G. Ramjee, M. Deers, S. A. Fiscus, S. Abdool-Karim, and D. C. Montefiori. 2002. Regional clustering of shared neutralization determinants on primary isolates of clade C human immunodeficiency virus type 1 from South Africa. J. Virol. **76:**2233-2244.

Burton, D. R. 2002. Antibodies, viruses and vaccines. Nat. Rev. Immunol. **2:**706-713.

Bush, C. E., R. M. Donovan, N. Markowitz, D. Baxa, P. Kvale, and L. D. Saravolatz. 1996. Gender is not a factor in serum human immunodeficiency virus type 1 RNA levels in patients with viremia. J. Clin. Microbiol. **34:**970-972.

University of Cape Town

Cannon, P. M., S. Matthews, N. Clark, E. D. Byles, O. Iourin, D. J. Hockley, S. M. Kingsman, and A. J. Kingsman. 1997. Structure-function studies of the human immunodeficiency virus type 1 matrix protein, p17. J. Virol. **71:**3474-3483.

Canque, B., M. Rosenzwajg, S. Camus, M. Yagello, M. L. Bonnet, M. Guigon, and J. C. Gluckman. 1996. The effect of in vitro human immunodeficiency virus infection on dendritic-cell differentiation and function. Blood **88:**4215-4228.

Carr, J. K., M. O. Salminen, C. Koch, D. Gotte, A. W. Artenstein, P. A. Hegerich, D. St Louis, D. S. Burke, and F. E. McCutchan. 1996. Full-length sequence and mosaic structure of a human immunodeficiency virus type 1 isolate from Thailand. J. Virol. **70:**5935-5943.

Carr, J. K., T. Laukkanen, M. O. Salminen, J. Albert, A. Alaeus, B. Kim, E. Sanders-Buell, D. L. Birx, and F. E. McCutchan. 1999. Characterization of subtype A HIV-1 from Africa by full genome sequencing. AIDS **13:**1819-1826.

Charneau, P., A. M. Borman, C. Quillent, D. Guetard, S. Chamaret, J. Cohen, G. Remy, L. Montagnier, and F. Clavel. 1994. Isolation and envelope sequence of a highly divergent HIV-1 isolate: definition of a new HIV-1 group. Virology **205:**247-253.

Chen, Z., P. Telfer, P. Reed, L. Zhang, A. Getti, D. D. Ho, and P. A. Marx. 1995. Isolation and characterization of the first simian immunodeficiency virus from a feral sooty mangabey (Cercocebus atys) in West Africa. J. Med. Primatol. **24:**108-115.

Chen, Z., P. Telfier, A. Gettie, P. Reed, L. Zhang, D. D. Ho, and P. A. Marx. 1996. Genetic characterization of new West African simian immunodeficiency virus SIVsm: geographic clustering of household-derived SIV strains with human immunodeficiency virus type 2 subtypes and genetically diverse viruses from a single feral sooty mangabey troop. J. Virol. **70:**3617-3627.

Chen, Z., A. Luckay, D. L. Sodora, P. Telfer, P. Reed, A. Gettie, J. M. Kanu, R. F. Sadek, J. Yee, D. D. Ho, L. Zhang, and P. A. Marx. 1997. Human immunodeficiency virus type 2 (HIV-2) seroprevalence and characterization of a distinct HIV-2 genetic subtype from the natural range of simian immunodeficiency virus-infected sooty mangabeys. J. Virol. **71:**3953-3960.

Chen, Z., D. Kwon, Z. Jin, S. Monard, P. Telfer, M. S. Jones, C. Y. Lu, R. F. Aguilar, D. D. Ho, and P. A. Marx. 1998. Natural infection of a homozygous delta24 CCR5 red-capped mangabey with an R2b-tropic simian immunodeficiency virus. J. Exp. Med. **188:**2057-2065.

Cheng-Mayer, C., C. Weiss, D. Seto, and J. A. Levy. 1989. Isolates of human immunodeficiency virus type 1 from the brain may constitute a special group of the AIDS virus. Proc. Natl. Acad. Sci. U. S. A **86:**8575-8579.

University of Cape Town

Chesebro, B., R. Buller, J. Portis, and K. Wehrly. 1990. Failure of human immunodeficiency virus entry and infection in CD4-positive human brain and skin cells. J. Virol. **64:**215-221.

Clavel, F., F. Brun-Vezinet, D. Guetard, S. Chamaret, A. Laurent, C. Rouzioux, M. Rey, C. Katlama, F. Rey, J. L. Champelinaud. 1986. [LAV type II: a second retrovirus associated with AIDS in West Africa]. C. R. Acad. Sci. III **302:**485-488.

Clewley, J. P., C. Arnold, K. L. Barlow, P. R. Grant, and J. V. Parry. 1996. Diverse HIV-1 genetic subtypes in UK. Lancet **347:**1487.

Coffin, J., A. Haase, J. A. Levy, L. Montagnier, S. Oroszlan, N. Teich, H. Temin, K. Toyoshima, H. Varmus, P. Vogt, and . 1986. What to call the AIDS virus? Nature **321:**10.

Coffin, J. M. 1979. Structure, replication, and recombination of retrovirus genomes: some unifying hypotheses. J. Gen. Virol. **42:**1-26.

Coffin, J. M. 1995. HIV population dynamics in vivo: implications for genetic variation, pathogenesis, and therapy. Science **267:**483-489.

Connor, E. M., R. S. Sperling, R. Gelber, P. Kiselev, G. Scott, M. J. O'Sullivan, R. VanDyke, M. Bey, W. Shearer, R. L. Jacobson. 1994. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. N. Engl. J. Med. **331:**1173-1180.

Connor, R. I. and D. D. Ho. 1994. Human immunodeficiency virus type 1 variants with increased replicative capacity develop during the asymptomatic stage before disease progression. J. Virol. **68:**4400-4408.

Connor, R. I., K. E. Sheridan, D. Ceradini, S. Choe, and N. R. Landau. 1997. Change in coreceptor use coreceptor use correlates with disease progression in HIV-1--infected individuals. J. Exp. Med. **185:**621-628.

Corbitt, G., A. S. Bailey, and G. Williams. 1990. HIV infection in Manchester, 1959. Lancet **336:**51.

Cornelissen, M., G. Mulder-Kampinga, J. Veenstra, F. Zorgdrager, C. Kuiken, S. Hartman, J. Dekker, H. L. van der, C. Sol, R. Coutinho. 1995. Syncytium-inducing (SI) phenotype suppression at seroconversion after intramuscular inoculation of a non-syncytium-inducing/SI phenotypically mixed human immunodeficiency virus population. J. Virol. **69:** 1810-1818.

Cornelissen, M., G. Kampinga, F. Zorgdrager, and J. Goudsmit. 1996. Human immunodeficiency virus type 1 subtypes defined by env show high frequency of recombinant gag genes. The UNAIDS Network for HIV Isolation and Characterization. J. Virol. **70:**8209-8212.

University of Cape Town

Cornelissen, M., B. R. van den, F. Zorgdrager, V. Lukashov, and J. Goudsmit. 1997. pol gene diversity of five human immunodeficiency virus type 1 subtypes: evidence for naturally occurring mutations that contribute to drug resistance, limited recombination patterns, and common ancestry for subtypes B and D. J. Virol. **71:**6348-6358.

Couturier, E., F. Damond, P. Roques, H. Fleury, F. Barin, J. B. Brunet, F. Brun-Vezinet, and F. Simon. 2000. HIV-1 diversity in France, 1996-1998. The AC 11 laboratory network. AIDS **14:**289-296.

Cullen, B. R. 1990. The HIV-1 Tat protein: an RNA sequence-specific processivity factor? Cell **63:**655-657.

Cullen, B. R. 1993. Does HIV-1 Tat induce a change in viral initiation rights? Cell **73:**417-420.

De Cock, K. M. 1996. The emergence of HIV/AIDS in Africa. Rev. Epidemiol. Sante Publique **44:**511-518.

De Francesco, M. A., A. Caruso, F. Fallacara, A. D. Canaris, F. Dima, C. Poiesi, S. Licenziati, M. Corulli, F. Martinelli, S. Fiorentini, and A. Turano. 1998. HIV p17 enhances lymphocyte proliferation and HIV-1 replication after binding to a human serum factor. AIDS **12:** 245-252.

Dean, M., M. Carrington, C. Winkler, G. A. Huttley, M. W. Smith, R. Allikmets, J. J. Goedert, S. P. Buchbinder, E. Vittinghoff, E. Gomperts, S. Donfield, D. Vlahov, R. Kaslow, A. Saah, C. Rinaldo, R. Detels, and S. J. O'Brien. 1996. Genetic restriction of HIV-1 infection and progression to AIDS by a deletion allele of the CKR5 structural gene. Hemophilia Growth and Development Study, Multicenter AIDS Cohort Study, Multicenter Hemophilia Cohort Study, San Francisco City Cohort, ALIVE Study. Science **273:**1856-1862.

Deeks, S. G. 2001. International perspectives on antiretroviral resistance. Nonnucleoside reverse transcriptase inhibitor resistance. J. Acquir. Immune. Defic. Syndr. **26 Suppl 1:**S25-S33.

Delwart, E., M. Magierowska, M. Royz, B. Foley, L. Peddada, R. Smith, C. Heldebrant, A. Conrad, and M. Busch. 2002. Homogeneous quasispecies in 16 out of 17 individuals during very early HIV-1 primary infection. AIDS **16:**189-195.

Delwart, E. L., E. G. Shpaer, J. Louwagie, F. E. McCutchan, M. Grez, H. Rubsamen-Waigmann, and J. I. Mullins. 1993. Genetic relationships determined by a DNA heteroduplex mobility assay: analysis of HIV-1 env genes. Science **262:**1257-1261.

Delwart, E. L., H. W. Sheppard, B. D. Walker, J. Goudsmit, and J. I. Mullins. 1994. Human immunodeficiency virus type 1 evolution in vivo tracked by DNA heteroduplex mobility assays. J. Virol. **68:**6672-6683.

University of Cape Town

Delwart, E. L., J. I. Mullins, P. Gupta, G. H. Learn, Jr., M. Holodniy, D. Katzenstein, B. D. Walker, and M. K. Singh. 1998. Human immunodeficiency virus type 1 populations in blood and semen. J. Virol. **72:**617-623.

Deng, H., R. Liu, W. Ellmeier, S. Choe, D. Unutmaz, M. Burkhart, P. Di Marzio, S. Marmon, R. E. Sutton, C. M. Hill, C. B. Davis, S. C. Peiper, T. J. Schall, D. R. Littman, and N. R. Landau. 1996. Identification of a major co-receptor for primary isolates of HIV-1. Nature **381:**661-666.

Di Marzio, P., S. Choe, M. Ebright, R. Knoblauch, and N. R. Landau. 1995. Mutational analysis of cell cycle arrest, nuclear localization and virion packaging of human immunodeficiency virus type 1 Vpr. J. Virol. **69:**7909-7916.

Dietrich, U., M. Grez, H. von Briesen, B. Panhans, M. Geissendorfer, H. Kuhnel, J. Maniar, G. Mahambre, W. B. Becker, M. L. Becker, and . 1993. HIV-1 strains from India are highly divergent from prototypic African and US/European strains, but are linked to a South African isolate. AIDS **7:**23-27.

Dimmock, N.J. 1995. Update on the neutralisation of animal viruses. Rev. Med. Virol. **5:** 165-179.

Dittmar, M. T., G. Simmons, S. Hibbitts, M. O'Hare, S. Louisirirotchanakul, S. Beddows, J. Weber, P. R. Clapham, and R. A. Weiss. 1997. Langerhans cell tropism of human immunodeficiency virus type 1 subtype A through F isolates derived from different transmission groups. J. Virol. **71:**8008-8013.

Doranz, B. J., J. Rucker, Y. Yi, R. J. Smyth, M. Samson, S. C. Peiper, M. Parmentier, R. G. Collman, and R. W. Doms. 1996. A dual-tropic primary HIV-1 isolate that uses fusin and the beta-chemokine receptors CKR-5, CKR-3, and CKR-2b as fusion cofactors. Cell **85:**1149-1158.

Dowdle, W. R. 1999. Influenza A virus recycling revisited. Bull. World Health Organ **77:**820-828.

Ellerbrock, T. V., J. L. Lennox, K. A. Clancy, R. F. Schinazi, T. C. Wright, M. Pratt-Palmore, T. Evans-Strickfaden, C. Schnell, R. Pai, L. J. Conley, E. E. Parrish-Kohler, T. J. Bush, K. Tatti, and C. E. Hart. 2001. Cellular replication of human immunodeficiency virus type 1 occurs in vaginal secretions. J. Infect. Dis. **184:**28-36.

