Genetic Diversity of Subtype C HIV-1 *env* Variants in Peripheral Blood Mononuclear Cell (PBMC) DNA from Infected Mother-Child Pairs: A Comparison of Heteroduplex Mobility Assay (HMA) and Base Excision Sequence Scanning (BESS) Methods

University of Cape Town

A dissertation submitted to the
University of Cape Town
for the degree of
Master of Science

By

Mr A. S. Loubser

Division of Medical Virology
Institute for Infectious Diseases and Molecular Medicine
University of Cape Town
Faculty of Health Sciences

The copyright of this thesis vests in the author. No quotation from it or information derived from it is to be published without full acknowledgement of the source. The thesis is to be used for private study or non-commercial research purposes only.

Published by the University of Cape Town (UCT) in terms of the non-exclusive license granted to UCT by the author.

University of Cape Town

University of Cape Town

University of Cape Town

University of Cape Town

University of Cape Town

Abstract

Immune system pressure on HIV-1 replication drives the antigenic changes seen over time. The monitoring of changes in viral sequences can provide important information on the nature of the immune response and the correlates of protection. Viral diversification may also occur due to other selective pressures such as cell availability and differences in viral fitness. Information on the genetic characteristics of HIV-1 variants present in the mother and her infected infant are useful data for establishing whether any common features exist between source infection and transmitted genotypes. This helps in the understanding of the mechanism of transmission and the selective pressures occurring during and following transmission.

The overall aim of this study was to explore alternative methods other than DNA sequencing for the monitoring of genetic diversity in the third variable region (V3) of the HIV-1 *env* gene of integrated HIV-1 variants in peripheral blood mononuclear cells (PBMC's) derived from infected mother-child pairs.

Two methods for displaying DNA differences were compared: Heteroduplex Mobility Assay (HMA) and Base Excision Sequence Scanning (BESS). These methods were validated using sequence data. Extracted PBMC DNA from infected mother-child pairs were used to amplify the V3 region by nested PCR. DNA fragments were cloned into plasmid vectors and analyzed by HMA and BESS to establish subtype and intrasample genetic diversity. In addition, a PCR-ELISA quantitation system was developed to measure copy numbers of integrated HIV-1 genomes in order to confirm whether a sufficient number of template molecules were present to be representative of the total viral quasispecies.

In conclusion, this study compared two methods (HMA and BESS) as cost-effective alternatives to DNA sequencing for HIV-1 diversity studies. In addition, a novel application of the BESS assay was demonstrated. Diversity studies are reliant on estimation of adequate input of amplifiable copies. The PCR-ELISA quantitation system developed provided an efficient and specific method for determining DNA copy number.

University of Cape Town

Abbreviations

µg	microgram
µl	microlitre
°C	degrees centigrade
aa	amino acid
ag	antigen
A	adenine
AIDS	acquired immunodeficiency syndrome
APS	ammonium persulphate
ATP	adenosine triphosphate
AZT	azidothymidine
BESS	base excision sequence scanning
bp	base pair
C	cytosine
C1-C5	constant regions 1-5
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
CTL	cytotoxic T-lymphocyte
dNTP	deoxyribonucleoside triphosphate
DTT	dithiothreitol
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
env	envelope protein gene
Env	envelope protein
EthBr	ethidium bromide
FCS	fetal calf serum
G	guanine
gag	group associated antigens gene
Gag	group associated antigens protein
H_2O	water

University of Cape Town

HAART	highly active antiretroviral therapy
HIV-1	human immunodeficiency virus type-1
HIV-2	human immunodeficiency virus type-2
HLA	human leukocyte antigen
HMA	heteroduplex mobility assay
hr	hour
HTA	heteroduplex tracking assay
IL-2	interleukin-2
IPTG	isopropylethio-β-D-galactoside
IVDU	intravenous drug users
LB	luria broth
LTNP	long term non-progressor
LTR	long terminal repeat
m	molar
mM	millimolar
M group	major group
MA	milliampere
Mg	milligram
$MgCl_2$	magnesium chloride
MHC	mouse histocompatibility complex
min	minute
ml	millilitre
MTCT	mother to child transmission
N group	novel variant
NaCl	sodium chloride
ND	not done
ng	nanogram
NSI	non-syncytium inducing
O group	outlier group
PBMC	peripheral blood mononuclear cell
PBS	phosphate buffered saline

University of Cape Town

pmol	picomol
PCR	polymerase chain reaction
R5	CCR5 using viruses
RANTES	regulated on activation normal T-cell expressed and activated
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
RNAse	ribonuclease
rpm	revolutions per minute
RT	reverse transcriptase
RT-PCR	reverse transcriptase-polymerase chain reaction
s	second
SABTS	South African blood transfusion services
SI	syncytium inducing
SIV	simian immunodeficiency virus
STD	sexually transmitted disease
T	thymine
TBE	tris-borate ethylenediaminetetraacetic acid
$TCID_{50}$	tissue culture infectious dose 50%
TEMED	N,N,N',N'- tetramethylethylenediamine
U	unit
UV	ultraviolet
V	volt
V1/V2	variable regions 1-2
W	watt
WHO	world health organization
X4	CXCR4 using viruses
X-gal	5-bromo-4-chloro-3-idolyl-β-D-galactosidase

University of Cape Town

Chapter 1: LITERATURE REVIEW

1.1 **INTRODUCTION**

More than 40 million individuals are currently infected with HIV-1 of which 28.1 million reside in sub-Saharan Africa (UNAIDS, 2002). South Africa has one of the fastest growing HIV-1 epidemics with an estimated 4.7 million infected individuals out of a population of 40.6 million. Vertical transmission of HIV-1, first described in 1983 (Rubinstein *et al.*, 1983.); (Oleske *et al.*, 1983), accounts for the vast majority of paediatric infections and the current global estimate of the number of newly infected children (<15 years of age) is 2,192 per day (UNAIDS, 2002). The national prevalence of HIV positive pregnant women attending antenatal clinics in South Africa at the end of 2001 was 24.8% (Figure 1), [South African Dept. of Health., 2002]. In specific regions of the country the number of HIV positive pregnant women attending antenatal clinics varied from 32.5% in KwaZulu-Natal to 7.1% in the Western Cape (Table 1). In developed countries a significant reduction in the rate of mother-to-child transmission has been achieved largely due to the introduction of anti-retroviral therapy, the avoidance of breastfeeding and delivery by caesarean section. In South Africa, access to medical facilities and antiretroviral treatment is limited and breastfeeding is a common practice.

University of Cape Town

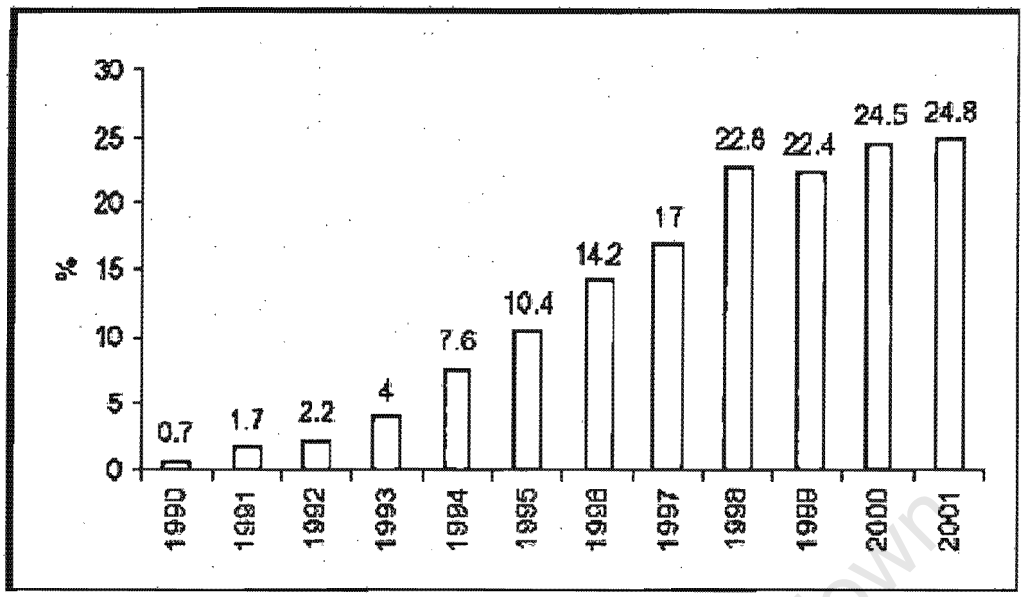


Figure 1: National HIV prevalence trends among antenatal clinic attendees in South Africa: 1990-2001 (South African Dept. of Health., 2002).

University of Cape Town

Table 1: Provincial HIV prevalence estimates: Antenatal clinic attendees, South Africa 1999-2001 (South African Dept. of Health., 2002).

PROVINCE	HIV pos. 95% CI 1999	HIV pos. 95% CI 2000	HIV pos. 95% CI 2001
KwaZulu-Natal (KZN)	32.5 (30.1–35.0)	36.2 (33.4–39.0)	33.5 (30.6–36.4)
Mpumalanga (MP)	27.3 (25.2–30.7)	29.7 (25.9–33.6)	29.2 (25.6–32.8)
Gauteng (GP)	23.9 (21.7–26.0)	29.4 (27.2–31.5)	29.8 (27.5–32.1)
Free State (FS)	27.9 (24.7–29.8)	27.9 (24.6–31.3)	30.1 (26.5–33.7)
North West (NW)	23.0 (19.7–26.3)	22.9 (20.1–25.7)	25.2 (21.9–28.6)
Eastern Cape (EC)	18.0 (14.9–21.1)	20.2 (17.2-23.1)	21.7 (19.0–24.4)
Limpopo (LP)	11.4 (9.1-13.5)	13.2 (11.7–14.8)	14.5 (12.2–16.9)
Northern Cape (NC)	10.1 (6.6-13.5)	11.2 (8.5–13.8)	15.9(10.1–21.6)
Western Cape (WC)	7.1 (4.4-9.9)	8.7 (6.0–11.4)	8.6 (5.8– 11.5)
National	22.4 (21.3–23.6)	24.5 (23.4–25.6)	24.8 (23.6 – 26.1)

N.B. The true value is estimated to fall within the two confidence limits, thus the confidence interval is important to refer to when interpreting data.

1.2 RATE OF TRANSMISSION

Estimation of transmission rates in untreated populations is dependent on the cohort, length of follow-up and the criteria used to define pediatric HIV-1 infection. Studies based on different methods cannot be directly compared. A re-assessment of published data, using a standardized method was undertaken by the Working Group on Mother-to-Child Transmission of HIV (1995), showing a range of 12.7% to 42.1% (Table 2). Generally lower rates of transmission are reported in Europe and higher rates in Africa. The difference is most likely attributable to the inclusion of some of the infants infected shortly after birth by postnatal transmission since the number of breastfeeding mothers in African studies is higher. In South Africa, Bobat *et al.*, (1996), using the same criteria as used by the Working Group, reported a 34% transmission rate which was similar to the 35.1% rate reported by Lyon *et al.*, (1996), also from South Africa.

University of Cape Town

Table 2: Re-assessment of reported vertical transmission rates for direct comparisons (The working group on mother-to-child transmission of HIV., 1995)

Study location.	Total children in study	Total No. (HIV-1 status unknown)	Intermediate transmission estimate (%)
Kenya	365	125	42.1
Congo	118	19	40.4
South Africa*	181	40	34.0
Zaire	323	58	27.9
USA (New York)	245	64	24.9
Ivory Coast	101	12	24.7
Rwanda (Kigali)	218	32	24.7
Uganda	402	92	24.2
France	946	101	20.4
Rwanda (Butare)	198	36	19.8
Switzerland	300	60	18.3
European Collaborative Study	1025	130	14.1
Haiti	480	267	12.7

*included from Bobat *et al.*, 1996

University of Cape Town

1.3 TIMING AND RELATIVE TRANSMISSION RATES

Transmission can occur either *in utero,* intrapartum or post-partum via breastfeeding (Simonon *et al.*, 1994). The relative frequency of *in utero,* intrapartum or post-partum transmission is difficult to determine since the accuracy of assessment is dependent on precise determination of the timing of transmission which relies on HIV-1 positivity testing. Diagnosis of HIV-1 infection in infants is made by virus isolation, detection of HIV-1 DNA, RNA, p24 antigen or the persistence of HIV-1 antibodies beyond the age of 18 months (Peckham and Gibb., 1995).

1.3.1 *IN UTERO* TRANSMISSION

Transmission during the first and second trimester has been suggested by studies of aborted fetuses from seropositive women. The presence of virus infected cells was shown in 8-week fetuses (Lewis *et al.*, 1990) and virus was isolated from a 15-week fetus (Sprecher *et al.*, 1986). HIV has been detected by polymerase chain reaction (PCR) in several organs of 11-24 week old fetuses including brain, liver or lung tissue in 7 of 23 fetuses (Soeiro *et al.*, 1992). Simonon *et al.*, (1997), using cord blood PCR test results, estimated a 7.7% *in utero* transmission rate.

1.3.2 INTRAPARTUM TRANSMISSION

Most vertical transmissions seem to occur during the peripartum period (Peckham and Gibb 1995) and are presumed to result from direct exposure of the infant to contaminated blood and/or cervical-vaginal secretions. This is supported by the lower frequencies of transmission observed in infants delivered by caesarean section and an apparent higher likelihood of infection associated with the firstborn child in a multiple pregnancy (European Collaborative Study, 1994). In South Africa, Bobat *et al.* (1997) reported a 27% intrapartum transmission rate.

1.3.3 POSTNATAL TRANSMISSION

Infection via the oral route by breastfeeding was first described by Ziegler *et al.* (1985). The presence of HIV-1 in breast milk in both cell-free (Lewis *et al.*, 1998; Thiry *et al.*, 1985) and cell-associated forms (Ruff *et al.*, 1994; Buranasin *et al.*, 1993) has been demonstrated. Ruff *et al.* (1994) showed that within the first 4 days of delivery, 70% of HIV-1 seropositive women showed the presence of viral DNA in their breast milk which could still be detected in 53% of samples taken a year later.

Conclusive evidence demonstrating postnatal transmission was reported in an early study by Van de Perre *et al.*, (1991), where mothers who became HIV-1 infected after delivery

University of Cape Town

transmitted to there infants. Breast milk was the most likely source of infection since all mothers were known to be breastfeeding their infants. A meta-analysis by Dunn *et al.*, (1992), calculated that the risk of postnatal transmission in infected women was 14% which increased to 29% if women became infected after delivery.

In comparative studies of breastfeeding and formula-feeding by seropositive mothers, more breastfed infants than formula-fed infants are infected by the end of follow up (European Collaborative Study, 1992); (Blanche *et al.*, 1989). A South African report from Soweto, showed a 46% postnatal infection rate in breastfed infants compared to 18% in formula-fed infants (Gray *et al.*, 1996). In addition, 85% of the infections had occurred before 6 months of age. In societies where breastfeeding is common due to cultural or economic factors, the risk of postnatal HIV-1 infection is highly significant, particularly when breastfeeding is prolonged since the risk of transmission increases with duration of breastfeeding (de Martino *et al.*, 1992);(Italian register for HIV infection in children, 1994b). A Durban study, observed a 39% transmission rate in an exclusive breastfeeding group compared to 24% in an exclusively formula-fed group (Bobat *et al.*, 1997) and showed that in the exclusive breastfed group, duration of breastfeeding was associated with an increase in transmission risk since the rate at 1, 2 and 3 months increased from 45%, 64% to 75% respectively.

Concern has also been raised with respect to the practice of mixed breast-feeding which according to Coutsoudis *et al.*, (2002) increases the risk of transmission by close to 50% when compared to the risk of exclusively breastfeeding. Their data showed the highest risk of transmission in the sub-group of children surviving to 3 months of age. Similar transmission rates were seen between the exclusive breastfed and never breastfed groups up to the age where exclusive breastfeeding was discontinued.

University of Cape Town

1.4 **RISK FACTORS OF VERTICAL TRANSMISSION**

1.4.1 EXPOSURE TO CONTAMINATED FLUIDS IN THE BIRTH CANAL

The birth environment may be highly significant with respect to the risk of infection of the child. This is inferred from data collected on infected mothers delivering twins showing that there is an increased risk for the first-born child. In an analysis of birth order and route of delivery in 100 twin births, the frequency of transmission was found to be 50% for the first-born child delivered vaginally, followed by 38% for the first-born child delivered by caesarean section and 19% for the second-born child delivered by either route (Goedert *et al.*, 1991). Although genetic factors can contribute to susceptibility of infection, discordances in infection status are similar in both monozygotic (Menez-Bautista *et al.*, 1986) and dizygotic twins (Young *et al.*, 1990); (Park *et al.*, 1987); (Barlow *et al.*, 1993). This suggests that transmission may occur at the time of delivery due to exposure of the infant to contaminated blood and/or secretions. Support of this stems from the observation that caesarean delivery reduces the risk of transmission. The European Collaborative Study, reported a reduction of 51% in the rate of vertical transmission when comparing caesarean delivery to vaginal delivery (European Collaborative Study, 1994). Two reports from South Africa have found similar decreases in transmission frequency in the caesarean delivery group (Bobat *et al.*, 1996); (Lyons *et al.*, 1996). Virus particles and viral DNA is detectable in cervical and vaginal secretions (Henin *et al.*, 1993); (Clemetson *et al.*, 1993), yet, attempts to disinfect the birth canal before delivery with chlorohexidine was not shown to affect the vertical transmission rate significantly in a trial in Malawi (Biggar *et al.*, 1996).

University of Cape Town

1.4.2 PREMATURITY

Elevated risk of vertical transmission in premature births has been described (Tovo *et al.*, 1996); (European Collaborative Study, 1992); (Goedert *et al.*, 1989). The European Collaborative Study (1992) reported a higher risk of infection in children born before 34 weeks. A 33% infection rate was observed in 33 children born before 34 weeks of

gestation and 14% for children born after 34 weeks. Similarly, Tovo *et al.* (1996) observed a pattern of highest transmission at less than 32 weeks of gestation followed by a decline in the remaining weeks of pregnancy. It is unclear why this pattern is evident, but may be a result of infection *in utero* before 32 weeks of gestation leading to abnormal fetal development and hence premature birth or alternatively, the health status of the mother may both influence the risk of premature delivery and the risk of vertical transmission.

1.4.3 MATERNAL IMMUNE STATUS

1.4.3.1 **Neutralizing antibodies**

The relationship between humoral immunity and transmission of HIV-1 is not clearly defined. It is known that all infants born to HIV-1 seropositive mothers carry maternal antibodies in their circulation for up to between 15 and 18 months (European Collaborative Study, 1991). The ability of anti-V3 immunoglobulins to neutralize virus infectivity has been documented in humans (Kenealy *et al.*, 1989); (Palker *et al.*, 1988); (Rusche *et al.*, 1988), as well as chimpanzee studies (Emini *et al.*, 1992). Experiments using rhesus macaques susceptible to a chimeric simian/human immunodeficiency virus (SHIV) incorporating the HIV-1 envelope, intravenous inoculation with neutralizing monoclonal antibodies 24 hours before intravenous challenge with virus showed complete protection in 3 animals and reduced viraemia with normal CD4+ cell counts in 3 animals that became infected (Mascola *et al.*, 1999). Hofmann-Lehmann *et al.*, (2001) were able to demonstrate that pre- and post-natal administration of three monoclonal neutralization antibodies to SHIV were able to protect neonatal rhesus macaques from oral challenge of virus.

In humans, antibodies directed towards the V3 region of HIV-1 gp120 have been associated with lower rates of heterosexual transmission from male-to-female and slower disease progression in infected individuals (Fiore *et al.*, 1993); (Page *et al.*, 1992). The presence of antibodies in maternal blood capable of preventing virus infectivity has been

University of Cape Town

investigated as a risk factor in mother-to-child transmission, however, the true significance is difficult to establish since the laboratory methods of detection are not standardized and results are dependent on the choice of isolates used in the assays.

Scarlatti *et al.* (1993) originally shown an association between maternal autologous neutralizing antibodies and vertical transmission, however, later studies have disagreed (Guevara *et al.*, 2002); (Calarota and Libonatti *et al.*, 2000); (Husson *et al.*, 1995). Lack of correlation between studies may be related to mutations in epitopes and sub-optimal choice of isolates. It is known that substitutions in the V3 loop region give rise to neutralization escape mutants (Masuda *et al.*, 1990); (McKeating *et al.*, 1989). This factor was taken into account by a study from Kinshasa, Zaire, where St. Louis *et al.* (1994) characterized the V3 loop sequences present in their mother-child cohort before selecting peptides for the antibody assays, yet, despite their use of highly relevant peptides, they found no correlation between anti-V3 loop antibody titers and risk of mother-to-child transmission. Similarly, secretory anti-HIV antibodies in breast milk were not found to be a factor of protection against viral transmission through breast milk (Becquart *et al.*, 2000). Maternal antibody levels and neutralizing ability do not appear to be involved in reducing mother-to-child transmission risk.

University of Cape Town

1.4.3.2 **CD4+ cell count**

An inverse relationship between CD4+ count and risk of transmission has been reported by several investigators (Dickover *et al.*, 2001); (Mayaux *et al.*,1995); (Bredberg-Raden *et al.*, 1995); (Scarlatti *et al.*, 1993); (St. Louis *et al.*, 1993). In South Africa, Lyons *et al.* (1996) found that mothers with reduced CD4+ counts at the time of delivery had increased risk of transmitting. The study by Dickover *et al.* (2001) showed a mean CD4+ count of 398 cells/mm^3 in the transmitter group (n=18) vs. 726 cells/mm^3 in the non-transmitter group (n=18). In addition, comparison of CD4+ cell count in the *in utero* transmission group (n=13) vs. the intrapartum transmission group (n=9) was 259 cells/mm^3 vs. 503 cells/mm^3 respectively, suggesting increased risk of early transmission linked to CD4+ cell count. The relevance of CD4+ count may be at the level of

competence of the maternal immune system in controlling viral replication since a lower CD4+ count implies a weaker immune response and hence the inability of the mother to control virus replication and exposure to the infant.

1.4.3.3 **Viral load**

Virus replication can be measured either by the quantitation of integrated viral genomes (Ferre *et al.*, 1992), the quantity of viral RNA (Lin *et al.*, 1994) or the total number of infectious units of virus per volume of maternal blood (Weiser *et al.*, 1994). Using more than a single measure of viral load, Dickover *et al.* (2001) found significant differences when comparing viral load data between transmitter (n=19) and non-transmitter groups (n=18) the average RNA copies/ml was 61,960 vs. 5,757 and the average DNA copies/µg of PBMC DNA was 233 vs. 20 and number of infected PBMCs/10^6 cells was 25 vs. 1, respectively. The study also showed higher average viral load figures for *in utero* vs. intrapartum transmitters, i.e. 146,098 copies/ml vs. 54,307 copies/ml.

1.4.4 MATERNAL HEALTH

Other risk factors for vertical transmission include infection during pregnancy such as chorioamnionitus and sexually transmitted diseases (Peckham and Gibb., 1995). Inflammation of the genital tract is associated with influx of target cells and increased viral loads.

1.4.5 HOST GENETICS

Investigations of whether sharing of class I HLA alleles between mother and infant influence risk of transmission have been carried out. MacDonald *et al.* (1998) reported that concordant HLA class I allele sharing was associated with increased risk of transmission and furthermore, showed that a stepwise increased risk for additional concordant alleles. Polymorphisms in the coding and non-coding regions of significant genes may also play a role in risk of transmission. Aikhionbare *et al.* (2001), analyzed

University of Cape Town

exons 2, 3, 6 and 7 in of HLA-G, the non-classical MHC class Ib molecule uniquely expressed in extravillous cytotrophoblast cells at the maternal-fetal interface, and found that concordance in a polymorphism in exon 2 between mother and child was associated with increased risk of transmission. Likewise, several mutations in the CCR5 chemokine receptor which serves as the major co-receptor for HIV-1 R5 viruses, have been shown to be natural polymorphisms that influence the risk of becoming infected with HIV-1. A 32-base pair deletion in CCR5 has been shown to reduce the risk of HIV-1 infection. CCR5-δ32 homozygotes are highly resistant to infection by R5 HIV-1 viruses (Huang Y *et al.*, 1996);(Samson *et al.*, 1996) whereas in adults and children, CCR5-δ32 heterozygotes are not protected against infection but progress more slowly to AIDS and death compared to wild-type individuals (Huang *et al.*, 1996), (Misrahi *et al.*, 1998). Mutations in the promoter region of CCR5 have also been associated with increased or retarded disease progression. The CCR5-59029-G/T polymorphism slows down disease progression (McDermott *et al.*, 1998) whereas CCR5-59029-A and the CCR5P1 combination (CCR5 59353-C, 59356-C and 59402-A) accelerates disease progression (McDermott *et al.*, 1998); (Martin *et al.*, 1998). A CCR5 59353-C polymorphism was linked to a slower decline in CD4+ cells (Easterbrook *et al.*, 1999). An association between a CCR5-59653-T polymorphism and a substitution of isoleucine at position 64 in the CCR2b gene has been associated with delayed disease progression (Kostrikis *et al.*, 1998). Children homozygous for the CCR5-59653-T polymorphism have increased risk of becoming infected by vertical transmission (Kostrikis *et al.*, 1999).

University of Cape Town

1.5 **POSSIBLE MECHANISMS OF TRANSMISSION**

Whether *in utero*, intrapartum or postnatal infection of infants born to HIV-1 positive mothers is due to exposure of the infant to infectious virions or infected cells or a combination has not been elucidated. If cell-associated virus is transmitted, it is unknown whether infected macrophages or CD4+ T-lymphocytes or both can be involved. In addition to systemic blood, cell-free and cell-associated virus can be detected in amniotic fluid (Mundy *et al.*, 1987), cervico-vaginal secretions (Overbaugh *et al.*, 1996) and breast milk (Lewis *et al.*, 1998); (Ruff *et al.*, 1994); (Buranasin *et al.*,

1993). Some studies have suggested infection of placental cells such as trophoblasts and Hofbauer cells as a possible transplacental route of infection (Douglas *et al.*, 1992); (Kesson *et al.*, 1994). Nielsen *et al.* (1996) have detected HIV-1 in cervico-vaginal secretions in infected mothers and in gastric aspirates of their infants, suggesting that the ingestion of contaminated maternal fluids or secretions may provide an opportunity for infectious virus to breach epithelial cell barriers in the infant. In the intestinal gut, the potential for the active transport of HIV-1 through intestinal M-cells has been postulated (Clark *et al.*, 1998) as well as a demonstration by Morgane Bomsel (1997), that *in vitro,* infected lymphocytes in contact with an epithelial cell monolayer can promote transcytosis of virus through the cytoplasm and cause budding of infectious virus from the internal membrane.

1.6 NATURE OF TRANSMITTED VIRUS

1.6.1 PHENOTYPE

Cell tropism and host-cell susceptibility may play a role in vertical transmission. Neonatal monocyte-derived macrophages from cord blood samples are more susceptible to HIV-1 infection than those of adults (Ho *et al.*, 1992); (Sperduto *et al.,* 1993). Some studies show that the majority of viral isolates from infected infants soon after birth are highly tropic for monocyte-derived macrophages (Dmetto *et al.*, 1995); (De Rossi *et al.*, 1993). The major determinants that mediate tropism of HIV-1 isolates is the specificity of the virus for chemokine receptors and the distribution and expression of them on CD4+ target cells. R5 viruses use CCR5 and are macrophage-tropic while X4 viruses use CXCR4 and are T-lymphocyte-tropic (Berger *et al.*, 1998). In vertical transmission of HIV-1, the more frequently transmitted phenotypes are R5 viruses, although X4 viruses can be transmitted, but less efficiently (Salvatori and Scarlatti, 2001). Subtype C viruses almost exclusively utilize the CCR5 co-receptor and a change from NSI to SI is extremely rare (Abebe *et al.*, 1999); (Tien *et al.*, 1999); (Morris *et al.*, 2001).

University of Cape Town

1.6.2 GENOTYPE

Genetic characterization of HIV-1 variants present in the mother and her infected infant are useful in establishing whether any common features exist between source infection and transmitted genotypes. This provides information on the mechanism of transmission and potentially on selective pressures occurring during and following transmission. Several issues regarding the transmitted genotypes in comparison to the source genotypes are under study. These include whether a single or multiple set of genotypes can be transmitted, whether minor or major genotypes are preferentially transmitted and whether transmittants are derived from cell-free or cell associated virus.

Several studies of the HIV-1 *env* gene, analyzing the V1-V2, V3, V4-V5 and V3-V5 regions, have shown that transmitted variants can originate from major or minor populations in the mother at the time of transmission (Dickover *et al.*, 2001); (Sutthent *et al.*, 1998); (Ahmad *et al.*, 1995); (Lamerset *et al.*, 1994); (Scarlatti *et al.*,1993); (Wolinsky *et al.*, 1992). Studies looking at *env* V3 and *gag* p17, showed discordance in transmission of major and minor variants with respect to both regions and suggested transmission of more than one genotype (Wade *et al.*, 1998); (Simonon *et al.*, 1997); (Mulder-Kampinga *et al.*, 1995). In a clear case of transmission of multiple *env* genotypes, sequences corresponding to both SI phenotype as well as NSI strains were transmitted in a report by Pasquier *et al.* (1998). In addition, the source of the transmitted variants can be derived from either cell-free or cell-associated virus (Scarlatti *et al.*, 1993); (Pasquier *et al.*, 1998). It is therefore likely that there are different selective pressures depending on the timing and route of transmission that influences the variants transmitted.

University of Cape Town

1.7 GENETIC VARIATION OF HIV-1

1.7.1 HIV-1 SUBTYPES

Phylogenetic analysis of HIV-1 strains obtained from various geographical regions show a high degree of genetic diversity (Los Alamos National Laboratory HIV Sequence Database, http://hiv-web.lanl.gov). The clustering of strains has defined three groups (M, N and O). Group M is the major group and most strains responsible for the HIV-1 pandemic belong to this group. The M group is further subdivided into nine subtypes of HIV-1 (A, B, C, D, F, G, H, J and K) as well as circulating recombinant forms (CRF). Subtypes A and F have further divisions into subtypes A1, A2, F1 and F2. Groups O and N are minority groups containing strains highly divergent from those of group M. The most prevalent strain globally is subtype C (UNAIDS, www.unaids.org). In South Africa, subtype C is the predominant strain circulating within the heterosexual population (Van Harmelen *et al.*, 2001), although other subtypes such as A, D, G, AG and unclassified strains have also been identified (Bredell *et al.*, 2002) as well as complex and novel recombinant viruses (Papathanasopoulos *et al.*, 2002). Strains belonging to the same subtype can vary by up to 20% in their envelope proteins and up to 35% between subtypes (Gaschen *et al.*, 2002).

