THE DEVELOPMENT OF AN EARLY DETECTION METHOD FOR HIV INFECTION IN INFANTS

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FELICIA MOTSILISI BOPANE MAINO

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Supervisor: Prof. GHJ Pretorius (PhD Biochem)
Co- supervisor: Prof. WMJ van den Heever-Kriek (PhD)

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DECLARATION

I, **Felicia Motsilisi Bopane Maino**, identity number [redacted] and student number 20327307, do hereby declare that the research work submitted to the Central University of Technology, Free State, for the degree **Magister Technologiae: Biomedical Technology,** is an original and independent work.

This work has not been submitted to any institution by myself or, to the best of my knowledge, any other person in fulfillment of requirements for the attainment of any qualification.

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Signature of Student Date

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SUMMARY

Early detection of mother-to-child transfer of Human Immunodeficiency Virus (HIV-1) is of the utmost importance for monitoring the success of intervention strategies, as well as for optimal treatment of HIV-positive children. Serology can only be used confidently after 18 months, as remaining antibodies from the mother may give false positive results. This leaves only molecular methods for early detection of the virus; unfortunately, the technology is still too expensive for general use.

The aim of this project was to develop and validate a cost-effective, fast, early detection method for HIV infection in infants. PCR was chosen as the developmental method, a technique that amplifies proviral sequences of HIV DNA, detecting HIV infection in peripheral blood mononuclear cells (PBMC) from infants of seropositive women during neonatal (age less than 28 days) and post-neonatal periods.

A method based on the commercial Roche HIV-1 DNA assay was chosen for implementation on the Roche LightCycler instrument. The published primer set was used to detect both HIV-1 DNA and an internal control. The target DNA for use as internal control was constructed from the plasmid pBR322 so that an AT-rich part of the plasmid was flanked by the HIV-1 primer-binding sites. The resulting amplicon was cloned into a vector and multiplied in *E. coli.* Amplification of the plasmid by PCR in the Roche LightCycler in the presence of SYBR Green created an amplicon having a Tm different (81 ± 1°C) from that of the HIV-1 amplicon (84 ± 1°C) so that post-amplification melting can be used to differentiate between HIV-1 and internal control.

After construction of the internal control, the reaction conditions were optimised so that the internal control would amplify strongly only in the absence of HIV-1 target DNA. Then 50 previously tested patient samples were analysed using the assay developed here. Only half of the known positive samples came up positive in the assay, indicating that it is not sensitive enough for diagnostic use in its current form. Various ways of improving the sensitivity are suggested for further development of the assay as described here.

OPSOMMING

Vroeë opsporing van moeder-na-kind-oordrag van Menslike Immuunge-brekvirus (MIV-1) is belangrik vir die monitering van intervensieprogramme asook vir optimale behandeling van MIV-1-positiewe kinders. Serologiese diagnose kan eers ná 18 maande vertrou word, aangesien die kind tot op daardie stadium nog moederlike antiliggaampies in die bloedstroom kan hê. Dit laat slegs molekulêre metodes oor vir vroeë diagnose, maar ongelukkig is die tegnologie nog steeds te duur vir algemene gebruik.

Die doel van die projek was die ontwikkeling en validasie van ’n koste-effektiewe en vinnige metode vir die vroeë diagnose van moeder-na-kind-oordrag van MIV-1. Polimerase-kettingreaksie-(PKR) amplifikasie van provirale MIV-1-DNS in perifere witselkerne is die voorkeurmetode van diagnose in neonate (<28 dae) en post-neonate.

Die algemeen gebruikte kommersiële kwalitatiewe MIV-1-DNS-metode van Roche is as basis gebruik vir die ontwikkeling van die metode wat hier beskryf word. Die gepubliseerde inleierstel soos deur Roche gebruik, is gebruik om beide MIV-1-DNS en ’n interne kontrole te teiken. As interne kontrole is ’n deel van die plasmied pBR322 gekies sodat dit ’n laer AT-inhoud as die MIV-1-teiken het. Deur middel van amplifikasie met hibried-inleiers is ’n DNS-molekuul geskep wat die interne kontrole intern het, maar aan weerskante bindingsgebiede het vir die MIV-1 inleiers. Die nuwe konstruk is in ’n vektor gekloon en in *E. coli* vermeerder. Amplifikasie van hierdie teiken in die Roche LightCycler in die teenwoordigheid van SYBR Green het ’n amplikon opgelewer met ’n smeltpunt van 81 ± 1°C , wat onderskeibaar is van dié van MIV-1-DNS (84 ± 1°C).

Ná konstruksie van die interne kontrole is die reaksiekondisies geoptimiseer sodat dit slegs sterk amplifiseer in die afwesigheid van MIV-1-DNS. Hierna is 50 monsters wat voorheen getoets is, getoets volgens die metode hierbo beskryf. Slegs die helfte van die bekende positiewe monsters het positief opgekom, wat beteken dat die metode in sy huidige vorm nie sensitief genoeg is vir diagnostiese gebruik nie. Verskeie voorstelle vir verbetering van die sensitiwiteit van die metode word aan die hand gedoen.

ABBREVIATIONS AND SYMBOLS

SYMBOLS AND MEASUREMENTS

℃	=	Degrees Celsius
%	=	Percentage
(-) c	=	Negative control
(+) c	=	Positive control
kg	=	kilogram
µl	=	microlitre
µg	=	microgram
µM	=	micromolar
mg	=	milligram
ng	=	nanogram
nm	=	nanometre
Tm	=	Melting point of amplicon
xg	=	Times gravity

A

A	=	Adenine
AIDS	=	Acquired Immune Deficiency Syndrome
ACTG	=	AIDS Clinical Trials Group
ALRI	=	Acute Lower Respiratory Infection
ARF	=	Acute Respiratory Failure

B

bp	=	Base pair

C

CA	=	Capsid protein
CI	=	Confidence Interval
CCR5	=	CC chemokine receptor 5
CD	=	Cluster of Differentiation
CNS	=	Central nervous system

Cp	=	Crossing point
CXCR4	=	CXC chemokine receptor 4

D

DNA	=	Deoxyribonucleic acid
DNS	=	Deoksieribonukleion suur
dNTPs	=	Deoxynucleoside triphosphates

E

EDTA	=	Ethylenediaminetetraacetic acid
e.g.	=	Example
env	=	Envelope glycoprotein
ELISA	=	Enzyme linked immunosorbent assay
EtOH	=	Ethanol

G

gag	=	Group antigen gene
gp	=	glycoprotein

H

H_2O	=	Water
HAART	=	Highly active antiretroviral therapy
HCl	=	Hydrochloric acid
HIV-1	=	Human Immune Deficiency Virus, type I
HIV-2	=	Human Immune Deficiency Virus, type II
HAART	=	Highly active antiretroviral therapy

I

IN	=	Integrase

K

kb	=	Kilobase pair

kD	=	Kilodalton

L

LTR	=	Long terminal repeat

M

M	=	Majority
MA	=	Matrix protein
$MgCl_2$	=	Magnesium Chloride
mg	=	microgram
MHC	=	Major histocompatibility complex
min	=	minute
MIV	=	Menslike Immuunge-brekvirus
ml	=	millilitre
mm	=	millimetre
mM	=	millimolar
mRNA	=	mitochondrial ribonucleic acid
MTCT	=	Mother to child transmission

N

N	=	non-M/non-O
NaAc	=	Sodium acetate
Nacl	=	Sodium chloride
NaOH	=	Sodium hydroxide
nef	=	negative regulatory factor
NC	=	Nucleocapsid protein