Emau, P., H. M. McClure, M. Isahakia, J. G. Else, and P. N. Fultz. 1991. Isolation from African Sykes' monkeys (Cercopithecus mitis) of a lentivirus related to human and simian immunodeficiency viruses. J. Virol. **65:**2135-2140.

Engelbrecht, S., I. Koulinska, T. L. Smith, J. Barreto, and E. J. van Rensburg. 1998. Variation in HIV type 1 V3 region env sequences from Mozambique. AIDS Res. Hum. Retroviruses **14:**803-805.

University of Cape Town

Engelbrecht, S., T. L. Smith, P. Kasper, E. Faatz, M. Zeier, D. Moodley, C. G. Clay, and E. J. van Rensburg. 1999. HIV type 1 V3 domain serotyping and genotyping in Gauteng, Mpumalanga, KwaZulu-Natal, and Western Cape Provinces of South Africa. AIDS Res. Hum. Retroviruses **15:**325-328.

Engelbrecht, S., T. de Villiers, C. C. Sampson, M. J. zur, S. W. Barnett, and E. J. van Rensburg. 2001. Genetic analysis of the complete gag and env genes of HIV type 1 subtype C primary isolates from South Africa. AIDS Res. Hum. Retroviruses **17:**1533-1547.

Enose, Y., M. Okada, T. Sata, W. Ma, T. Igarashi, K. Ibuki, E. Ido, and M. Hayami. 1997. Restriction of viral population by intravaginal infection of simian immunodeficiency viruses in macaque monkeys. Arch. Virol. **142:**37-51.

Epstein, L. G., C. Kuiken, B. M. Blumberg, S. Hartman, L. R. Sharer, M. Clement, and J. Goudsmit. 1991. HIV-1 V3 domain variation in brain and spleen of children with AIDS: tissue-specific evolution within host-determined quasispecies. Virology **180:**583-590.

Esparza, J. and N. Bhamarapravati. 2000. Accelerating the development and future availability of HIV-1 vaccines: why, when, where, and how? Lancet **355:**2061-2066.

Essex, M. 1995. Strategies of research for a vaccine against AIDS. Hist Philos. Life Sci. **17:**141-149.

Essex, M. 1999. Human immunodeficiency viruses in the developing world. Adv. Virus Res. **53:**71-88.

Evans, D. T., D. H. O'Connor, P. Jing, J. L. Dzuris, J. Sidney, J. da Silva, T. M. Allen, H. Horton, J. E. Venham, R. A. Rudersdorf, T. Vogel, C. D. Pauza, R. E. Bontrop, R. DeMars, A. Sette, A. L. Hughes, and D. I. Watkins. 1999. Virus-specific cytotoxic T-lymphocyte responses select for amino-acid variation in simian immunodeficiency virus Env and Nef. Nat. Med. **5:**1270-1276.

Ewart, G. D., T. Sutherland, P. W. Gage, and G. B. Cox. 1996. The Vpu protein of human immunodeficiency virus type 1 forms cation-selective ion channels. J. Virol. **70:**7108-7115.

Fantini, J., D. G. Cook, N. Nathanson, S. L. Spitalnik, and F. Gonzalez-Scarano. 1993. Infection of colonic epithelial cell lines by type 1 human immunodeficiency virus is associated with cell surface expression of galactosylceramide, a potential alternative gp120 receptor. Proc. Natl. Acad. Sci. U. S. A **90:**2700-2704.

Farmerie, W. G., D. D. Loeb, N. C. Casavant, C. A. Hutchison, III, M. H. Edgell, and R. Swanstrom. 1987. Expression and processing of the AIDS virus reverse transcriptase in Escherichia coli. Science **236:**305-308.

University of Cape Town

Farzadegan, H., D. R. Hoover, J. Astemborski, C. M. Lyles, J. B. Margolick, R. B. Markham, T. C. Quinn, and D. Vlahov. 1998. Sex differences in HIV-1 viral load and progression to AIDS. Lancet **352:**1510-1514.

Farzan, M., H. Choe, L. Vaca, K. Martin, Y. Sun, E. Desjardins, N. Ruffing, L. Wu, R. Wyatt, N. Gerard, C. Gerard, and J. Sodroski. 1998. A tyrosine-rich region in the N terminus of CCR5 is important for human immunodeficiency virus type 1 entry and mediates an association between gp120 and CCR5. J. Virol. **72:**1160-1164.

Fenyo, E. M., L. Morfeldt-Manson, F. Chiodi, B. Lind, A. von Gegerfelt, J. Albert, E. Olausson, and B. Asjo. 1988. Distinct replicative and cytopathic characteristics of human immunodeficiency virus isolates. J. Virol. **62:**4414-4419.

Fenyo, E. M., J. Albert, and J. McKeating. 1996. The role of the humoral immune response in HIV infection. AIDS **10 Suppl A:**S97-106.

Fouchier, R. A., M. Groenink, N. A. Kootstra, M. Tersmette, H. G. Huisman, F. Miedema, and H. Schuitemaker. 1992. Phenotype-associated sequence variation in the third variable domain of the human immunodeficiency virus type 1 gp120 molecule. J. Virol. **66:**3183-3187.

Franchini, G., E. Collalti, S. K. Arya, E. M. Fenyo, G. Biberfeld, J. F. Zagury, P. J. Kanki, F. Wong-Staal, and R. C. Gallo. 1987. Genetic analysis of a new subgroup of human and simian T-lymphotropic retroviruses: HTLV-IV, LAV-2, SBL-6669, and STLV-IIIAGM. AIDS Res. Hum. Retroviruses **3:**11-17.

Galai, N., A. Kalinkovich, R. Burstein, D. Vlahov, and Z. Bentwich. 1997. African HIV-1 subtype C and rate of progression among Ethiopian immigrants in Israel. Lancet **349:**180-181.

Gallo, R. C., S. Z. Salahuddin, M. Popovic, G. M. Shearer, M. Kaplan, B. F. Haynes, T. J. Palker, R. Redfield, J. Oleske, B. Safai, and . 1984. Frequent detection and isolation of cytopathic retroviruses (HTLV-III) from patients with AIDS and at risk for AIDS. Science **224:**500-503.

Gallo, R. C. 1987. The AIDS virus. Sci. Am. **256:**46-56.

Gallo, R. C., M. Robert-Guroff, F. Wong-Staal, M. S. Reitz, Jr., S. K. Arya, and H. Z. Streicher. 1987. HTLV-III/LAV and the origin and pathogenesis of AIDS. Int. Arch. Allergy Appl. Immunol. **82:**471-475.

Gallo, R. C. and L. Montagnier. 1988. AIDS in 1988. Sci. Am. **259:**41-48.

Gandhi, M., P. Bacchetti, P. Miotti, T. C. Quinn, F. Veronese, and R. M. Greenblatt. 2002. Does patient sex affect human immunodeficiency virus levels? Clin. Infect. Dis. **35:**313-322.

University of Cape Town

Gao, F., L. Yue, A. T. White, P. G. Pappas, J. Barchue, A. P. Hanson, B. M. Greene, P. M. Sharp, G. M. Shaw, and B. H. Hahn. 1992. Human infection by genetically diverse SIVSM-related HIV-2 in west Africa. Nature **358:**495-499.

Gao, F., S. G. Morrison, D. L. Robertson, C. L. Thornton, S. Craig, G. Karlsson, J. Sodroski, M. Morgado, B. Galvao-Castro, H. von Briesen, and . 1996. Molecular cloning and analysis of functional envelope genes from human immunodeficiency virus type 1 sequence subtypes A through G. The WHO and NIAID Networks for HIV Isolation and Characterization. J. Virol. **70:**1651-1667.

Gao, F., D. L. Robertson, C. D. Carruthers, Y. Li, E. Bailes, L. G. Kostrikis, M. O. Salminen, F. Bibollet-Ruche, M. Peeters, D. D. Ho, G. M. Shaw, P. M. Sharp, and B. H. Hahn. 1998. An isolate of human immunodeficiency virus type 1 originally classified as subtype I represents a complex mosaic comprising three different group M subtypes (A, G, and I). J. Virol. **72:**10234-10241.

Gao, F., E. Bailes, D. L. Robertson, Y. Chen, C. M. Rodenburg, S. F. Michael, L. B. Cummins, L. O. Arthur, M. Peeters, G. M. Shaw, P. M. Sharp, and B. H. Hahn. 1999. Origin of HIV-1 in the chimpanzee Pan troglodytes troglodytes. Nature **397:**436-441.

Gaschen, B., J. Taylor, K. Yusim, B. Foley, F. Gao, D. Lang, V. Novitsky, B. Haynes, B. H. Hahn, T. Bhattacharya, and B. Korber. 2002. Diversity considerations in HIV-1 vaccine selection. Science **296:**2354-2360.

aGeijtenbeek, T. B., D. S. Kwon, R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, J. Middel, I. L. Cornelissen, H. S. Nottet, V. N. KewalRamani, D. R. Littman, C. G. Figdor, and Y. van Kooyk. 2000. DC-SIGN, a dendritic cell-specific HIV-1-binding protein that enhances trans-infection of T cells. Cell **100:**587-597.

bGeijtenbeek, T. B., R. Torensma, S. J. van Vliet, G. C. van Duijnhoven, G. J. Adema, Y. van Kooyk, and C. G. Figdor. 2000. Identification of DC-SIGN, a novel dendritic cell-specific ICAM-3 receptor that supports primary immune responses. Cell **100:**575-585.

Gilbert, L. and L. Walker. 2002. HIV/AIDS in South Africa: an overview. Cad. Saude Publica **18:**651-660.

Goodrich, D. W. and P. H. Duesberg. 1990. Retroviral recombination during reverse transcription. Proc. Natl. Acad. Sci. U. S. A **87:**2052-2056.

Gotch, F., A. Gallimore, and A. McMichael. 1996. Cytotoxic T cells--protection from disease progression--protection from infection. Immunol. Lett. **51:**125-128.

University of Cape Town

Gottlieb, M. S., R. Schroff, H. M. Schanker, J. D. Weisman, P. T. Fan, R. A. Wolf, and A. Saxon. 1981. Pneumocystis carinii pneumonia and mucosal candidiasis in previously healthy homosexual men: evidence of a new acquired cellular immunodeficiency. N. Engl. J. Med. **305:**1425-1431.

Gottlinger, H. G., J. G. Sodroski, and W. A. Haseltine. 1989. Role of capsid precursor processing and myristoylation in morphogenesis and infectivity of human immunodeficiency virus type 1. Proc. Natl. Acad. Sci. U. S. A **86:**5781-5785.

Gottlinger, H. G., T. Dorfman, E. A. Cohen, and W. A. Haseltine. 1993. Vpu protein of human immunodeficiency virus type 1 enhances the release of capsids produced by gag gene constructs of widely divergent retroviruses. Proc. Natl. Acad. Sci. U. S. A **90:**7381-7385.

Goulder, P. J., R. E. Phillips, R. A. Colbert, S. McAdam, G. Ogg, M. A. Nowak, P. Giangrande, G. Luzzi, B. Morgan, A. Edwards, A. J. McMichael, and S. Rowland-Jones. 1997. Late escape from an immunodominant cytotoxic T-lymphocyte response associated with progression to AIDS. Nat. Med. **3:**212-217.

Gouws, E., B. G. Williams, H. W. Sheppard, B. Enge, and S. A. Karim. 2002. High incidence of HIV-1 in South Africa using a standardized algorithm for recent HIV seroconversion. J. Acquir. Immune. Defic. Syndr. **29:**531-535.

Granelli-Piperno, A., E. Delgado, V. Finkel, W. Paxton, and R. M. Steinman. 1998. Immature dendritic cells selectively replicate macrophagetropic (M-tropic) human immunodeficiency virus type 1, while mature cells efficiently transmit both M- and T-tropic virus to T cells. J. Virol. **72:**2733-2737.

Greenblatt, R. M., N. Ameli, R. M. Grant, P. Bacchetti, and R. N. Taylor. 2000. Impact of the ovulatory cycle on virologic and immunologic markers in HIV-infected women. J. Infect. Dis. **181:** 82-90.

Greenier, J. L., C. J. Miller, D. Lu, P. J. Dailey, F. X. Lu, K. J. Kunstman, S. M. Wolinsky, and M. L. Marthas. 2001. Route of simian immunodeficiency virus inoculation determines the complexity but not the identity of viral variant populations that infect rhesus macaques. J. Virol. **75:**3753-3765.

Grez, M., U. Dietrich, P. Balfe, H. von Briesen, J. K. Maniar, G. Mahambre, E. L. Delwart, J. I. Mullins, and H. Rubsamen-Waigmann. 1994. Genetic analysis of human immunodeficiency virus type 1 and 2 (HIV-1 and HIV-2) mixed infections in India reveals a recent spread of HIV-1 and HIV-2 from a single ancestor for each of these viruses. J. Virol. **68:** 2161-2168.