1.7.2 THE THIRD VARIABLE REGION (V3) OF HIV-1 ENVELOPE

The V3 loop contains determinants for co-receptor binding and cellular tropism (Shimizu *et al.*, 1999) and defines the capacity of the virus to induce syncytia (De wolf *et al.*, 1994). Although regions outside of the V3 loop may also determine coreceptor usage, it is possible to predict the phenotype of an isolate by calculating the number of positively charged amino acids in the region (Hoffman *et al.*, 2002). SI variants have the highest positive charge compared to NSI variants and charges at positions 11 and 15 are highly predictive of phenotype (Hoffman *et al.*, 2002). However, subtype C viruses predominantly use CCR5 as coreceptor and are different from subtype B viruses in that they generally do not switch to CXCR4 coreceptor usage associated with the onset of

University of Cape Town

AIDS (Adebe *et al.* 1999). Most vertical transmissions involve CCR5 using viruses, although the transmission of dual tropic and in rare cases, solely X4 utilizing viruses, have been reported (Salvatori and Scarlatti, 2001). Recognition sites for humoral and cell-mediated immunity also map to the V3 region (Palker *et al.*, 1988); (Takahashi *et al.*, 1992). Changes in glycosylation pattern of the envelope proteins influence antibody recognition epitopes (Wei *et al.*, 2003). With respect to subtype B viruses, it is known that most subtype C viruses lack a V3 glycosylation site and a basic amino acid residue at position 11 (Ping *et al.*, 1999); (Gordon *et al.*, 2003). Subtype B studies have suggested that this site may be involved in the interaction of gp120 with its coreceptors (Li *et al.*, 2001) and in perinatal transmission. Nakayama *et al.* (1998) found that the absence of this site reduced CXCR4-dependent but not CCR5-dependent viral entry. In addition to the functional importance of this domain, the V3 region has also been used by other investigators in mother-to-child transmission studies, particularly in phylogenetic analyses.

University of Cape Town

1.8 DISEASE PROGRESSION IN INFANTS INFECTED BY VERTICAL TRANSMISSION

The European Collaborative Study (1991), reported that an estimated 64% of infected children show laboratory or clinical features of HIV-1 infection by 3 months of age, 83% by 6 months and 90% by 1 year. In addition, 26% have developed AIDS at 12 months. Others develop AIDS at a reduced rate over a number years and a small proportion remain asymptomatic for 8-10 years (Andeweg *et al.*, 1992). No clear pattern of disease progression can be established since the course of disease in children infected with HIV-1 perinatally seems to vary considerably (European Collaborative Study, 1991), possibly as a result of factors such as the size of the inoculum, the time and route of transmission, the virulence of strain and the host immune response which may contribute in a combinatorial fashion to the outcome of disease in the infected child.

The time of transmission may influence disease progression based on reported associations of *in utero* transmission and rapid disease progression (Borkowsky *et al.*,

1994); (Mayaux *et al.*, 1996). Lambert *et al.* (1997) have shown that infants with a positive DNA PCR test within the first week of life and presumably infected late *in utero* or intrapartum, progressed more rapidly to AIDS. Shearer *et al.* (1997) have studied viral RNA levels in children infected perinatally and observed that the RNA load differed according to whether the infection was acquired early or late and noted that disease progression was associated with higher RNA levels not only at birth but also during the first few months of life. Although, a high viral RNA load in maternal serum at delivery and an associated rapid disease progression in the infected infant has been reported suggesting the role of the size of the inoculum (Coll *et al.*, 1997), no significant association between maternal viral load and disease progression in infected infants was noted by Lambert *et al.* (1997). Similarly, Jones *et al.* (1992) reported that infants infected by blood transfusion and presumably exposed to a higher inoculum than perinatally exposed infants still showed varying degrees of disease progression suggesting the involvement of additional factors.

The ability of the immune system of child to control the replication of the virus seems to play a critical role in disease progression. The level of plasma RNA load in infected children correlates with disease progression (De Rossi *et al.*, 1996); (Alimenti *et al.*, 1991). A retrospective, longitudinal study of HIV-1 infection in children by Zaknun *et al.* (1997) showed that high viral load, assessed by plasma RNA levels and p24 antigen detection, was associated with poor growth and similarly an investigation of HIV-1 proviral load and p24 antigenaemia in perinatally infected children by Dickover *et al.*, (1996) noted higher levels of cell-associated HIV-1 and p24 antigen in children with rapid progression. It is possible that the pressure of the host immune response on the replication of the virus may therefore be a critical factor that controls disease progression.

1.9 NATURAL HISTORY OF VIRAL DIVERSITY IN INFANTS

It is postulated that immune pressure on the replication of the virus is reflected by the antigenic changes seen over time. Variability in the V3 region compared to rates of disease progression in children infected perinatally have shown that a more antigenically

University of Cape Town

diverse virus population correlates with a slower decline of CD4+ cells (Ganeshan *et al.*, 1997); (Halapi *et al.*, 1997). A similar pattern has been reported in adult infections where DNA sequence studies showed greater diversification and a higher ratio of nonsynonomous to synonymous site mutations (dN/dS ratios) in slow versus more rapid progressors suggesting selection for phenotypic changes (Lukashov *et al.*, 1995); (Delwart *et al.*, 1993). There have, however, been contradictory results relating viral diversity to disease progression and a possible explanation was put forward by Shankarappa *et al.*, (1999) after completion of a longitudinal study of viral evolution from seroconversion to advanced disease in infected men with moderate disease progression. Based on their analysis at sequential timepoints of viral divergence within the HIV-1 Subtype B *env* C2-V5 region from the founder strain, intrasample diversity and outgrowth of CXCR4 using viruses, they were able to identify three distinct phases of virus divergence and diversification within the asymptomatic period. The first phase of variable duration was characterized by linear increases of approximately 1% per year in both divergence from the founder strain and intrasample diversity. The second phase of approximately 1.8 years was characterized by continued increase in divergence but a stabilization or decline in diversity. The third phase was characterized by stabilization or decline in divergence as well as the continued stability of decline in diversity. The emergence of CXCR4 using viruses was seen during the transition from the first to the second phase and were seen to decline at the transition between the second and third phase. T cell abnormalities and declining CD4+ cell counts were also evident at the transition to the third phase. This pattern of genetic variation seen in moderate progressors may help explain contradictory data observed in other studies of virus diversity during the asymptomatic period since the time of sampling could have fallen within one of the phases identified by Shankarappa *et al.* (1999).

Rates of disease progression are also dependent on the quality of the CTL response which is determined by HLA class I molecules. The binding of peptides to HLA class I molecules is critical for CTL recognition and killing of infected cells and is highly dependent on the primary sequence of the peptide. Amino-acid substitutions within the peptide sequence of epitopes can reduce the binding strength of peptides to HLA

University of Cape Town

molecules or abolish binding completely. Goulder *et al.* (1997) showed that in HLA identical hemophiliac twins infected from a common contaminated blood product, and presumably the same HIV-1 quasispecies, CTL studies revealed that one of the twins did not respond to two immunodominant epitopes in p17gag to which other twin did respond. The reason was due to genetic mutations observed in proviral sequences in the peptide coding regions. A similar finding in the context of mother-to-child transmission was reported by Goulder *et al.* (2001) where infected mothers expressing HLA-B27 who respond to a highly-conserved B27-restricted gag epitope transmitted variants to their children that failed to bind HLA-B27. The transmitted viruses were found to contain mutations within the epitope coding sequences, leading to immune escape in the mother. Transmitted variants remained stable and did not revert in the infected children. The study of virus genetic variation in infected children may thus be important in the context of disease progression and immune function.

University of Cape Town

CHAPTER 2: MATERIALS AND METHODS

2.1 INTRODUCTION

For subtyping and analysis of genetic diversity, DNA sequencing is preferred but is expensive and labour-intensive. Alternatively, heteroduplex mobility assay (HMA) developed by Delwart *et al.* (1993) offered a rapid and cost-effective method to subtype and display the degree of divergence between sequences amplified by PCR. A second alternative method available for the analysis of genetic diversity is Base Excision Sequence Scanning or BESS (Epicentre Technologies, Madison, USA) which identifies thymine residues on one strand of DNA amplified by PCR. This method can be used to map thymine residues on both strands and thereby generate partial sequence data reflecting adenine and thymine residues for genetic analysis.

To estimate the diversity of sequences amplified by PCR, it is important that input material contains sufficient copies of template so as to truly represent the viral quasispecies. Too few copies artificially lower viral diversity due to re-sampling. A minimum range of 20 to 50 proviral copies (measured by end-point dilution assay) was determined by Delwart *et al.* (1993). To control for input copy number a PCR-ELISA quantitation system was developed to measure template concentration in PBMC DNA.

In this chapter we will provide methods used in this study including: (i) Amplification and cloning of *env* fragments for use as reagents in the diversity study; (ii) DNA sequencing as the 'gold standard' for diversity studies; (iii) HMA and BESS as alternative methods to DNA sequencing; (iv) PCR-ELISA based quantitation system to estimate proviral copy number.

University of Cape Town

2.2 AMPLIFICATION AND CLONING

The strategy to generate DNA sequences for genetic characterisation and subtyping was as follows: (i) amplification of envelope sequences from PBMC DNA by nested PCR, (ii) cloning of PCR fragments into plasmid vectors and transformation into host bacteria, (iii) selection of positive clones by restriction fragment length polymorphism (RFLP) and colony hybridization, (iv) Re-amplification of correctly identified clones by colony PCR. These clones were used for the subtyping assays and as reagents for genetic diversity assessment. For quantitation of provirus copy number in PBMC DNA samples a PCR-ELISA was set up. PBMC DNA samples for the mother-child sets used for the amplification of the *env* sequences were also used in the PCR-ELISA. The PCR-ELISA system was set up by an initial optimization of the capture and detection of molecules labelled with biotin and digoxigenin (DIG), followed by calibration of the system using an external standard of known concentration before final application of the assay to the mother-child samples. Labelled molecules used were control-peptides (Boehringer-Mannheim, Mannheim, Germany) and DNA probes generated by PCR using DIG-labelled reverse primers and biotinylated forward primers. The system was tested and calibrated using biotinylated oligonucleotide probe sequences complementary to the DIG-labelled negative strand of DNA amplified by PCR using the HIV-1 DNA PCR standards (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: catalogue number ARP956).

University of Cape Town

2.2.1 SAMPLES

Blood samples, containing EDTA, were collected from eight HIV-1 infected mother-child pairs from the Red Cross War Memorial Children's Hospital in Cape Town (Professor G. Hussey). Blood was collected from infants aged between one and twenty-two months (Table 2.1). Genomic DNA was extracted from the PBMCs by the Dept. of Clinical Virology (University of Cape Town), using the proteinase K crude extraction method (Kawasaki *et al.*, 1990).

Table 2.1: Infant age and sex

Sample Identifier:	Age:	Sex:
RX1-b	8 months	male
RX2-b	Unknown	unknown
RX3-b	5 months	male
RX4-b	11 month	unknown
RX5-b	1 month	female
RX6-b	15 months	female
RX7-b	14 months	male
RX8-b	22 months	male

2.2.2 AMPLIFICATION BY PCR

The number of copies of integrated HIV-1 genomes in blood leukocyte DNA is generally low and two rounds (nested) of PCR amplification are necessary to generate sufficient quantities of amplification product for DNA sequencing. 'Nested' PCR utilizes two sets of primers where the second set of primers is located internal to the fragment generated by the first-round set of primers. This design also improves specificity.

The quality of the template is critical for efficient PCR amplification and DNA isolated by crude extraction methods may not be suitable due to protein contamination, particularly DNA-binding proteins. 'Hotstart' PCR was used to amplify some samples to increase specificity and sensitivity. 'Hotstart' PCR includes an initial heating step at 94 °C for 10 minutes before thermocycling to ensure that all protein is denatured, template DNA is single-stranded and non-specifically bound primer is removed. To avoid simultaneous inactivation of *Taq* polymerase, the enzyme is added after the denaturation step.

The PCR primers used, as described by Delwart *et al.* (1993), amplify regions of the HIV-1 *env* gene. The outer primer set (ED5 and ED12) generates a 1254 bp fragment

University of Cape Town

spanning the V1-V5 coding region of gp120 and the inner primer set (ED31 and ES8) generates a 851 bp fragment spanning the C2-V5 region (Box 2.1 and Box 2.2).

Box 2.1: PCR primers for amplification of 851bp C2-V5 region of the *env* gene (Delwart *et al.*, 1993)

ED5⇒ (outer forward primer)
*HXB2 numbering 6557-6582
5'-ATG GGA TCA AAG CCT AAA GCC ATG TG-3'
ED12⇐ (outer reverse primer)
*HXB2 numbering 7782-7811
5'-AGT GCT TCC TGC TGC TCC CAA GAA CCC AAG-3'
ED31⇒ (inner forward primer)
*HXB2 numbering 6817-6845
5'-CCT CAG CCA TTA CAC AGG CCT GTC CAA AG-3'
ES8⇐ (inner reverse primer)
*HXB2 numbering 7648-7668
5'-CAC TTC TCC AAT TGT CCC TCA-3'

*HXB2 numbering according to Los Alamos HIV database (http://hiv-web.lanl.gov)

Box 2.2: Location of PCR primers using the HIV-1 clone HXB2 sequence of the gp160 region of *env*

```
                                                ED5⇒
6557                                            ATGGGATC AAAGCCTAAA GCCATGTGTA
6585   AAATTAACCC CACTCTGTGT TAGTTTAAAG TGCACTGATT TGAAGAATGA TACTAATACC
6645   AATAGTAGTA GCGGGAGAAT GATAATGGAG AAAGGAGAGA TAAAAAACTG CTCTTTCAAT
6705  aATCAGCACAA GCATAAGAGG TAAGGTGCAG AAAGAATATG CATTTTTTTA TAAACTTGAT
                                                                   ED31⇒
6765   ATAATACCAA TAGATAATGA TACTACCAGC TATAAGTTGA CAAGTTGTAA CACCTCAGTC
6825   ATTACACAGG CCTGTCCAAA GGTATCCTTT GAGCCAATTC CCATACATTA TTGTGCCCCG
6885   GCTGGTTTTG CGATTCTAAA ATGTAATAAT AAGACGTTCA ATGGAACAGG ACCATGTACA
6945   AATGTCAGCA CAGTACAATG TACACATGGA ATTAGGCCAG TAGTATCAAC TCAACTGCTG
7005   TTAAATGGCA GTCTAGCAGA AGAAGAGGTA GTAATTAGAT CTGTCAATTT CACGGACAAT
7065   GCTAAAACCA TAATAGTACA GCTGAACACA TCTGTAGAAA TTAATTGTAC AAGACCCAAC
7125   AACAATACAA GAAAAAGAAT CCGTATCCAG AGAGGACCAG GGAGAGCATT TGTTACAATA
7185   GGAAAAATAG GAAATATGAG ACAAGCACAT TGTAACATTA GTAGAGCAAA ATGGAATAAC
7245   ACTTTAAAAC AGATAGCTAG CAAATTAAGA GAACAATTTG GAAATAATAA AACAATAATC
7305   TTTAAGCAAT CCTCAGGAGG GGACCCAGAA ATTGTAACGC ACAGTTTTAA TTGTGGAGGG
7365   GAATTTTTCT ACTGTAATTC AACACAACTG TTTAATAGTA CTTGGTTTAA TAGTACTTGG
7425   AGTACTGAAG GGTCAAATAA CACTGAAGGA AGTGACACAA TCACCCTCCC ATGCAGAATA
7485   AAACAAATTA TAAACATGTG GCAGAAAGTA GGAAAAGCAA TGTATGCCCC TCCCATCAGT
7545   GGACAAATTA GATGTTCATC AAATATTACA GGGCTGCTAT TAACAAGAGA TGGTGGTAAT
7605   AGCAACAATG AGTCCGAGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA CAATTGGAGA
         ES8⇐
7665   AGTGAATTAT ATAAATATAA AGTAGTAAAA ATTGAACCAT TAGGAGTAGC ACCCACCAAG
7725   GCAAAGAGAA GAGTGGTGCA GAGAGAAAAA AGAGCAGTGG GAATAGGAGC TTTGTTCCTT
                                  ED12⇐
7785   GGGTTCTTGG GAGCAGCAGG AAGCACT
```

University of Cape Town

2.2.2.1 'Nested' PCR

For the first-round reaction, 10 µl of PBMC DNA was used in a total volume of 50 µl containing 2 µl of each primer ED5 and ED12 (5 pmol/µl), 5 µl 10X PCR buffer, 2.5 µl $MgCl_2$ (25 mM), 0.5 µl DMSO, 0.5 µl glycerol, 0.1 µl each dNTP (100 mM), 0.5 µl *Taq* polymerase (5 U/µl) and 26.6 µl water.

Thermocycling parameters were as follows: Initial denaturation at 94 °C for 2 minutes; 3 cycles of 94 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s; 32 cycles of 94 °C for 15 s, 55 °C for 45 s, 72 °C for 60 s; final extension at 72 °C for 300 s.

For the second-round reaction, 2.5 µl or 5 µl of first-round reaction mixture was used in a total volume of 50 µl containing 2 µl of each primer ED31 and ES8 (5 pmol/µl), 5 µl 10X PCR buffer, 3.6 µl $MgCl_2$ (25 mM), 0.5 µl DMSO, 0.5 µl glycerol, 0.1 µl each dNTP (100 mM), 0.25 µl *Taq* polymerase (5 U/µl) and 33.25 µl or 30.75 µl water.

Thermocycling parameters were the same as used for the first-round amplification.

2.2.2.2 'Hotstart' PCR

'Hotstart' PCR was done on samples which failed to amplify using the standard nested PCR in section 2.2.2.1.

For the first-round reaction, the protocol was modified as follows: 10 µl of PBMC DNA was used in a preliminary volume of 45 µl containing 2 µl of each primer ED5 and ED12 (5 pmol/µl), 4.5 µl 10X PCR buffer, 2.5 µl $MgCl_2$ (25 mM), 0.5 µl DMSO, 0.5 µl glycerol, 0.1 µl each dNTP (100 mM) and 21.6 µl water. The reaction mixture was heated to 94 °C for 10 minutes. 5 µl of a solution made up of 0.5 µl *Taq* polymerase (5 U/µl), 0.5 µl 10X PCR buffer and 4 µl water was added and cycled as described in section 2.2.2.1.

University of Cape Town

2.2.3 LIGATION OF PCR PRODUCTS INTO PLASMID VECTORS FOR TRANSFORMATION INTO *E. COLI*

In order to produce sufficient quantities of template for manual DNA sequencing, amplicons generated by PCR are ligated into plasmid vectors that replicate to high copy numbers in *E. coli*. An improved cloning strategy takes advantage of thermostable DNA polymerases used in PCR that add an unpaired adenosine nucleotide to the 3'-end of double-stranded DNA. The use of a cloning vector with an unpaired thymidine nucleotide added to the 3'-end, such as the pMOS*Blue* T-vector (Amersham, Piscataway, USA), facilitates ligation of amplicons into the multiple cloning site of the beta-galactosidase reporter gene. Recombinant plasmids are transformed into competent *E. coli* and transformants are positively selected on agar plates containing antibiotic, X-gal and IPTG. Bacteria transformed with plasmid in which the beta-galactosidase gene has been insertionally inactivated are unable to catalyze the cleavage of X-gal and colonies appear white. The correct size insert is determined by restriction enzyme mapping of plasmid DNA extracted from overnight cultures of putative clones. The correct sequence of the insert is verified by additional analysis by restriction enzyme mapping or colony hybridization assay.

University of Cape Town

2.2.3.1 **Ligation of PCR products into pMOS*Blue* T-vector**

The 851 bp fragment spanning the C2-V5 region amplified by 'nested' PCR using the inner primer set (ED31 and ES8) was used for the ligation reactions as described below.

Amplicons were partially purified as follows: 5 µl of chloroform-isoamyl alcohol (24:1) was added to an equal volume of PCR reaction mixture, mixed by vortex for 60 s and centrifuged for 60 s. The ligation reaction was prepared, according to instructions supplied with the pMOS*Blue* T-vector kit (Amersham, Piscataway, USA), as follows: 2 µl of the supernatant was used in a total volume of 10 µl containing 1 µl 10X ligase buffer, 0.5 µl DTT (100 mM), 0.5 µl ATP (10 mM), 1 µl pMOS*Blue* T-vector (50 ng/µl),

0.5 µl T4 DNA ligase (10 U/µl) and 4.5 µl of water. The mixture was incubated at 16 °C for 2 hours.

2.2.3.2 **Transformation of recombinant plasmid into competent *E. coli***

2.2.3.2.1 Preparation of competent *E. coli*

Using a sterile loop, *E. coli* DHα bacteria from a glycerol stock stored at -70 °C were streaked onto a Luria agar (Appendix A) plate and incubated without antibiotic selection at 37 °C overnight. The following day, a single colony was transferred from the plate to 25 ml of Luria broth (Appendix A) and incubated on a shaker at 37 °C overnight. 5 ml of the overnight culture was inoculated into 500 ml of Luria broth and incubated at 37 °C until the OD_{600} was between 0.45 and 0.55. The culture was divided into equal volumes of 250 ml and cooled on ice for 2 hours. The cells were concentrated by centrifugation at 5,000 rpm for 20 minutes at 4 °C. The supernatant was discarded and the cells resuspended in ice-cold trituration buffer (Appendix A). 250 ml of ice-cold trituration buffer was added and the mixture cooled on ice for 45 minutes. The cells were concentrated by centrifugation at 4,000 rpm for 10 minutes at 4 °C. The supernatant was discarded and the cells resuspended in 25 ml of trituration buffer. The two resuspensions were pooled and 11.5 ml of 80% (v/v) glycerol added. 1 ml aliquots were transferred to cold 1.5 ml microfuge tubes and stored at -70 °C.

University of Cape Town

2.2.3.2.2 Measurement of transformation efficiency

The efficiency of transformation of competent cells is defined as the number of cells transformed per µg of plasmid and was measured by transforming a 200 µl aliquot of cells with 0.2 ng of plasmid. 1 ml of competent cells stored at -70 °C was thawed on ice. 1 µl of pMOS*Blue* test plasmid (0.0002 µg/µl) and 5 µl of DMSO was added to a 200 µl aliquot of thawed competent cells and incubated on ice for 30 minutes. The suspension was transferred to a 42 °C waterbath for 40 s and returned to ice for 2 minutes. 800 µl of

Luria broth was added and the mixture incubated at 37 °C for 1 hour. 5 µl, 50 µl and 100 µl of the transformation mixture was transferred to Luria agar plates containing 50 µg/ml ampicillin, 10 µg/ml IPTG and 50 µg/ml X-gal and incubated at 37 °C overnight.

The efficiency of transformation was calculated using the following formula:

$$\frac{\text{(no. of colonies) X (dilution factor)}}{\text{no. of µg of plasmid}}$$

2.2.3.2.3 Transformation of *E. coli*

The method of transformation described in section 2.2.3.2.2 was repeated using 1 µl of ligation mixture. The transformation mixture was plated in aliquots of 250 µl on large agar plates containing 50 µg/ml ampicillin, 10 µg/ml IPTG and 50 µg/ml X-gal and incubated at 37 °C overnight.

2.2.4 SCREENING OF TRANSFORMANTS FOR RECOMBINANT PLASMIDS

2.2.4.1 **Restriction fragment length polymorphism (RFLP) analysis**

2.2.4.1.1 Plasmid extraction

Plasmid DNA was extracted from cultured bacteria using the boiling method described by Berghammer and Auer (1993) as follows:

Using a sterile loop, white colonies were transferred from agar plates into sterile test tubes containing 5 ml of Luria broth with ampicillin (50 µg/ml). The test tubes were capped and incubated on a shaker at 37 °C overnight. 1.5 ml of the culture was transferred to microfuge tubes and centrifuged at maximum speed for 60 s. The

University of Cape Town

supernatant was discarded and 100 µl of lysis buffer (Appendix A) was added. The tubes were incubated at room temperature for 10 minutes on a shaker, transferred to a boiling waterbath for 60 s, cooled on ice for 60 s and centrifuged at maximum speed for 20 minutes. The supernatant containing plasmid DNA was recovered for further applications.

2.2.4.1.2 Identification of correct size insert by restriction enzyme mapping of plasmid

10 µl of supernatant was used in a reaction volume of 15 µl containing 1.5 µl 10X buffer H, 0.5 µl *Pst*I (5 U/µl), 0.5 µl *Eco*RI (5 U/µl) and 2.5 µl of water. Reaction mixtures were incubated at 37 °C for 2 hours and 10 µl used for agarose gel electrophoresis.

The expected fragments of 918 bp and 2887 bp of digested plasmid DNA were resolved on a 2% (w/v) agarose gel containing ethidium bromide (10 ng/ml) and visualized under UV light at 256 nm.

University of Cape Town

2.2.4.2 **Colony hybridization assay**

A method to prepare denatured bacterial DNA fixed onto a nylon membrane for hybridization assays described by Buluwela *et al.* (1989), was used to screen colonies for the correct insert using a digoxigenin (DIG)-labelled probe and a DIG-detection assay (Boehringer-Mannheim, Mannheim, Germany).

2.2.4.2.1 Preparation of agar plates

Putative clones identified by RFLP analysis of plasmid were plated onto duplicate agar gridded-plates. A Hybond-N nylon membrane (Amersham, Piscataway, USA) was placed on the surface of the agar layer of one plate before bacteria were streaked out. The plates were incubated at 37 °C overnight. The following day the membrane containing adhered bacteria was removed for probe hybridization assay.

2.2.4.2.2 Preparation of nylon membrane

Lysis of bacterial colonies, denaturation of DNA and fixation of single-stranded DNA to the membrane was achieved by placing the membrane with the colonies on the upper surface onto Whatman 3MM filter paper soaked in 2X SSC (Appendix A) containing 5% SDS for 5 minutes at room temperature. The membrane was then placed in a microwave oven for 3 minutes at maximum power (650W), soaked in 5X SSC containing 0.1% SDS and placed in a hybridization bag.

2.2.4.2.3 Preparation of DIG-labelled DNA probe

A DIG-labelled DNA probe was generated by PCR using a mixture dNTP's containing DIG-dUTP (Boehringer-Mannheim, Mannheim, Germany).

For PCR amplification, 2 µl of a subtype-C reference plasmid (50 pg/µl) taken from the Heteroduplex Mobility Analysis (HMA) HIV-1 *env* Genetic Subtyping Kit (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: catalogue number ARP961) was used in a total reaction volume of 50 µl containing 2 µl of each primer ED31 and ES8 (5 pmol/µl), 5 µl 10X PCR buffer, 3.6 µl $MgCl_2$ (25 mM), 0.5 µl DMSO, 0.5 µl glycerol, 10 µl dNTP DIG-dUTP mixture, 0.25 µl *Taq* polymerase (5 U/µl) and 24.15 µl water.

Thermocycling parameters were as follows: Initial denaturation at 94 °C for 2 minutes; 3 cycles of 94 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s; 32 cycles of 94 °C for 15 s, 55 °C for 45 s, 72 °C for 60 s; final extension at 72 °C for 300 s.

Incorporation of DIG-dUTP into amplicons was confirmed by agarose gel electrophoresis indicating a decrease in mobility due to the increased molecular weight.

University of Cape Town

2.2.4.2.4 Hybridization and DIG-detection assay

The membrane was blocked by prehybridizing for 1 hour at 42 °C in hybridization buffer (Appendix A). 5 µl of PCR reaction mixture containing DIG-labelled probe was heat-denatured at 94 °C for 2 minutes and rapidly cooled on dry ice. The membrane was incubated overnight at 42 °C in hybridization buffer containing denatured probe. The following day the membrane was rinsed in 2X SSC containing 0.1% SDS for 5 minutes at room temperature.

Detection of hybridized DIG-labelled probes was performed using the DIG Luminescent detection kit (Boehringer-Mannheim, Mannheim, Germany). The kit makes use of an anti-DIG antibody conjugated to alkaline phosphatase to bind DIG-labelled molecules. After rinsing the membrane free of unbound antibody, the chemilluminescent substrate was added to allow immobilized enzyme-conjugate to catalyze the chemical reaction that emits light. Emitted light was detected by autoradiography using X-ray film.

After addition of the substrate, the membrane was sealed in a plastic bag and placed in an X-ray cassette in contact with Agfa Curix RP1 X-Ray film. The film was developed after 10 minutes of exposure.

Bacterial clones containing recombinant plasmid with the correct insert were identified by viewing the autoradiograph of the probe hybridization assay under visible light and identifying positively-stained areas.

2.3 **DNA SEQUENCING**

2.3.1 MANUAL DNA SEQUENCING OF VECTOR INSERTS

In order to assess the reliability of heteroduplex mobility assay as a method of analyzing intrasample sequence diversity the eight clones (RX1-m1 to RX1-m8) previously identified by hybridization assay, were manually sequenced using the chain termination

University of Cape Town

method described by Sanger *et al.* (1977). Sequence reactions were performed using reagents supplied in the Sequenase 2.0 DNA sequencing kit (United States Biochemicals, Cleveland, USA).

2.3.1.1 **Extraction of template DNA**

A liquid culture of each clone was prepared by inoculating 100 ml of Luria broth containing ampicillin (50 µg/ml) with a sterile loop which was used to scrape the upper surface of frozen glycerol stocks of bacteria stored at -70 °C. The cultures were incubated at 37 °C overnight. Plasmid DNA was extracted from overnight cultures using a Nucleobond AX-100 plasmid purification kit (Macherey-Nagel, Duren, Germany) according to the instructions supplied with the kit.

Briefly, the cells were concentrated by centrifugation at 8000 rpm for 10 minutes in a Beckman J2-21 centrifuge using a JA20 rotor. The pelleted cells were resuspended in 4 ml of buffer provided (solution S1). 4 ml of NaOH/SDS lysis buffer (solution S2) was added, the suspension mixed gently and incubated at room temperature for 5 minutes. 4 ml of potassium acetate buffer (solution S3) was added, the suspension mixed gently and incubated on ice for 5 minutes. The suspension was centrifuged at 10,000 rpm for 38 minutes at 4 °C. The supernatant was carefully removed with a pipette and applied to an AX-100 cartridge pre-equilibrated with 2 ml of buffer supplied (solution N2). The cartridge was washed twice with 4 ml of supplied washing buffer (solution N3). 2 ml of elution buffer (solution N5) was added to the cartridge and equal volumes of 1 ml collected in each of two 2 ml microfuge tubes.

The eluted plasmid DNA in each tube was precipitated by addition of 700 µl of isopropanol and centrifugation in a microfuge at maximum speed for 10 minutes. The pelleted DNA was washed by adding 1 ml of 70% ethanol to each tube and centrifugation at maximum speed for 10 minutes. The ethanol was discarded and the pellets dried under vacuum for 5 minutes. The pelleted DNA was resuspended in 50 µl of TE buffer (Appendix A).