O

O	=	Outliers

P

PBMC	=	Peripheral blood mononuclear cell
pol	=	Poly-protein gene
PR	=	Protease
PCR	=	Polymerase chain reaction
PKR	=	Polimerase-kettingreaksie

R

rev	=	Regulator of viral expression
RNA	=	Ribonucleic acid
RNase	=	Ribonuclease
rpm	=	Revolutions per minute
RT	=	Reverse transcriptase
RT-PCR	=	Real-time polymerase chain reaction

S

SDS	=	Sodium dodecyl sulphate
sec	=	second
SIV	=	Simian immunodeficiency virus
SU	=	Surface envelope protein

T

T	=	Thymine
Ta	=	Annealing temperature
tat	=	Trans-activator of transcription
TATA	=	TATA box binding protein
TBE	=	Tris-Borate-EDTA
TE buffer	=	Tris-EDTA buffer
TM	=	Transmembrane
Tris	=	2 amino-2 (hydroxymethyl)-1,3-propanediol

U

U1	=	Unique region of the 1' LTR
U3	=	Unique region of the 3' LTR
U5	=	Unique region of the 5' LTR
UV	=	Ultraviolet

V

vif	=	Virion infectivity factor
vpr	=	Viral protein R
vpu	=	Viral protein U

W

WHO	=	World health organisation

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CHAPTER 1

INTRODUCTION

In Africa, especially Eastern and Southern Africa, which are most severely affected by the HIV/AIDS pandemic, the transmission of HIV from mother to child (MTCT) during pregnancy, delivery, and breastfeeding is the most common route for human immunodeficiency virus (HIV) infection in children (Newell, 1998). The estimated risk of infection is 5-10% during pregnancy, 10-20% during labour, and 10-20% during breastfeeding (De Cock *et al*., 2000; Harms *et al*., 2005). About 20% of the babies who are infected through mother-to-child transmission of HIV contract the virus through breastfeeding (De Cock *et al*., 2000). The baby might be at greater risk from breastfeeding if its mother was infected with HIV late in her pregnancy or in the months following birth, because of the higher virus count during the seroconversion phase of infection. Women are also more infective when they show symptoms of acquired immunodeficiency syndrome (AIDS) (De Cock *et al*., 2000). What increases the risk even more is the fact that between 80% and 90% of women in rural and remote areas in Africa breastfeed their babies for two years or longer. Some African studies have shown that breastfeeding increases the risk of infection by between 5% and 20% (De Cock *et al*., 2000; Van Dyk, 2001). Factors that may also affect mother-to-child transmission of HIV during breastfeeding are a vitamin A deficiency in the mother or child, breast diseases such as mastitis, cracked nipples, and diseases such as thrush and gastroenteritis in the infant (Van Dyk, 2001).

1.1 Passive immunity

During the first few months of life, the infant begins to acquire protection against specific pathogens. This type of immunity is known as acquired immunity and allows the child to mount a specific immune response towards

each pathogen the child encounters as it progresses through those first, vulnerable months and years (Adler, 1993). During pregnancy, antibodies from the mother pass to the child in the uterus. However, these antibodies are short-lived, and by the end of three months, the child must begin to acquire its own immunity. For the first three months of its life, the child is protected by the natural, passive immunity conferred to it by its mother (Adler, 1993).

1.2 Diagnosis of HIV infection in infants

The early diagnosis of HIV infection in infants born to mothers who are HIV-infected has been challenging, in that passive transfer of maternal antibodies to the child leads to detectable antibodies in uninfected children for up to 15 months of age/after birth. Polymerase chain reaction (PCR) has changed the approach and now allows diagnosis by 14 to 30 days of life in most infants (Young *et al*., 1998). Demonstration of virus by PCR of HIV deoxyribonucleic acid (DNA) from peripheral blood mononuclear cells (PBMC), reverse transcription polymerase chain reaction (RT-PCR) of ribonucleic acid (RNA) from plasma, or detection of virus by PBMC co-culture in peripheral blood from the infant (not cord blood), is presumptive evidence of infection. A positive test must be confirmed with a separate sample as soon as possible. Two positive assays drawn at two separate time points are considered diagnostic of infection.

Detection of virus within a few days is considered evidence of in utero infection, occurring for 30-45% of infected infants (Bryson *et al*., 1993). In a meta-analysis of data from 271 infected infants, HIV DNA PCR was only moderately sensitive within 48 hours of birth (38%; 90% confidence interval [CI], 29-46%). There was little change in the first week of life, but by day 14, the sensitivity was 93% (90% CI 76-97%) (Dunn *et al.,*1995). The increase in detection reflects the ability to detect infected children in the peripartum period. The usage of zidovudine monotherapy during the first 4 to 6 weeks of life does not seem to interfere with diagnosis (Connor *et al.,* 1994).

1.3 Management of HIV-exposed infants

A reasonable diagnostic strategy is to perform PCR testing within 48 hours of birth, then at 14 to 30 days of life. However, many experts prefer to test between 14 days and 1 month to allow earlier diagnosis, and there is no evidence that this reduces sensitivity. Infants with repeatedly negative viral tests should be followed for evidence of loss of HIV antibodies. All children born to HIV-infected mothers should be treated using the ACTG 076 protocol, with 2mg/kg being administered every 6 hours beginning within a few hours of birth and continuing for 4-6 weeks after being given zidovudine. In women for whom intrapartum nevirapine is being considered because of inadequate prenatal treatment or failure to suppress maternal viraemia, it is reasonable to also include a dose for the infants within 48 hours of birth. This provides nevirapine concentration in the infants that remains for more than 7 days (Mirochnick *et al.,* 1998).

There is no consensus on the management of children born to mothers with documented resistance to zidovudine. Increased rates of transmission have been documented when the mother has a circulating resistant virus (Walles *et al.,* 2000), and it is logical to use drugs that would be predicted to be active against the maternal strain. *Pneumocystis* prophylaxis is recommended for all HIV-infected infants, as well as those of unknown status, beginning at 4-6 weeks of age. For children who are documented to be uninfected and have normal CD4 cell counts, prophylaxis can be stopped by 4 to 6 months of age (Walles *et al., 2000*).

1.4 Viral load

The viral load is the amount of virus found in the bloodstream. The quantitative HIV-1 RNA testing (HIV-1 viral load) detects and quantifies the HIV-1 RNA in plasma. The viral load is given as a value representing RNA copies per millilitre, as well as a log value, e.g. 100 = 2 log, 1000 = 3 log, 10000 = 4 log etc. The log value provides an easier number to work with and

better indicates significant changes in viral load (Powderly *et al*., 1999; Spencer, 2005; National Department of Health, 2004).

As seen in the graph **(Figure 1.1)**, viral load is highest during the acute phase of primary infection (before antibodies are developed) and at end stage AIDS. Viral load tests indicate the effectiveness (or lack of effects) of treatment and the expected rate of disease progression; the higher the viral load, the faster the progression (The viral load count, 2009).