Gross, I., H. Hohenberg, C. Huckhagel, and H. G. Krausslich. 1998. N-Terminal extension of human immunodeficiency virus capsid protein converts the in vitro assembly phenotype from tubular to spherical particles. J. Virol. **72:**4798-4810.

University of Cape Town

Guo, J., L. E. Henderson, J. Bess, B. Kane, and J. G. Levin. 1997. Human immunodeficiency virus type 1 nucleocapsid protein promotes efficient strand transfer and specific viral DNA synthesis by inhibiting TAR-dependent self-priming from minus-strand strong-stop DNA. J. Virol. **71:**5178-5188.

Gupta, P., J. Mellors, L. Kingsley, S. Riddler, M. K. Singh, S. Schreiber, M. Cronin, and C. R. Rinaldo. 1997. High viral load in semen of human immunodeficiency virus type 1-infected men at all stages of disease and its reduction by therapy with protease and nonnucleoside reverse transcriptase inhibitors. J. Virol. **71:**6271-6275.

Gupta, P., K. B. Collins, D. Ratner, S. Watkins, G. J. Naus, D. V. Landers, and B. K. Patterson. 2002. Memory CD4(+) T cells are the earliest detectable human immunodeficiency virus type 1 (HIV-1)-infected cells in the female genital mucosal tissue during HIV-1 transmission in an organ culture system. J. Virol. **76:**9868-9876.

Gurtler, L. G., L. Zekeng, J. M. Tsague, A. van Brunn, Z. E. Afane, J. Eberle, and L. Kaptue. 1996. HIV-1 subtype O: epidemiology, pathogenesis, diagnosis, and perspectives of the evolution of HIV. Arch. Virol. Suppl **11:**195-202.

Hahn, B. H., G. M. Shaw, K. M. De Cock, and P. M. Sharp. 2000. AIDS as a zoonosis: scientific and public health implications. Science **287:**607-614.

Hall, T.A. 1999. BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. Nucl. Acids. Symp. Ser. **41:**95-98.

Hamed, K. A., M. A. Winters, M. Holodniy, D. A. Katzenstein, and T. C. Merigan. 1993. Detection of human immunodeficiency virus type 1 in semen: effects of disease stage and nucleoside therapy. J. Infect. Dis. **167:**798-802.

Hart, C. E., J. L. Lennox, M. Pratt-Palmore, T. C. Wright, R. F. Schinazi, T. Evans-Strickfaden, T. J. Bush, C. Schnell, L. J. Conley, K. A. Clancy, and T. V. Ellerbrock. 1999. Correlation of human immunodeficiency virus type 1 RNA levels in blood and the female genital tract. J. Infect. Dis. **179:**871-882.

Haseltine, W. A. 1988. Replication and pathogenesis of the AIDS virus. J. Acquir. Immune. Defic. Syndr. **1:**217-240.

He, J., S. Choe, R. Walker, P. Di Marzio, D. O. Morgan, and N. R. Landau. 1995. Human immunodeficiency virus type 1 viral protein R (Vpr) arrests cells in the G2 phase of the cell cycle by inhibiting p34cdc2 activity. J. Virol. **69:**6705-6711.

University of Cape Town

He, J., Y. Chen, M. Farzan, H. Choe, A. Ohagen, S. Gartner, J. Busciglio, X. Yang, W. Hofmann, W. Newman, C. R. Mackay, J. Sodroski, and D. Gabuzda. 1997. CCR3 and CCR5 are co-receptors for HIV-1 infection of microglia. Nature **385:**645-649.

Heinzinger, N. K., M. I. Bukinsky, S. A. Haggerty, A. M. Ragland, V. Kewalramani, M. A. Lee, H. E. Gendelman, L. Ratner, M. Stevenson, and M. Emerman. 1994. The Vpr protein of human immunodeficiency virus type 1 influences nuclear localization of viral nucleic acids in nondividing host cells. Proc. Natl. Acad. Sci. U. S. A **91:**7311-7315.

Hirsch, V. M., R. A. Olmsted, M. Murphey-Corb, R. H. Purcell, and P. R. Johnson. 1989. An African primate lentivirus (SIVsm) closely related to HIV-2. Nature **339:**389-392.

Hirsch, V. M., B. J. Campbell, E. Bailes, R. Goeken, C. Brown, W. R. Elkins, M. Axthelm, M. Murphey-Corb, and P. M. Sharp. 1999. Characterization of a novel simian immunodeficiency virus (SIV) from L'Hoest monkeys (Cercopithecus l'hoesti): implications for the origins of SIVmnd and other primate lentiviruses. J. Virol. **73:**1036-1045.

Ho, D. D., A. U. Neumann, A. S. Perelson, W. Chen, J. M. Leonard, and M. Markowitz. 1995. Rapid turnover of plasma virions and CD4 lymphocytes in HIV-1 infection. Nature **373:**123-126.

Hoelscher, M., W. E. Dowling, E. Sanders-Buell, J. K. Carr, M. E. Harris, A. Thomschke, M. L. Robb, D. L. Birx, and F. E. McCutchan. 2002. Detection of HIV-1 subtypes, recombinants, and dual infections in east Africa by a multi-region hybridization assay. AIDS **16:**2055-2064.

Hoffman, N. G., F. Seillier-Moiseiwitsch, J. Ahn, J. M. Walker, and R. Swanstrom. 2002. Variability in the human immunodeficiency virus type 1 gp120 Env protein linked to phenotype-associated changes in the V3 loop. J. Virol. **76:**3852-3864.

Hu, D. J., T. J. Dondero, M. A. Rayfield, J. R. George, G. Schochetman, H. W. Jaffe, C. C. Luo, M. L. Kalish, B. G. Weniger, C. P. Pau, C. A. Schable, and J. W. Curran. 1996. The emerging genetic diversity of HIV. The importance of global surveillance for diagnostics, research, and prevention. JAMA **275:**210-216.

Hu, J., M. B. Gardner, and C. J. Miller. 2000. Simian immunodeficiency virus rapidly penetrates the cervicovaginal mucosa after intravaginal inoculation and infects intraepithelial dendritic cells. J. Virol. **74:**6087-6095.

Hu, W. S. and H. M. Temin. 1990. Retroviral recombination and reverse transcription. Science **250:**1227-1233.

University of Cape Town

Huang, M. and M. A. Martin. 1997. Incorporation of Pr160(gag-pol) into virus particles requires the presence of both the major homology region and adjacent C-terminal capsid sequences within the Gag-Pol polyprotein. J. Virol. **71:**4472-4478.

Huang, Y., W. A. Paxton, S. M. Wolinsky, A. U. Neumann, L. Zhang, T. He, S. Kang, D. Ceradini, Z. Jin, K. Yazdanbakhsh, K. Kunstman, D. Erickson, E. Dragon, N. R. Landau, J. Phair, D. D. Ho, and R. A. Koup. 1996. The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. Nat. Med. **2:**1240-1243.

Huet, T., R. Cheynier, A. Meyerhans, G. Roelants, and S. Wain-Hobson. 1990. Genetic organization of a chimpanzee lentivirus related to HIV-1. Nature **345:**356-359.

Hughes, E. S., J. E. Bell, and P. Simmonds. 1997. Investigation of the dynamics of the spread of human immunodeficiency virus to brain and other tissues by evolutionary analysis of sequences from the p17gag and env genes. J. Virol. **71:**1272-1280.

Hymes, K. B., T. Cheung, J. B. Greene, N. S. Prose, A. Marcus, H. Ballard, D. C. William, and L. J. Laubenstein. 1981. Kaposi's sarcoma in homosexual men-a report of eight cases. Lancet **2:**598-600.

Itescu, S., P. F. Simonelli, R. J. Winchester, and H. S. Ginsberg. 1994. Human immunodeficiency virus type 1 strains in the lungs of infected individuals evolve independently from those in peripheral blood and are highly conserved in the C-terminal region of the envelope V3 loop. Proc. Natl. Acad. Sci. U. S. A **91:**11378-11382.

Iversen, A. K., L. Fugger, J. Eugen-Olsen, U. Balslev, T. Jensen, S. Wahl, J. Gerstoft, J. I. Mullins, and P. Skinhoj. 1998. Cervical human immunodeficiency virus type 1 shedding is associated with genital beta-chemokine secretion. J. Infect. Dis. **178:**1334-1342.

Iversen, A. K., A. R. Larsen, T. Jensen, L. Fugger, U. Balslev, S. Wahl, J. Gerstoft, J. I. Mullins, and P. Skinhoj. 1998. Distinct determinants of human immunodeficiency virus type 1 RNA and DNA loads in vaginal and cervical secretions. J. Infect. Dis. **177:**1214-1220.

Janssens, W., J. N. Nkengasong, L. Heyndrickx, K. Fransen, P. M. Ndumbe, E. Delaporte, M. Peeters, J. L. Perret, A. Ndoumou, C. Atende, and . 1994. Further evidence of the presence of genetically aberrant HIV-1 strains in Cameroon and Gabon. AIDS **8:**1012-1013.

Janssens, W., A. Buve, and J. N. Nkengasong. 1997. The puzzle of HIV-1 subtypes in Africa. AIDS **11:**705-712.

Jetzt, A. E., H. Yu, G. J. Klarmann, Y. Ron, B. D. Preston, and J. P. Dougherty. 2000. High rate of recombination throughout the human immunodeficiency virus type 1 genome. J. Virol. **74:**1234-1240.

University of Cape Town

Johansson, B., K. Sherefa, and A. Sonnerborg. 1995. Multiple enhancer motifs in HIV type 1 strains from Ethiopia. AIDS Res. Hum. Retroviruses **11:**761-764.

Jonassen, T. O., B. Grinde, B. Asjo, G. Hasle, and O. Hungnes. 2000. Intersubtype recombinant HIV type 1 involving HIV-MAL-like and subtype H-like sequence in four Norwegian cases. AIDS Res. Hum. Retroviruses **16:**49-58.

Jones, K. A. and B. M. Peterlin. 1994. Control of RNA initiation and elongation at the HIV-1 promoter. Annu. Rev. Biochem. **63:**717-743.

Jowett, J. B., V. Planelles, B. Poon, N. P. Shah, M. L. Chen, and I. S. Chen. 1995. The human immunodeficiency virus type 1 vpr gene arrests infected T cells in the G2 + M phase of the cell cycle. J. Virol. **69:**6304-6313.

Jung, A., R. Maier, J. P. Vartanian, G. Bocharov, V. Jung, U. Fischer, E. Meese, S. Wain-Hobson, and A. Meyerhans. 2002. Multiply infected spleen cells in HIV patients. Nature **418:**144.

Junghans, R. P., L. R. Boone, and A. M. Skalka. 1982. Retroviral DNA H structures: displacement-assimilation model of recombination. Cell **30:**53-62.

Kajumo, F., D. A. Thompson, Y. Guo, and T. Dragic. 2000. Entry of R5X4 and X4 human immunodeficiency virus type 1 strains is mediated by negatively charged and tyrosine residues in the amino-terminal domain and the second extracellular loop of CXCR4. Virology **271:**240-247.

Kampinga, G. A., A. Simonon, P. P. Van de, E. Karita, P. Msellati, and J. Goudsmit. 1997. Primary infections with HIV-1 of women and their offspring in Rwanda: findings of heterogeneity at seroconversion, coinfection, and recombinants of HIV-1 subtypes A and C. Virology **227:**63-76.

Kanki, P. J., K. U. Travers, S. Mboup, C. C. Hsieh, R. G. Marlink, A. Gueye-Ndiaye, T. Siby, I. Thior, M. Hernandez-Avila, J. L. Sankale, and . 1994. Slower heterosexual spread of HIV-2 than HIV-1. Lancet **343:**943-946.

Kanki, P. J., D. J. Hamel, J. L. Sankale, C. Hsieh, I. Thior, F. Barin, S. A. Woodcock, A. Gueye-Ndiaye, E. Zhang, M. Montano, T. Siby, R. Marlink, I. NDoye, M. E. Essex, and S. Mboup. 1999. Human immunodeficiency virus type 1 subtypes differ in disease progression. J. Infect. Dis. **179:**68-73.

Karczewski, M. K. and K. Strebel. 1996. Cytoskeleton association and virion incorporation of the human immunodeficiency virus type 1 Vif protein. J. Virol. **70:**494-507.

Karim, S. S. and G. Ramjee. 1998. Anal sex and HIV transmission in women. Am. J. Public Health **88:**1265-1266.

University of Cape Town

Kawai, S. and H. Hanafusa. 1972. Genetic recombination with avian tumor virus. Virology **49:**37-44.

Kawamura, T., M. Qualbani, E. K. Thomas, J. M. Orenstein, and A. Blauvelt. 2001. Low levels of productive HIV infection in Langerhans cell-like dendritic cells differentiated in the presence of TGF-beta1 and increased viral replication with CD40 ligand-induced maturation. Eur. J. Immunol. **31:**360-368.