University of Cape Town

The concentration and purity of each plasmid isolation was calculated from the OD_{260} and OD_{280} absorbance readings using a 1:100 dilution of sample in a spectrophotometer.

2.3.1.2 **Preparation of template for manual sequencing**

An appropriate aliquot of sample containing between 5 and 10 µg of plasmid DNA (sufficient for two sequencing reactions) was made up to a volume of 32 µl with water. The DNA was denatured by adding 8 µl of 2M NaOH and incubated at room temperature for 10 minutes. The alkali was neutralized by adding 7 µl of 3M sodium acetate (pH 4.8). The DNA was precipitated by adding 4 µl of water and 120 µl of 100% ethanol and cooled on dry ice for 15 minutes. The mixture was centrifuged for 15 minutes in a microfuge and the supernatant fluid discarded. The ethanol was discarded and the pellet dried under vacuum for 5 minutes. The DNA was resuspended in 14 µl of water and divided into equal volumes of 7 µl in separate microfuge tubes. 2 µl of 5X annealing reaction buffer supplied in the sequencing kit was added to each tube as well as 1 µl containing 5 pmol of forward or reverse primer (Box 2.3 and 2.4). The reactions were incubated at 65 °C for 2 minutes and allowed to cool to below 35 °C. The samples were cooled on ice before proceeding to the sequencing reactions.

University of Cape Town

Box 2.3: Sequencing primers used

T7⇒ (vector forward primer)
5'-TAA TAC GAC TCA CTA TAG GG-3'
ES7⇒ (insert forward primer)
*HXB2 numbering 7002-7021
5'-CTG TTA AAT GGC AGT CTA GC-3'
U19-mer⇐ (vector reverse primer)
5'-GTT TTC CCA GTC ACG ACG T-3'
SLR⇐ (insert reverse primer)
*HXB2 numbering 7362-7380
5'-TAC AGT AGA AAA ATT CCC C-3'

*HXB2 numbering according to Los Alamos HIV database (http://hiv-web.lanl.gov)

Box 2.4: Location of sequencing primers ES7 and SLR onto the HIV-1 clone HXB2 sequence of the partial gp120 region of *env*

```
        ED31⇒
6817    CCTCAGTCAT TACACAGGCC TGTCCAAAGG TATCCTTTGA GCCAATTCCC ATACATTATT
6877    GTGCCCCGGC TGGTTTTGCG ATTCTAAAAT GTAATAATAA GACGTTCAAT GGAACAGGAC
6937    CATGTACAAA TGTCAGCACA GTACAATGTA CACATGGAAT TAGGCCAGTA GTATCAACTC
             ES7⇒
6997    AACTGCTGTT AAATGGCAGT CTAGCAGAAG AAGAGGTAGT AATTAGATCT GTCAATTTCA
7057    CGGACAATGC TAAAACCATA ATAGTACAGC TGAACACATC TGTAGAAATT AATTGTACAA
7117    GACCCAACAA CAATACAAGA AAAAGAATCC GTATCCAGAG AGGACCAGGG AGAGCATTTG
7177    TTACAATAGG AAAAATAGGA AATATGAGAC AAGCACATTG TAACATTAGT AGAGCAAAAT
7237    GGAATAACAC TTTAAAACAG ATAGCTAGCA AATTAAGAGA ACAATTTGGA AATAATAAAA
7297    CAATAATCTT TAAGCAATCC TCAGGAGGGG ACCCAGAAAT TGTAACGCAC AGTTTTAATT
                                 SLR⇐
7357    GTGGAGGGGA ATTTTTCTAC TGTAATTCAA CACAACTGTT TAATAGTACT TGGTTTAATA
7417    GTACTTGGAG TACTGAAGGG TCAAATAACA CTGAAGGAAG TGACACAATC ACCCTCCCAT
7477    GCAGAATAAA ACAAATTATA AACATGTGGC AGAAAGTAGG AAAAGCAATG TATGCCCCTC
7537    CCATCAGTGG ACAAATTAGA TGTTCATCAA ATATTACAGG GCTGCTATTA ACAAGAGATG
7597    GTGGTAATAG CAACAATGAG TCCGAGATCT TCAGACCTGG AGGAGGAGAT ATGAGGGACA
                   ES8⇐
7657    ATTGGAGAAG TG
```

University of Cape Town

2.3.1.3 **Sequencing reactions**

Sequencing reactions were performed according to the instructions supplied. Briefly, the labelling reaction was performed by adding 0.4 µl labelling mixture, 1 µl DTT, 0.5 µl [α-S^{35}]-dATP (Amersham, Piscataway, USA), 2 µl diluted enzyme and 1.6 µl of water to the 10 µl of annealing reaction mixture. The reaction mixture was incubated for 5 minutes at room temperature for 5 minutes. 3.5 µl of the labelling reaction was added to each of four tubes containing 2.5 µl of termination mixture corresponding to a different ddNTP. The termination reactions were incubated at 37 °C for 5 minutes before adding 4 µl of stop solution.

2.3.1.4 **Polyacrylamide gel electrophoresis**

DNA fragments generated by the sequencing reactions were resolved by electrophoresis on a 6% (w/v) denaturing polyacrylamide gel. The gel apparatus used was made up of two rectangular glass plates separated by two 0.5 mm plastic spacers placed between the

plates at the sides along the length. A section of Whatman 3MM filter paper was placed between the plates at the base along the breadth and the assembly held together with metal clamps. 100 ml of a 6% polyacrylamide denaturing gel solution was prepared using 12 ml of 50% Longranger (AT Biochem, Malvern, USA), 10 ml of 10X NNB (Appendix A) and 42 g of urea made up to a volume of 100 ml with water. Polymerization was initiated by adding 100 µl of TEMED and 100 µl of a freshly prepared 25% APS solution. After polymerization the gel was pre-run in 1X NNB buffer for 30 minutes at 80W before loading of samples. Samples were heated at 80°C for 2 minutes before loading to generate single-stranded DNA molecules. 1 µl, 2.5 µl and 5 µl of each sample was loaded at different stages during electrophoresis corresponding to a short, medium and long run so as to obtain maximum sequence information. After electrophoresis, the gel was allowed to cool and the apparatus disassembled. The glass plates were separated and the gel transferred to a sheet of Whatman 3MM filter paper and dried for 1 hour under vacuum on a heated gel dryer.

University of Cape Town

2.3.1.5 **Autoradiography**

Once dry, the gel was placed in contact with a sheet of Agfa Curix RP1 X-ray film inside an X-ray cassette overnight. The following day, the exposed X-ray film was developed for 2 minutes, stopped in 2% acetic acid for 2 minutes, fixed for 5 minutes and rinsed in running water for 5 minutes. The film was then air dried.

2.3.1.6 **Sequence analysis**

Sequences were aligned using Clustal X software (Thompson *et al.*, 1997). Subtyping of sequences using the V3 loop reference subtypes was done by phylogenetic tree construction using Treecon software (Van de Peer and De Wachter, 1994) and the Kimura two-parameter algorithm. Sequences of the V3 loop from reference subtypes were downloaded from the Los Alamos HIV sequence database (http://hiv-web.lanl.gov).

2.4 HETERODUPLEX MOBILITY ASSAY (HMA) AND BASE EXCISION SEQUENCE SCANNING (BESS)

2.4.1 HETERODUPLEX MOBILITY ASSAY (HMA)

Heteroduplex mobility assay (HMA) developed by Delwart *et al.* (1993) offers a rapid and cost-effective method to subtype and display the degree of divergence between sequences amplified by PCR. Briefly, the method is based on the observation that hybrid molecules formed between related sequences (heteroduplexes) show reduced mobility with respect to the migrating homoduplexes when resolved in polyacrylamide gels. This shift in mobility is directly proportional to the degree of divergence between the positive and negative strands forming the heteroduplex. Subtyping of unknown samples can be achieved by generating heteroduplexes between known subtypes and the sample and observing the smallest shift in heteroduplex mobility. Similarly intrasample sequence diversity can be displayed by comparison of individual sequences hybridized to a common driver sequence. The limitation of using HMA as a display of DNA difference is the resolution limit of 1-2% as well as the influence on mobility shifts due insertions and deletions.

2.4.1.1 **Subtyping**

For subtyping of sample unknowns, one cloned sequence from each sample was selected for comparison with reference plasmids supplied in the HMA kit (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: catalogue number ARP961). The reference plasmids used are listed in Table 2.2

2.4.1.1.1 PCR amplification of reference HIV-1 subtypes

The primer pair ES7 and ES8 (Box 2.7) was used for the amplification of a 667 bp fragment of the *env* gene (Box 2.8). For the PCR amplification of reference plasmids, 1 µl of plasmid standard (10 ng/µl) was used in a total reaction volume of 100 µl

University of Cape Town

containing 4 µl of each primer (5 pmol/µl), 10 µl 10X PCR buffer, 7.2 µl $MgCl_2$ (25 mM), 1 µl DMSO, 1 µl glycerol, 0.2 µl each dNTP (100 mM), 0.5 µl *Taq* polymerase (5 U/µl) and 70.5 µl water.

Thermocycling parameters were as follows: initial denaturation at 94 °C for 2 minutes; 3 cycles of 94 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s; 32 cycles of 94°C for 15s, 55°C for 45s, 72°C for 60s; final extension at 72°C for 300s.

Box 2.7: List of PCR primers used (Delwart *et al.*, 1993)

```
ES7⇒ (insert forward primer)
*HXB2 numbering 7002-7021
5'-CTG TTA AAT GGC AGT CTA GC-3'
ES8⇐ (inner reverse primer)
*HXB2 numbering 7648-7668
5'-CAC TTC TCC AAT TGT CCC TCA-3'
```

*HXB2 numbering according to Los Alamos HIV database (http://hiv-web.lanl.gov)

Box 2.8: Mapping of PCR primers onto the sequence of HIV-1 clone HXB2 partial *env* region

```
                                                                ED31⇒
6765   ATAATACCAA TAGATAATGA TACTACCAGC TATAAGTTGA CAAGTTGTAA CACCTCAGTC
6825   ATTACACAGG CCTGTCCAAA GGTATCCTTT GAGCCAATTC CCATACATTA TTGTGCCCCG
6885   GCTGGTTTTG CGATTCTAAA ATGTAATAAT AAGACGTTCA ATGGAACAGG ACCATGTACA
                                                                ES7⇒
6945   AATGTCAGCA CAGTACAATG TACACATGGA ATTAGGCCAG TAGTATCAAC TCAACTGCTG
7005   TTAAATGGCA GTCTAGCAGA AGAAGAGGTA GTAATTAGAT CTGTCAATTT CACGGACAAT
7065   GCTAAAACCA TAATAGTACA GCTGAACACA TCTGTAGAAA TTAATTGTAC AAGACCCAAC
7125   AACAATACAA GAAAAAGAAT CCGTATCCAG AGAGGACCAG GGAGAGCATT TGTTACAATA
7185   GGAAAAATAG GAAATATGAG ACAAGCACAT TGTAACATTA GTAGAGCAAA ATGGAATAAC
7245   ACTTTAAAAC AGATAGCTAG CAAATTAAGA GAACAATTTG GAAATAATAA AACAATAATC
7305   TTTAAGCAAT CCTCAGGAGG GGACCCAGAA ATTGTAACGC ACAGTTTTAA TTGTGGAGGG
7365   GAATTTTTCT ACTGTAATTC AACACAACTG TTTAATAGTA CTTGGTTTAA TAGTACTTGG
7425   AGTACTGAAG GGTCAAATAA CACTGAAGGA AGTGACACAA TCACCCTCCC ATGCAGAATA
7485   AAACAAATTA TAAACATGTG GCAGAAAGTA GGAAAAGCAA TGTATGCCCC TCCCATCAGT
7545   GGACAAATTA GATGTTCATC AAATATTACA GGGCTGCTAT TAACAAGAGA TGGTGGTAAT
7605   AGCAACAATG AGTCCGAGAT CTTCAGACCT GGAGGAGGAG ATATGAGGGA CAATTGGAGA
     ⇐ES8
7665   AGTGAATTAT ATAAATATAA AGTAGTAAAA ATTGAACCAT TAGGAGTAGC ACCCACCAAG
```

University of Cape Town

Table 2.2: List of reference plasmids used for subtyping of sample unknowns by HMA (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: catalogue number ARP961)

Subtype:	Strain:	Country of origin:
A	RW20	Rwanda
B	BR20	Brazil
B	TH14	Thailand
C	MA959	Malawi
C	ZM18	Zambia
D	UG21	Uganda

2.4.1.1.2 PCR amplification of sample unknowns

The unknown samples were available as plasmids in glycerol stocks of transformed bacteria stored at -70 °C. Colony PCR was found to be a simple way of generating amplified DNA for HMA purposes.

For the PCR amplification of unknown samples, 2.5 µl of thawed glycerol stock suspension was used in a total reaction volume of 25 µl containing 1ul of each primer (5 pmol/µl), 2.5 µl 10X PCR buffer, 1.8 µl $MgCl_2$ (25 mM), 0.25 µl DMSO, 0.25 µl glycerol, 0.05µl each dNTP (100mM), 0.125µl *Taq* polymerase (5U/µl) and 15.375µl water.

Thermocycling parameters were the same as described in section 2.4.1.1.1.

2.4.1.1.3 Generation of heteroduplexes

Heteroduplexes were formed by combining 5 µl of each reference sample and 5 µl of the unknown sample with 1.1 µl of HMA annealing buffer (Appendix A) in a microfuge tube. A control tube using 5 µl of water and 5 µl of unknown sample was included. The

University of Cape Town

samples were heated to 94 °C for 2 minutes and then cooled on wet ice for 2 minutes before resolving on a polyacrylamide gel.

2.4.1.1.4 Polyacrylamide gel electrophoresis

A 6% polyacrylamide gel solution was prepared by adding 12 ml of 50% Longranger (AT Biochem, Malvern, USA) to 10 ml 10X NNB buffer and making the volume up to 100 ml with water. Polymerization was initiated by adding 100 µl of TEMED and 100 µl of fresh 25% APS. A 19 cm by 16 cm by 1.5 mm set of glass plates were used to cast the gel. The entire sample was used for electrophoresis a 200 V constant voltage for 5 hours on a Hoefer SE600 gel apparatus. Gels were stained with ethidium bromide after electrophoresis and DNA bands visualized under UV light.

2.4.1.2 **Analysis of genetic diversity**

In order to assess the genetic diversity between sequences, HMA was used by comparing the mobility of heteroduplexes formed between a single reference sequence (driver sequence) and the sequences of each clone. Each set of clones was assayed using an unrelated subtype C driver sequence. In addition the sequences of the child were assayed using a driver sequence derived from the mother and *vice versa*.

2.4.1.2.1 PCR amplification of driver sequence

The unrelated subtype C driver sequence selected was amplified from the MA959 HIV-1 strain from Malawi using the same PCR parameters as described in section 2.2.2.1. In addition, the most common sequence and therefore the dominant species observed in the HMA pattern of each mother using the driver sequence, was selected for re-amplification and used as a driver sequence for analysis of the sequences from the child and *vice versa*.

University of Cape Town

2.4.1.2.2 PCR amplification, formation of heteroduplexes and polyacrylamide gel electrophoresis of samples

For amplification of the sample sequences, 2.5 µl of thawed glycerol stock suspension was used in a colony PCR and the samples analyzed as described in section 2.4.1.1.2.

Box 2.9: PCR primers used (Delwart *et al.*, 1993)

ED31⇒ (outer forward primer)
*HXB2 numbering 6817-6845
5'-CCT CAG CCA TTA CAC AGG CCT GTC CAA AG-3'
ES8⇐ (outer reverse primer)
*HXB2 numbering 7648-7668
5'-CAC TTC TCC AAT TGT CCC TCA-3'
ES7⇒ (inner forward primer)
*HXB2 numbering 7002-7021
5'-CTG TTA AAT GGC AGT CTA GC-3'
V3R⇐ (inner reverse primer)
*HXB2 numbering 7273-7292
5'-TTT ATT AGG GAA GTG TTC TC-3'

*HXB2 numbering according to Los Alamos HIV database (http://hiv-web.lanl.gov)

Box 2.10: Mapping of PCR primers onto the sequence of the HIV-1 clone RX1-m1 partial *env* region

```
        ED31⇒
1       CCTCAGCCAT TACACAGGCC TGTCCAAAGG TCTCTTTTGA CCCGATTCCT ATACATTATT
61      GTGCTCCAGC TGGCTATGCG ATTCTAAAGT GTAATAATAA GACATTCAGT GGGAGAGGAC
121     CATGCCGTAA TGTCAGCACA GTCCAATGTA CACATGGAAT TAAGCCTGTG GTATCAACTC
             ES7⇒
181     AACTACTGTT AAATGGTAGC CTAGCAGAAG GGGAGATAAT AATTAGATCT GAGAATCTGA
                                                   V3 loop⇒
241     CAGACAATGT CAAAATAATA ATAGTACACC CTAATGAATC TGTAGAAATT GTGTGTACAA
301     GACCCAACAA TAATACAAGA AAAAGTATAA GGATAGGACC AGGACAAACA TTCTTTGCAA
361     CAAATGACAT AATAGGAGAC ATAAGACAAG CACATTGTAA CATTAGTACA ACAAAATGGA
                                                         V3R⇐
421     ACACAACTTT AGAAAGGGTA AGGAAGAAAT TAGGAGAACA CTTCCCTAAT AAAACAATAA
481     CCTTTAAACA GCCCTCAGGA GGGGACCTAG AAATTACAAC ACATAGCTTT AATTGTAGAG
541     GAGAGTTTTT TTATTGCAAT ACATCAAATC TGTTTCCTAA TGACAATGGG TCAAACCCAA
601     CCACCATCCC ATGCAAGATA AAACAAATTA TAAACATGTG GCAGGGGGTA GGACGAGCAA
661     TGTATGCCCC TCCCATTAAA GGGAACATAA CACGTAACTC AAGTATCACA GGATTACTAT
721     TGACACGTGA TGGTGGCGAC GGGAATAACA TAGAGGAGAT ATTCAGACCT GGAGGAGGAG
                              ES8⇐
781     ATATGAGGGA CAATTGGAGA AGTG
```

University of Cape Town

2.4.2 BASE EXCISION SEQUENCE SCANNING (BESS) ANALYSIS

An alternative rapid method available for the analysis of genetic diversity is Base Excision Sequence Scanning or BESS (Epicentre Technologies, Madison, USA) which identifies thymine residues on one strand of DNA amplified by PCR. This method can be used to map thymine residues on both strands and thereby generate partial sequence data for analysis. Briefly, the method uses PCR to amplify the DNA sequence of interest and incorporates limiting amounts of dUTP into the amplicons. Amplicons are then incubated with uracil N-glycosylase which removes uracil from the sugar-phosphate backbone leaving an abasic site. The sugar-phosphate backbone is cleaved at the abasic site by a second enzyme, exonuclease IV. End-labelled radioactive cleavage fragments can be resolved on a denaturing polyacrylamide gel and exposed to X-ray film resulting in a sequence pattern that resembles a T-ladder typical of manual S^{35} DNA sequencing autoradiographs. Sequence data concerning adenine and thymine residues can be accumulated by mapping the thymine residues of both strands. The method was applied using a P^{32} radio-labelled PCR primer and then adapted for analysis by an automated DNA-sequencer using a PCR primer labelled with fluorescein.

University of Cape Town

2.4.2.1 **BESS analysis using radio-labelled PCR primers**

PCR primers were labelled with [gamma-P^{32}]-dATP using PNK enzyme (Boehringer-Mannheim, Mannheim, Germany). The labelling-reaction was performed according to the instructions supplied with the enzyme.

Briefly, the labelling-reaction was performed in a total volume of 20 µl containing 2 µl 10X reaction buffer, 1 µl [gamma-P^{32}]-dATP (Amersham, Piscataway, USA), 1 µl of ES7 forward primer (20 pmol/µl), 1 µl T4 PNK (10 U/µl) and 15 µl water. The reaction mixture was incubated at 37 °C for 30 minutes. 1µl of the labelling-mixture containing labelled primer was used for PCR.

2.4.2.1.1 Amplification of sample DNA by PCR

The primer pair ES7 and ES8 was used for the amplification of a 667 bp fragment of the *env* gene. Glycerol stocks of transformed bacteria stored at -70 °C were used in a colony PCR to amplify DNA for BESS analysis.

For the amplification of unknown samples, 2.5 µl of thawed glycerol stock suspension was used in a total reaction volume of 25 µl containing 1 µl of radio-labelled forward primer (1 pmol/µl), 1 µl of unlabelled reverse primer (5 pmol/ul), 2.5 µl 10X PCR buffer, 1.8 µl $MgCl_2$ (25 mM), 2 µl BESS T-Scan dNTP Mix (Epicentre Technologies, Madison, USA), 0.125 µl *Taq* polymerase (5 U/µl) and 14.075 µl water.

Thermocycling parameters were as follows: initial denaturation at 94 °C for 2 minutes; 3 cycles of 94 °C for 60 s, 55 °C for 60 s, 72 °C for 60 s; 32 cycles of 94 °C for 15 s, 55 °C for 45 s, 72 °C for 60 s; final extension at 72 °C for 300 s.

2.4.2.1.2 Enzymatic cleavage of amplified DNA

According to the instructions of the suppliers, 10 µl PCR product was recommended in a 20 µl total reaction volume containing 2 µl BESS T-Scan 10X Excision Enzyme buffer, 1 µl BESS T-Scan Excision Enzyme Mix and 7 µl water. The reactions were incubated at 37 °C for 45 minutes. Reactions were stopped by addition of 10 µl of supplied stop/loading buffer.

2.4.2.1.3 Polyacrylamide gel electrophoresis of samples

DNA fragments generated by the cleavage reactions were resolved by electrophoresis on a 6% denaturing polyacrylamide gel as described for manual sequencing. Samples were heated at 80 °C for 2 minutes before loading to generate single-stranded DNA molecules and 2.5 µl of each sample was loaded.

University of Cape Town

After electrophoresis, the gel was allowed to cool and the apparatus disassembled. The glass plates were separated and the gel transferred to a sheet of Whatman 3MM filter paper and dried for 1 hour under vacuum on a heated gel dryer.

2.4.2.1.4 Autoradiography

Once dry, the gel was placed in contact with a sheet of Agfa Curix RP1 X-ray film inside an X-ray cassette overnight. The following day, the exposed X-ray film was developed for 2 minutes, stopped in 2% acetic acid for 2 minutes, fixed for 5 minutes and rinsed in running water for 5 minutes. The film was then air dried.

2.4.2.2 **BESS analysis using fluorescein-labelled PCR primers**

2.4.2.2.1 Amplification of samples by PCR

The primer pair ED31 and V3R was used for the amplification of a 473 bp fragment of the *env* gene. The reverse primer, V3R, was ordered with a 5'-fluorescein label from the Department of Biochemistry, UCT. Glycerol stocks of transformed bacteria stored at -70 °C were used in a colony PCR to amplify DNA for BESS analysis.

For the amplification of sample unknowns, 2.5 µl of thawed glycerol stock suspension was used in a total reaction volume of 25 µl containing 1 µl of unlabelled forward primer (5 pmol/µl), 1 µl of fluorescein-labelled reverse primer (5 pmol/µl), 2.5 µl 10X PCR buffer, 1.8 µl $MgCl_2$ (25 mM), 2 µl BESS T-Scan dNTP Mix (Epicentre Technologies, Madison, USA), 0.125 µl *Taq* polymerase (5 U/µl) and 14.075 µl water.

Thermocycling parameters were the same as described in section 2.4.2.1.1.

2.4.2.2.2 Enzymatic cleavage of PCR products

The same procedure was used as described in section 2.4.2.1.2.

University of Cape Town

2.4.2.2.3 Analysis of samples by an automated sequencer

2 µl of cleavage product was supplied to the Core Facility, Department of Chemical Pathology, UCT, for analysis on a Perkin-Elmer 377 automated sequencer.

2.5 **PCR-ELISA QUANTITATION**

2.5.1 PCR-ELISA QUANTITATION ASSAY

To measure the concentration of provirus in PBMC DNA samples a PCR-ELISA system (Kwok *et al.*, 1988) was used. Briefly, the method involves amplification of the viral target sequence by PCR using DIG-labelled reverse primers and unlabelled forward primers. The amplicons are alkali-denatured and hybridized to complimentary biotin-labelled oligonucleotide probe sequences in a conserved region on the negative-sense strand. Hybridized DNA molecules are captured in a streptavidin coated microtitre plate and assayed for the DIG-label by conventional ELISA methods. The copy number of target molecules in the unknown sample is inferred from a standard curve generated using an external standard of known copy number.

University of Cape Town

2.5.1.1 **Optimization of capture and detection of labelled molecules**

2.5.1.1.1 Homogeneity of streptavidin-coated plates

To determine whether the streptavidin-coated wells of the microtitre plate were coated with equivalent concentration of streptavidin, sixteen identical wells were assayed for bound control-peptide at two different concentrations of DIG-label and the OD_{450} values compared.

Streptavidin-coated microtitre plates were prepared by adding 100 µl of a 1 µg/ml stock of streptavidin (Sigma, St. Louis, USA) dissolved in carbonate/bicarbonate buffer (Appendix A) to each well and the plate incubated overnight at 4 °C. The wells were

then rinsed five times with PBS (Appendix A). 200 µl of blocking solution containing PBS and 1% (w/v) powdered milk was added to each well and the microtitre plate was incubated at 37 °C for 1 hour. The wells were then rinsed as before. 100 µl of control-peptide containing the equivalent of 1.67 fmol of DIG-label was added to sixteen successive wells and a second dilution containing the equivalent of 0.835 fmol of DIG-label was also included. The microtitre plate was incubated at 37 °C for 1 hour and then rinsed as before. 100 µl of anti-DIG POD conjugate (Boehringer-Mannheim, Mannheim, Germany) diluted 1:100 in blocking solution was added to each well. The microtitre plate was incubated at 37 °C for 1 hour and then rinsed as before. 100 µl of TMB substrate (Boehringer-Mannheim, Mannheim, Germany) was added to each well and the colour reaction allowed to develop at room temperature in the dark. The colour reaction was stopped after 30 minutes by adding 100 µl of 1M sulphuric acid to each well. OD_{450} values were measured for each well using a spectrophotometer with a reference filter of 620 nm.

2.5.1.1.2 Sensitivity of detection of labelled molecules

The sensitivity of detection of DIG- and biotin-labelled molecules captured by streptavidin was tested using a dilution series of control-peptide of known concentration of DIG-label. The assay was run in parallel with a dilution series of a labelled DNA probe generated by PCR using DIG- and biotin-labelled primers.

Streptavidin-coated microtitre plates were prepared as described in section 2.5.1.1.1. A two-fold dilution series of control-peptide in PBS was prepared ranging from the equivalent of 10 fmol to 0.3125 fmol of DIG-label. The DNA probe of unknown concentration was diluted in PBS in two-fold steps ranging from the undiluted sample to a 1:8192 dilution. 100 µl of each dilution was added to successive wells. Two wells were filled with PBS only to be used as controls for non-specific binding of the conjugate. Each dilution series was performed in triplicate to check the reproducibility of the assay. The microtitre plate was incubated at 37 °C for 1 hour and the wells rinsed as

University of Cape Town

before. 100 µl of anti-DIG POD conjugate diluted 1:100 in blocking solution was added to each well, except one well containing no sample, which was filled with 100 µl of blocking solution only. The assay was completed as described in section 2.5.1.1.1.

2.5.1.2 **Calibration of detection system**

The PCR-ELISA system was first tested with a PCR generated DIG-labelled amplicon before calibration of detection using a known copy number control sample. Thereafter, a standard curve was constructed by plotting copy number against optical density values obtained by PCR-ELISA of a two-fold dilution series starting at 100 copies of template molecules. A sample obtained from the diagnostic laboratory at UCT (Groote Schuur Hospital) was included as a positive control and for future use as a control standard.

2.5.1.2.1 PCR amplification

The PCR primers (SK431 and SK462), designed by Kwok *et al.* (1988), amplify a sequence of the p24 region of the HIV-1 *gag* gene and generates a fragment of 142 bp. The probe sequence (SK102) is complementary to a conserved sequence on the negative-strand. The sequences of the probe and primers used are listed in Box 2.5 and the region targeted is listed in Box 2.6. The use of DIG-labelled reverse primers and biotin-labelled probes complementary to the negative-strand allows hybridized molecules to be captured by streptavidin and quantified by ELISA for the DIG-label in a microtitre plate.

University of Cape Town

For PCR amplification, 10 µl of sample was used in a total reaction volume of 25 µl containing 0.5 µl of each primer (50 pmol/µl), 2.5 µl 10X PCR buffer, 1.8 µl $MgCl_2$ (25 mM), 1.25 µl DMSO, 0.05 µl each dNTP (100 mM), 0.025 µl *Taq* polymerase (5 U/µl) and 8.225 µl water.

Thermocycling parameters were as follows: initial denaturation at 94 °C for 120 s; 5 cycles of 93 °C for 20 s, 55 °C for 20 s, 72 °C for 20 s; 35 cycles of 92 °C for 20 s, 60 °C for 20 s, 72 °C for 20 s; final extension at 72 °C for 60 s.

Box 2.5: PCR primers and oligonucleotide probe (Kwok *et al.*, 1988)

SK462⇒ (forward primer)
*HXB2 numbering 1359-1388
5'-AGT TGG AGG ACA TCA AGC AGC CAT GCA AAT-3'
SK431⇐ (reverse primer)
*HXB2 numbering 1474-1500
5'-TGC TAT GTC AGT TCC CCT TGG TTC TCT-3'
SK102⇔ (oligonucleotide probe)
*HXB2 numbering 1396-1428
5'-GAG ACC ATC AAT GAG GAA GCT GCA GAA TGG GAT-3'

*HXB2 numbering according to Los Alamos HIV database (http://hiv-web.lanl.gov)

Box 2.6: Mapping of PCR primers onto the sequence of the HIV-1 clone HXB2 p24 region of *gag*

```
1       CCTATAGTGC AGAACATCCA GGGGCAAATG GTACATCAGG CCATATCACC TAGAACTTTA
61      AATGCATGGG TAAAAGTAGT AGAAGAGAAG GCTTTCAGCC CAGAAGTGAT ACCCATGTTT
                                                                SK462⇒
121     TCAGCATTAT CAGAAGGAGC CACCCCACAA GATTTAAACA CCATGCTAAA CACAGTGGGG
                                          SK102⇔
181     GGACATCAAG CAGCCATGCA AATGTTAAAA GAGACCATCA ATGAGGAAGC TGCAGAATGG
241     GATAGAGTGC ATCCAGTGCA TGCAGGGCCT ATTGCACCAG GCCAGATGAG AGAACCAAGG
              ⇐SK431
301     GGAAGTGACA TAGCAGGAAC TACTAGTACC CTTCAGGAAC AAATAGGATG GATGACAAAT
361     AATCCACCTA TCCCAGTAGG AGAAATTTAT AAAAGATGGA TAATCCTGGG ATTAAATAAA
421     ATAGTAAGAA TGTATAGCCC TACCAGCATT CTGGACATAA GACAAGGACC AAAGGAACCC
481     TTTAGAGACT ATGTAGACCG GTTCTATAAA ACTCTAAGAG CCGAGCAAGC TTCACAGGAG
541     GTAAAAAATT GGATGACAGA AACCTTGTTG GTCCAAAATG CGAACCCAGA TTGTAAGACT
601     ATTTTAAAAG CATTGGGACC AGCGGCTACA CTAGAAGAAA TGATGACAGC ATGTCAGGGA
661     GTAGGAGGAC CCGGCCATAA GGCAAGAGTT TTG
```

University of Cape Town

2.5.1.2.2 Preparation of sample DNA for DIG-detection

Amplicons were denatured by diluting 20 µl of PCR product in 70 µl of hybridization buffer (Appendix A) and adding 10 µl of 2M NaOH. The solution was incubated at room temperature for 5 minutes. The alkali was neutralized by adding 11 µl of 2M acetic acid and the solution made up to 125 µl volume by adding an additional 14 µl of buffer. 100 µl was used for the DIG-detection assay.