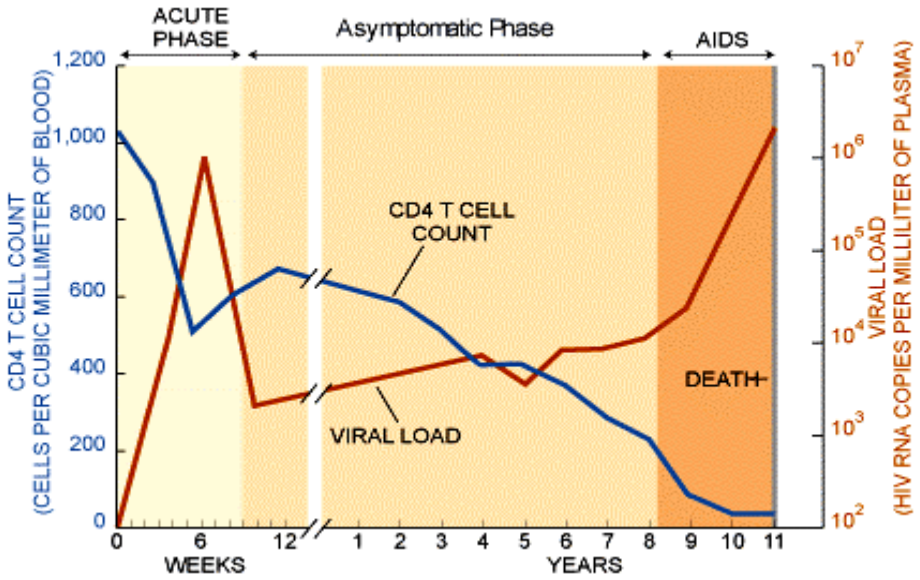

Figure 1.1. The viral load count

(Adapted from The viral load count, 2009)

1.5 The need for Antiretroviral Therapy

In spite of the advances in the antiretroviral drug therapy, HIV/AIDS remains a force to be reckoned with, even in developed countries. Antiretroviral therapy is associated with improved clinical well-being in children. Monotheraphy with zidovudine, didanosine, or stavudine demonstrated improved immunologic parameters, neurodevelopmental status and growth (Butler *et al.,* 1991; McKinney *et al.,* 1991; Kline *et al*., 1995). Combination therapy, with zidovudine and didanosine, zidovudine and lamivudine, or didanosine and stavudine, has subsequently been shown to be superior to monotherapy (Englund *et al*., 1997; McKinney *et al*., 1998; Kline *et al*., 1999).

The usage of protease inhibitor-containing triple combinations, either in treatment-experienced or treatment-naive children, confers significantly greater immunologic and virologic benefits than two-drug therapy (Nachman *et al*., 2000; Van Rossum *et al*., 2000; Wiznia *et al*., 2000). Triple combination therapy is associated with a significant survival benefit in children, as in adults, as demonstrated in prospective cohort studies (De Martino *et al*., 2000). The Italian HIV National AIDS registry study estimated that there was a reduction of almost 70% in the relative hazard of death associated with triple therapy compared to no antiretroviral therapy (De Martino *et al.,* 2000).

1.6 Immune reconstitution and when to start therapy in infants

The pattern of immune reconstitution in infants differs from that of adults (Borkowsky *et al*., 2000). It is likely that this is related to greater thymic activity in infants (Douek *et al*., 2000; Viganò *et al*., 2000). Compared to adults, children have an earlier and larger increase in naive CD4 cells and larger overall increases in CD4 count. In small studies, children have shown improvement in response to recall antigens and broadening of the T-cell receptor repertoire (Viganò *et al*., 1999).

Early initiation of therapy is associated with several potential problems, adherence to complex regimens with unpleasant formulations is difficult, yet adherence is the key determination of success (Watson & Farley, 1999; Reddington *et al*., 2000). It is becoming clear that metabolic complications of combination antiretroviral therapy, such as fat accumulation, fat atrophy, glucose intolerance, dyslipidemia, and lactic acidosis, affect a significant portion of infants, and risk factors and reversibility are poorly understood (Babl *et al.,* 1999; Jaquet *et al.,* 2000). When initiating therapy in infants, monitoring protease inhibitor concentrations should be strongly considered if available. For children older than one year, the decision on when to start therapy is more complicated. Antiretroviral therapy in all children, regardless of immunologic status or symptoms, should be initiated, or therapy should be deferred in children with normal immune status who are at low risk of disease

progression. Therapy should be considered when there is evidence of high viral load (Babl *et al.,* 1999; Jaquet *et al.,* 2000).

1.7 The aim of the study

The aim of this project was to develop and validate a cost-effective, fast, early detection method for HIV infection in infants. PCR was used as the developmental method, a technique that amplifies proviral sequences of HIV DNA, detecting HIV infection in peripheral blood mononuclear cells from infants of seropositive women during neonatal (age less than 28 days) and post-neonatal periods.

1.8 The objectives of the study

The final objective is to develop an alternative assay for testing babies for mother-to-child transfer of HIV-1. In order to reach this objective, the following has to be adhered to:

1. Identify a method satisfying the criteria of sensitivity, specificity, ease of use and speed.
2. Obtain the required oligonucleotides and other reagents to design and construct an internal control using the same set of primers as the primary target. This is to control for successful amplification and should be positive in the absence of HIV-1 DNA, otherwise it is impossible to distinguish between a true negative result and failed amplification. Ideally, an extraction control should be used which goes through the whole process together with the primary target, but for a proof-of-concept project like this, an amplification control should suffice.
3. Do the same for the assay itself.
4. Test the full reaction (target and internal control) in order to optimize it.
5. Test on a number of previously tested samples. It should perform on par or better than the current method before it can be implemented.

CHAPTER 2

LITERATURE REVIEW

One of the most important infectious diseases is acquired immune deficiency syndrome (AIDS). This disease is caused by infections with human immunodeficiency virus (HIV) and is characterized by profound immunosuppression with associated opportunistic infections and malignant tumours, wasting, and central nervous system (CNS) degeneration. The virus is transferred through blood and body fluids. Blood, semen, vaginal secretions, breast milk, and to a small extent, saliva of an infected individual, contain free virus or cells containing virus. Thus, HIV can be transmitted through sexual contact, sharing of needles, transfusion of blood or blood products, placental transfer, passage through the birth canal, and breast feeding (Benjamin *et al.,* 2000).

The UNAIDS Secretariat and the World Health Organisation estimated that at the end of 2007, approximately 33 million people were living with HIV infections globally **(Figure 2.1)**. It is also estimated that the annual number of new HIV infections declined from 3 million in 2001 to 2,7 million in 2007 and that 2 million people died due to HIV-related causes during 2007, compared with an estimated 1,7 million people in 2001.

Southern Africa continues to bear a disproportionate share of the global burden of HIV: 35% of HIV infections and 38% of AIDS deaths in 2007 occurred in that sub region. Altogether, sub-Saharan Africa is home to 67% of all people living with HIV. Half of

all people living with HIV worldwide are women, and nearly 60% of HIV infections are in Sub-Saharan Africa **(Figure 2.2)**.

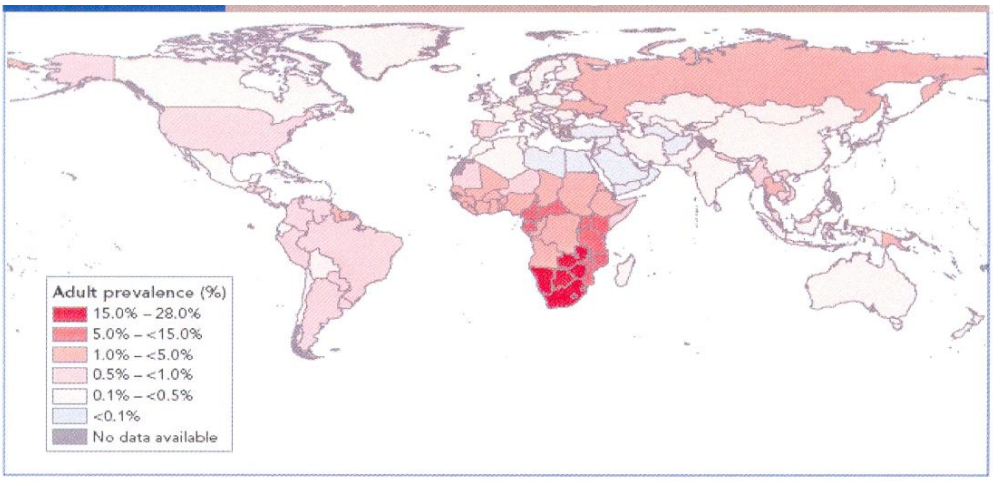

Figure 2.1 Global view of HIV infection, 2007

33 million people [30- 36 million] living with HIV, 2007

(http://data.unaids.org/pub/GlobalReport/2008/jc1510_2008_global_repoert_pp29_62_en.pdf)

Young people aged 15-24 years account for an estimated 45% of new HIV infections worldwide. An estimated 370 000 children younger than 15 became infected with HIV in 2007. Globally, the number of children younger than 15 living with HIV increased from 1,6 million in 2001 to 2 million in 2007 **(Figure 2.2)**. Almost 90% of children younger than 15 years live in Sub-Saharan Africa and acquired the virus during pregnancy, birth, or breastfeeding.