Kerkau, T., I. Bacik, J. R. Bennink, J. W. Yewdell, T. Hunig, A. Schimpl, and U. Schubert. 1997. The human immunodeficiency virus type 1 (HIV-1) Vpu protein interferes with an early step in the biosynthesis of major histocompatibility complex (MHC) class I molecules. J. Exp. Med. **185:**1295-1305.

Kestler, H. W., III, Y. Li, Y. M. Naidu, C. V. Butler, M. F. Ochs, G. Jaenel, N. W. King, M. D. Daniel, and R. C. Desrosiers. 1988. Comparison of simian immunodeficiency virus isolates. Nature **331:**619-622.

Kiernan, R. E., A. Ono, G. Englund, and E. O. Freed. 1998. Role of matrix in an early postentry step in the human immunodeficiency virus type 1 life cycle. J. Virol. **72:**4116-4126.

Kijak, G. H., V. Simon, P. Balfe, J. Vanderhoeven, S. E. Pampuro, C. Zala, C. Ochoa, P. Cahn, M. Markowitz, and H. Salomon. 2002. Origin of human immunodeficiency virus type 1 quasispecies emerging after antiretroviral treatment interruption in patients with therapeutic failure. J. Virol. **76:**7000-7009.

Kimura, M. 1980. A simple model for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J Mol Evol, **19:**111-120.

Kolchinsky, P., E. Kiprilov, P. Bartley, R. Rubinstein, and J. Sodroski. 2001. Loss of a single N-linked glycan allows CD4-independent human immunodeficiency virus type 1 infection by altering the position of the gp120 V1/V2 variable loops. J. Virol. **75:**3435-3443.

Kondo, E. and H. G. Gottlinger. 1996. A conserved LXXLF sequence is the major determinant in p6gag required for the incorporation of human immunodeficiency virus type 1 Vpr. J. Virol. **70:**159-164.

Kong, L. B., D. An, B. Ackerson, J. Canon, O. Rey, I. S. Chen, P. Krogstad, and P. L. Stewart. 1998. Cryoelectron microscopic examination of human immunodeficiency virus type 1 virions with mutations in the cyclophilin A binding loop. J. Virol. **72:**4403-4407.

Koot, M., I. P. Keet, A. H. Vos, R. E. de Goede, M. T. Roos, R. A. Coutinho, F. Miedema, P. T. Schellekens, and M. Tersmette. 1993. Prognostic value of HIV-1 syncytium-inducing phenotype for rate of CD4+ cell depletion and progression to AIDS. Ann. Intern. Med. **118:**681-688.

Kostrikis, L. G., E. Bagdades, Y. Cao, L. Zhang, D. Dimitriou, and D. D. Ho. 1995. Genetic analysis of human immunodeficiency virus type 1 strains from patients in Cyprus: identification of a new subtype designated subtype I. J. Virol. **69:**6122-6130.

University of Cape Town

Kramer, R. A., M. D. Schaber, A. M. Skalka, K. Ganguly, F. Wong-Staal, and E. P. Reddy. 1986. HTLV-III gag protein is processed in yeast cells by the virus pol-protease. Science **231:**1580-1584.

Krausslich, H. G., M. Facke, A. M. Heuser, J. Konvalinka, and H. Zentgraf. 1995. The spacer peptide between human immunodeficiency virus capsid and nucleocapsid proteins is essential for ordered assembly and viral infectivity. J. Virol. **69:**3407-3419.

Kroodsma, K. L., M. J. Kozal, K. A. Hamed, M. A. Winters, and T. C. Merigan. 1994. Detection of drug resistance mutations in the human immunodeficiency virus type 1 (HIV-1) pol gene: differences in semen and blood HIV-1 RNA and proviral DNA. J. Infect. Dis. **170:**1292-1295.

Kuiken, C.L., B. Foley, B. Hahn, B. Korber, F. McCutchan, P.A. Marx, J.W. Mellors, J.I. Mullins, J. Sodroski, S. Wolinsky. 2000. Human Retroviruses and AIDS. Theoretical and Biophysics Group, Los Alamos National Laboratory, Los Alamos, New Mexico.

Kuiken, C. L., J. Goudsmit, G. F. Weiller, J. S. Armstrong, S. Hartman, P. Portegies, J. Dekker, and M. Cornelissen. 1995. Differences in human immunodeficiency virus type 1 V3 sequences from patients with and without AIDS dementia complex. J. Gen. Virol. **76 (Pt 1):**175-180.

Kuiken, C. L., V. V. Lukashov, E. Baan, J. Dekker, J. A. Leunissen, and J. Goudsmit. 1996. Evidence for limited within-person evolution of the V3 domain of the HIV-1 envelope in the amsterdam population. AIDS **10:**31-37.

Kumar, S., K. Tamura, I. B. Jakobsen, and M. Nei. 2001. MEGA2: molecular evolutionary genetics analysis software. Bioinformatics. **17:**1244-1245.

Kwong, P. D., R. Wyatt, J. Robinson, R. W. Sweet, J. Sodroski, and W. A. Hendrickson. 1998. Structure of an HIV gp120 envelope glycoprotein in complex with the CD4 receptor and a neutralizing human antibody. Nature **393:**648-659.

Kwong, P. D., R. Wyatt, Q. J. Sattentau, J. Sodroski, and W. A. Hendrickson. 2000. Oligomeric modeling and electrostatic analysis of the gp120 envelope glycoprotein of human immunodeficiency virus. J. Virol. **74:**1961-1972.

Labrosse, B., C. Treboute, A. Brelot, and M. Alizon. 2001. Cooperation of the V1/V2 and V3 domains of human immunodeficiency virus type 1 gp120 for interaction with the CXCR4 receptor. J. Virol. **75:**5457-5464.

Larder, B. A. and S. D. Kemp. 1989. Multiple mutations in HIV-1 reverse transcriptase confer high-level resistance to zidovudine (AZT). Science **246:**1155-1158.

University of Cape Town

Laukkanen, T., J. K. Carr, W. Janssens, K. Liitsola, D. Gotte, F. E. McCutchan, d. C. Op, M. Cornelissen, L. Heyndrickx, G. G. van der, and M. O. Salminen. 2000. Virtually full-length subtype F and F/D recombinant HIV-1 from Africa and South America. Virology **269:**95-104.

Lawn, S. D., S. Subbarao, T. C. Wright, Jr., T. Evans-Strickfaden, T. V. Ellerbrock, J. L. Lennox, S. T. Butera, and C. E. Hart. 2000. Correlation between human immunodeficiency virus type 1 RNA levels in the female genital tract and immune activation associated with ulceration of the cervix. J. Infect. Dis. **181:**1950-1956.

Learn, G. H., D. Muthui, S. J. Brodie, T. Zhu, K. Diem, J. I. Mullins, and L. Corey. 2002. Virus population homogenization following acute human immunodeficiency virus type 1 infection. J. Virol. **76:**11953-11959.

Learn, G. H., Jr., B. T. Korber, B. Foley, B. H. Hahn, S. M. Wolinsky, and J. I. Mullins. 1996. Maintaining the integrity of human immunodeficiency virus sequence databases. J. Virol. **70:**5720-5730.

Lee, Y. M. and X. F. Yu. 1998. Identification and characterization of virus assembly intermediate complexes in HIV-1-infected CD4+ T cells. Virology **243:**78-93.

Leonard, C. K., M. W. Spellman, L. Riddle, R. J. Harris, J. N. Thomas, and T. J. Gregory. 1990. Assignment of intrachain disulfide bonds and characterization of potential glycosylation sites of the type 1 recombinant human immunodeficiency virus envelope glycoprotein (gp120) expressed in Chinese hamster ovary cells. J. Biol. Chem. **265:**10373-10382.

Levy J.A., H, Fraenkel-Conrat, and R.A. Owens. 1994. Chapter 5 – Viruses using reverse transcription during replication. pp125-141. In *Virology*. J.A Levy, H, Fraenkel-Conrat, and R.A. Owens (eds). Prentice-Hall, Inc., New Jersey.

Levy, J. A., A. D. Hoffman, S. M. Kramer, J. A. Landis, J. M. Shimabukuro, and L. S. Oshiro. 1984. Isolation of lymphocytopathic retroviruses from San Francisco patients with AIDS. Science **225:**840-842.

Liao, F., G. Alkhatib, K. W. Peden, G. Sharma, E. A. Berger, and J. M. Farber. 1997. STRL33, A novel chemokine receptor-like protein, functions as a fusion cofactor for both macrophage-tropic and T cell line-tropic HIV-1. J. Exp. Med. **185:**2015-2023.

Liitsola, K., I. Tashkinova, T. Laukkanen, G. Korovina, T. Smolskaja, O. Momot, N. Mashkilleyson, S. Chaplinskas, H. Brummer-Korvenkontio, J. Vanhatalo, P. Leinikki, and M. O. Salminen. 1998. HIV-1 genetic subtype A/B recombinant strain causing an explosive epidemic in injecting drug users in Kaliningrad. AIDS **12:**1907-1919.

University of Cape Town

Linial, M. and S. Brown. 1979. High-frequency recombination within the gag gene of Rous sarcoma virus. J. Virol. **31**:257-260.

Liu, R., W. A. Paxton, S. Choe, D. Ceradini, S. R. Martin, R. Horuk, M. E. MacDonald, H. Stuhlmann, R. A. Koup, and N. R. Landau. 1996. Homozygous defect in HIV-1 coreceptor accounts for resistance of some multiply-exposed individuals to HIV-1 infection. Cell **86**:367-377.

Liu, S. L., A. G. Rodrigo, R. Shankarappa, G. H. Learn, L. Hsu, O. Davidov, L. P. Zhao, and J. I. Mullins. 1996. HIV quasispecies and resampling. Science **273**:415-416.

Long, E. M., H. L. Martin, Jr., J. K. Kreiss, S. M. Rainwater, L. Lavreys, D. J. Jackson, J. Rakwar, K. Mandaliya, and J. Overbaugh. 2000. Gender differences in HIV-1 diversity at time of infection. Nat. Med. **6**:71-75.

Long, E. M., S. M. Rainwater, L. Lavreys, K. Mandaliya, and J. Overbaugh. 2002. HIV type 1 variants transmitted to women in Kenya require the CCR5 coreceptor for entry, regardless of the genetic complexity of the infecting virus. AIDS Res. Hum. Retroviruses **18**:567-576.

Loussert-Ajaka, I., M. L. Chaix, B. Korber, F. Letourneur, E. Gomas, E. Allen, T. D. Ly, F. Brun-Vezinet, F. Simon, and S. Saragosti. 1995. Variability of human immunodeficiency virus type 1 group O strains isolated from Cameroonian patients living in France. J. Virol. **69**:5640-5649.

Louwagie, J., F. E. McCutchan, M. Peeters, T. P. Brennan, E. Sanders-Buell, G. A. Eddy, G. G. van der, K. Fransen, G. M. Gershy-Damet, R. Deleys, and . 1993. Phylogenetic analysis of gag genes from 70 international HIV-1 isolates provides evidence for multiple genotypes. AIDS **7**:769-780.

Louwagie, J., W. Janssens, J. Mascola, L. Heyndrickx, P. Hegerich, G. G. van der, F. E. McCutchan, and D. S. Burke. 1995. Genetic diversity of the envelope glycoprotein from human immunodeficiency virus type 1 isolates of African origin. J. Virol. **69**:263-271.

Loveday, C. 2001. International perspectives on antiretroviral resistance. Nucleoside reverse transcriptase inhibitor resistance. J. Acquir. Immune. Defic. Syndr. **26 Suppl 1**:S10-S24.

Lowenstine, L. J., N. C. Pedersen, J. Higgins, K. C. Pallis, A. Uyeda, P. Marx, N. W. Lerche, R. J. Munn, and M. B. Gardner. 1986. Seroepidemiologic survey of captive Old-World primates for antibodies to human and simian retroviruses, and isolation of a lentivirus from sooty mangabeys (Cercocebus atys). Int. J. Cancer **38**:563-574.

Lu, Y. L., R. P. Bennett, J. W. Wills, R. Gorelick, and L. Ratner. 1995. A leucine triplet repeat sequence (LXX)4 in p6gag is important for Vpr incorporation into human immunodeficiency virus type 1 particles. J. Virol. **69**:6873-6879.

University of Cape Town

Lue, J., M. Hsu, D. Yang, P. Marx, Z. Chen, and C. Cheng-Mayer. 2002. Addition of a single gp120 glycan confers increased binding to dendritic cell-specific ICAM-3-grabbing nonintegrin and neutralization escape to human immunodeficiency virus type 1. J. Virol. **76:**10299-10306.