2.5.1.2.3 DIG-detection ELISA

The DIG detection assay was performed with streptavidin-coated microtitre plates prepared as described in section 2.5.1.1.1. After rinsing each well as before, 100 µl of PBS containing 5 pmol of SK102 probe was added to each well and the microtitre plate incubated at 37 °C for 1 hour. The wells were rinsed and blocked as before. 100 µl of denatured PCR product from each sample in the dilution series was added to successive wells. Hybridization was performed at 55 °C for 1 hour and the DIG-detection performed as described in section 2.5.1.1.1.

2.5.1.3 **Application to sample unknowns**

10 µl of PBMC DNA from each mother-child (RX-3, RX-6 and RX-8) sample pair was used in the PCR amplification step as described in section 2.5.1.2.1. The positive control previously used was included as a standard of known copy number.

University of Cape Town

CHAPTER 3: RESULTS

3.1 RESULTS

Results obtained in this study will be presented in four sections. The first section will describe the amplification, cloning and sequencing of samples to be used as reagents. These reagents were used in the evaluation of alternative methods for monitoring sequence diversity. The second section discusses results from a PCR-ELISA quantitation system, developed to measure provirus load in PBMC DNA. This was necessary to control for number of input copies used for PCR amplification in order to ensure adequate sampling of viral quasispecies. The third section describes the use of HMA for subtyping as well as its use in visually estimating intrasample diversity. The fourth and final section describes the use of BESS to generate partial sequence data for use in phylogenetic analyses. These assays would be useful in studies designed to investigate molecular changes in the envelope sequences associated with vertical transmission as well as other HIV-1 diversity studies.

3.1.1 PCR AMPLIFICATION, CLONING AND SEQUENCING

3.1.1.2 PCR Amplification

A total of fourteen out of sixteen samples (RX1-m and –b, RX2-m and -b, RX3-m and b, RX4-m, RX5-m and -b, RX6-m and -b, RX7-b, RX8-m and -b) were amplified using primers spanning the 851 bp C2-V3 region (Figure 3.1) Two samples (RX4-b and RX7-m) consistently failed to amplify, either due to sample degradation, low proviral load or PCR primer mismatches and due to this the two mother-child pairs (RX4 and RX7) were excluded from the study. The 851 bp C2-V5 *env* products spanning the C2-V5 region of *env* amplified from RX1-m were cloned for use as reagents in setting up the HMA.

University of Cape Town

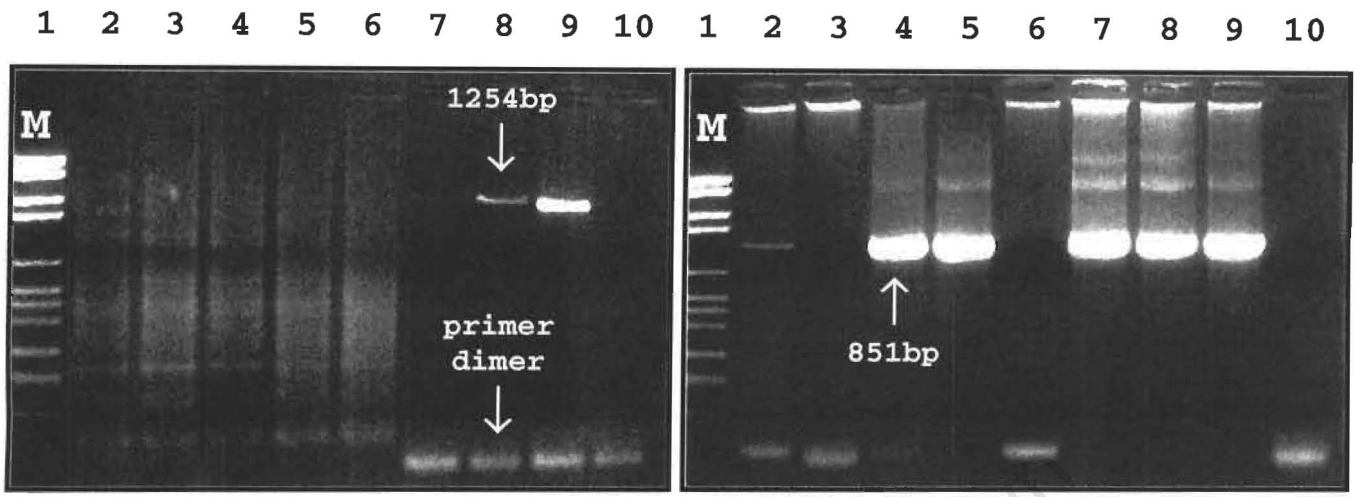


Figure 3.1: 2% (w/v) agarose gels stained with ethidium bromide of DNA fragments amplified by PCR. (A): Outer reaction; (B): Inner reaction.

A: The 1254 bp outer fragment size amplified using the ED5-ED12 primer set is indicated by the arrow.

B: The 851 bp inner fragment size amplified using the ED31-ES8 primer set is indicated by the arrow.

lane 1: DNA molecular weight marker VI (Boehringer-Mannheim, Mannheim, Germany), lane 2: RX1-m, lane 3: RX1-b, lane 4: RX2-m, lane 5: RX2-b, lane 6: HIV-1 negative human DNA control, lane 7: positive plasmid control (1 copy), lane 8: positive plasmid control (10 copies), lane 9: positive plasmid control (100 copies), lane 10: negative water control.

University of Cape Town

3.1.1.2 **RFLP analysis and colony hybridization assay**

The C2-V5 PCR products were cloned from samples RX1-m, RX6-m and -b and RX8-m and –b. Colonies containing recombinant plasmids with the correct insert were identified by colony probe hybridization assay (Figure 3.2) and confirmed by RFLP analysis on extracted plasmid DNA (Figure 3.3). Eight clones were generated from RX1-m (numbered RX1-m1 to RX1-m8) and ten clones were generated from samples RX6-m (numbered RX6-m1 to RX6-m10), RX6-b (numbered RX6-b1 to RX6-b10), RX8-m (numbered RX8-m1 to RX8-m10) and RX8-b (numbered RX8-b1 to RX8-b10).

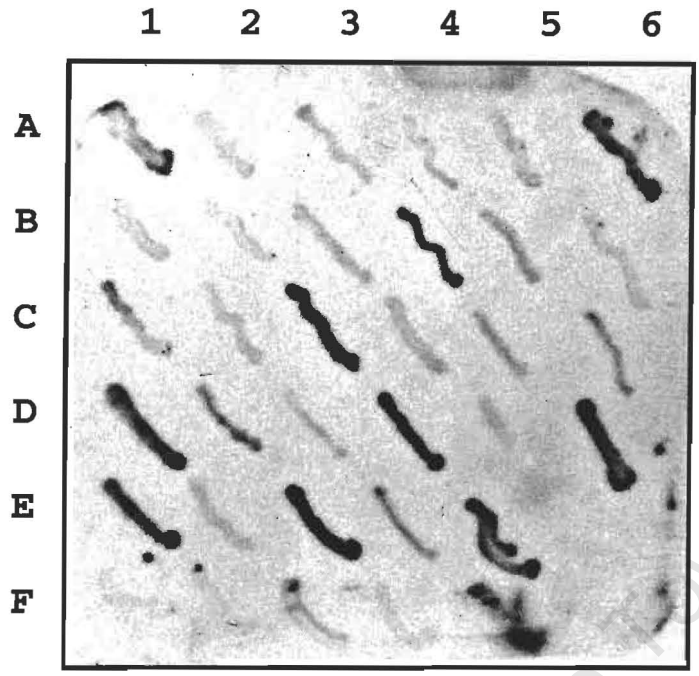


Figure 3.2: Autoradiograph of colony hybridization assay of *E.coli* transformed with recombinant plasmid DNA and probed with a DIG-labeled reference probe generated by PCR.

Grid F5: +ve control.
Grid F6: -ve control.

University of Cape Town

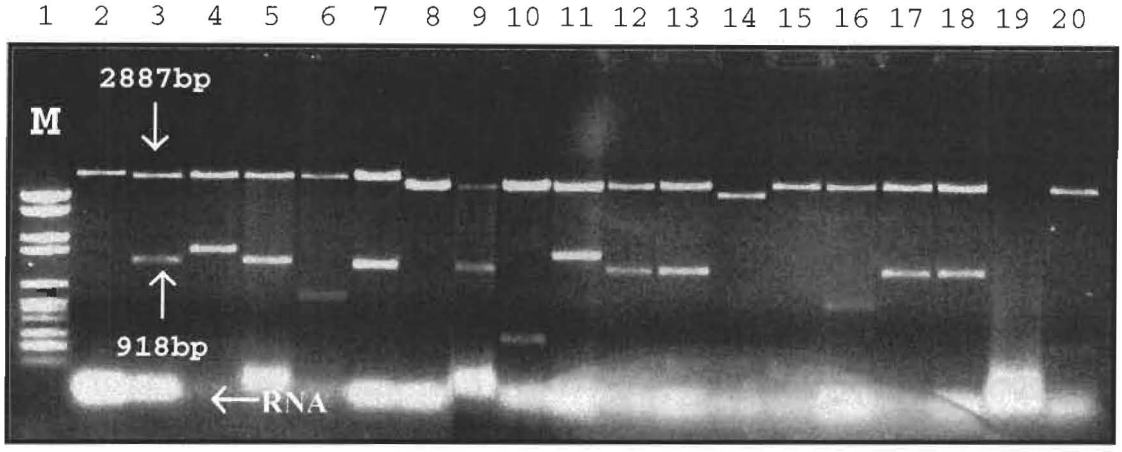


Figure 3.3: Composite image of a 2% (w/v) agarose gel containing ethidium bromide of *Pst*I and *Eco*RI restriction enzyme digests of plasmid DNA extracted from transformants (Lanes 2-20). M: Molecular weight marker VI (Boehringer-Mannheim, Mannheim, Germany)

3.1.1.3 **Manual DNA sequencing of clones**

The 851 bp C2-V5 insert from each of the eight clones from sample RX1-m was manually sequenced (Figure 3.4) and the forward and reverse sequences aligned to resolve ambiguities. Manual sequencing was performed at this time because automated sequencing was not yet available. Sequence data was obtained for all clones and is displayed as an alignment in Figure 3.6 and deduced protein sequence alignment in Figure 3.8. Nucleotide variability between sequences ranged from no difference to 1% constituting a highly homogeneous viral population (Figure 3.5-A) and amino-acid variability between deduced protein sequences ranged from no difference to 3% (Figure 3.5-B). Phylogenetic analysis of V3 sequences from clones derived from sample RX1-m showed clustering with the subtype C reference sequences (Figure 3.7). Six out of eight sequences had identical V3 loops although differences were present outside of this region (Figure 3.6). In addition, analysis of the deduced protein sequence identified fourteen potential N-linked glycosylation sites (N-X-S or N-X-T) numbered from one to fourteen. The loss of an N-linked glycosylation site was noted at position eight in sequence RX1-m5 in the C4 region and an additional site was found at position ten in sequence RX1-m7 in the C4 region. Twenty-five nucleotide changes were observed of which twenty resulted in amino acid changes (nonsynonomous mutation). The non-synonymous mutations together with the changes in glycosylation pattern could be a result of positive

University of Cape Town

selection for virus envelope escape mutants. The V3 loop region of all clones sequenced retained the GPGQ motif characteristic of subtype C HIV-1 viruses.

University of Cape Town

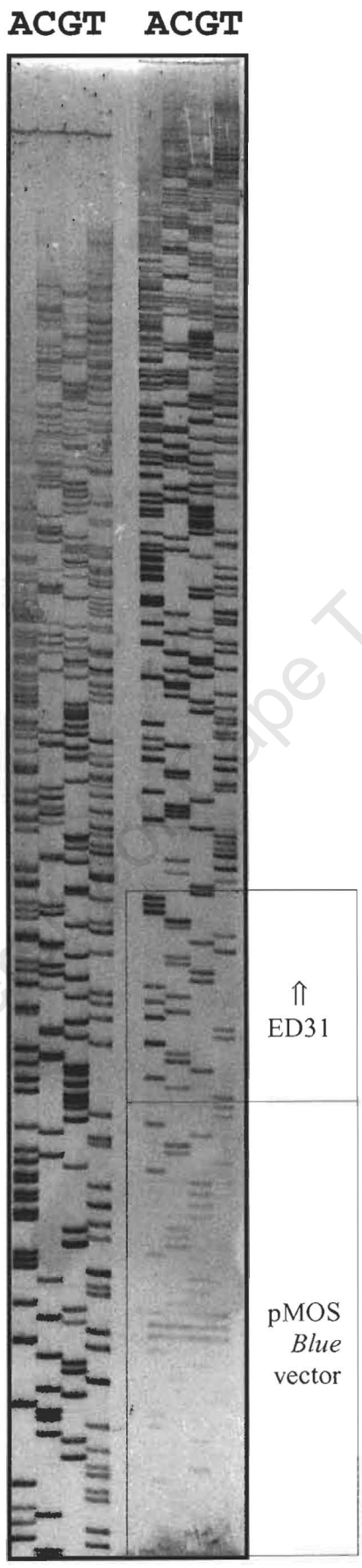


Figure 3.4:
Autoradiograph of 6% (w/v) polyacrylamide gel containing S^{35}-labelled DNA fragments generated by manual dideoxynucleotide sequencing of plasmid DNA from sample RX1-m.

University of Cape Town

Figure 3.5: (A) Distance table of percentage DNA difference of 804 bp nucleotide sequences and (B) Distance table of percentage amino acid difference of 267 residues of deduced protein sequences derived from sample RX1-m. The eight clones are labelled RX1-m1 to RX1-m8.

(A)

	RX1-m1	RX1-m2	RX1-m3	RX1-m4	RX1-m5	RX1-m6	RX1-m7	RX1-m8
RX1-m1	0							
RX1-m2	0.000	0						
RX1-m3	0.007	0.007	0					
RX1-m4	0.010	0.010	0.010	0				
RX1-m5	0.007	0.007	0.007	0.010	0			
RX1-m6	0.007	0.007	0.000	0.010	0.007	0		
RX1-m7	0.010	0.010	0.007	0.010	0.010	0.007	0	
RX1-m8	0.007	0.007	0.007	0.007	0.007	0.007	0.007	0

(B)

	RX1-m1	RX1-m2	RX1-m3	RX1-m4	RX1-m5	RX1-m6	RX1-m7	RX1-m8
RX1-m1	0							
RX1-m2	0.000	0						
RX1-m3	0.011	0.011	0					
RX1-m4	0.022	0.022	0.019	0				
RX1-m5	0.019	0.019	0.015	0.026	0			
RX1-m6	0.011	0.011	0.000	0.019	0.015	0		
RX1-m7	0.026	0.026	0.015	0.026	0.030	0.015	0	
RX1-m8	0.015	0.015	0.011	0.015	0.019	0.011	0.019	0

University of Cape Town

Figure 3.6: 804 bp C2-V5 nucleotide sequence alignment of eight clones derived from PBMC DNA from sample RX1-m.

```
     ED31⇒
m1   CCTCAGCCATTACACAGGCCTGTCCAAAGGTCTCTTTTGACCCGATTCCTATACATTATT 60
m2   CCTCAGCCATTACACAGGCCTGTCCAAAGGTCTCTTTTGACCCGATTCCTATACATTATT
m3   CCTCAGCCATTACACAGGCCTGTCCAAAGGTCTCTTTTGACCCGATTCCTATACATTATT
m4   CCTCAGCCATTACACAGGCCTGTCCAAAGGTCTCTTTTGACCCGATCCCTATACATTATT
m5   CCTCAGCCATTACACAGGCCTGTCCAAAGGTCTCTCTTGACCCGATTCCTGTACATTATT
m6   CCTCAGCCATTACACAGGCCTGTCCAAAGGTCTCTTTTGACCCGATTCCTATACATTATT
m7   CCTCAGCCATTACACAGGCCTGTCCAAAGGTCTCTTTTGACCCGATTCCTATACATTATT
m8   CCTCAGCCATTACACAGGCCTGTCCAAAGGTCTCTTTTGACCCGATTCCTATACATTATT
     ************************************ ********** *** *********

m1   GTGCTCCAGCTGGCTATGCGATTCTAAAGTGTAATAATAAGACATTCAGTGGGAGAGGAC 120
m2   GTGCTCCAGCTGGCTATGCGATTCTAAAGTGTAATAATAAGACATTCAGTGGGAGAGGAC
m3   GTGCTCCAGCTGGCTATGCGATTCTAAAGTGTAATAATAAGACATTCAGTGGGAGAGGAC
m4   GTGCTCCAGCTGGCTATGCGATTCTAAAGTGTAATAATAAGACACTCAGTGGGAGAGGAC
m5   GTGCTCCAGCTGGCTATGCGATTCTAAAGTGTAATAATAAGACATTCAGTGGGAGAGGAC
m6   GTGCTCCAGCTGGCTATGCGATTCTAAAGTGTAATAATAAGACATTCAGTGGGAGAGGAC
m7   GTGCTCCAGCTGGCTATGCGATTCTAAAGTGTAATAATAAGACATTCTGTGGGAGAGGAC
m8   GTGCTCCAGCTGGCTATGCGATTCTAAAGTGTAATAATAAGACATTCAGTGGGAGAGGAC
     ********************************************** ** ************

m1   CATGCCGTAATGTCAGCACAGTCCAATGTACACATGGAATTAAGCCTGTGGTATCAACTC 180
m2   CATGCCGTAATGTCAGCACAGTCCAATGTACACATGGAATTAAGCCTGTGGTATCAACTC
m3   CATGCCGTAATGTCAGCACAGTCCAATGTACACATGGAATTAAGCCTGTGGTATCAACTC
m4   CATGCCGTAATGTCAGCACAGTCCAATGTACACATGGAATTAAGCCTGTGGTATCAACTC
m5   CATGCCGTAATGTCAGCACAGTCCAATGTACACATGGAATTAAGCCTGTGGTATCAACTC
m6   CATGCCGTAATGTCAGCACAGTCCAATGTACACATGGAATTAAGCCTGTGGTATCAACTC
m7   CATGCCGTAATGTCAGCACAGTCCAATGTACACATGGAATTAAGCCTGTGGTATCAACTC
m8   CATGCCGTAATGTCAGCACAGTCCAATGTACACATGGAATTAAGCCTGTGGTATCAACTC
     ************************************************************

m1   AACTACTGTTAAATGGTAGCCTAGCAGAAGGGGAGATAATAATTAGATCTGAGAATCTGA 240
m2   AACTACTGTTAAATGGTAGCCTAGCAGAAGGGGAGATAATAATTAGATCTGAGAATCTGA
m3   AACTACTGTTAAATGGTAGCCTAGCAGAAGGGGAGATAATAATTAGATCTGAGAATCTGA
m4   AACTACCGTTAAATGGTAGCCTAGCAGAAGGGGAGATAATAATTAGATCTGAGAATCTGA
m5   AACTACTGTTAAATGGTAGCCTAGCAGAAGGGGAGATAATAATTAGATCTGAGAATCTGA
m6   AACTACTGTTAAATGGTAGCCTAGCAGAAGGGGAGATAATAATTAGATCTGAGAATCTGA
m7   AACTACCGTTAAATGGTAGCCTAGCAGAAGGGGAGATAATAATTAGATCTGAGAATCTGA
m8   AACTACCGTTAAATGGTAGCCTAGCAGAAGGGGAGATAATAATTAGATCTGAGAATCTGA
     ****** ****************************************************

m1   CAGACAATGTCAAAATAATAATAGTACACCCTAATGAATCTGTAGAAATTGTGTGTACAA 300
m2   CAGACAATGTCAAAATAATAATAGTACACCCTAATGAATCTGTAGAAATTGTGTGTACAA
m3   CAGACAATGTCAAAATAATAATAGTACACCTTAATGAATCTGTAGAAATTGTGTGTACAA
m4   CAGACAATGTCAAAATAATAATAGTACACCTTAATGAATCTGTAGAAATTGTGTGTACAA
m5   CAGACAATGTCAAAATAATAATAGTACACCTTAATGAATCTGTAGAAATTGTGTGTACAA
m6   CAGACAATGTCAAAATAATAATAGTACACCTTAATGAATCTGTAGAAATTGTGTGTACAA
m7   CAGACAATGTCAAAATAATAATAGTACACCTTAATGAATCTGTAGAAATTGTGTGTACAA
m8   CAGACAATGTCAAAATAATAATAGTACACCTTAATGAATCTGTAGAAATTGTGTGTACAA
     ****************************** ******************************
```

University of Cape Town

```
                                ⇒V3 region⇐
m1  GACCCAACAATAATACAAGAAAAAGTATAAGGATAGGACCAGGACAAACATTCTTTGCAA 360
m2  GACCCAACAATAATACAAGAAAAAGTATAAGGATAGGACCAGGACAAACATTCTTTGCAA
m3  GACCCAACAATAATACAAGAAAAAGTATAAGGATAGGACCAGGACAAACATTCTTTGCAA
m4  GACCCAACAATAATACAAGAAAAAGTATAAGGATAGGACCAGGACAAACATTCTTTGCAA
m5  GACCCAACAATAATACAAGAAAAAGTATAAGGATAGGACCAGGACAAACATTCTTTGCAA
m6  GACCCAACAATAATACAAGAAAAAGTATAAGGATAGGACCAGGACAAACATTCTTTGCAA
m7  GACCCATCAATAATACAAGAAAAAGTATAAGGATAGGACCAGGACAAACATTCTTTGCAA
m8  GACCCAACAATAATACAAGAAAAAGTATAAGGATAGGACCAGGACAAACATTCTTTGCAA
    ****** ************************************************************

m1  CAAATGACATAATAGGAGACATAAGACAAGCACATTGTAACATTAGTACAACAAAATGGA 420
m2  CAAATGACATAATAGGAGACATAAGACAAGCACATTGTAACATTAGTACAACAAAATGGA
m3  CAAATGACATAATAGGAGACATAAGACAAGCACATTGTAACATTAGTACAACAAGATGGA
m4  CAAATGACATAATAGGAGACATAAGACAAGCACATTGTAACATTAGTACAACAAAATGGA
m5  CAAATGACATAATAGGAGACATAAGACAAGCACATTGTAACATTAGTACAACAAAATGGA
m6  CAAATGACATAATAGGAGACATAAGACAAGCACATTGTAACATTAGTACAACAAGATGGA
m7  CAAATGACATAATAGGAGACATAAGACAAGCACATTGTAACATTAGTACAACAAGATGGA
m8  CAGATGACATAATAGGAGACATAAGACAAGCACATTGTAACATTAGTACAACAAAATGGA
    ** ************************************************** *****

m1  ACACAACTTTAGAAAGGGTAAGGAAGAAATTAGGAGAACACTTCCCTAATAAAACAATAA 480
m2  ACACAACTTTAGAAAGGGTAAGGAAGAAATTAGGAGAACACTTCCCTAATAAAACAATAA
m3  ACACAACTTTAGAAAGGGTAAGGAAGAAATTAGGAGAACACTTCCCTAATAAGACAATAA
m4  ACACAACTTTAGAAAGGGTAAGGAAGAAATTAGGAGAACACTTCCCTAATAAAACAATAA
m5  GCACAACTTTAGAAAGGGTAAGGAAGAAATTAGGAGAACACTTCCCTAATAAAACAATAA
m6  ACACAACTTTAGAAAGGGTAAGGAAGAAATTAGGAGAACACTTCCCTAATAAGACAATAA
m7  ACACAACTTTAGAAAGGGTAAGGAAGAAATTAGGAGAACACTTCCCTAATAAAACAATAA
m8  ACACAACTTTAGAAAGGGTAAGGAAGAAATTAGGAGAACACTTCCCTAATAAAACAATAA
     ************************************************** *******

m1  CCTTTAAACAGCCCTCAGGAGGGGACCTAGAAATTACAACACATAGCTTTAATTGTAGAG 540
m2  CCTTTAAACAGCCCTCAGGAGGGGACCTAGAAATTACAACACATAGCTTTAATTGTAGAG
m3  CCTTTAAACAGCCCTCAGGAGGGGACCTAGAAATTACAACACATAGCTTTAATTGTAGAG
m4  CCTTTAAACAGCCCTCAGGAGGGGACCTAGAAATTACAACACATAGCTTTAATCGTAGAG
m5  CCTTTAAACAGCCCTCAGGAGGGGACCTAGAAATTACAACACATAGCTTTAATTGTAGAG
m6  CCTTTAAACAGCCCTCAGGAGGGGACCTAGAAATTACAACACATAGCTTTAATTGTAGAG
m7  CCTTTAAACAGCCCTCAGGAGGGGACCTAGAAAATACAACACATAGCTTTAATTGTAGAG
m8  CCTTTAAACAGCCCTCAGGAGGGGACCTAGAAATTACAACACATAGCTTTAATTGTAGAG
    ********************************** ****************** ******

                                              ⇒V4 region⇐
m1  GAGAGTTTTTTTATTGCAATACATCAAATCTGTTTCCTAATGACAATGGGTCAAACCCAA 600
m2  GAGAGTTTTTTTATTGCAATACATCAAATCTGTTTCCTAATGACAATGGGTCAAACCCAA
m3  GAGAGTTTTTTTATTGCAATACATCAAATCTGTTTCCTAATGACAATGGGTCAAACCCAA
m4  GAGAGTTTTTTTATTGCAATACATCAAATCTGTTTCCTAATGACAATGGGTCAAACCCAA
m5  GAGAGTTTTTTTATTGCAATACATCAAATCTGTTTCCTAATGACAATGGGTCAAACCCAA
m6  GAGAGTTTTTTTATTGCAATACATCAAATCTGTTTCCTAATGACAATGGGTCAAACCCAA
m7  GAGAGTTTTTTTATTGCAATACATCAAATCTGTTTCCTAATGACAATGGGTCAAACCCAA
m8  GAGAGTTTTTTTATTGCAATACATCAAATCTGTTTCCTAATGACAATGGGTCAAACCCAA
    ************************************************************
```

University of Cape Town

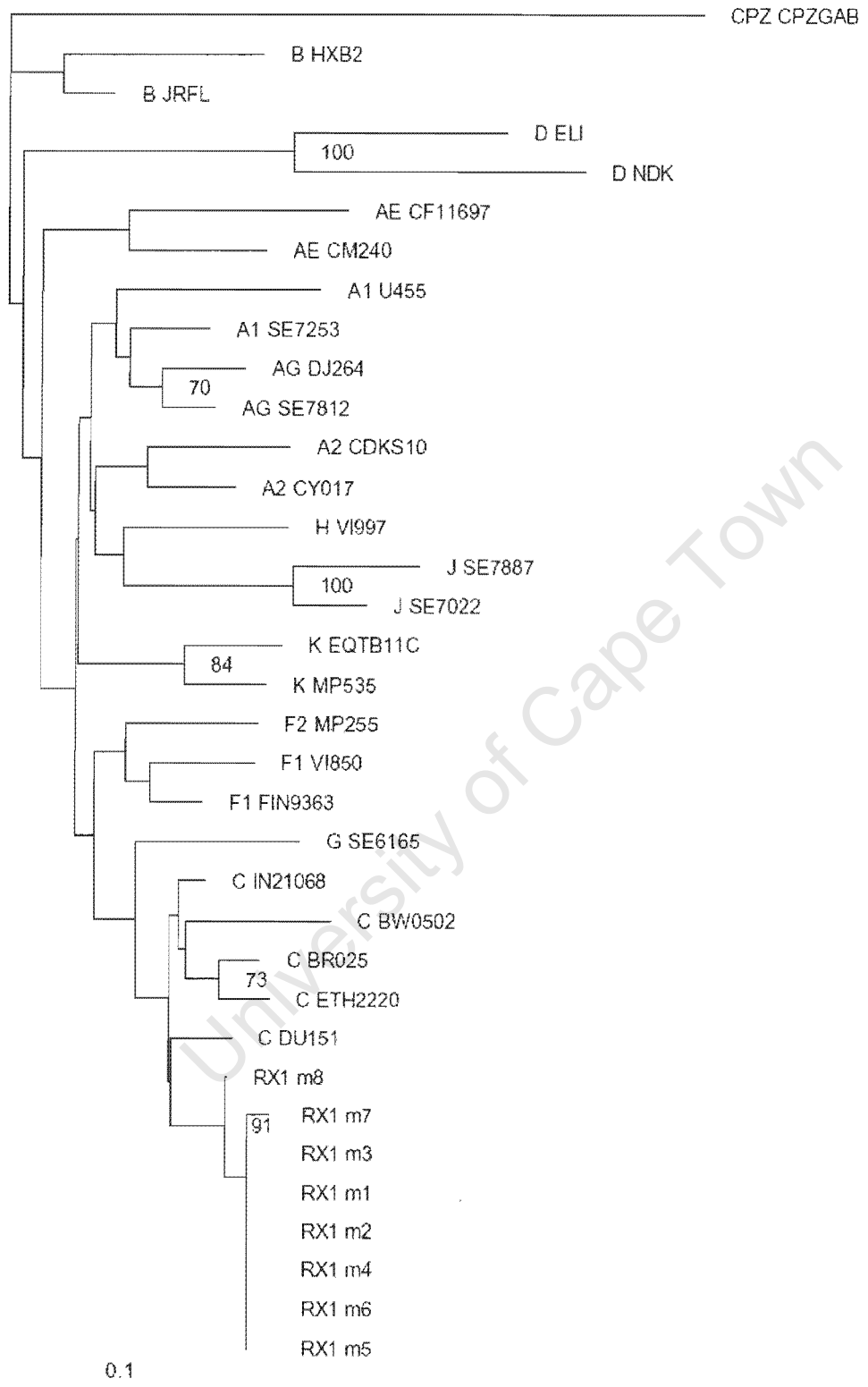
```
m1  CCACCATCCCATGCAAGATAAAACAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAA 660
m2  CCACCATCCCATGCAAGATAAAACAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAA
m3  CCACCATCCCATGCAAGATAAAACAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAA
m4  CCGCCATCCCATGCAAGATAAAACAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAA
m5  CCACCATCCCATGCAAGATAAAACAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAA
m6  CCACCATCCCATGCAAGATAAAACAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAA
m7  CCACCATCCCATGCAAGATAAAACAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAA
m8  CCACCATCCCATGCAAGATAAAACAAATTATAAACATGTGGCAGGGGGTAGGACGAGCAA
    ** ************************************************************

m1  TGTATGCCCCTCCCATTAAAGGGAACATAACACGTAACTCAAGTATCACAGGATTACTAT 720
m2  TGTATGCCCCTCCCATTAAAGGGAACATAACACGTAACTCAAGTATCACAGGATTACTAT
m3  TGTATGCCCCTCCCATTAAGGGAAACATAACATGTAACTCAAGTATCACAGGATTACTAT
m4  TGTATGCCCCTCCCATTAAAGGAAACATAACATGTAACTCAAGTATCACAGGATTACTAT
m5  TGTATGCCCCTCCCATTAAAGGAAACATAACATGTAACTCAAGTATCACAGGATTACTAT
m6  TGTATGCCCCTCCCATTAAGGGAAACATAACATGTAACTCAAGTATCACAGGATTACTAT
m7  TGTATGCCCCTCCCATTAAAGGAAACATAACATGTAACTCAAGTATCACAGGATTACTAT
m8  TGTACGCCCCTCCCATTAAAGGAAACATAACATGTAACTCAAGTATCACAGGATTACTAT
    **** ************** ** ********* ****************************

                                        ⇒V5 region⇐
m1  TGACACGTGATGGTGGCGACGGGAATAACATAGAGGAGATATTCAGACCTGGAGGAGGAG 780
m2  TGACACGTGATGGTGGCGACGGGAATAACATAGAGGAGATATTCAGACCTGGAGGAGGAG
m3  TGACACGTGATGGTGGCGACGGGAATAACATAGAGGAGATATTCAGACCTGGAGGAGGAG
m4  TGACACGTGATGGTGGCGACGGGAATAACATAGAGGAGATATTCAGACCTGGAGGAGGAG
m5  TGACACGTGATGGTGGCGACGGGAATAACATAGAGGAGATATTCAGACCTGGAGGAGGAG
m6  TGACACGTGATGGTGGCGACGGGAATAACATAGAGGAGATATTCAGACCTGGAGGAGGAG
m7  TGACACGTGATGGTGGCGACGGGAATAACATAGAGGAGATATTCAGACCTGGAGGAGGAG
m8  TGACACGTGATGGTGGCGACGGGAATAACATAGAGGAGATATTCAGACCTGGAGGAGGAG
    ************************************************************

                          ⇐ES8
m1  ATATGAGGGACAATTGGAGAAGTG 804
m2  ATATGAGGGACAATTGGAGAAGTG
m3  ATATGAGGGACAATTGGAGAAGTG
m4  ATATGAGGGACAATTGGAGAAGTG
m5  ATATGAGGGACAATTGGAGAAGTG
m6  ATATGAGGGACAATTGGAGAAGTG
m7  ATATGAGGGACAATTGGAGAAGTG
m8  ATATGAGGGACAATTGGAGAAGTG
    ************************
```

University of Cape Town



University of Cape Town

Figure 3.7: 105 bp V3 phylogenetic tree representing eight clones generated from RX1-m as well as V3 reference sequences from HIV-1 clades A to J. A South African subtype C strain DU151 is also included. Trees were constructed using the neighbour-joining method, SIVcpz was used as an outlier and bootstrap values >70% are indicated.