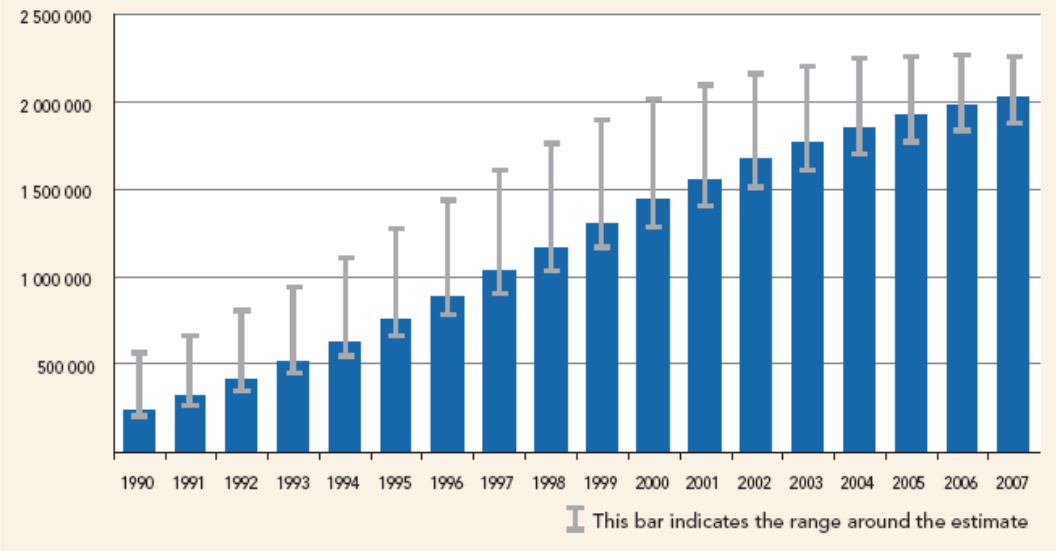

Figure 2.2 Children living with HIV globally, 1990-2007

(Adapted from UNAIDS, 2008)

A small fraction of HIV infections in children are caused by contaminated injections, the transfusion of infected blood or blood products, sexual abuse, sexual intercourse, or scarification (Hauri *et al*., 2004; Kiwanuka *et al.,* 2004; Schmid *et al.,* 2004).

In **Figure 2.3**, new HIV infections in children appear to have peaked in 2000-2002, due to the stabilization of the prevalence of HIV among women overall, and to the increasing coverage of programmes for preventing mother-to-child transmission of HIV. Since 2003-2007 the HIV prevalence in infected children has slowly been decreasing. In 2005 alone, 700 000 children acquired HIV infection through mother–to-child transmission before, during, and after delivery. HIV infections in childbearing women is as high as 57%, and vertically acquired HIV infection is now reversing positive downwards trend in infant and child mortality previously seen in Sub-Sahara Africa (Giaquinto *et al*., 2006).

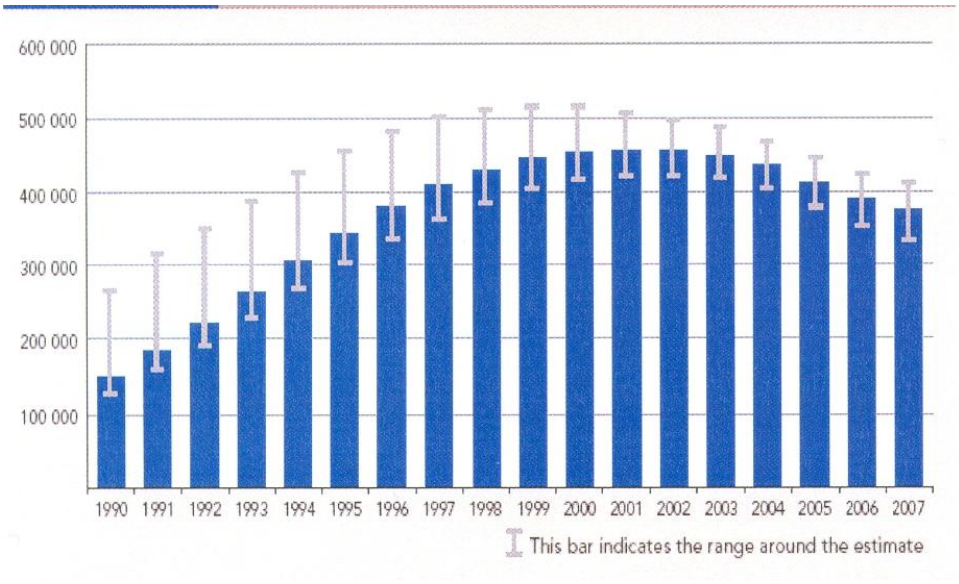

Figure 2.3 New HIV infections among children, 1990-2007

(Adapted from UNAIDS, 2008)

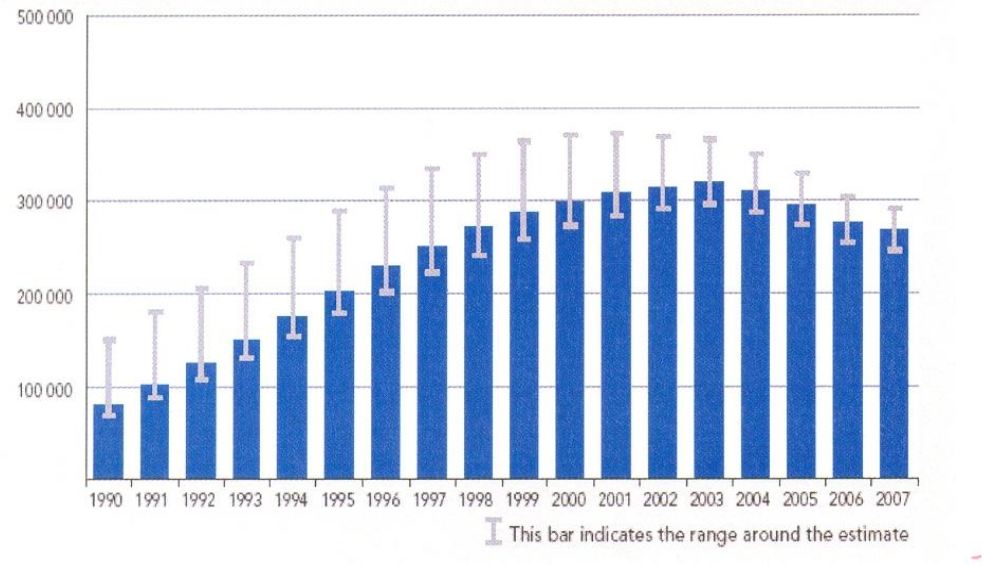

Figure 2.4 Child deaths due to AIDS, 1990-2007

(Adapted from UNAIDS, 2008)

As **Figure 2.4** illustrates, the total number of AIDS death in children peaked in 2003 and has been decreasing since. This decline

mainly reflects the drop in new infections seen earlier **(**visible in **Figure 2.3),** as well as increased access to antiretroviral treatment.