Maartens, G., R. Wood, E. O'Keefe, and C. Byrne. 1997. Independent epidemics of heterosexual and homosexual HIV infection in South Africa--survival differences. QJM. **90:**449-454.

Mann, J. M., N. Nzilambi, P. Piot, N. Bosenge, M. Kalala, H. Francis, R. C. Colebunders, P. K. Azila, J. W. Curran, and T. C. Quinn. 1988. HIV infection and associated risk factors in female prostitutes in Kinshasa, Zaire. AIDS **2:**249-254.

Martin, M. P., M. Carrington, M. Dean, S. J. O'Brien, H. W. Sheppard, S. A. Wegner, and N. L. Michael. 1998. CXCR4 polymorphisms and HIV-1 pathogenesis. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. **19:**430.

Marx, P. A., Y. Li, N. W. Lerche, S. Sutjipto, A. Gettie, J. A. Yee, B. H. Brotman, A. M. Prince, A. Hanson, R. G. Webster, and . 1991. Isolation of a simian immunodeficiency virus related to human immunodeficiency virus type 2 from a west African pet sooty mangabey. J. Virol. **65:**4480-4485.

Marx, P. A., A. I. Spira, A. Gettie, P. J. Dailey, R. S. Veazey, A. A. Lackner, C. J. Mahoney, C. J. Miller, L. E. Claypool, D. D. Ho, and N. J. Alexander. 1996. Progesterone implants enhance SIV vaginal transmission and early virus load. Nat. Med. **2:**1084-1089.

Masur, H., M. A. Michelis, J. B. Greene, I. Onorato, R. A. Stouwe, R. S. Holzman, G. Wormser, L. Brettman, M. Lange, H. W. Murray, and S. Cunningham-Rundles. 1981. An outbreak of community-acquired Pneumocystis carinii pneumonia: initial manifestation of cellular immune dysfunction. N. Engl. J. Med. **305:**1431-1438.

Mauclere, P., I. Loussert-Ajaka, F. Damond, P. Fagot, S. Souquieres, L. M. Monny, F. X. Mbopi Keou, F. Barre-Sinoussi, S. Saragosti, F. Brun-Vezinet, and F. Simon. 1997. Serological and virological characterization of HIV-1 group O infection in Cameroon. AIDS **11:**445-453.

McCormack, G. P., J. R. Glynn, A. C. Crampin, F. Sibande, D. Mulawa, L. Bliss, P. Broadbent, K. Abarca, J. M. Ponnighaus, P. E. Fine, and J. P. Clewley. 2002. Early evolution of the human immunodeficiency virus type 1 subtype C epidemic in rural Malawi. J. Virol. **76:**12890-12899.

McCutchan, F. E., A. W. Artenstein, E. Sanders-Buell, M. O. Salminen, J. K. Carr, J. R. Mascola, X. F. Yu, K. E. Nelson, C. Khamboonruang, D. Schmitt, M. P. Kieny, J. G. McNeil, and D. S. Burke. 1996. Diversity of the envelope glycoprotein among human immunodeficiency virus type 1 isolates of clade E from Asia and Africa. J. Virol. **70:**3331-3338.

University of Cape Town

McCutchan, F. E., J. K. Carr, M. Bajani, E. Sanders-Buell, T. O. Harry, T. C. Stoeckli, K. E. Robbins, W. Gashau, A. Nasidi, W. Janssens, and M. L. Kalish. 1999. Subtype G and multiple forms of A/G intersubtype recombinant human immunodeficiency virus type 1 in Nigeria. Virology **254:**226-234.

McCutchan, F. E. 2000. Understanding the genetic diversity of HIV-1. AIDS **14 Suppl 3:**S31-S44.

McMichael, A. J. and S. L. Rowland-Jones. 2001. Cellular immune responses to HIV. Nature **410:**980-987.

McNearney, T., Z. Hornickova, R. Markham, A. Birdwell, M. Arens, A. Saah, and L. Ratner. 1992. Relationship of human immunodeficiency virus type 1 sequence heterogeneity to stage of disease. Proc. Natl. Acad. Sci. U. S. A **89:**10247-10251.

Mellors, J. W., B. P. Griffith, M. A. Ortiz, M. L. Landry, and J. L. Ryan. 1991. Tumor necrosis factor-alpha/cachectin enhances human immunodeficiency virus type 1 replication in primary macrophages. J. Infect. Dis. **163:**78-82.

Mellors, J. W., A. Munoz, J. V. Giorgi, J. B. Margolick, C. J. Tassoni, P. Gupta, L. A. Kingsley, J. A. Todd, A. J. Saah, R. Detels, J. P. Phair, and C. R. Rinaldo, Jr. 1997. Plasma viral load and CD4+ lymphocytes as prognostic markers of HIV-1 infection. Ann. Intern. Med. **126:**946-954.

Meng, G., X. Wei, X. Wu, M. T. Sellers, J. M. Decker, Z. Moldoveanu, J. M. Orenstein, M. F. Graham, J. C. Kappes, J. Mestecky, G. M. Shaw, and P. D. Smith. 2002. Primary intestinal epithelial cells selectively transfer R5 HIV-1 to CCR5+ cells. Nat. Med. **8:**150-156.

Mervis, R. J., N. Ahmad, E. P. Lillehoj, M. G. Raum, F. H. Salazar, H. W. Chan, and S. Venkatesan. 1988. The gag gene products of human immunodeficiency virus type 1: alignment within the gag open reading frame, identification of posttranslational modifications, and evidence for alternative gag precursors. J. Virol. **62:**3993-4002.

Michael, NL. 1999. Host genetic influences on HIV-1 pathogenesis. Curr. Opin. Immunol. **11:**466-474.

Miller, C. J., N. J. Alexander, S. Sutjipto, A. A. Lackner, A. Gettie, A. G. Hendrickx, L. J. Lowenstine, M. Jennings, and P. A. Marx. 1989. Genital mucosal transmission of simian immunodeficiency virus: animal model for heterosexual transmission of human immunodeficiency virus. J. Virol. **63:**4277-4284.

Miller, R. H. and N. Sarver. 1997. HIV accessory proteins as therapeutic targets. Nat. Med. **3:**389-394.

Miller, V. 2001. International perspectives on antiretroviral resistance. Resistance to protease inhibitors. J. Acquir. Immune. Defic. Syndr. **26 Suppl 1:**S34-S50.

Mofenson, L. M., J. S. Lambert, E. R. Stiehm, J. Bethel, W. A. Meyer, III, J. Whitehouse, J. Moye, Jr., P. Reichelderfer, D. R. Harris, M. G. Fowler, B. J. Mathieson, and G. J. Nemo. 1999. Risk factors

University of Cape Town

for perinatal transmission of human immunodeficiency virus type 1 in women treated with zidovudine. Pediatric AIDS Clinical Trials Group Study 185 Team. N. Engl. J. Med. **341:**385-393.

Montagnier, L. 1988. Origin and evolution of HIVs and their role in AIDS pathogenesis. J. Acquir. Immune. Defic. Syndr. **1:**517-520.

Montavon, C., F. Bibollet-Ruche, D. Robertson, B. Koumare, C. Mulanga, E. Esu-Williams, C. Toure, S. Mboup, E. Saman, E. Delaporte, and M. Peeters. 1999. The identification of a complex A/G/I/J recombinant HIV type 1 virus in various West African countries. AIDS Res. Hum. Retroviruses **15:**1707-1712.

Moodley, D., T. L. Smith, E. J. van Rensburg, J. Moodley, and S. Engelbrecht. 1998. HIV type 1 V3 region subtyping in KwaZulu-Natal, a high-seroprevalence South African region. AIDS Res. Hum. Retroviruses **14:**1015-1018.

Moore, C. B., M. John, I. R. James, F. T. Christiansen, C. S. Witt, and S. A. Mallal. 2002. Evidence of HIV-1 adaptation to HLA-restricted immune responses at a population level. Science **296:**1439-1443.

Moore, J. P. and D. R. Burton. 1999. HIV-1 neutralizing antibodies: how full is the bottle? Nat. Med. **5:**142-144.

Moore, J. P., P. W. Parren, and D. R. Burton. 2001. Genetic subtypes, humoral immunity, and human immunodeficiency virus type 1 vaccine development. J. Virol. **75:**5721-5729.

Morgado, M., M. Guimaraes, and B. Galvao-Castro. 2002. HIV-1 Polymorphism: a Challenge for Vaccine Development - A Review. Mem. Inst. Oswaldo Cruz **97:**143-150.

Morris, L., T. Cilliers, H. Bredell, M. Phoswa, and D. J. Martin. 2001. CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis. AIDS Res. Hum. Retroviruses **17:**697-701.

Mostad, S. B., S. Jackson, J. Overbaugh, M. Reilly, B. Chohan, K. Mandaliya, P. Nyange, J. Ndinya-Achola, J. J. Bwayo, and J. K. Kreiss. 1998. Cervical and vaginal shedding of human immunodeficiency virus type 1-infected cells throughout the menstrual cycle. J. Infect. Dis. **178:**983-991.

Mulder, J., N. McKinney, C. Christopherson, J. Sninsky, L. Greenfield, and S. Kwok. 1994. Rapid and simple PCR assay for quantitation of human immunodeficiency virus type 1 RNA in plasma: application to acute retroviral infection. J. Clin. Microbiol. **32:**292-300.

Musicco, M., A. Lazzarin, A. Nicolosi, M. Gasparini, P. Costigliola, C. Arici, and A. Saracco. 1994. Antiretroviral treatment of men infected with human immunodeficiency virus type 1 reduces the incidence

University of Cape Town

of heterosexual transmission. Italian Study Group on HIV Heterosexual Transmission. Arch. Intern. Med. **154:**1971-1976.

Nasioulas, G., D. Paraskevis, E. Magiorkinis, M. Theodoridou, and A. Hatzakis. 1999. Molecular analysis of the full-length genome of HIV type 1 subtype I: evidence of A/G/I recombination. AIDS Res. Hum. Retroviruses **15:**745-758.

Nei, M. and T. Gojobori. 1986. Simple methods for estimating the numbers of synonymous and nonsynonymous nucleotide substitutions. Mol. Biol. Evol. **3:**418-426.

Neilson, J. R., G. C. John, J. K. Carr, P. Lewis, J. K. Kreiss, S. Jackson, R. W. Nduati, D. Mbori-Ngacha, D. D. Panteleeff, S. Bodrug, C. Giachetti, M. A. Bott, B. A. Richardson, J. Bwayo, J. Ndinya-Achola, and J. Overbaugh. 1999. Subtypes of human immunodeficiency virus type 1 and disease stage among women in Nairobi, Kenya. J. Virol. **73:**4393-4403.

Nelson, J. A., S. A. Fiscus, and R. Swanstrom. 1997. Evolutionary variants of the human immunodeficiency virus type 1 V3 region characterized by using a heteroduplex tracking assay. J. Virol. **71:**8750-8758.

Nkengasong, J. N., W. Janssens, L. Heyndrickx, K. Fransen, P. M. Ndumbe, J. Motte, A. Leonaers, M. Ngolle, J. Ayuk, P. Piot, and . 1994. Genotypic subtypes of HIV-1 in Cameroon. AIDS **8:**1405-1412.

Novitsky, V., P. O. Flores-Villanueva, P. Chigwedere, S. Gaolekwe, H. Bussman, G. Sebetso, R. Marlink, E. J. Yunis, and M. Essex. 2001. Identification of most frequent HLA class I antigen specificities in Botswana: relevance for HIV vaccine design. Hum. Immunol. **62:**146-156.

Novitsky, V., U. R. Smith, P. Gilbert, M. F. McLane, P. Chigwedere, C. Williamson, T. Ndung'u, I. Klein, S. Y. Chang, T. Peter, I. Thior, B. T. Foley, S. Gaolekwe, N. Rybak, S. Gaseitsiwe, F. Vannberg, R. Marlink, T. H. Lee, and M. Essex. 2002. Human immunodeficiency virus type 1 subtype C molecular phylogeny: consensus sequence for an AIDS vaccine design? J. Virol. **76:**5435-5451.

Nowak, M. A. 1992. Variability of HIV infections. J. Theor. Biol. **155:**1-20.

Nowak, M. A., R. M. May, R. E. Phillips, S. Rowland-Jones, D. G. Lalloo, S. McAdam, P. Klenerman, B. Koppe, K. Sigmund, C. R. Bangham, and . 1995. Antigenic oscillations and shifting immunodominance in HIV-1 infections. Nature **375:**606-611.

Nutt, R. F., S. F. Brady, P. L. Darke, T. M. Ciccarone, C. D. Colton, E. M. Nutt, J. A. Rodkey, C. D. Bennett, L. H. Waxman, I. S. Sigal, and . 1988. Chemical synthesis and enzymatic activity of a 99-residue peptide with a sequence proposed for the human immunodeficiency virus protease. Proc. Natl. Acad. Sci. U. S. A **85:**7129-7133.