Figure 3.8: Alignment of deduced protein sequence of eight cloned sequences for sample RX1-m. Numbered arrows indicate potential N-linked glycosylation sites. Variable regions are labelled above and printed in bold. The V3 tetrameric tip is underlined.

```
m1    S A I T Q A C P K V S F D P I P I H Y C 20
m2    S A I T Q A C P K V S F D P I P I H Y C
m3    S A I T Q A C P K V S F D P I P I H Y C
m4    S A I T Q A C P K V S F D P I P I H Y C
m5    S A I T Q A C P K V S L D P I P V H Y C
m6    S A I T Q A C P K V S F D P I P I H Y C
m7    S A I T Q A C P K V S F D P I P I H Y C
m8    S A I T Q A C P K V S F D P I P I H Y C
      * * * * * * * * * * *   * * * *   * * *
                            1↓
m1    A P A G Y A I L K C N N K T F S G R G P 40
m2    A P A G Y A I L K C N N K T F S G R G P
m3    A P A G Y A I L K C N N K T F S G R G P
m4    A P A G Y A I L K C N N K T L S G R G P
m5    A P A G Y A I L K C N N K T F S G R G P
m6    A P A G Y A I L K C N N K T F S G R G P
m7    A P A G Y A I L K C N N K T F C G R G P
m8    A P A G Y A I L K C N N K T F S G R G P
      * * * * * * * * * * * * * *   * * * * *
          2↓
m1    C R N V S T V Q C T H G I K P V V S T Q 60
m2    C R N V S T V Q C T H G I K P V V S T Q
m3    C R N V S T V Q C T H G I K P V V S T Q
m4    C R N V S T V Q C T H G I K P V V S T Q
m5    C R N V S T V Q C T H G I K P V V S T Q
m6    C R N V S T V Q C T H G I K P V V S T Q
m7    C R N V S T V Q C T H G I K P V V S T Q
m8    C R N V S T V Q C T H G I K P V V S T Q
      * * * * * * * * * * * * * * * * * * * *
              3↓                        4↓
m1    L L L N G S L A E G E I I I R S E N L T 80
m2    L L L N G S L A E G E I I I R S E N L T
m3    L L L N G S L A E G E I I I R S E N L T
m4    L P L N G S L A E G E I I I R S E N L T
m5    L L L N G S L A E G E I I I R S E N L T
m6    L L L N G S L A E G E I I I R S E N L T
m7    L P L N G S L A E G E I I I R S E N L T
m8    L P L N G S L A E G E I I I R S E N L T
      *   * * * * * * * * * * * * * * * * * *
                          5↓
m1    D N V K I I I V H P N E S V E I V C T R 100
m2    D N V K I I I V H P N E S V E I V C T R
m3    D N V K I I I V H L N E S V E I V C T R
m4    D N V K I I I V H L N E S V E I V C T R
m5    D N V K I I I V H L N E S V E I V C T R
m6    D N V K I I I V H L N E S V E I V C T R
m7    D N V K I I I V H L N E S V E I V C T R
m8    D N V K I I I V H L N E S V E I V C T R
      * * * * * * * * *   * * * * * * * * * *
```

University of Cape Town

```
          6↓                                ⇒V3 region⇐
m1    P  N  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  F  A  T 120
m2    P  N  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  F  A  T
m3    P  N  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  F  A  T
m4    P  N  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  F  A  T
m5    P  N  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  F  A  T
m6    P  N  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  F  A  T
m7    P  I  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  F  A  T
m8    P  N  N  N  T  R  K  S  I  R  I  G  P  G  Q  T  F  F  A  T
      *     *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

                                                7↓                8↓
m1    N  D  I  I  G  D  I  R  Q  A  H  C  N  I  S  T  T  K  W  N 140
m2    N  D  I  I  G  D  I  R  Q  A  H  C  N  I  S  T  T  K  W  N
m3    N  D  I  I  G  D  I  R  Q  A  H  C  N  I  S  T  T  R  W  N
m4    N  D  I  I  G  D  I  R  Q  A  H  C  N  I  S  T  T  K  W  N
m5    N  D  I  I  G  D  I  R  Q  A  H  C  N  I  S  T  T  K  W  S
m6    N  D  I  I  G  D  I  R  Q  A  H  C  N  I  S  T  T  R  W  N
m7    N  D  I  I  G  D  I  R  Q  A  H  C  N  I  S  T  T  R  W  N
m8    D  D  I  I  G  D  I  R  Q  A  H  C  N  I  S  T  T  K  W  N
         *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

                                                      9↓
m1    T  T  L  E  R  V  R  K  K  L  G  E  H  F  P  N  K  T  I  T 160
m2    T  T  L  E  R  V  R  K  K  L  G  E  H  F  P  N  K  T  I  T
m3    T  T  L  E  R  V  R  K  K  L  G  E  H  F  P  N  K  T  I  T
m4    T  T  L  E  R  V  R  K  K  L  G  E  H  F  P  N  K  T  I  T
m5    T  T  L  E  R  V  R  K  K  L  G  E  H  F  P  N  K  T  I  T
m6    T  T  L  E  R  V  R  K  K  L  G  E  H  F  P  N  K  T  I  T
m7    T  T  L  E  R  V  R  K  K  L  G  E  H  F  P  N  K  T  I  T
m8    T  T  L  E  R  V  R  K  K  L  G  E  H  F  P  N  K  T  I  T
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

                                   10↓
m1    F  K  Q  P  S  G  G  D  L  E  I  T  T  H  S  F  N  C  R  G 180
m2    F  K  Q  P  S  G  G  D  L  E  I  T  T  H  S  F  N  C  R  G
m3    F  K  Q  P  S  G  G  D  L  E  I  T  T  H  S  F  N  C  R  G
m4    F  K  Q  P  S  G  G  D  L  E  I  T  T  H  S  F  N  R  R  G
m5    F  K  Q  P  S  G  G  D  L  E  I  T  T  H  S  F  N  C  R  G
m6    F  K  Q  P  S  G  G  D  L  E  I  T  T  H  S  F  N  C  R  G
m7    F  K  Q  P  S  G  G  D  L  E  N  T  T  H  S  F  N  C  R  G
m8    F  K  Q  P  S  G  G  D  L  E  I  T  T  H  S  F  N  C  R  G
      *  *  *  *  *  *  *  *  *  *     *  *  *  *  *  *     *  *

             11↓                ⇒V4 region⇐          12↓      13↓
m1    E  F  F  Y  C  N  T  S  N  L  F  P  N  D  N  G  S  N  P  T 200
m2    E  F  F  Y  C  N  T  S  N  L  F  P  N  D  N  G  S  N  P  T
m3    E  F  F  Y  C  N  T  S  N  L  F  P  N  D  N  G  S  N  P  T
m4    E  F  F  Y  C  N  T  S  N  L  F  P  N  D  N  G  S  N  P  T
m5    E  F  F  Y  C  N  T  S  N  L  F  P  N  D  N  G  S  N  P  T
m6    E  F  F  Y  C  N  T  S  N  L  F  P  N  D  N  G  S  N  P  T
m7    E  F  F  Y  C  N  T  S  N  L  F  P  N  D  N  G  S  N  P  T
m8    E  F  F  Y  C  N  T  S  N  L  F  P  N  D  N  G  S  N  P  T
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
```

University of Cape Town

```
m1    T  I  P  C  K  I  K  Q  I  I  N  M  W  Q  G  V  G  R  A  M 220
m2    T  I  P  C  K  I  K  Q  I  I  N  M  W  Q  G  V  G  R  A  M
m3    T  I  P  C  K  I  K  Q  I  I  N  M  W  Q  G  V  G  R  A  M
m4    A  I  P  C  K  I  K  Q  I  I  N  M  W  Q  G  V  G  R  A  M
m5    T  I  P  C  K  I  K  Q  I  I  N  M  W  Q  G  V  G  R  A  M
m6    T  I  P  C  K  I  K  Q  I  I  N  M  W  Q  G  V  G  R  A  M
m7    T  I  P  C  K  I  K  Q  I  I  N  M  W  Q  G  V  G  R  A  M
m8    T  I  P  C  K  I  K  Q  I  I  N  M  W  Q  G  V  G  R  A  M
         *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *
                              14↓                 15↓
m1    Y  A  P  P  I  K  G  N  I  T  R  N  S  S  I  T  G  L  L  L 240
m2    Y  A  P  P  I  K  G  N  I  T  R  N  S  S  I  T  G  L  L  L
m3    Y  A  P  P  I  K  G  N  I  T  C  N  S  S  I  T  G  L  L  L
m4    Y  A  P  P  I  K  G  N  I  T  C  N  S  S  I  T  G  L  L  L
m5    Y  A  P  P  I  K  G  N  I  T  C  N  S  S  I  T  G  L  L  L
m6    Y  A  P  P  I  K  G  N  I  T  C  N  S  S  I  T  G  L  L  L
m7    Y  A  P  P  I  K  G  N  I  T  C  N  S  S  I  T  G  L  L  L
m8    Y  A  P  P  I  K  G  N  I  T  C  N  S  S  I  T  G  L  L  L
      *  *  *  *  *  *  *  *  *  *     *  *  *  *  *  *  *  *  *
                                    ⇒V5 region⇐
m1    T  R  D  G  G  D  G  N  N  I  E  E  I  F  R  P  G  G  G  D 260
m2    T  R  D  G  G  D  G  N  N  I  E  E  I  F  R  P  G  G  G  D
m3    T  R  D  G  G  D  G  N  N  I  E  E  I  F  R  P  G  G  G  D
m4    T  R  D  G  G  D  G  N  N  I  E  E  I  F  R  P  G  G  G  D
m5    T  R  D  G  G  D  G  N  N  I  E  E  I  F  R  P  G  G  G  D
m6    T  R  D  G  G  D  G  N  N  I  E  E  I  F  R  P  G  G  G  D
m7    T  R  D  G  G  D  G  N  N  I  E  E  I  F  R  P  G  G  G  D
m8    T  R  D  G  G  D  G  N  N  I  E  E  I  F  R  P  G  G  G  D
      *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *  *

m1    M  R  D  N  W  R  S 267
m2    M  R  D  N  W  R  S
m3    M  R  D  N  W  R  S
m4    M  R  D  N  W  R  S
m5    M  R  D  N  W  R  S
m6    M  R  D  N  W  R  S
m7    M  R  D  N  W  R  S
m8    M  R  D  N  W  R  S
      *  *  *  *  *  *  *
```

University of Cape Town

3.1.2 ESTIMATION OF PROVIRAL COPY NUMBER BY PCR-ELISA

A PCR-ELISA method was developed to estimate the provirus copy number to ensure that an adequate number of template molecules are used for PCR amplification in order to estimate the genetic diversity of the sample more accurately. Low input copy number can result in artificially low diversity due to re-sampling. To quantitate the proviral DNA, the viral target sequence was amplified using DIG-labelled reverse primers, the amplicons alkali-denatured and hybridized to complementary biotin-labelled oligonucleotide probe sequences. The hybridized DNA molecules were captured in a streptavidin-coated microtitre plate and assayed for the DIG-label by conventional ELISA methods. The copy number of target molecules in the unknown sample was inferred from a standard curve generated using an external standard of known copy number.

3.1.2.1 **Homogeneity of streptavidin coating of microtitre plates**

For accurate and reproducible estimation of copy number, it is important that the streptavidin coating, which captures the biotin-labelled molecules, is homogenous. The OD_{450} of sixteen identical wells coated with streptavidin and probed with two different concentrations of DIG-label (1.670 fmol DIG/well and 0.835 fmol DIG/well) was measured. Results showed comparable values between wells (1.670 fmol DIG/well: mean OD_{450} and SD = 1.155 $\pm$ 0.03457; 0.835 fmol DIG/well: mean OD_{450} and SD = 0.5834 $\pm$ 0.02702 OD) and was indicative of equivalent streptavidin coating of each well (Table 3.3).

University of Cape Town

Table 3.3: OD_{450} values obtained for sixteen identical wells at two concentrations of DIG-label.

OD_{450} (1.670 fmol DIG/well)	OD_{450} (0.835 fmol DIG/well)
1.169	0.604
1.120	0.587
1.137	0.593
1.171	0.597
1.189	0.599
1.145	0.563
1.159	0.559
1.169	0.579
1.231	0.600
1.173	0.595
1.167	0.569
1.116	0.629
1.098	0.507
1.102	0.583
1.147	0.571
1.179	0.600
Mean and SD: 1.155 ± 0.03457	Mean and SD: 0.5834 ± 0.02702

University of Cape Town

3.1.2.2 **Sensitivity of detection of labelled molecules**

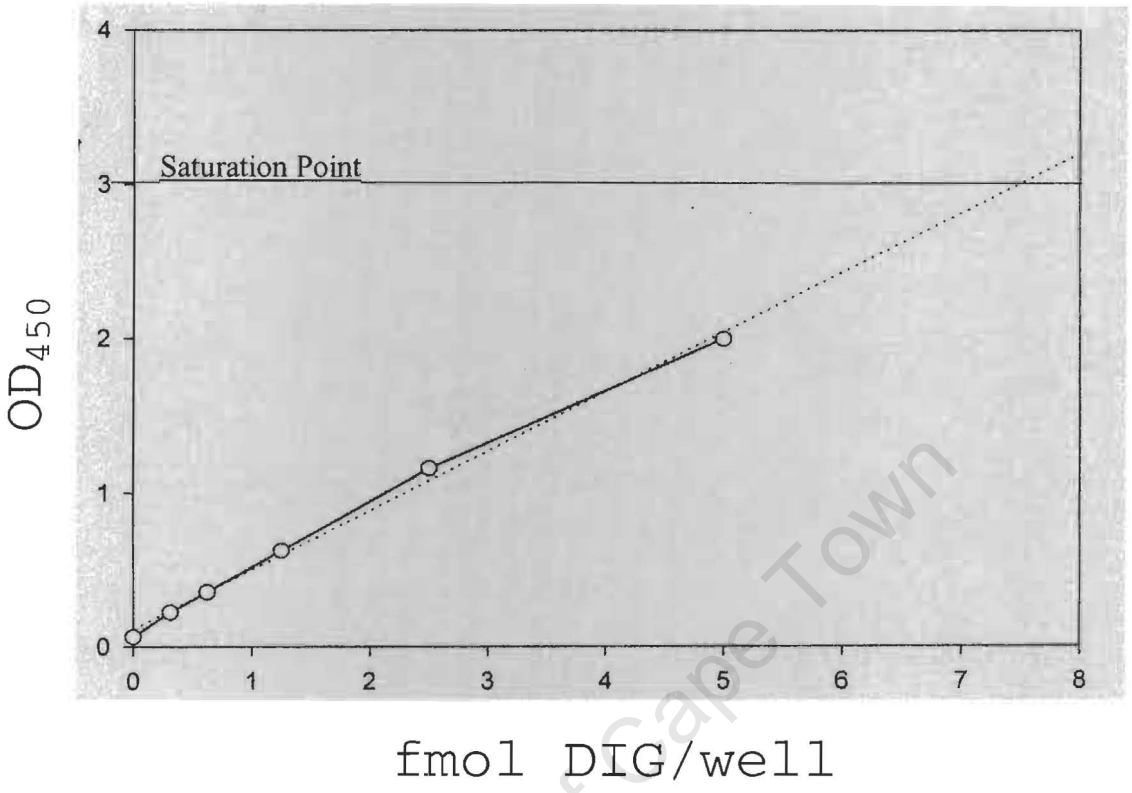
To determine the sensitivity of the PCR-ELISA, a two-fold dilution curve of DIG-labelled molecules from 10 fmol to 0.3125 fmol to was performed in triplicate (Table 3.4 and Graph 3.1). The lowest dilutions (0.3124 fmol) were 3.5 times greater than background showing a high degree of sensitivity. The concentration at which saturation was reached for the assay was shown to fall between 5 fmol and 10 fmol of DIG-label.

Table 3.4: Results of DIG-detection ELISA showing OD_{450} values obtained in triplicate from two-fold dilutions of control-peptide starting with the equivalent of 10 fmol of DIG-label

Sample: (fmol DIG/well)	OD_{450} (1)	OD_{450} (2)	OD_{450} (3)	* OD_{450}
10	>3	>3	>3	>3
5	1.966	2.052	1.971	1.996 ± 0.04827
2.5	1.149	1.122	1.197	1.156 ± 0.03799
1.25	0.631	0.615	0.621	0.6223 ± 0.008083
0.625	0.358	0.364	0.343	0.355 ±0.01082
0.3125	0.228	0.222	0.216	0.222 ± 0.006
no peptide (conjugate added)	0.064	0.067	0.058	0.063 ± 0.004583
no peptide (no conjugate added)	0.053	0.068	0.049	0.05667 ± 0.01002

*OD_{450} : mean and standard deviation of the 3 values obtained.

University of Cape Town



Graph 3.1: Linear plot of results obtained from DIG-detection ELISA using control-peptide showing the OD_{450} values versus two-fold dilutions of control-peptide. (Data listed in Table 3.4)

University of Cape Town

3.1.2.3 **Calibration of detection system**

The PCR-ELISA system was calibrated using an external standard of 100 HIV-1 proviral copies. PCR copy number standards were obtained from the NIH (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: catalogue number ARP956). Before using the dilution standards, an HIV-1 test sample of undetermined copy number was used as template to generate a labelled DNA control to confirm that the assay was functioning correctly. In addition, the test sample was used as a standard of known copy number since the calibration standards were in limited supply. The OD_{450} values obtained for a dilution curve of the test sample (Table 3.5) were used to calculate

the absolute values of DIG-label (Table 3.6) based on the standard curve obtained using the control-peptide of known concentration (Table 3.4). Successful generation and detection of labelled PCR products was demonstrated and the assay was repeated using the control standard of known HIV-1 DNA copy number. A standard curve was generated relating copy number to OD_{450} values (Table 3.7 and Graph 3.3).

Table 3.5: Results of DIG-detection ELISA showing OD_{450} values obtained in triplicate from two-fold dilutions of labelled DNA generated by PCR starting with the undiluted sample

dilution ratio	OD_{450} (1)	OD_{450} (2)	OD_{450} (3)	*OD_{450}
1:1	>3	>3	>3	>3
1:2	>3	>3	>3	>3
1:4	>3	>3	>3	>3
1:8	>3	>3	>3	>3
1:16	>3	>3	>3	>3
1:32	>3	>3	>3	>3
1:64	2.709	2.366	2.580	2.552 ± 0.1732
1:128	1.657	1.592	1.435	1.561 ± 0.1141
1:256	0.969	0.765	0.893	0.8757 ± 0.1031
1:512	0.518	0.512	0.496	0.5087 ± 0.01137
1:1024	0.300	0.284	0.239	0.2743 ± 0.03163
1:2048	0.179	0.191	0.194	0.1880 ± 0.007937
1:4096	0.117	0.122	0.125	0.1213 ± 0.004041
1:8192	0.095	0.105	0.100	0.1000 ± 0.005000
no DNA (conjugate added)	0.070	0.066	0.060	0.06533 ± 0.005033
no DNA (no conjugate added)	0.055	0.054	0.057	0.05533 ± 0.001528

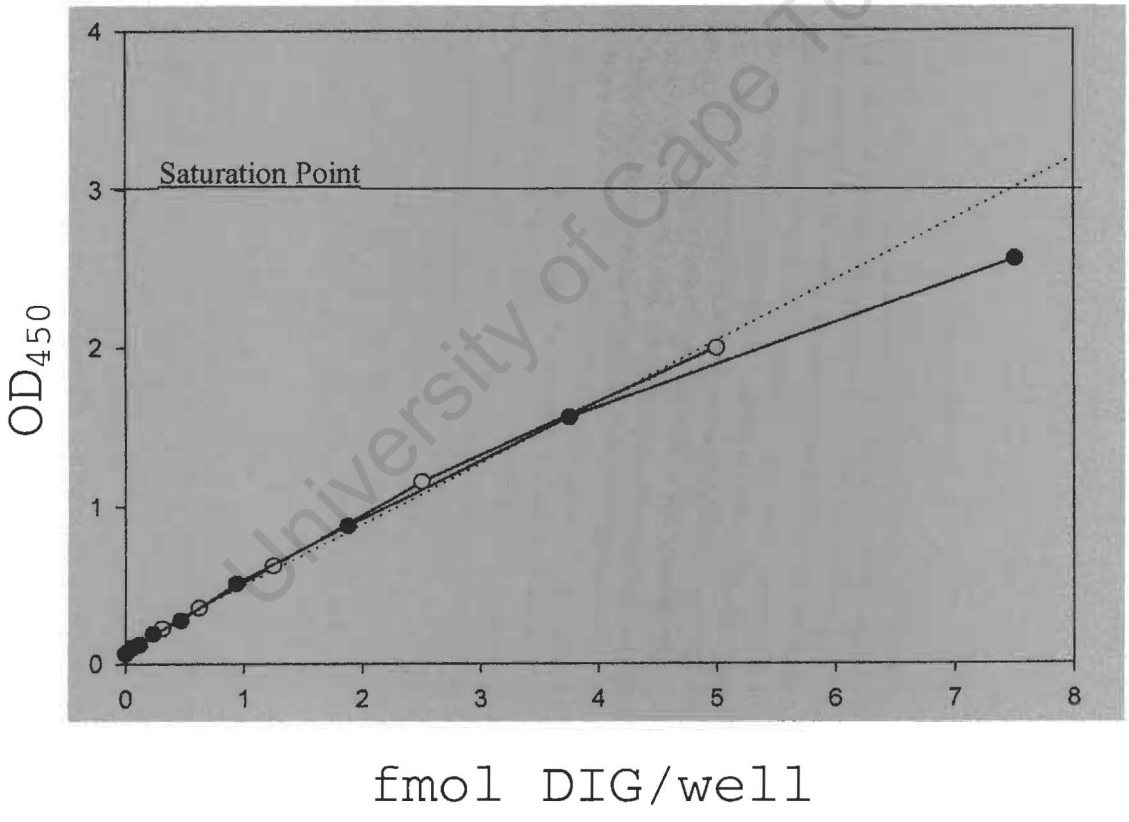
*OD_{450}: mean and standard deviation of the three values obtained.

University of Cape Town

Table 3.6: Deduced values of equivalent DIG-label calculated for the OD_{450} values listed in Table 3.4 using the standard curve constructed from data listed in Table 3.3.

fmol DIG/well	OD_{450}
6.322	2.552
3.161	1.561
1.581	0.876
0.527	0.509
0.264	0.274
0.132	0.188
0.660	0.121
0.330	0.100
no DNA (conjugate added)	0.065

University of Cape Town

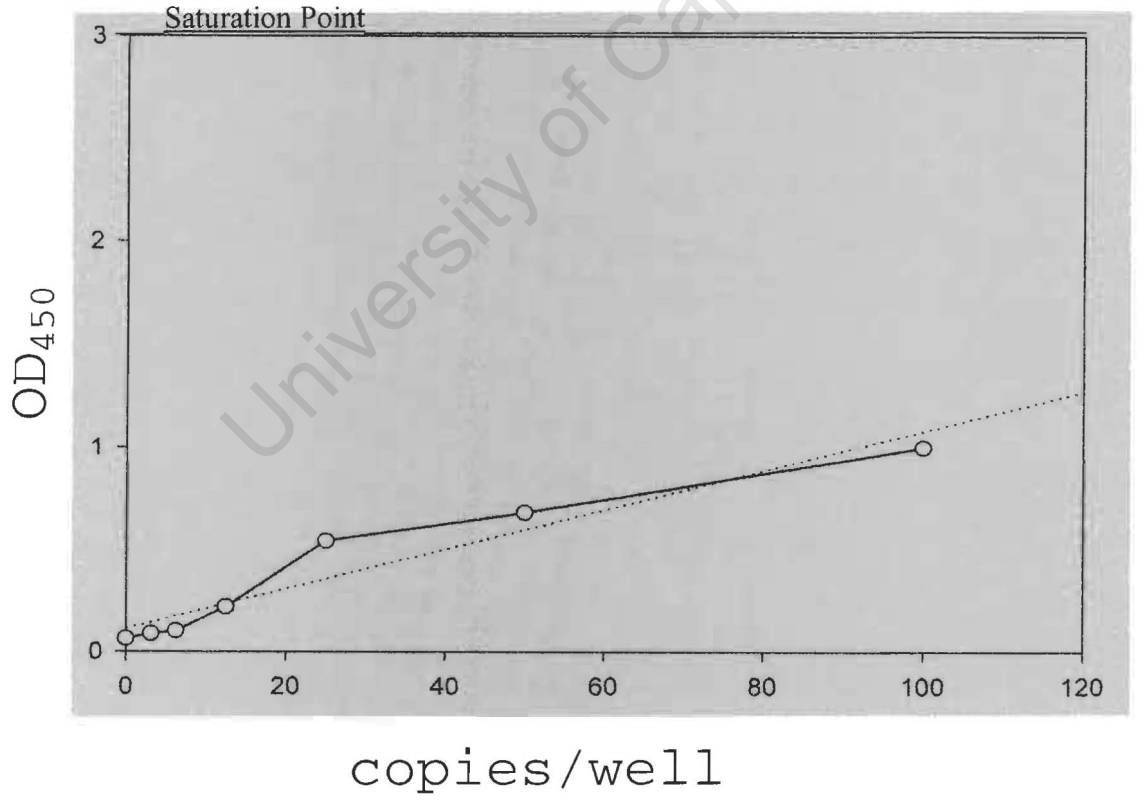


Graph 3.2: Solid-circles are a linear plot of results obtained from DIG-detection ELISA using DIG-labelled PCR amplicons showing the OD_{450} values versus two-fold dilutions of amplicon. (Data listed in Table 3.6). Open-circles correspond to values plotted on Graph 3.1.

Table 3.7: Results of DIG-detection PCR-ELISA showing OD_{450} values obtained from two-fold dilutions of control template molecules starting with one hundred copies.

Sample: (number of template copies)	OD_{450}
100	0.988
50	0.680
25	0.540
12.5	0.218
6.25	0.102
3.125	0.088
-ve H_2O control	0.064
+ve DNA control	1.318

University of Cape Town



Graph 3.3: Linear plot of results obtained from DIG-detection ELISA using labelled DNA generated by PCR showing the OD_{450} values versus two-fold dilutions of template starting with the equivalent of 100 copies/well (Data listed in Table 3.7).

3.1.2.4 **DIG-detection ELISA applied to sample unknowns**

Using a positive control of known copy number as inferred from the standard curve generated previously, samples from three mother-child sets (RX3, RX6 and RX8) were assayed for provirus copy number. The number of copies ranged from 2,100 to 13,200 per ml with between 21 and 132 copies added per amplification reaction (Table 3.9). As the number of amplifiable copies fell within the range of 20 to 50 amplifiable copies recommended by Delwart *et al.* (1993), the proviral copy number was considered sufficient for accurate estimation of intraperson sequence diversity.

Table 3.8: Results of DIG-detection PCR-ELISA showing OD_{450} values obtained by using PBMC DNA from infected mother-child pairs as the source of template molecules.