2.1 The HIV epidemic in South Africa

In Sub-Saharan Africa, there are an estimate of 1,9 million people who are infected with HIV, bringing to 22 million the number of people living with HIV. Two thirds (67%) of the global total of 33 million people living with HIV, live in this region, and three quarters (75%) **(Figure 2.5)** of all AIDS deaths occurred there in 2007 (UNAIDS, 2008).

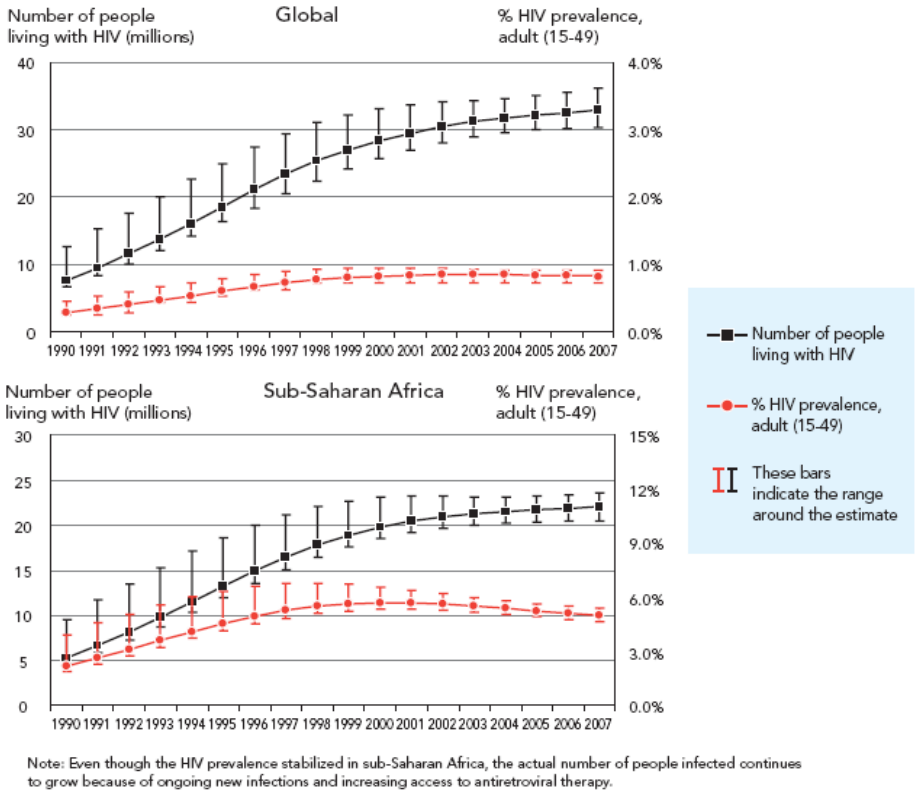

Figure 2.5 Estimated number of adults living with HIV.

Global HIV epidemic, 1990-2007; and HIV epidemic in Sub-Saharan Africa, 1990-2007. (Adapted from UNAIDS, 2008)

Since 2003, surveys have confirmed that the trend has changed, so that the level of HIV prevalence is now growing slowly. The most encouraging finding concerns teenage girls (15-24 years of age), among whom prevalence has been declining since 1999 (Avert, 2008). Based on the sample of 33 488 women attending 1 415 antenatal clinics across all nine provinces, the South African Department study estimates that 28% of pregnant women were living with HIV in 2007. The provinces that recorded the highest HIV rates were KwaZulu-Natal, Free State, and Gauteng **(Table 2.1)** The Northern Cape and Western Cape recorded the lowest prevalence (Avert, 2008). HIV data from antenatal clinics in South Africa suggests that the country's epidemic might be decreasing in some provinces, but there is no evidence yet of major changes of HIV-related behaviour. The estimated 5,7 million South Africans living with HIV in 2007 makes this the largest HIV epidemic in the world (UNAIDS, 2008).

Table 2.1 Estimated HIV prevalence among antenatal clinic attendees, according to province (Avert, 2008).

Province	2000 Prev.%	2001 Prev.%	2002 Prev.%	2003 Prev.%	2004 Prev.%	2005 Prev. %	2006 Prev. %
KwaZulu-Natal	36,2	33,5	36,5	37,5	40,7	39,1	39,1
Gauteng	29,4	29,8	31,6	29,6	33,1	32,4	30,8
Free State	27,9	30,1	28,8	30,1	29,5	30,3	31,1
Mpumalanga	29,7	29,2	28,6	32,6	30,8	34,8	32,1
North West	22,9	25,2	26,2	29,9	26,7	31,8	29,0
Eastern Cape	20,2	21,7	23,6	27,1	28,0	29,5	29,0
Limpopo	13,2	14,5	15,6	17,5	19,3	21,5	20,7
Northern Cape	11,2	15,9	151	16,7	17,6	18,5	15,6
Western Cape	8,7	86	12,4	13,1	15,4	15,7	15,2
National	24,5	24,8	26,5	27,9	29,5	30,2	29,1

Prev. = Prevalence

2.2 Transmission of HIV from mother to child

The most complex and difficult challenge in the field of maternal and child health is the need to protect children from this virus, for which there is no vaccine (Holmes & Kwarteng, 2001). Mother-to-child transmission occurs during pregnancy, at the time of labour or delivery and afterwards through breastfeeding. The estimated rate

of mother-to-child transmission of HIV ranges from 15-30% in the non-breastfeeding population and from 25-45% in breastfed children, which means that over half of all children born to HIV-infected women are not infected (De Cock *et al.,* 2000).

Even when antiretroviral prophylaxis successfully reduces the risk of mother-to-child transmission late in pregnancy and during delivery, postnatal transmission through breastfeeding remains an important risk (De Cock *et al.,* 2000). Mother-to-child transmission is responsible for more than 90% of these infections. Mother-to-child transmission is the most dominant mode of HIV infection among young children worldwide, whereby there are at least 1 700 new infections each day and more than one paediatric infection every minute (Peter, 2006).

HIV transmission from mother to infant can occur antepartum (in utero), intrapartum (during labour or delivery), or postpartum (through breastfeeding). It is suggested that in the absence of breastfeeding, 30% of infant infections occur during pregnancy, 70% occur during labour and delivery, and during breastfeeding. Where formula feeding is not available, breastfeeding contributes substantially to the risk of transmission, accounting for approximately one third of cases (Giaquinto *et al.,* 2006).

Babies of HIV positive mothers have a higher risk of low weight, prematurity, stillbirth, and perinatal mortality. Clinical features which are common of HIV infection in children are recurrent and persistent diarrhoea, oral thrush, generalized lymphadenopathy, itchy rashes, chronic cough, developmental delay, neurological problems, parotitis, recurrent bacterial infections, and especially pneumonia. Children developing signs of infections in their first

year have a poor prognosis and most die within 3 years (Holmes & Kwarteng, 2001). Some develop symptoms for the first time in their second or third year and continue to grow well, although they may have frequent minor illnesses (Holmes & Kwarteng, 2001).