University of Cape Town

Oelrichs, R. B., C. Workman, T. Laukkanen, F. E. McCutchan, and N. J. Deacon. 1998. A novel subtype A/G/J recombinant full-length HIV type 1 genome from Burkina Faso. AIDS Res. Hum. Retroviruses **14:**1495-1500.

Oelrichs, R. B., V. A. Lawson, K. M. Coates, C. Chatfield, N. J. Deacon, and D. A. McPhee. 2000. Rapid full-length genomic sequencing of two cytopathically heterogeneous Australian primary HIV-1 isolates. J. Biomed. Sci. **7:**128-135.

Oelrichs, R. B., I. L. Shrestha, D. A. Anderson, and N. J. Deacon. 2000. The explosive human immunodeficiency virus type 1 epidemic among injecting drug users of Kathmandu, Nepal, is caused by a subtype C virus of restricted genetic diversity. J. Virol. **74:**1149-1157.

Ogert, R. A., M. K. Lee, W. Ross, A. Buckler-White, M. A. Martin, and M. W. Cho. 2001. N-linked glycosylation sites adjacent to and within the V1/V2 and the V3 loops of dualtropic human immunodeficiency virus type 1 isolate DH12 gp120 affect coreceptor usage and cellular tropism. J. Virol. **75:**5998-6006.

Op, d. C., S. A. van der, J. Goudsmit, B. R. van den, W. Janssens, L. Heyndrickx, G. G. van der, and M. Cornelissen. 2000. Independent introduction of transmissible F/D recombinant HIV-1 from Africa into Belgium and The Netherlands. Virology **270:**267-277.

Overbaugh, J. and C. R. Bangham. 2001. Selection forces and constraints on retroviral sequence variation. Science **292:**1106-1109.

Paladin, F. J., O. T. Monzon, H. Tsuchie, M. R. Aplasca, G. H. Learn, Jr., and T. Kurimura. 1998. Genetic subtypes of HIV-1 in the Philippines. AIDS **12:**291-300.

Palker, T. J., M. E. Clark, A. J. Langlois, T. J. Matthews, K. J. Weinhold, R. R. Randall, D. P. Bolognesi, and B. F. Haynes. 1988. Type-specific neutralization of the human immunodeficiency virus with antibodies to env-encoded synthetic peptides. Proc. Natl. Acad. Sci. U. S. A **85:**1932-1936.

Pantaleo, G. and A. S. Fauci. 1995. New concepts in the immunopathogenesis of HIV infection. Annu. Rev. Immunol. **13:**487-512.

Panther, L. A., L. Tucker, C. Xu, R. E. Tuomala, J. I. Mullins, and D. J. Anderson. 2000. Genital tract human immunodeficiency virus type 1 (HIV-1) shedding and inflammation and HIV-1 env diversity in perinatal HIV-1 transmission. J. Infect. Dis. **181:**555-563.

Parren, P. W., J. P. Moore, D. R. Burton, and Q. J. Sattentau. 1999. The neutralizing antibody response to HIV-1: viral evasion and escape from humoral immunity. AIDS **13 Suppl A:**S137-S162.

University of Cape Town

Parren, P. W. and D. R. Burton. 2001. The antiviral activity of antibodies in vitro and in vivo. Adv. Immunol. **77:**195-262.

Pathak, V. K. and H. M. Temin. 1990. Broad spectrum of in vivo forward mutations, hypermutations, and mutational hotspots in a retroviral shuttle vector after a single replication cycle: substitutions, frameshifts, and hypermutations. Proc. Natl. Acad. Sci. U. S. A **87:**6019-6023.

Peeters, M., C. Honore, T. Huet, L. Bedjabaga, S. Ossari, P. Bussi, R. W. Cooper, and E. Delaporte. 1989. Isolation and partial characterization of an HIV-related virus occurring naturally in chimpanzees in Gabon. AIDS **3:**625-630.

Peeters, M., A. Gueye, S. Mboup, F. Bibollet-Ruche, E. Ekaza, C. Mulanga, R. Ouedrago, R. Gandji, P. Mpele, G. Dibanga, B. Koumare, M. Saidou, E. Esu-Williams, J. P. Lombart, W. Badombena, N. Luo, M. Vanden Haesevelde, and E. Delaporte. 1997. Geographical distribution of HIV-1 group O viruses in Africa. AIDS **11:**493-498.

Peeters, M., F. Liegeois, N. Torimiro, A. Bourgeois, E. Mpoudi, L. Vergne, E. Saman, E. Delaporte, and S. Saragosti. 1999. Characterization of a highly replicative intergroup M/O human immunodeficiency virus type 1 recombinant isolated from a Cameroonian patient. J. Virol. **73:**7368-7375.

Pettit, S. C., M. D. Moody, R. S. Wehbie, A. H. Kaplan, P. V. Nantermet, C. A. Klein, and R. Swanstrom. 1994. The p2 domain of human immunodeficiency virus type 1 Gag regulates sequential proteolytic processing and is required to produce fully infectious virions. J. Virol. **68:**8017-8027.

Phillips, R. E., S. Rowland-Jones, D. F. Nixon, F. M. Gotch, J. P. Edwards, A. O. Ogunlesi, J. G. Elvin, J. A. Rothbard, C. R. Bangham, C. R. Rizza, and . 1991. Human immunodeficiency virus genetic variation that can escape cytotoxic T cell recognition. Nature **354:**453-459.

Piguet, V. and A. Blauvelt. 2002. Essential roles for dendritic cells in the pathogenesis and potential treatment of HIV disease. J. Invest Dermatol. **119:**365-369.

Ping, L. H., J. A. Nelson, I. F. Hoffman, J. Schock, S. L. Lamers, M. Goodman, P. Vernazza, P. Kazembe, M. Maida, D. Zimba, M. M. Goodenow, J. J. Eron, Jr., S. A. Fiscus, M. S. Cohen, and R. Swanstrom. 1999. Characterization of V3 sequence heterogeneity in subtype C human immunodeficiency virus type 1 isolates from Malawi: underrepresentation of X4 variants. J. Virol. **73:**6271-6281.

Piyasirisilp, S., F. E. McCutchan, J. K. Carr, E. Sanders-Buell, W. Liu, J. Chen, R. Wagner, H. Wolf, Y. Shao, S. Lai, C. Beyrer, and X. F. Yu. 2000. A recent outbreak of human immunodeficiency virus type 1 infection in southern China was initiated by two highly homogeneous, geographically separated strains, circulating recombinant form AE and a novel BC recombinant. J. Virol. **74:**11286-11295.

University of Cape Town

Poiesz, B. J., F. W. Ruscetti, A. F. Gazdar, P. A. Bunn, J. D. Minna, and R. C. Gallo. 1980. Detection and isolation of type C retrovirus particles from fresh and cultured lymphocytes of a patient with cutaneous T-cell lymphoma. Proc. Natl. Acad. Sci. U. S. A **77:**7415-7419.

Poignard, P., T. Fouts, D. Naniche, J. P. Moore, and Q. J. Sattentau. 1996. Neutralizing antibodies to human immunodeficiency virus type-1 gp120 induce envelope glycoprotein subunit dissociation. J. Exp. Med. **183:**473-484.

Pomerantz, R. J., S. M. de la Monte, S. P. Donegan, T. R. Rota, M. W. Vogt, D. E. Craven, and M. S. Hirsch. 1988. Human immunodeficiency virus (HIV) infection of the uterine cervix. Ann. Intern. Med. **108:**321-327.

Poon, D. T., J. Wu, and A. Aldovini. 1996. Charged amino acid residues of human immunodeficiency virus type 1 nucleocapsid p7 protein involved in RNA packaging and infectivity. J. Virol. **70:** 6607-6616.

Poss, M., H. L. Martin, J. K. Kreiss, L. Granville, B. Chohan, P. Nyange, K. Mandaliya, and J. Overbaugh. 1995. Diversity in virus populations from genital secretions and peripheral blood from women recently infected with human immunodeficiency virus type 1. J. Virol. **69:**8118-8122.

Poss, M., A. G. Rodrigo, J. J. Gosink, G. H. Learn, P. D. de Vange, H. L. Martin, Jr., J. Bwayo, J. K. Kreiss, and J. Overbaugh. 1998. Evolution of envelope sequences from the genital tract and peripheral blood of women infected with clade A human immunodeficiency virus type 1. J. Virol. **72:**8240-8251.

Pratt, R. D., J. F. Shapiro, N. McKinney, S. Kwok, and S. A. Spector. 1995. Virologic characterization of primary human immunodeficiency virus type 1 infection in a health care worker following needlestick injury. J. Infect. Dis. **172:**851-854.

Preston, B. D., B. J. Poiesz, and L. A. Loeb. 1988. Fidelity of HIV-1 reverse transcriptase. Science **242:**1168-1171.

Pudney, J. and D. Anderson. 1991. Orchitis and human immunodeficiency virus type 1 infected cells in reproductive tissues from men with the acquired immune deficiency syndrome. Am. J. Pathol. **139:**149-160.

Quillent, C., E. Oberlin, J. Braun, D. Rousset, G. Gonzalez-Canali, P. Metais, L. Montagnier, J. L. Virelizier, F. Arenzana-Seisdedos, and A. Beretta. 1998. HIV-1-resistance phenotype conferred by combination of two separate inherited mutations of CCR5 gene. Lancet **351:** 14-18.

Quinn, T. C. 1996. Global burden of the HIV pandemic. Lancet **348:**99-106.

University of Cape Town

Quinn, T. C., M. J. Wawer, N. Sewankambo, D. Serwadda, C. Li, F. Wabwire-Mangen, M. O. Meehan, T. Lutalo, and R. H. Gray. 2000. Viral load and heterosexual transmission of human immunodeficiency virus type 1. Rakai Project Study Group. N. Engl. J. Med. **342:**921-929.

Quinones-Mateu, M. E., Y. Gao, S. C. Ball, A. J. Marozsan, A. Abraha, and E. J. Arts. 2002. In vitro intersubtype recombinants of human immunodeficiency virus type 1: comparison to recent and circulating in vivo recombinant forms. J. Virol. **76:**9600-9613.

Ramjee, G., N. S. Morar, M. Alary, L. Mukenge-Tshibaka, B. Vuylsteke, V. Ettiegne-Traore, V. Chandeying, S. A. Karim, and L. Van Damme. 2000. Challenges in the conduct of vaginal microbicide effectiveness trials in the developing world. AIDS **14:**2553-2557.

Ramjee, G. and a. E. Gouws. 2002. Prevalence of HIV among truck drivers visiting sex workers in KwaZulu-Natal, South Africa. Sex Transm. Dis. **29:**44-49.

Ratner, L., R. C. Gallo, and F. Wong-Staal. 1985. HTLV-III, LAV, ARV are variants of same AIDS virus. Nature **313:**636-637.

Ray, S. C. and T. C. Quinn. 2000. Sex and the genetic diversity of HIV-1. Nat. Med. **6:**23-25.

Raymond, F. L., A. J. Caton, N. J. Cox, A. P. Kendal, and G. G. Brownlee. 1986. The antigenicity and evolution of influenza H1 haemagglutinin, from 1950-1957 and 1977-1983: two pathways from one gene. Virology **148:**275-287.

Reece, J. C., A. J. Handley, E. J. Anstee, W. A. Morrison, S. M. Crowe, and P. U. Cameron. 1998. HIV-1 selection by epidermal dendritic cells during transmission across human skin. J. Exp. Med. **187:**1623-1631.

Reichelderfer, P. S., R. W. Coombs, D. J. Wright, J. Cohn, D. N. Burns, S. Cu-Uvin, P. A. Baron, M. H. Coheng, A. L. Landay, S. K. Beckner, S. R. Lewis, and A. A. Kovacs. 2000. Effect of menstrual cycle on HIV-1 levels in the peripheral blood and genital tract. WHS 001 Study Team. AIDS **14:**2101-2107.

Report from the European Commission (DG XII, INCO-DC) and the Joint United Nations Programme on HIV/AIDS 1997. HIV-1 subtypes: implications for epidemiology, pathogenicity, vaccines and diagnostics. Workshop. AIDS **11:**17-36.

Robertson, D. L., P. M. Sharp, F. E. McCutchan, and B. H. Hahn. 1995. Recombination in HIV-1. Nature **374:**124-126.

**Robertson, D. L., J. P. Anderson, J. A. Bradac, J. K. Carr, B. Foley, R. K. Funkhouser, F. Gao, B. H. Hahn, M. L. Kalish, C. Kuiken, G. H. Learn, T. Leitner, F. McCutchan, S. Osmanov, M. Peeters, D.