Sample:	OD_{450}
RX3-m	0.318
RX3-c	1.393
RX6-m	0.450
RX6-c	1.209
RX8-m	0.421
RX8-c	0.840
-ve H_2O control	0.054
+ve DNA control	1.532

Table 3.9: Deduced number of template molecules calculated from the OD_{450} values listed in Table 3.8 using the standard curve constructed from data listed in Table 3.7.

Sample:	Number of template molecules/10 µl:
RX3-m	21
RX3-c	132
RX6-m	35
RX6-c	113
RX8-m	32
RX8-c	75

University of Cape Town

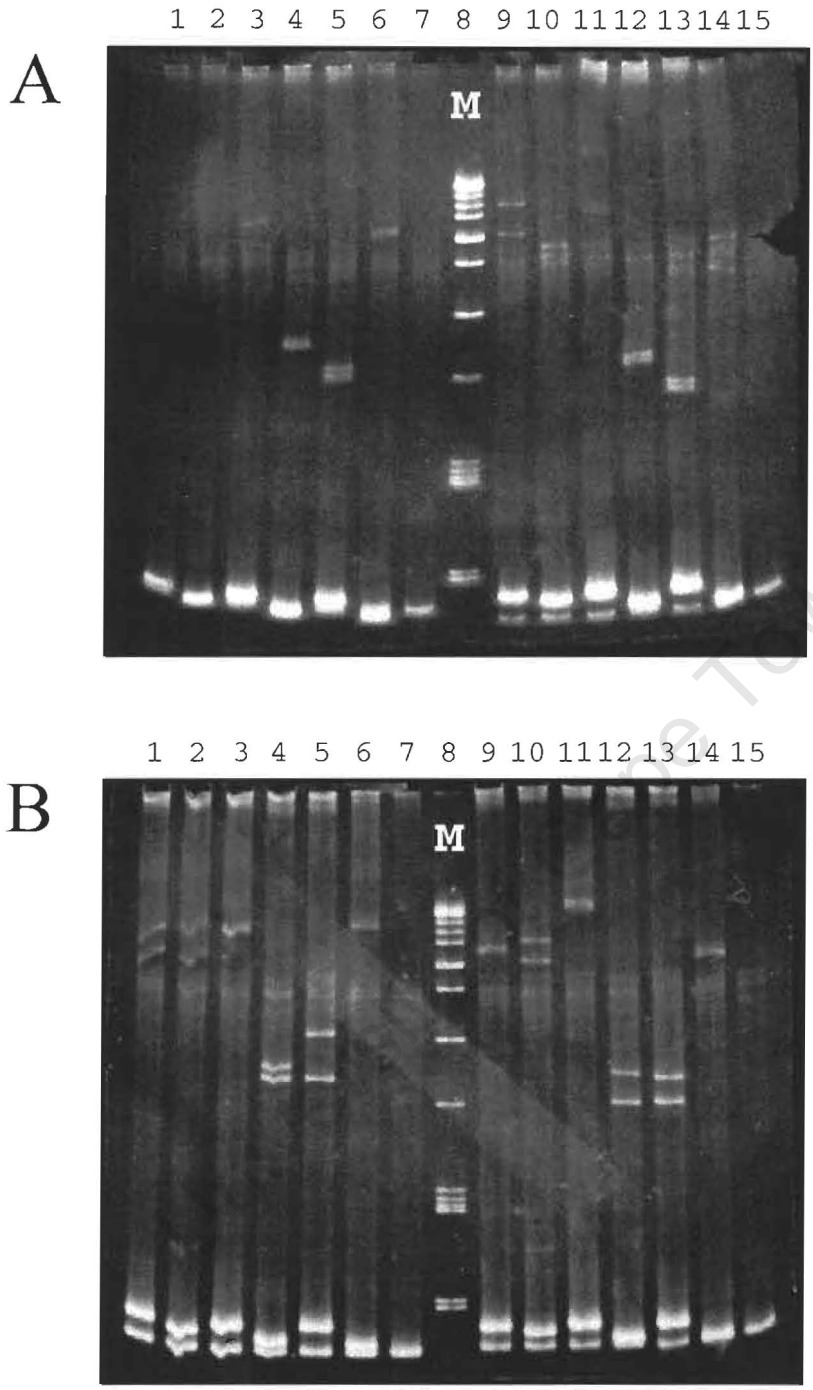
3.1.3 HETERODUPLEX MOBILITY ASSAY (HMA)

Heteroduplex mobility assay (HMA) was used for subtyping and was assessed as a method for estimating intraperson sequence diversity. HMA involves melting and re-annealing related PCR fragments and exploits the property that hybrid molecules (heteroduplexes) show reduced mobility with respect to the migrating homoduplexes when resolved in polyacrylamide gels. This shift in mobility is proportional to the degree of genetic divergence.

3.1.3.1 **Subtyping by HMA**

Subtyping of unknown samples can be achieved by generating heteroduplexes with known subtypes generated from reference plasmids. Heteroduplexes show the least mobility shift relative to the homoduplex (fastest migrating fragments) when annealed to the same subtype. For subtyping of samples RX6 and RX8, a selected clone from RX6-m, RX6-b, RX8-m and RX8-b was hybridized with reference subtypes A, B, C and D (Figure 3.10). As expected, heteroduplexes formed between clones and reference sequences showed the lowest shift in mobility when the subtype C reference sequence was used as a driver indicating that all samples belonged to subtype C.

University of Cape Town



University of Cape Town

Figure 3.9: Polyacrylamide gels of heteroduplexes (lanes 1-6) formed between 851 bp C2-V4 fragments amplified from clone RX6-m4 mixed with reference plasmids RW20 (subtype A), BR20 (subtype B), TH14 (subtype B), MA959 (subtype C), ZM18 (subtype C) and UG21 (subtype D); lane 7: clone RX6-m4 only; lane 8: low molecular weight marker (Promega, Madison, USA); lanes 9-14: clone RX6-b10 and reference subtypes; lane 15: clone RX6-b10 only; (B): lanes 1-6: clone RX8-m2 sequence mixed with reference subtype sequences as in (A); lane 7: clone RX8-m2 only; lane 8: low molecular weight marker (Promega, Madison, USA); lanes 9-14: clone RX8-b6 mixed with reference subtypes as above; lane 15: clone RX8-b6 only.

3.1.3.2 **Genetic diversity estimation by HMA analysis**

HMA can be used to estimate intrasample sequence diversity by a comparison between individual sequences hybridized to a common driver sequence. Using the eight clones generated from sample RX1-m, the HMA method was set-up and tested with a driver sequence amplified from the MA959 subtype C plasmid provided in the HMA subtyping kit (AIDS Research and Reference Reagent Program, Division of AIDS, NIAID, NIH: catalogue number ARP961) [Figure 3.10]. The driver sequence is a homologous DNA sequence used in excess concentration to form heteroduplexes with the unknown sequences. It was observed that samples could be visually distinguished from each other by differences in banding pattern when percentage DNA differences were as low as 0.07%. The distance of migration of the heteroduplexes relative to the homduplexes, however, did not correlate with percentage DNA differences. This finding was also reported by Delwart *et al.*, (1993) who determined that the assay could not be used reliably for estimation of percentage DNA difference in the 1-2% range.

HMA was applied to the clones generated from mother-child pairs RX6 and RX8 to visualize intrasample genetic diversity. Heteroduplexes were generated between clones from each mother and child and an unrelated subtype C driver sequence (MA959). Additional analysis was performed using a clone selected from each mother and child as a closer related driver sequence. As expected mobility shifts were lower in magnitude when using the phylogenetically linked samples than the unlinked subtype C driver sequences indicating that there was a greater degree of similarity between the sequences of the mother and child than between the unrelated C subtype sequence. There was significant divergence of the infant's sequences compared to the mother's sequences as visualized by the large mobility shifts of the heteroduplexes (Figures 3.11 and 3.12).

For sample RX6-m, 10 clones were analyzed and showed seven different banding patterns and for sample RX6-b, ten clones were analyzed showing eight different banding patterns (Figure 3.11). Mobility shifts of the heteroduplex bands were similar to each other in both mother and child which is indicative of low intrasample percentage DNA

University of Cape Town

difference, approximately 1%. For sample RX8-m, ten clones were analyzed and showed eight different banding patterns and for sample RX8-b, ten clones were analyzed showing nine different banding patterns (Figure 3.12). Mobility shifts of the heteroduplex bands were more varied than in RX6 and is indicative of a greater degree of heterogeneity in this sample pair. The use of an unrelated or related driver sequence did not influence the banding patterns other than the mobility shifts of the heteroduplexes depending of the homology between the samples and the driver sequence.

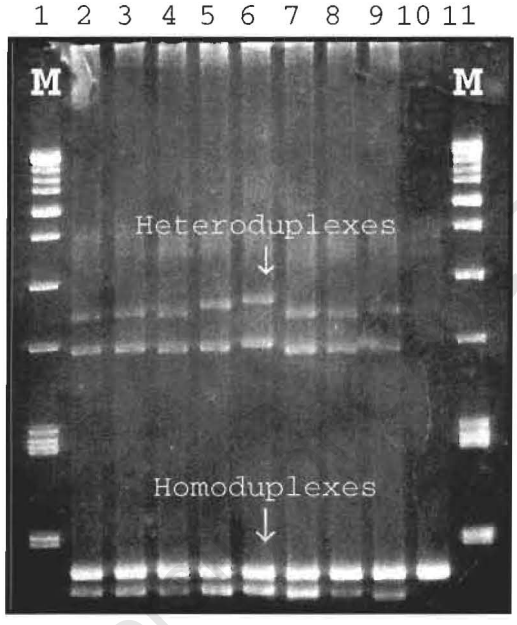


Figure 3.10: Polyacrylamide gel of heteroduplexes generated by mixing of 851 bp C2-V4 PCR product amplified from 8 clones derived from sample RX1-m with a related subtype C reference sequence MA959. Lane 1 and 11: Low molecular weight marker (Amersham, Piscataway, USA); lane 2: RX1-m1; lane 3: RX1-m2; lane 4: RX1-m3; lane 5: RX1-m4; lane 6: RX1-m5; lane 7: RX1-m6; lane 8: RX1-m7; lane 9: RX1-m8; lane 10: reference MA979 only.

University of Cape Town

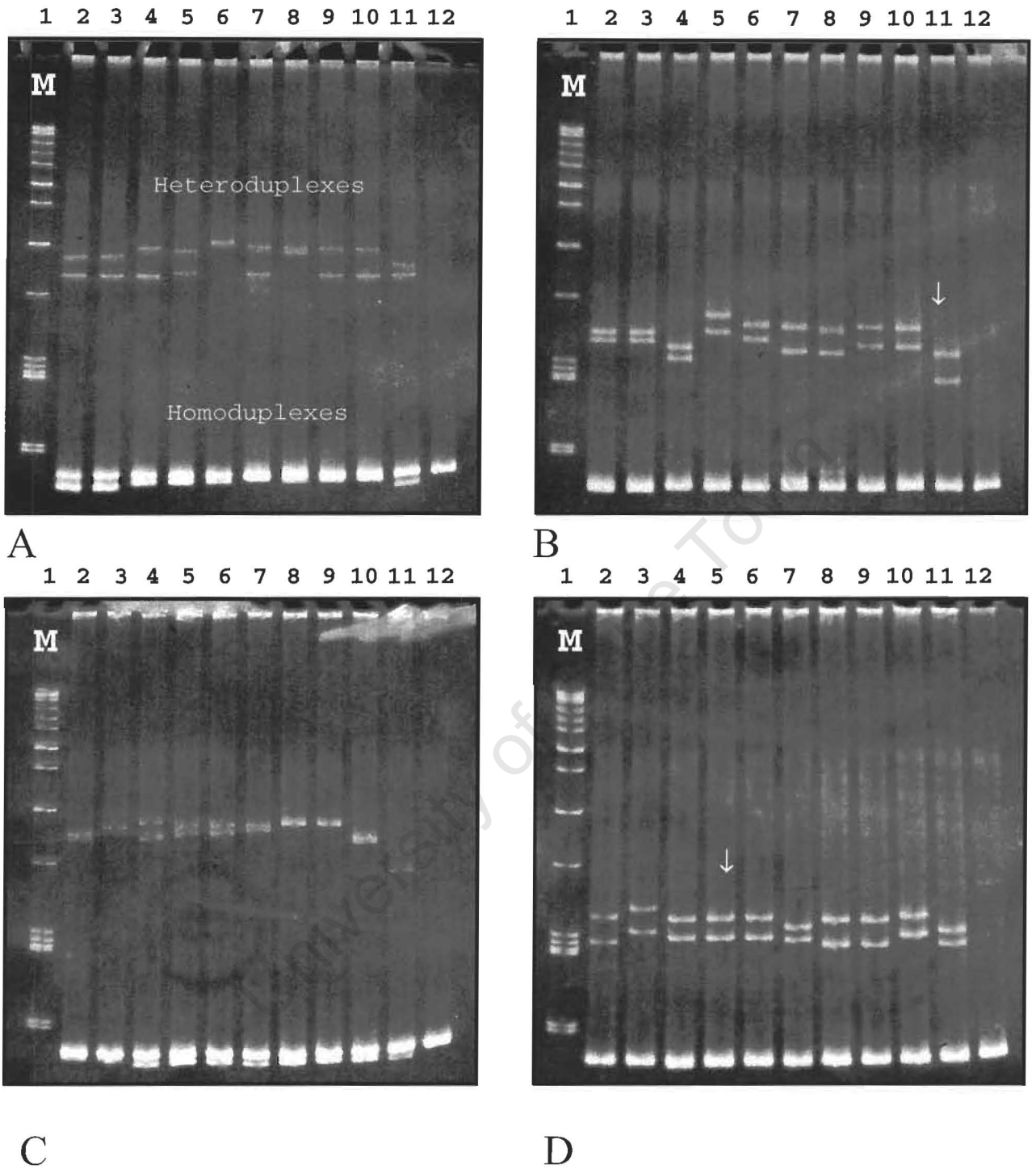
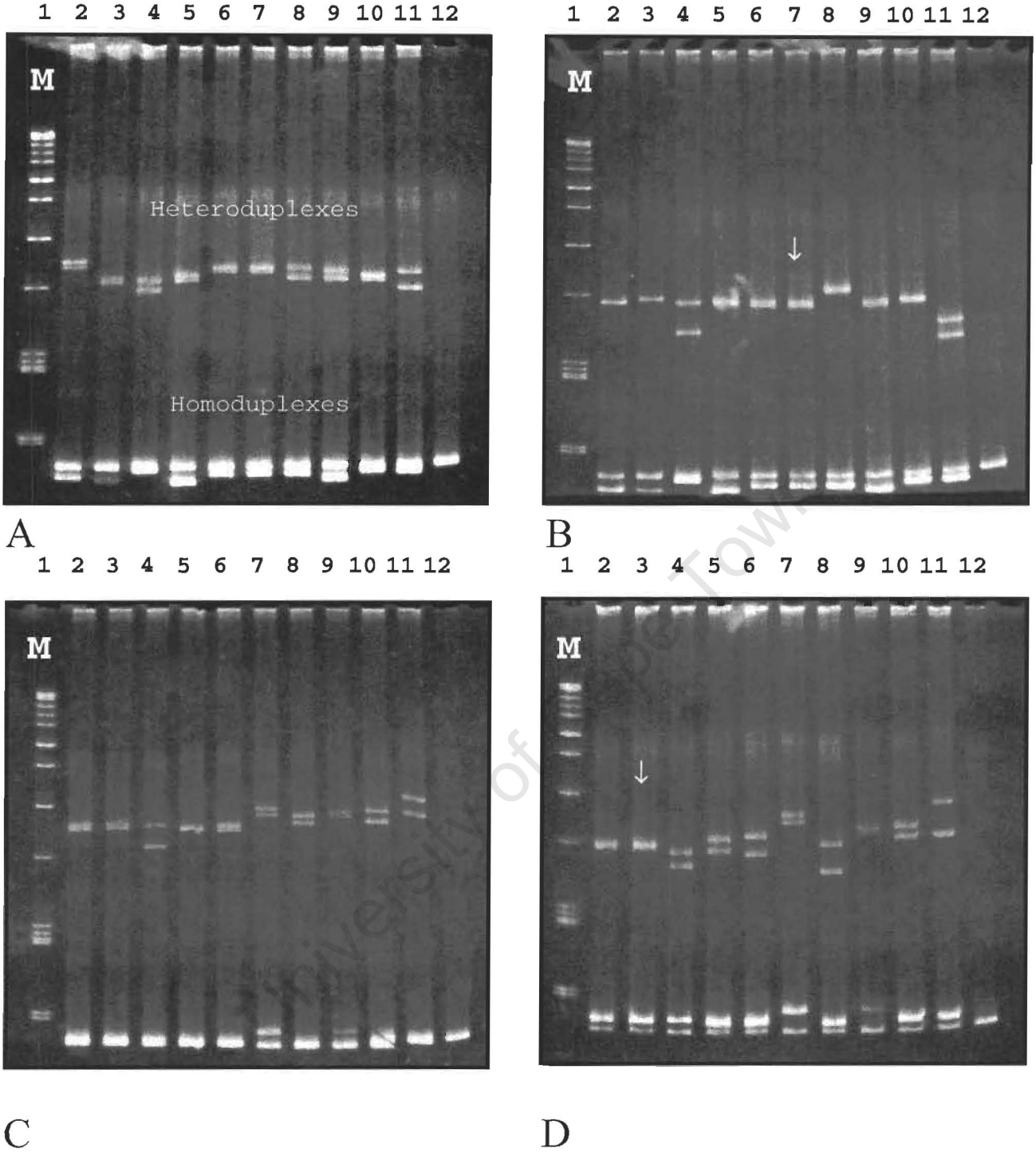


Figure 3.11: (A) Polyacrylamide gels of heteroduplexes generated between 851 bp C2-V4 fragments amplified by PCR from clones generated from sample RX6-b and reference C subtype MA979; (B): clones 1-10 from sample RX6-b and selected sequence RX6-m4; (C): clones 1-10 from sample RX6-m and reference C subtype MA979; (D): clones 1-10 from sample RX6-m and a selected sequence RX6-b10. Positions of heteroduplexes and homoduplexes are labeled and arrows indicate samples containing the same mixture of clones. In all images lane 1: Low molecular weight marker (Promega, Madison, USA); lane 11: driver sequence DNA only; lanes 2-11: clones 1-10 mixed with driver sequence DNA.



University of Cape Town

Figure 3.12: (A) Polyacrylamide gels of heteroduplexes generated between 851 bp C2-V4 fragments amplified by PCR from clones generated from sample RX8-b and reference C subtype MA979; (B): clones 1-10 from sample RX8-b and selected sequence RX8-m2; (C): clones 1-10 from sample RX8-m and reference C subtype MA979; (D): clones 1-10 from sample RX8-m and a selected sequence RX8-b6. Positions of heteroduplexes and homoduplexes are labeled and arrows indicate samples containing the same mixture of clones. In all images lane 1: Low molecular weight marker (Promega, Madison, USA); lane 11: driver sequence DNA only; lanes 2-11: clones 1-10 mixed with driver sequence DNA.

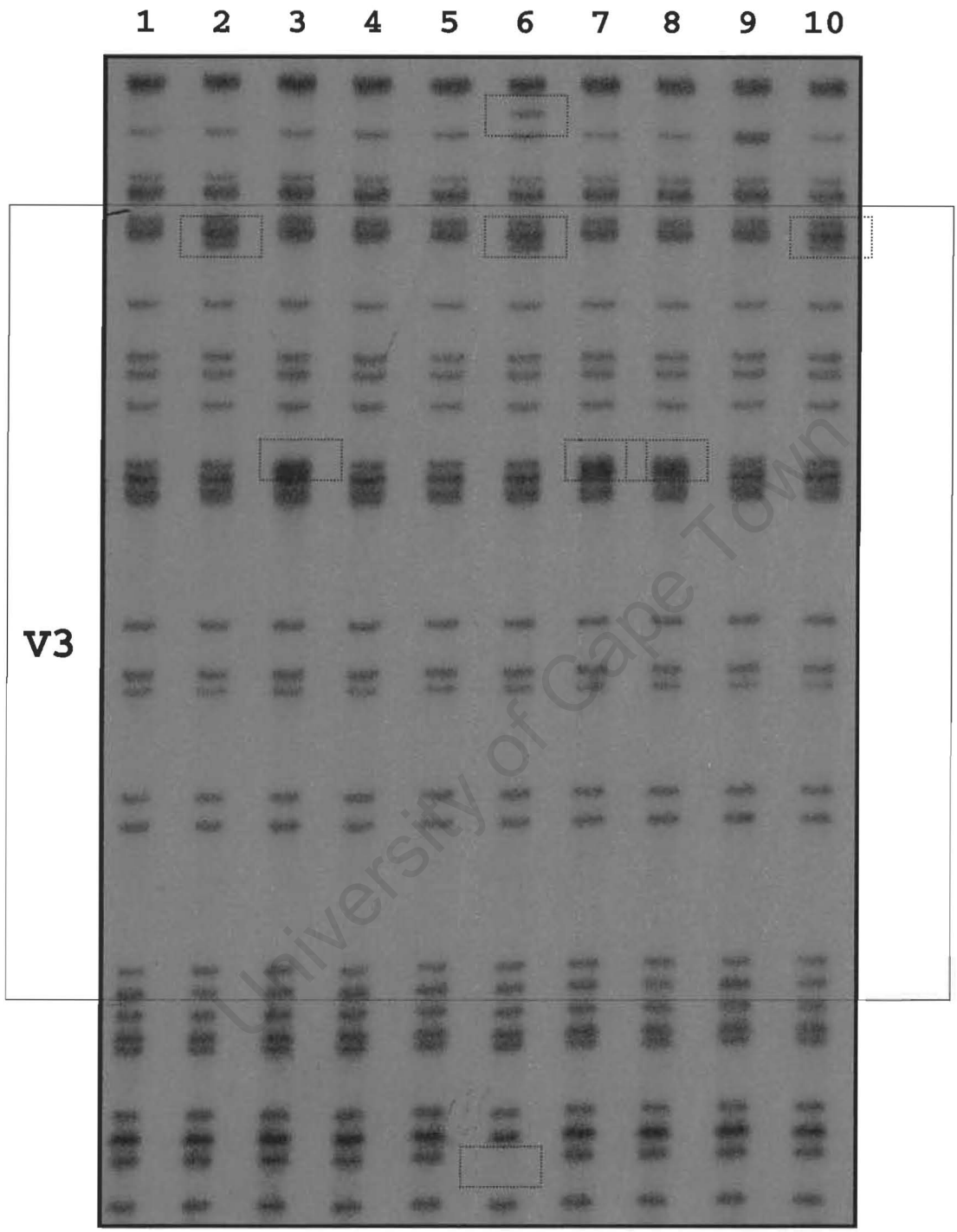
3.1.4 BASE EXCISION SEQUENCE SCANNING (BESS)

Base Excision Sequence Scanning or BESS (Epicentre Technologies, Madison, USA) generates partial sequence by mapping thymine residues. In this method, amplification is performed with limiting amounts of dUTP which are incorporated into the amplicons. Incubation with uracil N-glycosylase results in removal of the uracil leaving an abasic site which is then cleaved by a second enzyme, exonuclease IV. End-labelled radioactive cleavage fragments can be resolved on a denaturing polyacrylamide gel. In this study, the method was optimized and applied using a P^{32} radio-labelled PCR primer and then adapted for analysis by an automated DNA-sequencer using a PCR primer labelled with fluorescein. Analysis of positive and negative strands results in the generation of an AT sequence profile.

3.1.4.1 **BESS analysis using radio-labelled PCR primers**

The BESS method was applied to the clones generated previously from two mother-child pairs (RX6 and RX8). An example of the autoradiographs obtained is depicted in Figure 3.13. The presence of a band is indicative of a thymine residue at a specific position on the DNA strand, identical to the T-ladder obtained from DNA sequencing autoradiograph. The loss or gain of a band is indicative of a single nucleotide mutation contributing to sequence variation. Using BESS, sequence data representing thymine residues in the V3 loop was generated for the positive strand for each clone.

University of Cape Town



University of Cape Town

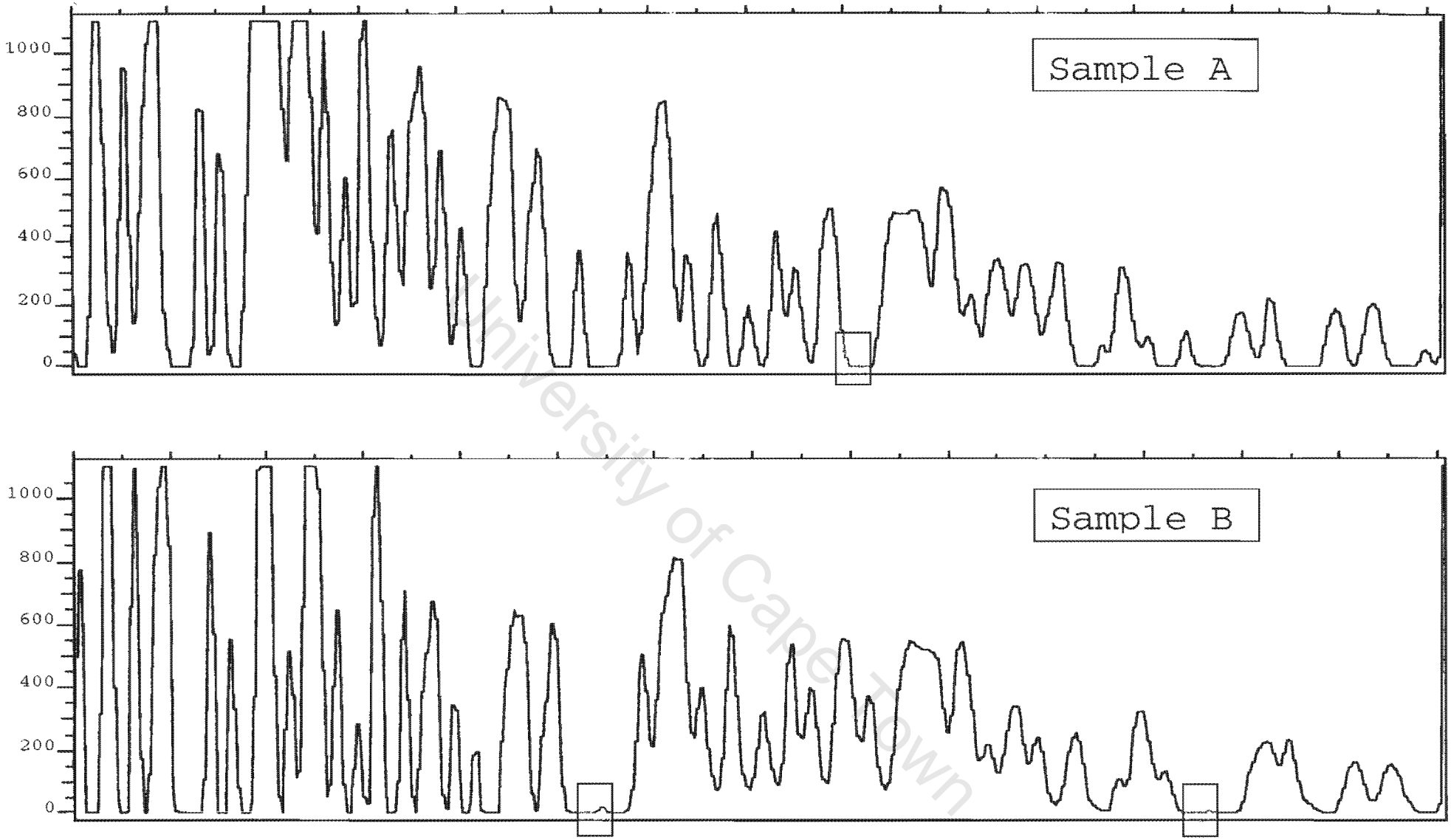
Figure 3.13: Autoradiograph of gamma-P^{32} labeled cleavage products generated by BESS and resolved on a 6% polyacrylamide gel. The V3 region is indicated by the dotted line. Small rectangles indicate differences in banding patterns.

3.1.4.2 **BESS analysis using fluorescein-labelled PCR primers**

To improve efficiency, the BESS method was modified for reading of samples by an automated sequencer by using a fluorescent primer in the PCR amplification step. This modification was applied to clones generated from two mother-child pairs (RX6 and RX8) using a fluorescein-labelled reverse primer. Sequence data representing thymine residues in the V3 loop were generated for the negative strand for each clone. An example of the electropherograms obtained is depicted in Figure 3.14. The presence of a peak is indicative of a thymine residue at a specific position on the DNA strand, identical to the T-pattern obtained from automated DNA sequencing chromatograms. The loss or gain of a peak is indicative of a single nucleotide mutation contributing to sequence variation.

It was noted that although the use of radio-labelled primers was more labour intensive, the analysis of data on the autoradiograph was clearer and less ambiguous than using the electropherogram reports.

University of Cape Town



University of Cape Town

Figure 3.14: Peaks of fluorescence monitored over time representing thymidine residues from two DNA clones amplified by PCR and used for BESS analysis. The absence of a peak is shown by a rectangle.

3.1.4.3 **Re-constructed sequences of mother-child pairs**

Using data generated by BESS, partial sequences reflecting A and T residues were re-constructed for clones generated from two mother-child pairs (Figure 3.15 and Figure 3.16). Ten sequences were re-constructed for sample RX6-m, nine for sample RX6-b, ten for sample RX8-m and eight for sample RX8-b. A single clone from each mother and child was selected for automated sequencing and used as a reference in the re-constructed alignments.

Analysis of partial sequence alignments for sample pair RX6 (Figure 3.15) showed that for RX6-m, six positions showed a loss or gain of a T or an A residue involving fourteen residues in total. A potential glycosylation site (N-X-T) was conserved in all maternal sequences at amino-acid residue six of the V3 loop. In an analysis of partial sequence alignments generated for the child RX6-b, 7 positions showed a loss or gain of a T or an A residue involving nine residues in total. Assuming that a G to C mutation had not occurred at nucleotide position sixteen of the V3 sequence RX6-b8, it was noted that the predicted glycosylation site at amino-acid residue six was lost in two of the child's sequences (RX6-b8 and RX6-b10).

Analysis of partial sequence alignments for sample pair RX8 (Figure 3.16) showed that for RX8-m, six positions showed a loss or gain of a T or an A residue involving fourteen residues. Five sequences (RX8-m1, RX8-m2, RX8-m6, RX8-m9 and RX8-m10) had a deletion of a codon at amino acid position twenty-three in V3 loop. A predicted glycosylation site (N-X-T) at amino-acid position six was conserved in all of the maternal sequences. Analysis of sequence alignments for the child RX8-b showed nine positions with a loss or gain of a T or an A residue involving twenty-three residues. No sequences in the child had a deletion of a codon at amino acid position twenty-three of V3. Assuming a G to C mutation had not occurred at nucleotide position seventeen in sequence RX8-b8, the predicted glycosylation site at amino acid position six of V3 was lost in two of the child's sequences (RX8-b6 and RX8-b8).