HIV transmission occurs early through breast milk, although some risks continue throughout the period of breastfeeding (Nduati *et al.,* 2000). Most babies, who are born to HIV-infected mothers and are breastfed, do not become infected with HIV, and these infants probably benefit from the general and HIV-specific antibodies in breast milk (Tozzi *et al.,* 1990). Some African studies have shown that breastfeeding increases the risk of infection by between 15% and 30% (Van Dyk, 2001). Factors that may also affect mother-to-child transmission of HIV during breastfeeding are a vitamin A deficiency in the mother or child, breast diseases such as mastitis, cracked nipples, and diseases such as thrush and gastroenteritis in the infant (Van Dyk, 2001).

2.3 The specific immune response

During the first few months of life, the infant begins to acquire protection against specific pathogens. This type of immunity is known as acquired immunity, and it allows the child to mount a specific immune response towards each pathogen he or she encounters as it progresses through those first few vulnerable months and years. During pregnancy, antibodies from the mother pass to the child in the uterus. However, these, antibodies are short-lived, and by the end of three months, the child must begin to acquire its own immunity. For the first three months of its life, the child is protected by the natural, passive immunity conferred to it by its mother (Adler, 1993). Because of the maternal HIV antibodies

crossing the placenta, it is difficult to diagnose HIV infection in infants up to 18 months of age without access to advanced tests such as PCR (Holmes & Kwarteng, 2001).

2.4 Types and Subtypes of HIV

There are two types of human AIDS viruses, HIV-1 and HIV-2, which are distinguished according to their genome organization and phylogenetic relationships with other primate lentiviruses **(Figure 2.6).** HIV-1 has three distinct virus groups: M (majority), O (outliers) and N (non-M/non-O). The majority of infected individuals in the world have HIV-1 group M, which comprises of ten different subtypes, designated clades A through K on the basis of phylogenetic analysis of genomic or sub-genomic proviral sequences (Machuca *et al.,* 2001; Choisy *et al.,* 2004; Glass, 2006). The O and N virus groups are found in Africa, with a low prevalence (Simon *et al.,* 1998; Liitsola, 2000). Similarly, HIV-2 comprises of six subtypes, A through F. Comparison of HIV subtypes to simian immunodeficiency virus (SIV) provides clues as to the origins of HIV, as well as providing a valuable animal model. Five major lineages of primate lentiviruses have been fully sequenced, helping to place the origins of HIV in Central Western Africa (Hahn *et al.,* 2000; Choisy *et al.,* 2004).

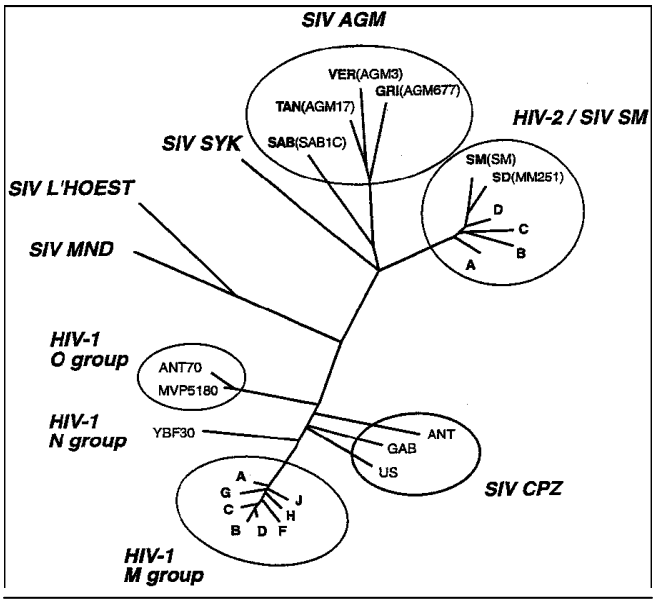

Figure 2.6 Phylogenetic relationships among primate lentiviruses

The tree is a composite of several analyses based on viral gag, pol and full-length genome alignment

(Adapted from Alaeus, 2000)

The HIV viruses are members of the lentivirus family of retroviruses (Clavel *et al.,* 1986; Keren & Warren, 1992; Marlink *et al.,* 1994; Witten & Perelson, 2004). Retroviruses are RNA viruses that replicate through DNA intermediates using the viral enzyme reverse transcriptase (Price & Perry, 1994). HIV-1 and HIV-2 contain a few different proteins. The clinically available enzyme linked immunosorbent assays (ELISA's) for HIV-1 will cross-react with from 59 to 91 percent of HIV-2 antibody-positive sera. HIV-2 appears to be transmitted in the same manner as HIV-1. Although clinical experience with HIV-2 is limited, HIV-2, like HIV-1, is known to produce decreased CD4 cell count, lymphadenopathy, immunosuppression, and AIDS. Some researchers have suggested that HIV-2 may have a longer incubation period than

HIV-1 infection and that HIV-2 infected patients may have a somewhat longer survival rate (Sigal & Ron, 1994).

2.5 Differences between subtypes

It is essential to understand the biological and geographical differences between subtypes. HIV-1 group M, subtypes A & D, are predominant in Sub-Saharan Africa, while subtype C is predominant in South Africa, India, and Nepal (Relucio & Holodniy, 2002). The populations of Japan, Australia, the Caribbean, Europe, and the Americas are mostly affected by subtype B (McCutchan, 1999; Michael *et al.,* 1999). Subtype E occurs in the Central African Republic, as well as Thailand and other countries of Southeast Asia, while subtype F (Brazil & Romania), subtypes G & H (Russia and Central Africa) and subtype I (Cyprus) clades are of a very low prevalence (Kahn, 2003). Primarily group O HIV-1 strains have been detected in Western African countries like Cameroon. The first group N HIV-1 strain was found in Cameroon in 1998, since viruses from this particular group are exceedingly rare (Relucio & Holodniy, 2002). Different subtypes vary in their effect on their host. Variance in disease progression is caused by the biological differences between strains and subtypes and the genetic difference between hosts. T-cell counts act as a measure of virus progression and are therefore a common way to estimate the virulence of subtypes (Kuiken, 1999).

2.6 Virology of HIV-1

HIV-1 is a single-stranded diploid RNA virus, 100-200μm in diameter (Adler *et al.,* 1993). The RNA component is 9749 nucleotides long (Ratner *et al.,* 1985). Two major viral-envelope proteins, gp120 and gp41, form spikes (Roux & Taylor, 2007). The HIV-1 lipid bilayers are made up of various host proteins, including class I and class II histocompatibility antigens, which are obtained during virion budding. The proteins p24, p17, p6, and p7 are the four nucleocapsid proteins contained in the care of HIV-1, each of which is cleaved from 53kD gag precursor by the HIV-1 protease. The main component of the inner layer of the nucleocapsid is formed by the phosphorylated p24 capsid **(Figure 2.7)**. The myristoylated p17 protein is associated with the inner surface of the lipid bi-layer and probably stabilizes the exterior and interior components of the virion.

The p7 matrix binds directly to the genomic RNA through a zinc-finger structural motif and, together with p9, forms the nucleoid core. This retroviral core contains two copies of the single stranded HIV-1 genomic RNA, which is associated with the various pre-formed viral enzymes, including two reverse transcriptase components, integrase and protease (Coffin, 1995; Parren *et al*., 1999; Turner & Summers, 1999).

CHAPTER 3

MATERIALS AND METHODS

3.1 MATERIALS

3.1.1 Patient samples

The ethical committee of CUT (ETOVS 32/03) cleared the project and fifty HIV positive DNA samples (from EDTA blood) were used to develop and evaluate the new PCR method. In March 2004, the samples were tested by PathCare Laboratories in Cape Town using the Roche Amplicor HIV-1 DNA kit. The samples were supplied without any way of linking them to specific patients, therefore it was not deemed necessary to obtain informed consent.