University of Cape Town

Pieniazek, M. Salminen, P. M. Sharp, S. Wolinsky, and B. Korber. 2000. HIV-1 nomenclature proposal. Science **288:**55-56.

Rodrigo, A. G., P. C. Goracke, K. Rowhanian, and J. I. Mullins. 1997. Quantitation of target molecules from polymerase chain reaction-based limiting dilution assays. AIDS Res. Hum. Retroviruses **13:**737-742.

Rucker, J., A. L. Edinger, M. Sharron, M. Samson, B. Lee, J. F. Berson, Y. Yi, B. Margulies, R. G. Collman, B. J. Doranz, M. Parmentier, and R. W. Doms. 1997. Utilization of chemokine receptors, orphan receptors, and herpesvirus-encoded receptors by diverse human and simian immunodeficiency viruses. J. Virol. **71:**8999-9007.

Samson, M., F. Libert, B. J. Doranz, J. Rucker, C. Liesnard, C. M. Farber, S. Saragosti, C. Lapoumeroulie, J. Cognaux, C. Forceille, G. Muyldermans, C. Verhofstede, G. Burtonboy, M. Georges, T. Imai, S. Rana, Y. Yi, R. J. Smyth, R. G. Collman, R. W. Doms, G. Vassart, and M. Parmentier. 1996. Resistance to HIV-1 infection in caucasian individuals bearing mutant alleles of the CCR-5 chemokine receptor gene. Nature **382:**722-725.

Sandefur, S., V. Varthakavi, and P. Spearman. 1998. The I domain is required for efficient plasma membrane binding of human immunodeficiency virus type 1 Pr55Gag. J. Virol. **72:**2723-2732.

Sanders, R. W., M. Venturi, L. Schiffner, R. Kalyanaraman, H. Katinger, K. O. Lloyd, P. D. Kwong, and J. P. Moore. 2002. The mannose-dependent epitope for neutralizing antibody 2G12 on human immunodeficiency virus type 1 glycoprotein gp120. J. Virol. **76:**7293-7305.

Sanger, F., S. Nicklen, and A. Coulson. 1977. DNA Sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. U.S.A. **74**:5463-5467

Scanlan, C. N., R. Pantophlet, M. R. Wormald, S. E. Ollmann, R. Stanfield, I. A. Wilson, H. Katinger, R. A. Dwek, P. M. Rudd, and D. R. Burton. 2002. The broadly neutralizing anti-human immunodeficiency virus type 1 antibody 2G12 recognizes a cluster of alpha1-->2 mannose residues on the outer face of gp120. J. Virol. **76:**7306-7321.

Scarlatti, G., E. Tresoldi, A. Bjorndal, R. Fredriksson, C. Colognesi, H. K. Deng, M. S. Malnati, A. Plebani, A. G. Siccardi, D. R. Littman, E. M. Fenyo, and P. Lusso. 1997. In vivo evolution of HIV-1 co-receptor usage and sensitivity to chemokine-mediated suppression. Nat. Med. **3:**1259-1265.

Schneider, T. D. and R. M. Stephens. 1990. Sequence logos: a new way to display consensus sequences. Nucleic Acids Res. **18:**6097-6100.

University of Cape Town

Schuitemaker, H., M. Koot, N. A. Kootstra, M. W. Dercksen, R. E. de Goede, R. P. van Steenwijk, J. M. Lange, J. K. Schattenkerk, F. Miedema, and M. Tersmette. 1992. Biological phenotype of human immunodeficiency virus type 1 clones at different stages of infection: progression of disease is associated with a shift from monocytotropic to T-cell-tropic virus population. J. Virol. **66:**1354-1360.

Schwartz, M. D., D. Fiore, and A. T. Panganiban. 1997. Distinct functions and requirements for the Cys-His boxes of the human immunodeficiency virus type 1 nucleocapsid protein during RNA encapsidation and replication. J. Virol. **71:**9295-9305.

Shaffer, N., R. Chuachoowong, P. A. Mock, C. Bhadrakom, W. Siriwasin, N. L. Young, T. Chotpitayasunondh, S. Chearskul, A. Roongpisuthipong, P. Chinayon, J. Karon, T. D. Mastro, and R. J. Simonds. 1999. Short-course zidovudine for perinatal HIV-1 transmission in Bangkok, Thailand: a randomised controlled trial. Bangkok Collaborative Perinatal HIV Transmission Study Group. Lancet **353:**773-780.

Shankarappa, R., J. B. Margolick, S. J. Gange, A. G. Rodrigo, D. Upchurch, H. Farzadegan, P. Gupta, C. R. Rinaldo, G. H. Learn, X. He, X. L. Huang, and J. I. Mullins. 1999. Consistent viral evolutionary changes associated with the progression of human immunodeficiency virus type 1 infection. J. Virol. **73:**10489-10502.

Shankarappa, R., R. Chatterjee, G. H. Learn, D. Neogi, M. Ding, P. Roy, A. Ghosh, L. Kingsley, L. Harrison, J. I. Mullins, and P. Gupta. 2001. Human immunodeficiency virus type 1 env sequences from Calcutta in eastern India: identification of features that distinguish subtype C sequences in India from other subtype C sequences. J. Virol. **75:**10479-10487.

Shanker, G., M. Sorci-Thomas, and M. R. Adams. 1994. Estrogen modulates the expression of tumor necrosis factor alpha mRNA in phorbol ester-stimulated human monocytic THP-1 cells. Lymphokine Cytokine Res. **13:**377-382.

Sharp, P. M., D. L. Robertson, and B. H. Hahn. 1995. Cross-species transmission and recombination of 'AIDS' viruses. Philos. Trans. R. Soc. Lond B Biol. Sci. **349:**41-47.

Shattock, R. J., G. E. Griffin, and G. I. Gorodeski. 2000. In vitro models of mucosal HIV transmission. Nat. Med. **6:**607-608.

Sher, R. 1989. HIV infection in South Africa, 1982-1988--a review. S. Afr. Med. J. **76:**314-318.

Simon, F., P. Mauclere, P. Roques, I. Loussert-Ajaka, M. C. Muller-Trutwin, S. Saragosti, M. C. Georges-Courbot, F. Barre-Sinoussi, and F. Brun-Vezinet. 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. Nat. Med. **4:**1032-1037.

University of Cape Town

Simon, J. H. and M. H. Malim. 1996. The human immunodeficiency virus type 1 Vif protein modulates the postpenetration stability of viral nucleoprotein complexes. J. Virol. **70:**5297-5305.

Smith, T. L., E. J. van Rensburg, and S. Engelbrecht. 1998. Neutralization of HIV-1 subtypes: implications for vaccine formulations. J. Med. Virol. **56:**264-268.

Soriano, V., M. Gutierrez, G. Garcia-Lerma, O. Aguilera, A. Mas, R. Bravo, M. L. Perez-Labad, M. Baquero, and J. Gonzalez-Lahoz. 1996. First case of HIV-1 group O infection in Spain. Vox Sang. **71:**66.

Soto-Ramirez, L. E., B. Renjifo, M. F. McLane, R. Marlink, C. O'Hara, R. Sutthent, C. Wasi, P. Vithayasai, V. Vithayasai, C. Apichartpiyakul, P. Auewarakul, C. Pena, V, D. S. Chui, R. Osathanondh, K. Mayer, T. H. Lee, and M. Essex. 1996. HIV-1 Langerhans' cell tropism associated with heterosexual transmission of HIV. Science **271:**1291-1293.

Spearman, P., R. Horton, L. Ratner, and I. Kuli-Zade. 1997. Membrane binding of human immunodeficiency virus type 1 matrix protein in vivo supports a conformational myristyl switch mechanism. J. Virol. **71:**6582-6592.

Sperling, R. S., D. E. Shapiro, R. W. Coombs, J. A. Todd, S. A. Herman, G. D. McSherry, M. J. O'Sullivan, R. B. Van Dyke, E. Jimenez, C. Rouzioux, P. M. Flynn, and J. L. Sullivan. 1996. Maternal viral load, zidovudine treatment, and the risk of transmission of human immunodeficiency virus type 1 from mother to infant. Pediatric AIDS Clinical Trials Group Protocol 076 Study Group. N. Engl. J. Med. **335:**1621-1629.

Spira, A. I., P. A. Marx, B. K. Patterson, J. Mahoney, R. A. Koup, S. M. Wolinsky, and D. D. Ho. 1996. Cellular targets of infection and route of viral dissemination after an intravaginal inoculation of simian immunodeficiency virus into rhesus macaques. J. Exp. Med. **183:**215-225.

Sterling, T. R., C. M. Lyles, D. Vlahov, J. Astemborski, J. B. Margolick, and T. C. Quinn. 1999. Sex differences in longitudinal human immunodeficiency virus type 1 RNA levels among seroconverters. J. Infect. Dis. **180:**666-672.

Sterling, T. R., D. Vlahov, J. Astemborski, D. R. Hoover, J. B. Margolick, and T. C. Quinn. 2001. Initial plasma HIV-1 RNA levels and progression to AIDS in women and men. N. Engl. J. Med. **344:**720-725.

Su, L., M. Graf, Y. Zhang, H. von Briesen, H. Xing, J. Kostler, H. Melzl, H. Wolf, Y. Shao, and R. Wagner. 2000. Characterization of a virtually full-length human immunodeficiency virus type 1 genome of a prevalent intersubtype (C/B') recombinant strain in China. J. Virol. **74:**11367-11376.

University of Cape Town

Subbarao, S., K. Limpakarnjanarat, T. D. Mastro, J. Bhumisawasdi, P. Warachit, C. Jayavasu, N. L. Young, C. C. Luo, N. Shaffer, M. L. Kalish, and G. Schochetman. 1998. HIV type 1 in Thailand, 1994-1995: persistence of two subtypes with low genetic diversity. AIDS Res. Hum. Retroviruses **14:**319-327.

Takehisa, J., L. Zekeng, E. Ido, Y. Yamaguchi-Kabata, I. Mboudjeka, Y. Harada, T. Miura, L. Kaptu, and M. Hayami. 1999. Human immunodeficiency virus type 1 intergroup (M/O) recombination in cameroon. J. Virol. **73:**6810-6820.

Taubenberger, J. K., A. H. Reid, and T. G. Fanning. 2000. The 1918 influenza virus: A killer comes into view. Virology **274:**241-245.

Tersmette, M., R. E. de Goede, B. J. Al, I. N. Winkel, R. A. Gruters, H. T. Cuypers, H. G. Huisman, and F. Miedema. 1988. Differential syncytium-inducing capacity of human immunodeficiency virus isolates: frequent detection of syncytium-inducing isolates in patients with acquired immunodeficiency syndrome (AIDS) and AIDS-related complex. J. Virol. **62:**2026-2032.

Thompson, J. D., T. J. Gibson, F. Plewniak, F. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL_X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. **25:**4876-4882.

Tien, P. C., T. Chiu, A. Latif, S. Ray, M. Batra, C. H. Contag, L. Zejena, M. Mbizvo, E. L. Delwart, J. I. Mullins, and D. A. Katzenstein. 1999. Primary subtype C HIV-1 infection in Harare, Zimbabwe. J. Acquir. Immune. Defic. Syndr. Hum. Retrovirol. **20:**147-153.

Trask, S. A., C. A. Derdeyn, U. Fideli, Y. Chen, S. Meleth, F. Kasolo, R. Musonda, E. Hunter, F. Gao, S. Allen, and B. H. Hahn. 2002. Molecular epidemiology of human immunodeficiency virus type 1 transmission in a heterosexual cohort of discordant couples in Zambia. J. Virol. **76:**397-405.

Trkola, A., A. B. Pomales, H. Yuan, B. Korber, P. J. Maddon, G. P. Allaway, H. Katinger, C. F. Barbas, III, D. R. Burton, D. D. Ho, and . 1995. Cross-clade neutralization of primary isolates of human immunodeficiency virus type 1 by human monoclonal antibodies and tetrameric CD4-IgG. J. Virol. **69:**6609-6617.

Trkola, A., T. Dragic, J. Arthos, J. M. Binley, W. C. Olson, G. P. Allaway, C. Cheng-Mayer, J. Robinson, P. J. Maddon, and J. P. Moore. 1996. CD4-dependent, antibody-sensitive interactions between HIV-1 and its co-receptor CCR-5. Nature **384:**184-187.

Tscherning, C., A. Alaeus, R. Fredriksson, A. Bjorndal, H. Deng, D. R. Littman, E. M. Fenyo, and J. Albert. 1998. Differences in chemokine coreceptor usage between genetic subtypes of HIV-1. Virology **241:**181-188.

University of Cape Town

Tsujimoto, H., R. W. Cooper, T. Kodama, M. Fukasawa, T. Miura, Y. Ohta, K. Ishikawa, M. Nakai, E. Frost, G. E. Roelants, and . 1988. Isolation and characterization of simian immunodeficiency virus from mandrills in Africa and its relationship to other human and simian immunodeficiency viruses. J. Virol. **62:**4044-4050.