University of Cape Town

Figure 3.15: 105 bp partial sequence alignment of V3 derived from BESS analysis of cloned *env* sequences from mother-child pair RX6. The first sequence is a clone selected for automated sequencing, all other sequences do not contain G or C data. Predicted protein sequence for the sequenced clone is printed above. Arrow indicates a potential site for N-linked glycosylation. Differences observed in thymine and adenine residues are highlighted in grey.

University of Cape Town

Figure 3.16: 105 bp partial sequence alignment of V3 derived from BESS analysis of cloned *env* sequences from mother-child pair RX8. The first sequence is a clone selected for automated sequencing, all other sequences do not contain G or C data. Predicted protein sequence for the sequenced clone is printed above. Arrow indicates a potential site for N-linked glycosylation. Differences observed in thymine and adenine residues are highlighted in grey.

3.1.4.4 **Phylogenetic analysis**

The BESS system does not detect G or C residues. Based on an assumption, not necessarily true, that no G to C or C to G mutations had occurred, the missing G and C residues were included in the re-constructed partial sequences generated by BESS according to the following criteria: (i) the sequenced clone for each mother and child was used as a primary reference and unknown bases were filled with the base present in the sequenced clone; (ii) the consensus base for the child's sequences was used if the base was uncertain in the mother and *vice versa*; (iii) the consensus base in the reference subtypes, particularly subtype C, was used if neither the mother nor the child's sequenced clone could be referred to; (iv) a default G was used arbitrarily if all other criteria could not be used.

The filling in of missing G and C residues was a necessary step in order to create an input file for sequence analysis software. Sequence alignments and distance matrices were generated using Clustal X software (Thompson *et al.*, 1997) with gaps included. A phylogenetic neighbour-joining tree was constructed based on the Kimura two-parameter algorithm using TreeCon software (Van de Peer and De Wachter., 1994). Sequences of reference HIV-1 subtypes downloaded from the Los Alamos sequence database (http://hiv-web.lanl.gov) were included for subtyping of samples. Distance matrices for the V3 clones are listed in Figure 3.17.

The phylogenetic trees of mother-child pairs RX6 (Figure 3.18) and RX8 (Figure 3.19) showed correct clustering of sequences within the subtype C reference group as well as correct clustering of mother and infant sequences indicating that there were sufficient informative sites contained within the partial sequences to allow correct discrimination of related genotypes. In addition, as expected, mother-child sequences clustered most closely to the local South African strain of HIV-1, DU151. However, clustering was not always supported by high bootstrap values due to the reduced number of informative sites available from short fragments (105bp).

University of Cape Town

The phylogenetic tree for mother-child pair RX6 (Figure 3.18), depicted eight different genotypes for RX6-m in the ten sequences included and for the child RX6-b, seven different genotypes were depicted in the nine sequences included. In the mother, sequences RX6-m4 and RX6-m5 were identical in V3 as well as sequences RX6-m7 and RX6-m8. In the child, sequences RX6-b1 and RX6-b2 were identical in V3. RX6-b8 and RX6-b10 were identical in V3 and RX6-b3, RX6-b5 and RX6-b6 were identical in V3. The child's sequences were more homogeneous compared to the mother's sequences

The phylogenetic tree for mother-child pair RX8 (Figure 3.19), depicted five different genotypes for RX8-m in the ten sequences included and for the child RX8-b, all eight sequences included were different. In the mother, sequences RX8-m4 and RX8-m8 were identical in V3. The maternal sequences RX8-m1, RX8-m2, RX8-m6, RX8-m7 and RX8-m9 (the sequences with the codon deletion) were also identical in V3. Converse to the findings for mother-child pair RX6, the mother's sequences were more homogeneous compared to the child's sequences.

University of Cape Town

Figure 3.17: Distance tables of percentage DNA difference of 105 bp re-constructed nucleotide sequences for mother-child pairs RX6 and RX8.

(A)

	RX6-m1	RX6-m2	RX6-m3	RX6-m4	RX6-m5	RX6-m6	RX6-m7	RX6-m8	RX6-m9	RX6-m10
RX6-m1	0									
RX6-m2	0.029	0								
RX6-m3	0.038	0.029	0							
RX6-m4	0.019	0.010	0.019	0						
RX6-m5	0.019	0.010	0.019	0.000	0					
RX6-m6	0.038	0.010	0.038	0.019	0.019	0				
RX6-m7	0.038	0.029	0.010	0.019	0.019	0.038	0			
RX6-m8	0.038	0.029	0.010	0.019	0.019	0.038	0.000	0		
RX6-m9	0.029	0.019	0.019	0.010	0.010	0.029	0.010	0.010	0	
RX6-m10	0.038	0.010	0.029	0.019	0.019	0.019	0.019	0.019	0.010	0

(B)

	RX6-b1	RX6-b2	RX6-b3	RX6-b4	RX6-b5	RX6-b6	RX6-b8	RX6-b9	RX6-b10
RX6-b1	0								
RX6-b2	0.000	0							
RX6-b3	0.019	0.019	0						
RX6-b4	0.038	0.038	0.019	0					
RX6-b5	0.019	0.019	0.000	0.019	0				
RX6-b6	0.019	0.019	0.000	0.019	0.000	0			
RX6-b8	0.038	0.038	0.019	0.038	0.019	0.019	0		
RX6-b9	0.029	0.029	0.010	0.029	0.010	0.010	0.029	0	
RX6-b10	0.038	0.038	0.019	0.038	0.019	0.019	0.000	0.029	0

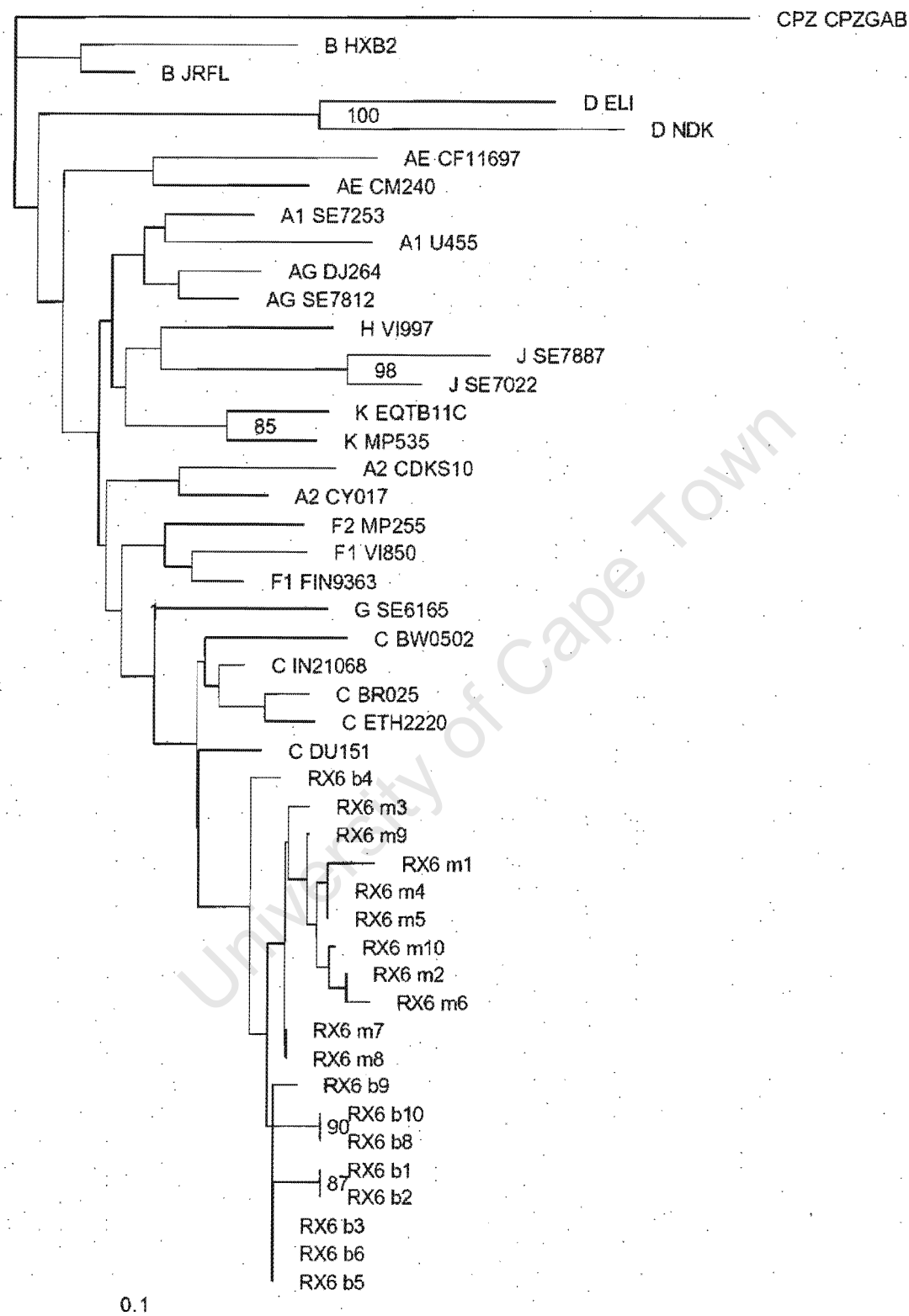
(C)

	RX8-m1	RX8-m2	RX8-m3	RX8-m4	RX8-m5	RX8-m6	RX8-m7	RX8-m8	RX8-m9	RX8-m10
RX8-m1	0									
RX8-m2	0.000	0								
RX8-m3	0.029	0.029	0							
RX8-m4	0.010	0.010	0.019	0						
RX8-m5	0.000	0.000	0.029	0.010	0					
RX8-m6	0.000	0.000	0.029	0.010	0.000	0				
RX8-m7	0.000	0.000	0.029	0.010	0.000	0.000	0			
RX8-m8	0.010	0.010	0.019	0.000	0.010	0.010	0.010	0		
RX8-m9	0.000	0.000	0.029	0.010	0.000	0.000	0.000	0.010	0	
RX8-m10	0.039	0.039	0.049	0.049	0.039	0.039	0.039	0.049	0.039	0

(D)

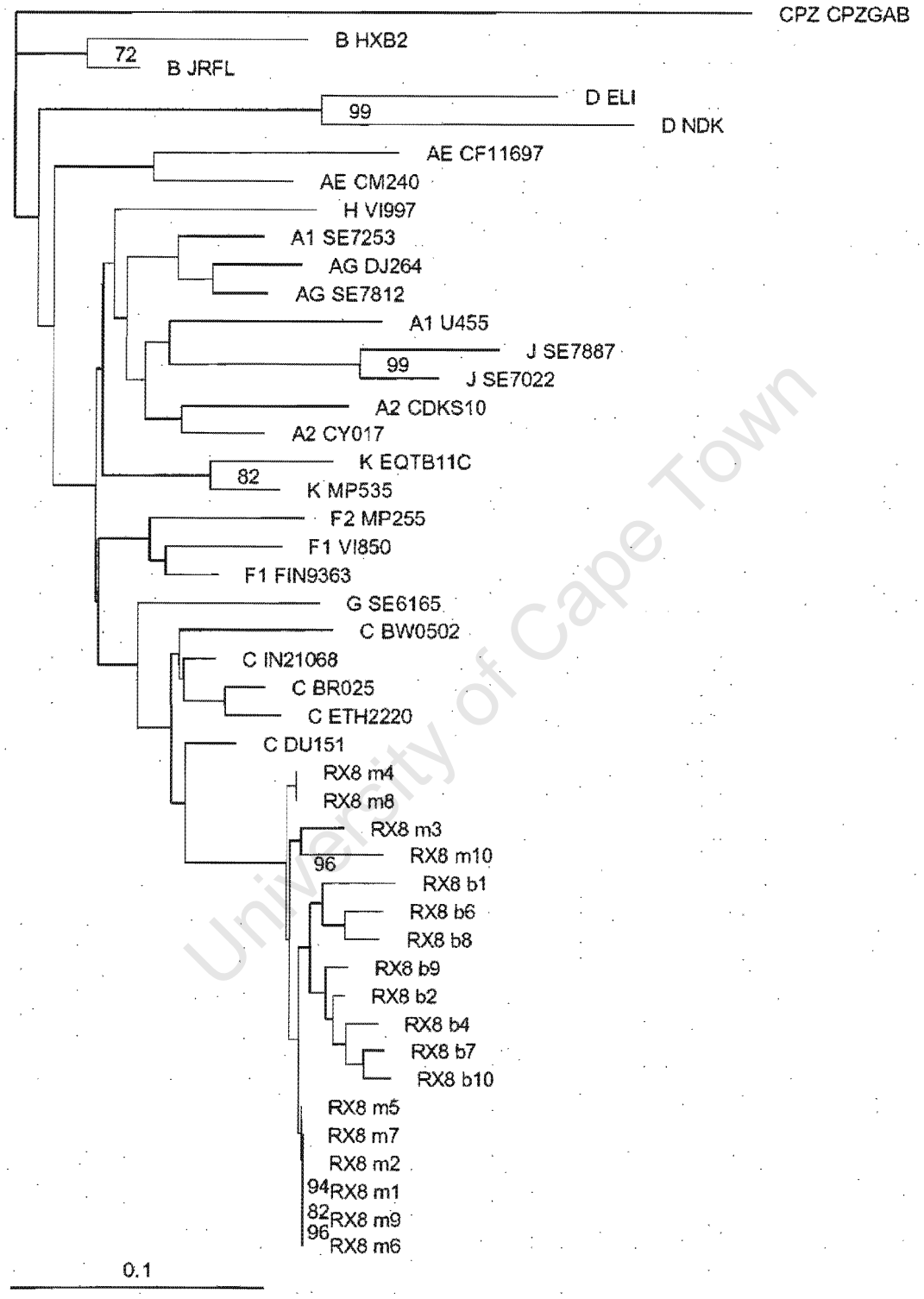
	RX8-b1	RX8-b2	RX8-b4	RX8-b6	RX8-b7	RX8-b8	RX8-b9	RX8-b10
RX8-b1	0							
RX8-b2	0.057	0						
RX8-b4	0.057	0.019	0					
RX8-b6	0.057	0.019	0.038	0				
RX8-b7	0.057	0.019	0.019	0.038	0			
RX8-b8	0.048	0.048	0.067	0.029	0.067	0		
RX8-b9	0.057	0.019	0.038	0.038	0.019	0.048	0	
RX8-b10	0.038	0.038	0.038	0.057	0.019	0.067	0.019	0

University of Cape Town



University of Cape Town

Figure 3.18: Phylogenetic neighbour-joining tree based on partial sequence data covering the 105 bp V3 region of representing nineteen clones generated from RX6-m and –b as well as V3 reference sequences from HIV-1 clades A to J. A South African subtype C strain DU151 is also included. Trees were constructed using the neighbour-joining method, SIVcpz was used as an outlier and bootstrap values >70% are indicated.



University of Cape Town

Figure 3.19: Phylogenetic neighbour-joining tree based on partial sequence data covering the 105 bp V3 region of *env* representing eighteen clones generated from RX8-m and –b as well as V3 reference sequences from HIV-1 clades A to J. A South African subtype C strain DU151 is also included. Trees were constructed using the neighbour-joining method, SIVcpz was used as an outlier and bootstrap values >70% are indicated.

CHAPTER 4: DISCUSSION

In summary, the C2-V5 region of the HIV-1 *env* gene was amplified from fourteen samples from infected mother-child pairs. PCR products were cloned for use as reagents in the development of heteroduplex mobility assay (HMA) and base excision sequence scanning (BESS) assays to monitor genetic changes as an alternative to DNA sequencing methods. In addition, an ELISA-PCR quantitation system was developed to estimate proviral copy number in DNA extracted from PBMC's. The quantitation of proviral sequences was necessary to control for the number of copies of template used in PCR amplification to avoid re-sampling errors so that amplified products generated were truly representative of the viral quasispecies.

The eight clones generated from sample RX1-m were manually sequenced and used as reagents for testing of the HMA method. From the sequence data generated, the intrasample percentage DNA difference was less than 1% and this was reflected by the similar shifts in heteroduplex mobility between clones in the HMA. For this reason intrasample DNA difference could not be estimated by measurement of mobility shift. Delwart *et al.* (1993) reported that the mobility shift was not necessarily proportional to the percentage DNA difference in the 1-2% range. It was, however, possible to visually estimate the number of different genotypes by comparing banding patterns. The assay was applied to cloned sequences from two mother-child pairs (RX6 and RX8) and as observed in the RX1-m HMA, mobility shifts were also similar for each clone included. It was therefore most likely that the intrasample diversity in these samples was also less than 2%. Other possibilities affecting mobility shift include sub-optimal length of the sequences used or length polymorphisms introduced by insertions and deletions between reference and sample sequences. Delwart *et al.* (1993) reported that insertions and deletions introduce bulges in the heteroduplex which have a more dramatic influence on mobility shifts than point mutation do. Sequence length polymorphisms were commonly seen in the sample sets used in the HMA as evidenced by two migrating bands at the position of the homoduplexes. It is likely that this factor as well as the low degree of intrasample diversity has affected the relationship between reduced mobility and

University of Cape Town

percentage DNA divergence. The position of mismatches may also vary in the effect on heteroduplex mobility. This finding was reported by Upchurch *et al.* (2000), who showed that in the absence of length polymorphisms the assay was only reliable at a resolution of greater than 4.5% due to the exaggerated influences on heteroduplex mobilities related to mismatches positioned near the centre of the molecule or the clustering of mismatches.

The HMA results from this study did indicate the reliability of the assay to show dissimilarity or similarity between sequences based on observed banding patterns and in addition the method was accurate for subtyping of samples. Epidemiological linkage between samples, such as mother-child pairs, could also be shown since smaller mobility shifts were observed when a maternal sequence was annealed to a sequence from the child and *vice versa*. Mobility shifts were greater when an external unrelated subtype C sequence was used as the driver. The method was successfully used to subtype a cloned sequence from each mother and child and all samples were classified as subtype C.

The BESS method was found to be suited for both subtyping and phylogenetic analysis. Radio-labelled primers as well as fluorescein-labelled primers for automated sequencer analysis were tested. Although the use of automated sequencing was less labour intensive than autoradiography, the interpretation of data in an elecropherogram format was more prone to reading errors in comparison to reading autoradiographs. Using this method, which excludes detection of G to C or C to G mutations, phylogenetic analysis of the V3 sequence data could be achieved by re-construction of V3 loop sequences using A and T positions mapped by BESS. It was however necessary to assume that G and C residues were unchanged. Results from analysis of the phylogenetic trees generated from the sequence data showed that for sample pair RX6, the V3 sequences from the child were more homogeneous than was found in the mother. The converse was observed in sample pair RX8, which may be explained by the ages of the children at the time of sampling. Child RX6-b was fifteen months old at the time of sampling and child RX8-b was twenty-two months old. It is likely that independent evolution of viral sequences in the older child had lead to a greater level of diversification compared to the mother. The

University of Cape Town

younger child, having been infected for a shorter time, retained a more homogeneous population of variants compared to the mother

In addition, results from the assay showed genetic changes in the V3 loop that could be associated with immune evasion. Conserved glycosylation sites in the sequences of both mothers (RX6-m and RX8-m) were found to have been lost in sequences in their corresponding child's sequences. A loss of a glycosylation site may potentially affect recognition of antibody epitopes. A deletion of a codon was detected in five of the sequences from RX8-mother, which was not found in any of the child's sequences. Changes in the primary structure of V3 may affect CTL, T-helper cell or antibody epitopes. Co-receptor binding may also be influenced by amino-acid changes in the V3 loop which influences infectivity.

In conclusion, a novel application of the BESS method was developed to generate partial V3 sequence data which could be used for subtyping and sequence diversity estimates. This method is cost-effective compared to DNA sequencing techniques and informative data can be generated. The HMA method was also tested and was useful for visual estimation of heterogeneity and for accurate subtyping of samples. The PCR-ELISA proviral load assay was an important method used to estimate the number of provirus copies for the accurate characterization of sequence variability of the viral quasispecies.

University of Cape Town

Appendix A: REAGENTS

Luria agar

10 g NaCl
5 g Yeast Extract
10 g Tryptone
15 g agar
Made up to 1 litre with distilled water

Luria broth

10 g NaCl
5 g Yeast Extract
10 g Tryptone
Made up to 1 litre with distilled water

Carbonate/bicarbonate buffer (0.1M pH 7.6)

sodium carbonate 1.36 g
sodium bicarbonate 7.35 g
distilled water 950 ml
The pH is adjusted to 7.6 and the volume made up to 1 litre with distilled water

Phosphate buffered saline (PBS)

NaCl 8.0 g
KCl 0.2 g
Na_2HPO_4 0.12 g
distilled water 900 ml
The pH is adjusted to 7.5 and the volume made up to 1 litre with distilled water

Trituration buffer (1M $CaCl_2$)

$CaCl_2.2H_2O$ 14.7 g
Made up to 100 ml with distilled water

Lysis buffer

10mM Tris-HCl (pH 8.0)
1mM EDTA
15% sucrose (w/v)
2 mg/ml lysozyme
0.2 mg/ml RNase A
0.1 mg/ml BSA

University of Cape Town

SSC (20X)

0.3M sodium citrate
3M NaCl
pH 7 with 10N NaOH

Hybridization buffer (colony hybridization)

30% formamide
5X SSC
0.02% SDS
2% milk powder (w/v)

TE buffer

1ml Tris-Cl (1M)
0.2ml EDTA (0.5M)
distilled water 90ml
pH is adjusted to 7.6 and the volume made up to 100ml

NNB buffer (10X)

162 g Tris
27.5 g Boric acid
9.3 g EDTA
volume made up to 1 litre with distilled water

HMA annealing buffer

100mM NaCl
10mM Tris, pH 7.8
2mM EDTA

University of Cape Town

References

Abebe A, Demissie D, Goudsmit J, Brouwer M, Kuiken CL, Pollakis G, Schuitemaker H, Fontanet AL, Rinke de Wit TF., HIV-1 subtype C syncytium- and non-syncytium-inducing phenotypes and co-receptor usage among Ethiopian patients with AIDS. AIDS, 1999:13;1305-11

Ahmad N, Baroudy BM, Baker RC, Chappey C., Genetic analysis of human immunodeficiency virus type 1 envelope V3 region isolates from mothers and infants after perinatal transmission. J Virol 1995;2:1001-12

Alimenti A, Luzuriaga K, Stechenberg B, Sullivan JL., Quantitation of human immunodeficiency virus in vertically infected infants and children. J Pediatr 1991;119:225-9

Andeweg AC, Groenink M, Leeflang P, de Goede RE, Osterhaus AD, Tersmette M, Bosch ML., Genetic and functional analysis of a set of HIV-1 envelope genes obtained from biological clones with varying syncytium-inducing capacities. AIDS Res Hum Retroviruses 1992;8:1803-10

Aoki-Sei S, Yarchoan R, Kageyama S, Hoekzema DT, Pluda JM, Wyvill KM, Broder S, Mitsuya H., Plasma HIV-1 viraemia in HIV-1 infected individuals assessed by polymerase chain reaction. AIDS Res Hum Retroviruses 1992;8:1263-70

Barlow KM, Mok JY., Dizygotic twins discordant for HIV and hepatitis C virus. Arch Dis Childhood 1993;68:507

Becquart P, Hocini H, Levy M, Sepou A, Kazatchkine MD, Belec L., Secretory Anti-Human Immunodeficiency Virus (HIV) Antibodies in Colostrum and Breast Milk Are not a Major Determinant of the Protection of Early Postnatal Transmission of HIV. J Inf Dis 2000:181;532-9

Berger EA, Doms RW, Fenyo EM, Korber BT, Littman DR, Moore JP, Sattentau QJ, Schuitemaker H, Sodroski J, Weiss RA., A new classification for HIV-1. Nature 1998;391:240

Berghammer H, Auer B, "Easypreps":fast and easy plasmid minipreparation for analysis of recombinant clones in *E. coli*. Biotechniques 1993;14:527-8

University of Cape Town

Biggar RJ, Miotti PG, Taha TE, Mtimavalye L, Broadhead R, Justesen A, Yellin F, Liomba G, Miley W, Waters D, Chiphangwi JD, Goedert JJ., Perinatal intervention trial in Africa: effect of a birth canal cleansing intervention to prevent HIV transmission. Lancet 1996;347:1647-50

Blanche S, Rouzioux C, Moscato ML, Veber F, Mayaux MJ, Jacomet C, Tricoire J, Deville A, Vial M, Firtion G, *et al.*, A prospective study of infants born to women seropositive for human immunodeficiency virus type 1. N Engl J Med 1989;320:1643-8

Bobat R, Moodley D, Coutsoudis A, Coovadia H., Breastfeeding by HIV-1-infected women and outcome in their infants: a cohort study from Durban, South Africa. AIDS 1997;11:1627-33

Bobat R, Coovadia H, Coutsoudis A, Moodley D., Determinants of mother-to-child transmission of human immunodeficiency virus type 1 infection in a cohort from Durban, South Africa. Pediatr Infect Dis J 1996;15:604-10

Bomsel M, Transcytosis of infectious human immunodeficiency virus across a tight human epithelial cell line barrier. Nat Med 1997;3:42-7

Borkowsky W, Krasinski K, Cao Y, Ho D, Pollack H, Moore T, Chen SH, Allen M, Tao PT., Correlation of perinatal transmission of human immunodeficiency virus type 1 with maternal viraemia and lymphocyte phenotypes. J Pediatr 1994;125:345-51

Bredberg-Raden U, Urassa W, Urassa E, Lyamuya E, Msemo G, Kawo G, Kazimoto T, Massawe A, Grankvist O, Mbena E, *et al.*, Predictive markers for mother-to-child transmission of HIV-1 in Dar es Salaam, Tanzania. J Acquir Immun Defic Syndr Hum Retrovirol 1995;8:182-7

Bredell H, Hunt G, Casteling A, Cilliers T, Rademeyer C, Coetzer M, Miller S, Johnson D, Tiemessen CT, Martin DJ, Williamson C, Morris L., HIV-1 Subtype A, D, G, AG and unclassified sequences identified in South Africa., AIDS Res Hum Retroviruses 2002. 10;18(9):681-3

Briant L, Wade CM, Puel J, Brown AJ, Guyader M., Analysis of envelope sequence variants suggests multiple mechanisms of mother-to-child transission of human immunodeficiency virus type 1. J Virol 1995;6:3778-88

University of Cape Town

Buluwela L, Forster A, Boehm T, Rabbitts TH., A rapid procedure for colony screening using nylon filters. Nuc Acids Res 1989;17:452

Buranasin P, Kunakorn M, Petchclai B, Raksakait K, Wichukchinda N, Jirapinyo M, Thongcharoen P., Detection of human immunodeficiency virus type 1 (HIV-1) proviral DNA in breast milk and colostrum of seropositive mothers. J Med Assoc Tha 1993;76:41-5

Calarota S and Libonatti O., Maternal antibodies to HIV-1 envelope domains: No correlation with HIV-1 vertical transmission from Argentina., Scand J Immunol, 2000 Sep;52(3):292-7

Clark MA, Hirst BH, Jepson MA., Inoculum composition and Salmonella pathogenicity island 1 regulate M-cell invasion and epithelial destruction by Salmonella typhimurium. Infect Immun 1998 Feb;66(2):724-31.

Clemetson DB, Moss GB, Willerford DM, Hensel M, Emonyi W, Holmes KK, Plummer F, Ndinya-Achola J, Roberts PL, Hillier S, *et al.*, Detection of HIV DNA in cervical and vaginal secretions. JAMA 1993;269:2860-4

Coll O, Hernandez M, Boucher CA, Fortuny C, de Tejada BM, Canet Y, Caragol I, Tijnagel J, Bertran JM, Espanol T., Vertical HIV-1 transmission correlates with a high maternal viral load at delivery. J Acqui Immune Def Syndr Hum Retrovirol 1997;14:26-30

Coutsoudis A, Kuhn L, Pillay K, Coovadia HM., Exclusive breast-feeding and HIV transmission. AIDS 2002;16(3):498-9.

de Martino M, Tovo PA, Tozzi AE, Pezzotti P, Galli L, Livadiotti S, Caselli D, Massironi E, Ruga E, Fioredda F, *et al.*, HIV-1 transmission through breastmilk: appraisal of risk according to duration of feeding. AIDS 1992;6:991-7

de Rossi A, Giaquinto C, Ometto L, Mammano F, Zanotto C, Dunn D, Chieco-Bianchi L., Replication and tropism of human immunodeficiency virus type 1 as predictors of disease outcome in infants with vertically acquired infection. J Pediatr 1993;123:929-36

de Rossi A, Ometto L, Masiero S, Zanchetta M, Chieco-Bianchi L., Viral phenotype in mother-to-child HIV-1 transmission and disease progression of vertically acquired HIV-1 infection. Acta Paediatr Suppl 1997;421:22-8

University of Cape Town

de Rossi A, Masiero S, Giaquinto C, Ruga E, Comar M, Giacca M, Chieco-Bianchi L., Dynamics of viral replication in infants with vertically acquired human immunodeficiency virus type 1 infection. J Clin Invest 1996;97:323-30

De Wolf F, Hogervorst E, Goudsmit J, Fenyo EM, Rubsamen-Waigmann H, Holmes H, Galvao-Castro B, Karita E, Wasi C, Sempala SD, *et al.*, Syncytium-inducing and non-syncytium-inducing capacity of human immunodeficiency virus type 1 subtypes other than B: phenotypic and genotypic characteristics. WHO Network for HIV isolation and Characterisation. AIDS Res Hum Retroviruses 1994:10; 1387-400

Delwart EL, Shpaer EG, Louwagie J, McCutchan FE, Grez M, Rubsamen-Waigmann H, Mullins JI., Genetic relationships determined by a DNA heteroduplex mobility assay: Analysis of HIV-1 *env* genes. Science 1993;262:1257-61

Dickover RE, Garratty EM, Herman SA, Sim MS, Plaeger S, Boyer PJ, Keller M, Deveikis A, Stiehm ER, Bryson YJ., Identification of levels of maternal HIV-1 RNA associated with risk of perinatal transmission: effect of maternal zidovudine treatment on viral load. JAMA 1996;275:599-605

Dickover RE, Garratty EM, Plaeger S, Bryson YJ., Perinatal Transmission of Major, Minor, and Multiple Maternal Human Immunodeficiency Virus Type 1 Variants In Utero and Intrapartum., J. Virol. 2001:Mar;2194-203.