3.1.2. Nucleic Acids (Plasmids and Primers)

Plasmid pBR322 purchased from Promega was taken from the frozen stocks of the Department of Haematology and Cell Biology. Plasmid pGEM-T Easy was purchased from Promega. The primers summurized in Table 3.1 were designed using the AlleleID (Biosearch Technologies) program, employing settings to search for an amplicon with a low Tm on pBR322. The binding sites of the primers are 4038-4062 for PS1 and 4127-4147 for PS2. Primers PS1 and PS2 were tailed with the HIV-1 primers SKCC1B and SK145 so that the amplicon may be amplified using the latter set of primers.

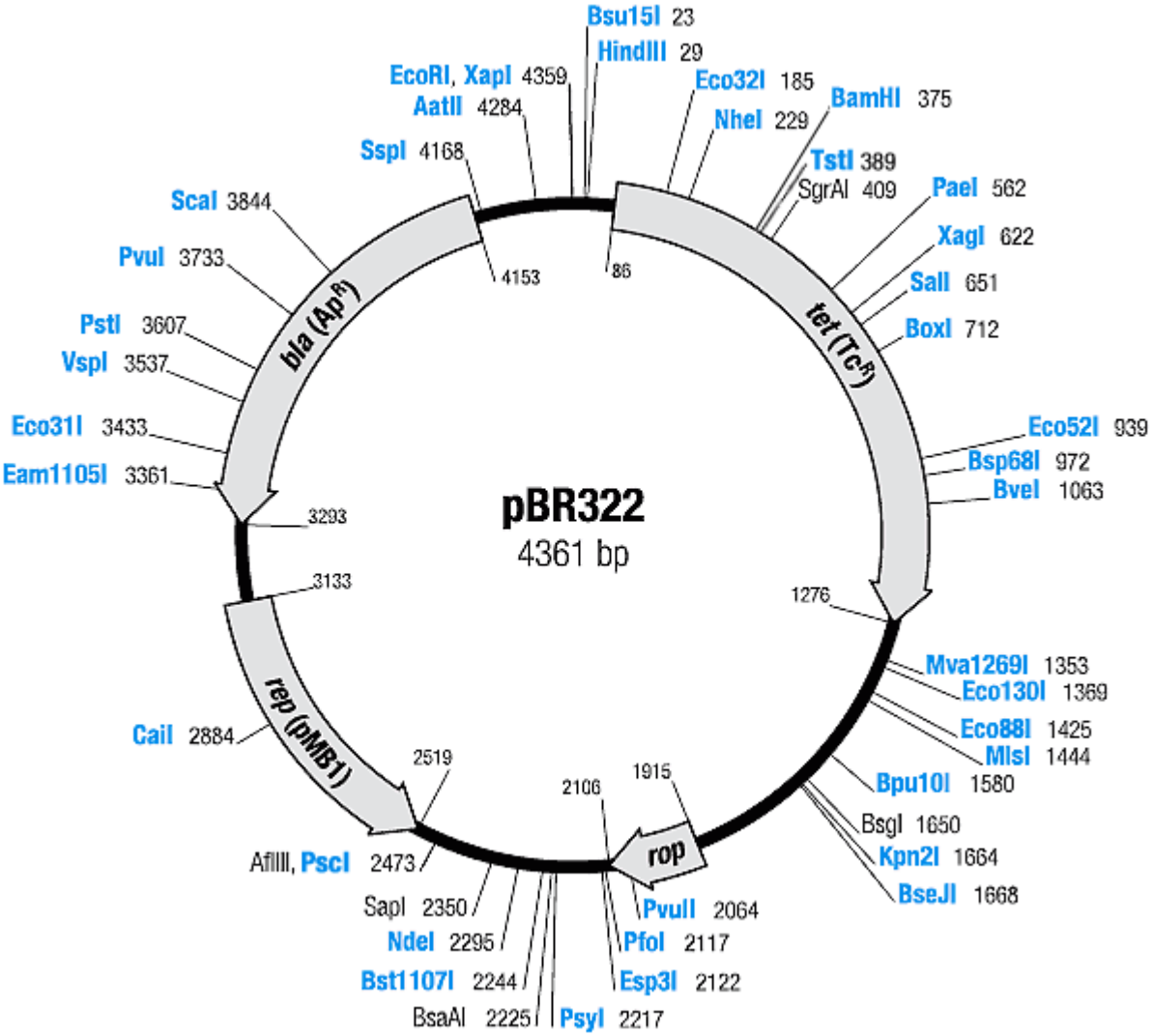

Figure 3.1 pBR322 plasmid (4361 kb)

(Adapted from Molecular cloning, vectors pBR322 DNA, 2009)

Table 3.1 Primers used for PCR methods

Primers	Number of bases	Sequence
PS1	55 bases	AGTGGGGGGACATCAAGCAGCCATGCAAATCCCAAC TGATCTTCAGCATGTTTTA
PS2	49 bases	TACTAGTAGTTCCTGCTATGTCACTTCCATTCAACATTT CCGTGTCGCC
SKCC1B	28 bases	TACTAGTAGTTCCTGCTATGTCACTTCC
SK145	30 bases	AGTGGGGGGACATCAAGCAGCCATGCAAAT

3.1.3 Chemicals and media

All chemicals were of analytical grade and were purchased from the following companies: Sigma ALDRICH, (Steinheim, Germany), Promega (Madison, USA) and Roche Diagnostics (Indianapolis, USA). Reagents for PCR were purchased from Roche. Microbiological media was supplied by Difco (Franklin Lakes, New Jersey, USA), and Merck (Darmstadt, Germany).

3.2 METHODS

3.2.1 Extraction of genomic DNA

The Promega Wizard Genomic Purification DNA kit was used to extract DNA from 300µl EDTA blood (see 3.1.1). The following procedure was followed; 900µl of cell lysis solution was pipetted into a sterile1,5ml microcentrifuge tube. The tube of blood was gently inverted until thoroughly mixed. Then 300µl of blood was transferred to the tube containing the cell lysis solution. The mixture of the blood cells was incubated for 10 minutes at room temperature and centrifuged at 14 000xg in a microcentrifuge for 1 minute. As much supernatant as possible was removed and discarded without disturbing the visible white pellets. If some of the red blood cells and red cell debris were visible along with the white blood cells the procedure was repeated.

The tube was vigorously vortexed until the white blood cells resuspended (10-15 seconds). Then 300µl of nuclei lysis solution was added to the tube containing the resuspended cells. The solution was pipetted 5-6 times to lyse the white blood cells until the solution became very viscous. If the clumps were visible after mixing, the solution was incubated at 37°C until the clumps were disrupted. If the clumps were still visible after 1 hour, an additional 100µl of nuclei lysis solution was added and the incubation was repeated. Then 100µl of protein precipitation solution was added to the nuclear lysate and vortexed vigorously for 20 seconds. Small protein clumps were occasionally visible after vortexing. The solution was centrifuged at 14 000xg for 3 minutes at room temperature. A dark brown protein pellet was visible and the supernatant was transferred to a clean microcentrifuge tube containing 300µl of room temperature isopropanol.