Turville, S. G., P. U. Cameron, J. Arthos, K. MacDonald, G. Clark, D. Hart, and A. L. Cunningham. 2001. Bitter-sweet symphony: defining the role of dendritic cell gp120 receptors in HIV infection. J. Clin. Virol. **22:**229-239.

Turville, S. G., P. U. Cameron, A. Handley, G. Lin, S. Pohlmann, R. W. Doms, and A. L. Cunningham. 2002. Diversity of receptors binding HIV on dendritic cell subsets. Nat. Immunol. **3:**975-983.

UNAIDS and WHO (World Health Organization). 2002. Report on the global HIV/AIDS epidemic.

Upchurch, D. A., R. Shankarappa, and J. I. Mullins. 2000. Position and degree of mismatches and the mobility of DNA heteroduplexes. Nucleic Acids Res. **28:**E69.

van't Wout, A. B., N. A. Kootstra, G. A. Mulder-Kampinga, N. Albrecht-van Lent, H. J. Scherpbier, J. Veenstra, K. Boer, R. A. Coutinho, F. Miedema, and H. Schuitemaker. 1994. Macrophage-tropic variants initiate human immunodeficiency virus type 1 infection after sexual, parenteral, and vertical transmission. J. Clin. Invest **94:**2060-2067.

Van Damme, L., G. Ramjee, M. Alary, B. Vuylsteke, V. Chandeying, H. Rees, P. Sirivongrangson, L. Mukenge-Tshibaka, V. Ettiegne-Traore, C. Uaheowitchai, S. S. Karim, B. Masse, J. Perriens, and M. Laga. 2002. Effectiveness of COL-1492, a nonoxynol-9 vaginal gel, on HIV-1 transmission in female sex workers: a randomised controlled trial. Lancet **360:**971-977.

Van de Peer, Y., de Wachter, R. 1994. TREECON for Windows: A softward package for the contruction and drawing of evolutionary trees for the Microsoft Windows environment. Comput. Applic. Biosci. **10:**569:570.

van Gemen, B., R. van Beuningen, A. Nabbe, D. van Strijp, S. Jurriaans, P. Lens, and T. Kievits. 1994. A one-tube quantitative HIV-1 RNA NASBA nucleic acid amplification assay using electrochemiluminescent (ECL) labelled probes. J. Virol. Methods **49:**157-167.

van Griensven, G. J., N. A. Hessol, B. A. Koblin, R. H. Byers, P. M. O'Malley, N. Albercht-van Lent, S. P. Buchbinder, P. E. Taylor, C. E. Stevens, and R. A. Coutinho. 1993. Epidemiology of human immunodeficiency virus type 1 infection among homosexual men participating in hepatitis B vaccine trials in Amsterdam, New York City, and San Francisco, 1978-1990. Am. J. Epidemiol. **137:**909-915.

University of Cape Town

van Harmelen, J., R. Wood, M. Lambrick, E. P. Rybicki, A. L. Williamson, and C. Williamson. 1997. An association between HIV-1 subtypes and mode of transmission in Cape Town, South Africa. AIDS **11:**81-87.

van Harmelen, J., C. Williamson, B. Kim, L. Morris, J. Carr, S. S. Karim, and F. McCutchan. 2001. Characterization of full-length HIV type 1 subtype C sequences from South Africa. AIDS Res. Hum. Retroviruses **17:**1527-1531.

Van Harmelen, J. H., R. E. Van der, A. S. Loubser, D. York, S. Madurai, S. Lyons, R. Wood, and C. Williamson. 1999. A predominantly HIV type 1 subtype C-restricted epidemic in South African urban populations. AIDS Res. Hum. Retroviruses **15:**395-398.

Vanden Haesevelde, M., J. L. Decourt, R. J. De Leys, B. Vanderborght, G. G. van der, H. van Heuverswijn, and E. Saman. 1994. Genomic cloning and complete sequence analysis of a highly divergent African human immunodeficiency virus isolate. J. Virol. **68:**1586-1596.

Vanden Haesevelde, M. M., M. Peeters, G. Jannes, W. Janssens, G. G. van der, P. M. Sharp, and E. Saman. 1996. Sequence analysis of a highly divergent HIV-1-related lentivirus isolated from a wild captured chimpanzee. Virology **221:**346-350.

Vassiliadou, N., L. Tucker, and D. J. Anderson. 1999. Progesterone-induced inhibition of chemokine receptor expression on peripheral blood mononuclear cells correlates with reduced HIV-1 infectability in vitro. J. Immunol. **162:**7510-7518.

Vernazza, P. L., B. L. Gilliam, J. Dyer, S. A. Fiscus, J. J. Eron, A. C. Frank, and M. S. Cohen. 1997. Quantification of HIV in semen: correlation with antiviral treatment and immune status. AIDS **11:**987-993.

Vidal, N., M. Peeters, C. Mulanga-Kabeya, N. Nzilambi, D. Robertson, W. Ilunga, H. Sema, K. Tshimanga, B. Bongo, and E. Delaporte. 2000. Unprecedented degree of human immunodeficiency virus type 1 (HIV-1) group M genetic diversity in the Democratic Republic of Congo suggests that the HIV-1 pandemic originated in Central Africa. J. Virol. **74:**10498-10507.

Villanueva, J. M., T. V. Ellerbrock, J. L. Lennox, T. J. Bush, T. C. Wright, M. Pratt-Palmore, T. Evans-Strickfaden, L. J. Conley, C. Schnell, and C. E. Hart. 2002. The menstrual cycle does not affect human immunodeficiency virus type 1 levels in vaginal secretions. J. Infect. Dis. **185:**170-177.

Vogt, P. K. 1971. Genetically stable reassortment of markers during mixed infection with avian tumor viruses. Virology **46:**947-952.

Watkins, B. A., S. Buge, K. Aldrich, A. E. Davis, J. Robinson, M. S. Reitz, Jr., and M. Robert-Guroff. 1996. Resistance of human immunodeficiency virus type 1 to neutralization by natural antisera occurs

University of Cape Town

through single amino acid substitutions that cause changes in antibody binding at multiple sites. J. Virol. **70:**8431-8437.

Wegner, S. A., P. K. Ehrenberg, G. Chang, D. E. Dayhoff, A. L. Sleeker, and N. L. Michael. 1998. Genomic organization and functional characterization of the chemokine receptor CXCR4, a major entry co-receptor for human immunodeficiency virus type 1. J. Biol. Chem. **273:**4754-4760.

Wei, X., S. K. Ghosh, M. E. Taylor, V. A. Johnson, E. A. Emini, P. Deutsch, J. D. Lifson, S. Bonhoeffer, M. A. Nowak, B. H. Hahn, and . 1995. Viral dynamics in human immunodeficiency virus type 1 infection. Nature **373:**117-122.

Wiegers, K., G. Rutter, H. Kottler, U. Tessmer, H. Hohenberg, and H. G. Krausslich. 1998. Sequential steps in human immunodeficiency virus particle maturation revealed by alterations of individual Gag polyprotein cleavage sites. J. Virol. **72:**2846-2854.

Williams, G., T. Stretton, and J. Leonard. 1983. AIDS in 1959? The Lancet, **1136**:11-12-9830.

Williamson, C., S. Engelbrecht, M. Lambrick, E. J. van Rensburg, R. Wood, W. Bredell, and A. L. Williamson. 1995. HIV-1 subtypes in different risk groups in South Africa. Lancet **346:**782.

Wolff, H. and D. J. Anderson. 1988. Potential human immunodeficiency virus-host cells in human semen. AIDS Res. Hum. Retroviruses **4:**1-2.

Wolff, H., K. Mayer, G. Seage, J. Politch, C. R. Horsburgh, and D. Anderson. 1992. A comparison of HIV-1 antibody classes, titers, and specificities in paired semen and blood samples from HIV-1 seropositive men. J. Acquir. Immune. Defic. Syndr. **5:**65-69.

Wolinsky, S. M., B. T. Korber, A. U. Neumann, M. Daniels, K. J. Kunstman, A. J. Whetsell, M. R. Furtado, Y. Cao, D. D. Ho, and J. T. Safrit. 1996. Adaptive evolution of human immunodeficiency virus-type 1 during the natural course of infection. Science **272:**537-542.

Wrobel, J. A., S. F. Chao, M. J. Conrad, J. D. Merker, R. Swanstrom, G. J. Pielak, and C. A. Hutchison, III. 1998. A genetic approach for identifying critical residues in the fingers and palm subdomains of HIV-1 reverse transcriptase. Proc. Natl. Acad. Sci. U. S. A **95:**638-645.

Wu, L., N. P. Gerard, R. Wyatt, H. Choe, C. Parolin, N. Ruffing, A. Borsetti, A. A. Cardoso, E. Desjardin, W. Newman, C. Gerard, and J. Sodroski. 1996. CD4-induced interaction of primary HIV-1 gp120 glycoproteins with the chemokine receptor CCR-5. Nature **384:**179-183.

Wyatt, R. and J. Sodroski. 1998. The HIV-1 envelope glycoproteins: fusogens, antigens, and immunogens. Science **280:**1884-1888.

University of Cape Town

Xiao, L., D. L. Rudolph, S. M. Owen, T. J. Spira, and R. B. Lal. 1998. Adaptation to promiscuous usage of CC and CXC-chemokine coreceptors in vivo correlates with HIV-1 disease progression. AIDS **12:**F137-F143.

Yu, X., X. Yuan, Z. Matsuda, T. H. Lee, and M. Essex. 1992. The matrix protein of human immunodeficiency virus type 1 is required for incorporation of viral envelope protein into mature virions. J. Virol. **66:**4966-4971.

Zaitseva, M., A. Blauvelt, S. Lee, C. K. Lapham, V. Klaus-Kovtun, H. Mostowski, J. Manischewitz, and H. Golding. 1997. Expression and function of CCR5 and CXCR4 on human Langerhans cells and macrophages: implications for HIV primary infection. Nat. Med. **3:**1369-1375.

Zekeng, L., L. Gurtler, Z. E. Afane, A. Sam-Abbenyi, G. Mbouni-Essomba, E. Mpoudi-Ngolle, M. Monny-Lobe, J. B. Tapka, and L. Kaptue. 1994. Prevalence of HIV-1 subtype O infection in Cameroon: preliminary results. AIDS **8:**1626-1628.

Zhang, L., P. J. Dailey, T. He, A. Gettie, S. Bonhoeffer, A. S. Perelson, and D. D. Ho. 1999. Rapid clearance of simian immunodeficiency virus particles from plasma of rhesus macaques. J. Virol. **73:**855-860.

Zhang, L. Q., P. MacKenzie, A. Cleland, E. C. Holmes, A. J. Brown, and P. Simmonds. 1993. Selection for specific sequences in the external envelope protein of human immunodeficiency virus type 1 upon primary infection. J. Virol. **67:**3345-3356.

Zhang, Y. and E. Barklis. 1995. Nucleocapsid protein effects on the specificity of retrovirus RNA encapsidation. J. Virol. **69:**5716-5722.

Zhou, W. and M. D. Resh. 1996. Differential membrane binding of the human immunodeficiency virus type 1 matrix protein. J. Virol. **70:**8540-8548.

Zhu, T., H. Mo, N. Wang, D. S. Nam, Y. Cao, R. A. Koup, and D. D. Ho. 1993. Genotypic and phenotypic characterization of HIV-1 patients with primary infection. Science **261:**1179-1181.

Zhu, T., N. Wang, A. Carr, S. Wolinsky, and D. D. Ho. 1995. Evidence for coinfection by multiple strains of human immunodeficiency virus type 1 subtype B in an acute seroconvertor. J. Virol. **69:**1324-1327.

Zhu, T. and D. D. Ho. 1995. Was HIV present in 1959? Nature **374:**503-504.

Zhu, T., N. Wang, A. Carr, D. S. Nam, R. Moor-Jankowski, D. A. Cooper, and D. D. Ho. 1996. Genetic characterization of human immunodeficiency virus type 1 in blood and genital secretions: evidence for viral compartmentalization and selection during sexual transmission. J. Virol. **70:**3098-3107.

University of Cape Town

Zhu, T., B. T. Korber, A. J. Nahmias, E. Hooper, P. M. Sharp, and D. D. Ho. 1998. An African HIV-1 sequence from 1959 and implications for the origin of the epidemic. Nature **391:**594-597.

Zhu, T., D. Muthui, S. Holte, D. Nickle, F. Feng, S. Brodie, Y. Hwangbo, J. I. Mullins, and L. Corey. 2002. Evidence for human immunodeficiency virus type 1 replication in vivo in CD14(+) monocytes and its potential role as a source of virus in patients on highly active antiretroviral therapy. J. Virol. **76:**707-716.

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