Douglas GC, King BF., Maternal-fetal transmission of human immunodeficiency virus: a review of possible routes and cellular mechanisms of infection. Clin Infect Dis 1992;15:678-91

Dunn DT, Newell ML, Ades AE, Peckham CS., Risk of human immunodeficiency virus type 1 transmission through breastfeeding. Lancet 1992;340:585-88

Easterbrook PJ, Rostron T, Ives N, Troop M, Gazzard BG, Rowland-Jones SL., Chemokine receptor polymorphisms and Human Immunodeficiency Virus Disease Progression J Inf Dis 1999, 180:1096-105

Emini EA, Schleif WA, Nunberg JH, Conley AJ, Eda Y, Tokiyoshi S, Putney SD, Matsushita S, Cobb KE, Jett CM, *et al.*, Prevention of HIV-1 infection in

University of Cape Town

chimpanzees by gp120 V3 domain-specific monoclonal antibody. Nature 1992;355:728-30

European Collaborative Study, Children born to women with HIV-1 infection: natural history and risk of transmission. Lancet 1991;337:253-60

European Collaborative Study, Risk factors for mother-to-child transmission of HIV. Lancet 1992;339:1007-12

European Collaborative Study, Caesarean section and risk of vertical transmission of HIV-1 infection. Lancet 1994;343:1464-7

Fang G, Burger H, Grimson R, Tropper P, Nachman S, Mayers D, Weislow O, Moore R, Reyelt C, Hutcheon N, *et al.*, Maternal plasma human immunodeficiency virus type 1 RNA level: a determinant and projected threshold for mother-to-child transmission. Proc Natl Acad Sci USA 1995;92:12100-4

Ferre F, Marchese A, Duffy PC, Lewis DE, Wallace MR, Beecham HJ, Burnett KG, Jensen FC, Carlo DJ., Quantitation of HIV viral burden by PCR in HIV seropositive navy personnel representing Walter Reed staging 1 to 6. AIDS Res Hum Retroviruses 1992;8:269-75

Fiore JR, Jansson M, Scarlatti G, Angarano G, Caputo SL, Buccoliero G, Rossi P, Fenyo EM, Pastore G., Correlation between seroreactivity to HIV-1 V3 loop peptides and male-to-female heterosexual transmission. AIDS 1993;7:29-31

Ganeshan S, Dickover RE, Korber BT, Bryson YJ, Wolinsky SM., Human immunodeficiency virus type 1 genetic evolution in children with different rates of development of disease. J Virol 1997;71:663-7

Gaschen B, Taylor J, Yusim K, Foley B, Gao F, Lang D, Novitsky V, Haynes B, Hahn BH, Bhattacharya T, Korber B., Diversity considerations in HIV-1 vaccine selection., Science 2002:296; 28 June

Goedert JJ, Duliege AM, Amos CI, Felton S, Biggar RJ., High risk of HIV-1 infection for first-born twins. Lancet 1991;338:1471-5

Goedert JJ, Mendez H, Drummond JE, Robert-Guroff M, Minkoff HL, Holman S, Stevens R, Rubinstein A, Blattner WA, Willoughby A, *et al.*, Mother-to-infant transmission of human immunodeficiency virus type 1: association with prematurity or low anti-gp120. Lancet 1989;2:1351-4

University of Cape Town

Gordon M, De Oliveira T, Bishop K, Coovadia HM, Madurai L, Engelbrecht S, Janse van Rensburg E, Mosam A, Smith A, Cassol S., Molecular Characteristics of Human Immunodeficiency Virus Type 1 Subtype C Viruses from KwaZulu-Natal, South Africa: Implications for Vaccine and Antiretroviral Control Strategies., J Virol 2003; 77(4):2587-99

Goulder PJ, Sewell AK, Lalloo DG, Price DA, Whelan JA, Evans J, Taylor GP, Luzzi G, Giangrande P, Phillips RE, McMichael AJ., Patterns of immunodominance in HIV-1 specific cytotoxic T lymphocyte responses in two human histocampatibility leukocyte antigens (HLA) identical siblings with HLA-A*0201 are influenced by epitope mutation. J Exp Med 1997;185(8):1423-33

Goulder PJ, Brander C, Tang Y, Tremblay C, Colbert RA, Addo MM, Rosenberg ES, Nguyen T, Allen R, Trocha A, Altfeld M, He S, Bunce M, Funkhouser R, Pelton SI, Burchett SK, McIntosh K, Korber BT, Walker BD., Evolution and transmission of stable CTL escape mutations in HIV infection. Nature 2001;412 Jul 19:334-38

Gray G, *et al.*, The effect of breastfeeding on vertical transmission of HIV-1 in Soweto, South Africa. XI International Conference on AIDS, 1996 [Th.C.415]

Guevara H, Casseb J, Zijenah LS, Mbizvo M, Oceguera LF 3rd, Hanson CV, Katzenstein DA, Hendry RM., Maternal HIV-1 antibody and vertical transmission in subtype C virus infection., J Acquir Immune Defic Syndr, 2002; Apr 15: 29(5); 435-40

Halapi E, Leitner T, Jansson M, Scarlatti G, Orlandi P, Plebani A, Romiti L, Albert J, Wigzell H, Rossi P., Correlation between HIV sequence evolution, specific immune response and clinical outcome in vertically infected infants. AIDS 1997;11:1709-17

Henin Y, Mandelbrot L, Henrion R, Pradinaud R, Coulaud JP, Montagnier L., HIV in the cervicovaginal secretions of pregnant and non-pregnant women. J Acquir Imun Defic Syndr 1993;6:72-5

Ho WZ, Lioy J, Song L, Cutilli JR, Polin RA, Douglas SD., Infection of cord blood monocyte-derived macrophages with human immunodeficiency virus type 1. J Virol 1992;66:573-579

University of Cape Town

Hoffman NG, Seillier-Moiseiwitsch F, Ahn J, Walker JM, Swanstrom R., Variability in the human immunodeficiency virus type 1 gp120 Env protein linked to phenotype-associated changes in the V3 loop. J Virol 2002;76:3852-64

Hofmann-Lehmann R, Vlasak J, Rasmussen RA, Smith BA, Baba TW, Liska V, Ferrantelli F, Montefiori DC, McClure HM, Anderson DC, Bernacky BJ, Rizvi TA, Schmidt R, Hill LR, Keeling ME, Katinger H, Stiegler G, Cavacini LA, Posner MR, Chou TC, Andersen J, Ruprecht RM., Postnatal Passive Immnization of Neonatal Macaques with a triple Combination of Human Monoclonal Antibodies against Oral Simian-Human Immunodeficiency Virus Challenge. J Virol 2001: 75(16):7470-80.

Huang Y, Paxton WA, Wolinsky SM, Neumann AU, Zhang L, He T, Kang S, Ceradini D, Jin Z, Yazdanbakhsh K, Kunstman K, Erickson D, Dragon E, Landau NR, Phair J, Ho DD, Koup RA., The role of a mutant CCR5 allele in HIV-1 transmission and disease progression. 1996 Nat Med 2:1240-3

Husson RN, Lan Y, Kojima E, Venzon D, Mitsuya H, McIntosh K., Vertical transmission of human immunodeficiency virus type 1: autologous neutralizing antibody, virus load, and virus phenotype., 1995, J Pediatr., Jun;126(6):865-71.

Italian register for HIV infection in children, Features of children perinatally infected with HIV-1 surviving longer than 5 years. Lancet 1994a;343:191-5

Italian register for HIV infection in children, Human immunodeficiency virus type 1 infection and breastmilk. Acta Paediatr 1994b (suppl 400):S51-S58

Jones DS, Byers RH, Bush TJ, Oxtoby MJ, Rogers MF., Epidemiology of transfusion associated acquired immunodeficiency syndrome in children in the United States. Pediatrics 1992;89:123-7

Kawasaki E, Sample preparation from blood, cells and other fluids. In PCR protocols: A guide to methods and applications. Edited by Innis M, *et al.*, Berkeley: Academic Press, Inc 1990:148

Kenealy WR, Matthews TJ, Ganfield MC, Langlois AJ, Waselefsky DM, Petteway SR Jr., Antibodies from human immunodeficiency virus-infected individuals bind to a short amino acid sequence that elicits neutralizing antibodies in animals. AIDS Res Hum Retroviruses 1989;5:173-81

University of Cape Town

Kesson AM, Fear WR, Williams L, Chang J, King NJ, Cunningham AL., HIV infection of placental macrophages: their potential role in vertical transmission. J Leukoc Biol 1994;56:241-6

Kliks SC, Wara DW, Landers DV, Levy JA., Features of HIV-1 that could influence mother-to-child transmission. JAMA 1994;272:467-74

Kostrikis LG, Huang Y, Moore JP, Wolinsky SM, Zhang L, Guo Y, Deutsch L, Phair J, Neumann AU, Ho DD., A chemokine receptor CCR2 allele delays HIV-1 disease progression and is associated with a CCR5 promoter mutation. Nat Med 4:350-3

Kostrikis LG, Neumann AU, Thomson B, Korber BT, McHardy P, Karanicolas R, Deutsch L, Huang Y, Lew JF, McIntosh K, Pollack H, Borkowsky W, Spiegel HM, Palumbo P, Oleske J, Bardeguez A, Luzuriaga K, Sullivan J, Wolinsky SM, Koup RA, Ho DD, Moore JP., A polymorphism in the regulatory region of the CC-chemokine receptor 5 gene influences perinatal transmission of human immunodeficiency virus type 1 to African-American Infants. J Virol 1999;73(12);10264-10271.

Ometto L, Zanotto C, Maccabruni A, Caselli D, Truscia D, Giaquinto C, Ruga E, Chieco-Bianchi L, De Rossi A., Viral phenotype and host-cell susceptibility to HIV-1 infection as risk factors for mother to child HIV-1 transmission. AIDS 1995;9:427-34

Ou CY, Kwok S, Mitchell SW, Mack DH, Sninsky JJ, Krebs JW, Feorino P, Warfield D, Schochetman G., DNA amplification for direct detection of HIV-1 in DNA of peripheral blood mononuclear cells. Science 1988;239:295-7

Lambert G, Thea DM, Pliner V, Steketee RW, Abrams EJ, Matheson P, Thomas PA, Greenberg B, Brown TM, Bamji M, Kalish ML., Effect of maternal CD4+ cell count, acquired immunodeficiency syndrome and viral load on disease progression in infants with perinatally acquired human immunodeficiency virus type 1 infection. J Pediatr 1997;130:890-7

Lamers SL, Sleasman JW, She JX, Barrie KA, Pomeroy SM, Barrett DJ, Goodenow MM., Independent variation and positive selection in *env* V1 and V2 domains

University of Cape Town

within maternal-infant strains of human immunodeficiency virus type 1 *in vivo*. J Virol 1993;7:3951-60

Lamers SL, Sleasman JW, She JX, Barrie KA, Pomeroy SM, Barrett DJ, Goodenow MM., Persistence of multiple maternal genotypes of human immunodeficiency virus type 1 in infants infected by vertical transmission. J Clin Invest 1994;93:380-90

Lewis P, Nduati R, Kreiss JK, John GC, Richardson BA, Mbori-Ngacha D, Ndinya-Achola J, Overbaugh J., Cell-free human immunodeficiency virus type 1 in breast milk. J Infect Dis 1998 Jan;177(1):34-9

Lewis SH, Reynolds-Kohler C, Fox HE, Nelson JA., HIV-1 in trophoblastic and villous Hofbauer cells and haematological precursors in 8-week fetuses. Lancet 1990;335:565-8

Li, Y., M.-A. Rey-Cuille, and S.-L. Hu.. N-linked glycosylation in the V3 region of HIV type 1 surface antigen modulates coreceptor usage in viral infection. AIDS Res. Hum. Retrovir. 2001;17:1473-1479

Lin HJ, Myers LE, Yen-Lieberman B, Hollinger FB, Henrard D, Hooper CJ, Kokka R, Kwok S, Rasheed S, Vahey M, *et al.*, Multicenter evaluation of quantification methods for plasma human immunodeficiency virus type 1 RNA. J Infect Dis 1994;170:553-62

Lukashov, V. V., Kuiken C. L., Goudsmit J., Intrahost human immunodeficiency virus type 1 evolution is related to length of the immunocompetent period. J. Virol. 1995 69:6911–6916.

Lyons S, *et al.*, Mother-to-infant transmission of HIV-1 in South Africa. XI International Conference on AIDS, 1996 [Tu.C.2579]

Martin MP, Dean M, Smith MW, Winkler C, Gerrard B, Michael NL, Lee B, Doms RW, Margolick J, Buchbinder S, Goedert JJ, O'Brien TR, Hilgartner MW, Vlahov D, O'Brien SJ, Carrington M., Genetic acceleration of AIDS progression by a promoter variant of CCR5 Science 1998, 282: 1907-11

McDermott DH, Zimmerman PA, Guignard F, Kleeberger CA, Leitman SF, Murphy PM., CCR5 promoter polymorphism and HIV-1 disease progression. Lancet 1998, 352:866-70

University of Cape Town

Masuda T, Matsushita S, Kuroda MJ, Kannagi M, Takatsuki K, Harada S., Generation of neutralization-resistant HIV-1 *in vitro* due to amino acid interchanges of third hypervariable *env* region. J Immunol 1990;148:3240-6

Mascola JR, Stiegler G, VanCott TC, Katinger H, Carpenter CB, Hanson CE, Beary H, Hayes D, Frankel SS, Birx DL, Lewis MG., Protection of Macaques against Pathogenic Simain/Human Immunodeficiency Virus 89.6PD by Passive Transfer of Neutralizing Antibodoes., J Virol 1999;73(5):4009-18

Mayaux MJ, Blanche S, Rouzioux C, Le Chenadec J, Chambrin V, Firtion G, Allemon MC, Vilmer E, Vigneron NC, Tricoire J, *et al.*, Maternal factors associated with perinatal HIV-1 transmission: The French cohort study: 7 years of follow-up observation. J Acquir Immun Defic Syndr Hum Retrovirol 1995;8:188-94

Mayaux MJ, Burgard M, Teglas JP, Cottalorda J, Krivine A, Simon F, Puel J, Tamalet C, Dormont D, Masquelier B, Doussin A, Rouzioux C, Blanche S., Neonatal characteristics in rapidly progressive perinatally acquired HIV-1 disease. JAMA 1996;275:606-10

McKeating JA, Gow J, Goudsmit J, Pearl LH, Mulder C, Weiss RA., Characterization of HIV-1 neutralization escape mutants. AIDS 1989;3:777-84

Menez-Bautista R, Fikrig SM, Pahwa S, Sarangadharan MG, Stoneburner RL., Monozygotic twins discordant for the acquired immunodeficiency syndrome. Am J Dis Child 1986;140:678-9

Misrahi M, Teglas JP, N'Go N, Burgard M, Mayaux MJ, Rouzioux C, Delfraissy JF, Blanche S., CCR5 chemokine receptor variant in HIV-1 mother-to-child transmission and disease progression in children. French Paediatric HIV infection study Group JAMA 279:277-80

Morris L, Cilliers T, Bredell H, Phoswa M, Martin DJ., CCR5 is the major coreceptor used by HIV-1 subtype C isolates from patients with active tuberculosis. AIDS Res Hum Retroviruses. 2001 May 20;17(8):697-701.

Mulder-Kampinga GA, Kuiken C, Dekker J, Scherpbier HJ, Boer K, Goudsmit J., Genomic human immunodeficiency virus type 1 RNA variation in mother and child following intra-uterine virus transmission. J Gen Virol 1993;74:1747-56

University of Cape Town

Mulder-Kampinga GA, Simonon A, Kuiken CL, Dekker J, Scherpbier HJ, van de Perre P, Boer K, Goudsmit J., Similarity in *env* and *gag* genes between genomic RNAs of human immunodeficiency virus type 1 (HIV-1) from mother and infant is unrelated to time of HIV-1 RNA positivity in the child. J Virol 1995;4:2285-96

Mundy DC, Schinazi RF, Gerber AR, Nahmias AJ, Randall HW Jr., Human immunodeficiency virus isolated from amniotic fluid. Lancet 1987;2;459-60

Nakayama EE, Shioda T, Tatsumi M, *et al.*, Importance of the N-glycan in the V3 loop of HIV-1 envelope protein for CXCR-4 but not CCR-5-dependent fusion. FEBS Lett. 1998;426:367-372

Nielsen K, Boyer P, Dillon M, Wafer D, Wei LS, Garratty E, Dickover RE, Bryson YJ., Presence of human immunodeficiency virus (HIV) type 1 and HIV-1 specific antibodies in cervicovaginal secretions of infected mothers and in the gastric aspirates of their infants. J Infect Dis 1996;173:1001-4

Oleske J, Minnefor A, Cooper R Jr, Thomas K, dela Cruz A, Ahdieh H, Guerrero I, Joshi VV, Desposito F., Immune deficiency syndrome in children. JAMA 1983;249:2345-9

Overbaugh J, Anderson RJ, Ndinya-Achola JO, Kreiss JK., Distinct but related human immunodeficiency virus type 1 variant populations in genital secretions and blood. AIDS Res Hum Retroviruses 1996;12;107-15

Page M, Vella C, Corcoran T, Dilger P, Ling C, Heath A, Thorpe R., Restriction of serum antibody reactivity to the V3 neutralizing domain of HIV gp120 with progression to AIDS. AIDS 1992;6:441-6

Page R, TreeView for Windows Version 1.2, Division of Environmental and evolutionary biology. IBLS, University of Glasgow 1996

Palker TJ, Clark ME, Langlois AJ, Matthews TJ, Weinhold KJ, Randall RR, Bolognesi DP, Haynes BF., Type-specific neutralization of the human immunodeficiency virus with antibodies to *env*-coded synthetic peptides. Proc Natl Acad Sci USA 1988;85:1932-6

Papathanasopoulos MA, Cilliers T, Morris L, Mokili JL, Dowling W, Birx DL, McCutchan FE., Full-length genome analysis of HIV-1 Subtype C utilizing

University of Cape Town

CXCR4 and intersubtype recombinants isolated in South Africa., AIDS Res Hum Retroviruses 2002:18(12); 879-886

Park CL, Streicher H, Rothberg R., Transmission of human immunodeficiency virus from parents to only one dizygotic twin. J Clin Microbiol 1987;25:1119-21

Pasquier C, Cayrou C, Blancher A, Tourne-Petheil C, Berrebi A, Tricoire J, Puel J, Izopet J., Molecular evidence for mother-to-child transmission of multiple variants by analysis of RNA and DNA sequences of human immunodeficiency virus type 1., J Virol., 1998.,Nov:8493-8501.

Peckham C, Gibb D., Mother-to-child transmission of the human immunodeficiency virus, N Engl J Med 1995, 333;298-302

Ping LH, Nelson JA, Hoffman IF, Schock J, Lamers SL, Goodman M, Vernazza P, Kazembe P, Maida M, Zimba D, Goodenow MM, Eron JJ Jr, Fiscus SA, Cohen MS, Swanstrom R., Characterization of V3 Sequence Heterogeneity in Subtype C Human Immunodeficiency Virus Type 1 Isolates from Malawi: Underrepresentation of X4 Variants., J Virol 1999 Aug;73(8):6271-81

Quillent C, Oberlin E, Braun J, Rousset D, Gonzalez-Canali G, Metais P, Montagnier L, Virelizier JL, Arenzana-Seisdedos F, Beretta A., HIV-1 resistance phenotype conferred by a combination of two separate inherited mutations of the CCR5 gene Lancet 1998, 351:14-8

Roques P, Marce D, Courpotin C, Mathieu FP, Herve F, Boussin FD, Narwa R, Meyohas MC, Dollfus C, Dormont D., Correlation between HIV provirus burden and in utero transmission., AIDS. 1993 Nov;7 Suppl 2:S39-43

Roth WW, Zuberi JA, Stringer HG Jr, Davidson SK, Bond VC., Examination of HIV type 1 Variants in mother-child pairs. AIDS Res Hum Retroviruses 1996;10:925-30

Rouzioux C, Costagliola D, Burgard M, Blanche S, Mayaux MJ, Griscelli C, Valleron AJ., Estimated Timing of mother-to-child human immunodeficiency virus type 1 transmission by use of a Markov model. Am J Epi 1995;12:1330-7

Rubinstein A, Sicklick M, Gupta A, Bernstein L, Klein N, Rubinstein E, Spigland I, Fruchter L, Litman N, Lee H, Hollander M., Acquired immunodeficiency with

University of Cape Town

reverse T4/T8 ratios in infants born to promiscuous and drug addicted mothers. JAMA 1983;249:2350-6

Ruff AJ, Coberly J, Halsey NA, Boulos R, Desormeaux J, Burnley A, Joseph DJ, McBrien M, Quinn T, Losikoff P, *et al.*, Prevalence of HIV-1 DNA and p24 antigen in breast milk and correlation with maternal factors. J Acqui Immune Defic Syn 1994;7:68-73

Rusche JR, Javaherian K, McDanal C, Petro J, Lynn DL, Grimaila R, Langlois A, Gallo RC, Arthur LO, Fischinger PJ, *et al.*, Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope gp120. Proc Natl Acad Sci USA 1988;85:3198-202

Salvatori F, Scarlatti G., HIV type 1 Chemokine Receptor Usage in Mother-to-child Transmission., AIDS Res Hum Retro 2001., 17;10:925-35

Samson M, Libert F, Doranz BJ, Rucker J, Liesnard C, Farber CM, Saragosti S, Lapoumeroulie C, Cognaux J, Forceille C, Muyldermans G, Verhofstede C, Burtonboy G, Georges M, Imai T, Rana S, Yi Y, Smyth RJ, Collman RG, Doms RW, Vassart G, Parmentier M., Resistance to HIV-1 infection in Caucasion Individuals bearing mutant alleles of the CCR-5 chemokine receptor gene 1996 Nature 382:722-5

Sanger F, Nicklen S, Coulson AR., DNA sequencing with chain-terminating inhibitors., Proc Natl Acad Sci USA 1977;74:5463

Scarlatti G, Leitner T, Halapi E, Wahlberg J, Jansson M, Wigzell H, Fenyo EM, Albert J, Uhlen M, Rossi P., Analysis of the HIV-1 envelope V3-loop sequences from ten mother-child pairs. Anna NY Acad Sci 1992;10:277-80

Scarlatti G, Leitner T, Halapi E, Wahlberg J, Marchisio P, Clerici-Schoeller MA, Wigzell H, Fenyo EM, Albert J, Uhlen M, *et al.*, Comparison of variable region 3 sequences of human immunodeficiency virus type 1 from infected children with the RNA and DNA sequences of the virus populations of their mothers. Proc Natl Acad Sci USA 1993:90;1721-5

Scarlatti G, Albert J, Rossi P, Hodara V, Biraghi P, Muggiasca L, Fenyo EM., Mother-to-child transmission of human immunodeficiency virus type 1:

University of Cape Town

correlation with neutralizing antibodies against primary isolates. J Infect Dis 1993;168:207-10

Scarlatti G, Hodara V, Rossi P, Muggiasca L, Bucceri A, Albert J, Fenyo EM., Transmission of human immunodeficiency virus type 1 (HIV-1) from mother to child correlates with viral phenotype. Virology 1993;197:624-9

Shankarappa R, Margolick JB, Gange SJ, Rodrigo AG, Upchurch D, Farzadegan H, Gupta P, Rinaldo CR, Learn GH, He X, Huang XL, Mullins JI., Consistent viral evolutionary changes associated with the progression of Human Immunodeficiency Virus Type 1 Infection. J Virol 1999;73(12):10489-502

Shearer WT, Quinn TC, LaRussa P, Lew JF, Mofenson L, Almy S, Rich K, Handelsman E, Diaz C, Pagano M, Smeriglio V, Kalish LA., Viral load and disease progression in infants infected with human immunodeficiency virus type 1. J Med 1997;336:1337-42

Shimizu N, Haraguchi Y, Takeuchi Y, Soda Y, Kanbe K, Hoshino H., Changes in and discrepancies between cell tropisms and coreceptor uses of human immunodeficiency virus type 1 induced by single point mutations at the V3 tip of the env protein. Virology 1999:259;324-33

Simonon A, Lepage P, Karita E, Hitimana DG, Dabis F, Msellati P, Van Goethem C, Nsengumuremyi F, Bazubagira A, Van de Perre P., An assessment of the timing of mother-to-child transmission of human immunodeficiency virus type 1 by means of polymerase chain reaction. J Acquir Immune Defic Syndr 1994;7:952-7

Simonon A, Mulder-Kampinga GA, van de Perre P, Karita E, Msellati P, Kuiken C, Goudsmit J., Evolution of human immunodeficiency virus subtype A in women seroconverting post partum and in their offspring post-natally infected by ingestion of breast milk. J Gen Virol 1997;78:2225-33

Soeiro R, Rubinstein A, Rashbaum WK, Lyman WD., Maternofetal transmission of AIDS: frequency of human immunodeficiency virus type 1 nucleic acid sequences in human fetal DNA. J Inf Dis 1992;166:699-703

University of Cape Town

South African Dept. of Health., National HIV sero-prevalence survey of women attending public antenatal clinics in South Africa., 2001., www.gov.za/reports/2002/hivsurvey01.pdf

Sperduto AR, Bryson YJ, Chen IS., Increased susceptibility of neonatal monocyte/macrophages to HIV-1 infection. AIDS Res Hum Retrovir 1993;9:1277-85

Sprecher S, Soumenkoff G, Puissant F, Degueldre M., Vertical transmission of HIV in a 15-week fetus. Lancet 1986;2:288

St Louis ME, Pau CP, Nsuami M, Ou CY, Matela B, Kashamuka M, Brown C, George JR, Heyward WL., Lack of association between anti-V3 loop antibody and perinatal HIV-1 transmission in Kinshasa, Zaire, despite use of assays based on local HIV-1 strains. J Acqui Immun Defic Syndr 1994;7:63-7

St Louis ME, Kamenga M, Brown C, Nelson AM, Manzila T, Batter V, Behets F, Kabagabo U, Ryder RW, Oxtoby M, *et al.*, Risk for perinatal HIV-1 transmission according to mataernal immunologic, virologic and placental factors. JAMA 1993;269:2853-9

Sutthent R, Foongladda S, Chearskul S, Wanprapa N, Likanonskul S, Kositanont U, Riengrojpitak S, Sahaphong S, Wasi C., V3 sequence diversity of HIV-1 subtype E in infected mothers and their infants. J Acquir Immune Def Syndr Hum Retrovirol 1998;18:323-31

Takahashi H, Nakagawa Y, Pendleton CD, Houghten RA, Yokomuro K, Germain RN, Berzofsky JA., 1992., Induction of broadly cross reactive cytotoxic T-cells recognizing an HIV-1 envelope determinant. Science 1992:255;333-336

The working group on mother-to-child transmission of HIV., Rates of mother-to-child transmission of HIV-1 in Africa, America and Europe: Results from 13 perinatal studies. J Acquir Immune Defic Syndr Hum Retrovirol 1995;5:506-10

Thiry L, Sprecher-Goldberger S, Jonckheer T, Levy J, Van de Perre P, Henrivaux P, Cogniaux-LeClerc J, Clumeck N., Isolation of AIDS virus from cell-free breast milk of three healthy virus carriers. Lancet 1985;2:891

University of Cape Town

Thompson JD, Gibson TJ, Plewniak F, Jeanmougin F, Higgins DG., The CLUSTAL-X Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res 1997;25:4876-82

Tien PC, Chiu T, Latif A, Ray S, Batra M, Contag CH, Zejena L, Mbizvo M, Delwart EL, Mullins JI, Katzenstein DA., Primary subtype C HIV-1 infection in Harare, Zimbabwe. J Aquir Immune Defic Syndr Hum Retrovirol 1999;20;147-53

Tovo PA, de Martino M, Gabiano C, Galli L, Cappello N, Ruga E, Tulisso S, Vierucci A, Loy A, Zuccotti GV, *et al.*, Mode of delivery and gestational age influence perinatal HIV-1 transmission. J Acquir Immun Defic Syndr Hum Retrovirol 1996;11:88-94

UNAIDS organisation., 2002., http://www.unaids.org/barcelona/presskit/barcelona%20report/contents_html.html

Upchurch DA, Shankarappa R, Mullins JI., Position and degree of mismatches and the mobility of DNA heteroduplexes., Nuc Acids Res 2000;28(12):e69-74

Van de Peer Y, De Wachter R., TREECON for Windows: A software package for the construction and drawing of evolutionary trees for the Microsoft Windows environment. Comput Applic Biosci 1994;10:569-70

Van de Perre P, Simonon A, Msellati P, Hitimana DG, Vaira D, Bazubagira A, Van Goethem C, Stevens AM, Karita E, Sondag-Thull D, *et al.*, Postnatal transmission of human immunodeficiency virus type 1 from mother to infant. A prospective cohort study in Kigali, Rwanda. N Engl J Med 1991 Aug 29;325(9):593-8

van Harmelen J, Williamson C, Kim B, Morris L, Carr J, Karim SS, McCutchan F., Characterization of full-length HIV type 1 subtype C sequences from South Africa., AIDS Res Hum Retroviruses 2001:17(16);1527-31

Wade CM, Lobidel D, Brown AJ., Analysis of human immunodeficiency virus type 1 env and gag sequence variants derived from a mother and two vertically infected children provides evidence for the transmission of multiple sequence variants., J Gen Virol., 1998., 79:1055-68

University of Cape Town

Wei X, Decker JM, Wang S, Hui H, Kappes JC, Wu X, Salazar-Gonzalez JF, Salazar MG, Kilby JM, Saag MS, Komarova NL, Nowak MA, Hahn BH, Kwong PD, Antibody neutralization and escape by HIV-1. Nature. 2003 Mar 20;422(6929):307-12.

Weiser B, Nachman S, Tropper P, Viscosi KH, Grimson R, Baxter G, Fang G, Reyelt C, Hutcheon N, Burger H., Quantitation of human immunodeficiency virus type 1 during pregnancy: Relationship of viral titer to mother-to-child transmission and stability of viral load. Proc Natl Acad Sci USA 1994;91:8037-41

Wolinsky SM, Wike CM, Korber BT, Hutto C, Parks WP, Rosenblum LL, Kunstman KJ, Furtado MR, Munoz JL., Selective Transmission of human immunodeficiency virus type-1 variants from mothers to infants. Science 1992;255;1134-7

Young KK, Nelson RP, Good RA., Discordant human immunodeficiency virus infection in dizygotic twins twins detected by polymerase chain reaction. Pediatr Infect Dis J 1990;9:454-6

Zaknun D, Orav J, Kornegay J, al-Attar I, Fuchs D, Zaknun J, Wachter H, Chatis P, Burchett SK, McIntosh K., Correlation of ribonucleic acid polymerase chain reaction, acid dissociated p24 antigen and neopterin with progression of disease. J Pediatr 1997;130:898-905

Ziegler JB, Cooper DA, Johnson RO, Gold J., Postnatal transmission of AIDS-associated retrovirus from mother to infant. Lancet 1985;1:896-8

University of Cape Town