The solution was gently mixed until the white thread-like strands of DNA were visible as a small white pellet. The supernatant was removed and 300 µl of 70% ethanol at room temperature was added to the DNA. The tube was gently inverted several times to wash the DNA pellet and the sides of

microcentrifuge tube, and then centrifuged for 1 minute at 14 000xg at room temperature. The ethanol was carefully aspirated using a drawn Pasteur pipette. Because the DNA pellet was very loose, care was taken to avoid aspiration of the pellet. The tube was inverted on to a clean absorbent paper and the pellet dried for 15 minutes. The 100µl of DNA rehydrating solution (10mM Tris-HCl/1mM EDTA, pH 7,4) was added to the tube and the DNA was dehydrated by incubating at 65°C for 1 hour. Then the solution was gently mixed by tapping the tube. The DNA was rehydrated by incubating the solution overnight at room temperature and storing it at 4°C.

3.2.2 Polymerase chain reaction

Amplification reactions were carried out in a total volume of 50µl which contained the following: primers (100-200nM), 200µM of dNTPs, 5µl of reaction buffer (10X), 5 units of *Taq* polymerase, 1.5-3mM $MgCl_2$ and a variable amount of pBR322 DNA as template. The PCR cycling was as follows: denaturation step at 95ºC for 2 minutes, 30 cycles at 95ºC for 10 seconds, 55-60ºC for 10 seconds and 72ºC for 10 seconds, with one cycle at 72ºC for 5 minutes.

3.2.3 Agarose gel electrophoresis

Two percent gels were prepared by adding an appropriate amount of agarose to 50ml TBE. This was heated in a microwave oven until all the agarose was dissolved. After being cooled down, 2,5µl ethidium bromide (10 mg/ml) was added. The gel was poured in the gel plate and left for 30 minutes to solidify. Then 5µl DNA was mixed with 5µl of loading buffer (0,25% bromophenol blue in 40% sucrose solution) and loaded in separate slots and electrophoresed at 80 volts for 1 hour in 1 x TBE buffer. Then the bands were visualized on a UV transilluminator. A 100 bp ladder (Promega) was used as size standard.

3.2.4 Purification of DNA from agarose gels

To purify the DNA a GenElute Agarose Spin Column kit (Sigma) was used. The column was placed into a collection tube and pre-washed by adding 1x TE (10mM Tris, pH8,0, 1mM EDTA). The spin column was capped and centrifuged at 14 000xg for 10 seconds. Then the GenElute Agarose Spin Column was transferred to a fresh collection tube. The bands of interest from the agarose gel of the amplified PCR product were cut and loaded into the pre-washed column. The GenElute Agarose Spin Column was centrifuged at 14 000xg for 10 minutes. The purified DNA in the collection tube was centrifuged for 15 minutes at maximum speed. The supernatant was then discarded and the pellet was washed with 20µl of 70% ethanol (room temperature). Subsequently, the DNA pellet was dried for 5 minutes and resuspended in 20µl TE buffer (10mM Tris, pH8,0, 1mM EDTA).

3.2.5 Preparation of LB medium

Table 3.2 Two mixtures of LB medium for plates (1) or liquid (2).

	MIXTURE 1	MIXTURE 2
H_2O	200ml	200ml
TRYPTONE	2,0g	2,0g
YEAST EXTRACT	1,0g	1,0g
NaCl	2,0g	2,0g
AGAR	3,0g	0,0g

The mixtures were autoclaved for 20 minutes at 121ºC. Two hundred microlitres of 50% ethanol was mixed in 25mg/ml Ampicillin to mixture 1 (**Table 3.2**), after which it was poured into the plates and left in the fume hood to solidify.

3.2.6 Cloning of PCR products

The pGEM-T Easy Vector System kit (Promega) was used for cloning of the PCR amplicon resulting from primers PS1 and PS2 and pBR322 as template.

Table 3.3 Cloning the fragment of amplified PCR product.

TUBES →	FRAGMENTS	CONTROL INSERT	NEGATIVE CONTROL
2X Rapid ligation buffer	5µl	5µl	5µl
pGEM-T Easy (50ng/µl)	1µl	1µl	1µl
PCR fragments	3µl	-	-
Control insert DNA (4ng/µl)	-	2µl	-
T4 DNA ligase (3u/µl)	1µl	1µl	1µl
H_20	-	1µl	3µl

Three Eppendorf tubes were prepared from the above reagents. They were incubated at room temperature for 1 hour.

3.2.7 Electroporation of ligated DNA

Three tubes of electrocompetent cells were thawed and 2,5µl of ligated mixture was added to each tube. Each was mixed well and kept on ice for 1 minute. The cell suspension was loaded into 2mm cuvettes, which were chilled beforehand on ice. Two thousand five hundred volts was selected as the output voltage of the electroporator (Bio-Rad). The pulse was triggered immediately. After the pulse, 500µl of liquid LB medium was added**.** The cells were transferred into Eppendorf tubes and incubated at 37ºC for 1 hour before being centrifuged at 4 000xg for 2 minutes. Three hundred and fifty µl of the supernatant of each mixture was removed and each pellet was carefully mixed. The cells were plated on selective media containing Ampicillin, and incubated overnight at 37°C.

3.2.8 Identification of insert-containing colonies

Twenty colonies were picked randomly from the plate using toothpicks and stabbed onto a new plate in a grid pattern. After overnight incubation at 37°C, a small fraction of each colony was picked up and used to inoculate 1 ml aliquots of LB + Amp medium. After overnight culture at 37°C, 10µl was taken from each culture at room temperature and then boiled for 5 minutes and centrifuged at 4 000xg for 5 minutes. Five microlitres of each supernatant was used in a PCR reaction, using primers SK145 and SKCC1B. Products were analysed on a 2% agarose gel and colonies showing a product of the correct size were identified as containing inserts.

3.2.9 Isolation of plasmid DNA from *E. coli*

The remaining liquid culture of an insert-positive colony from 3.2.8 was used to inoculate 1 litre of LB + Amp, which was incubated overnight with shaking at 37°C. The GenElute Plasmid Maxiprep Kit from Sigma was used as follows: The overnight mixture was divided evenly into the conical tubes, then centrifuged at 8 000xg for 10 minutes before the media supernatant was discarded. The pellet was washed with 40ml of H_2O and centrifuged at 8 000xg for 10 minutes, after which the supernatant was discarded. The bacterial pellet was resuspended with 6ml of resuspension solution (50mM Tris-HCl (pH7,5); 10mM EDTA) that was mixed with RNase A (100µg/ml). The pellet was vortexed until all the cells were completely resuspended as a homogenous suspension. The resuspended cells were lysed by adding 6ml of lysis solution (0,2M NaOH and 1% SDS). Then the cells were gently inverted 8-10 times until the mixture became clear and viscous. The cells were incubated at room temperature for 2 minutes.

The cell debris was precipitated by adding 8ml of neutralization/binding buffer (1,32M potassium acetate, pH4,8). The tube was gently inverted 4-6 times and centrifuged at 14 000xg for 10 minutes. The cell debris, proteins, lipids, SDS, and chromosomal DNA fell out as a cloudy, viscous precipitate. The supernatant was re-centrifuged if the floating supernatant contained a

large amount of floating particles after centrifugation. A GenElute Maxiprep Binding Column was inserted into a 50ml collection tube and the cleared lysate was transferred to the column, then centrifuged at 4 000xg for 2 minutes. The flow-through liquid was discarded. Eight millilitres of optional wash solution (80mM potassium acetate, 8,3mM Tris-HCl, pH7.5, 40µM EDTA) was added to the GenElute Maxiprep Binding Column, centrifuged at 4 000xg for 5 minutes, after which the flow-through liquid was discarded. Fifteen millilitres of diluted wash solution (80mM potassium acetate, 8,3mM Tris-HCl, pH7.5, 40µM EDTA) was added to the GenElute Maxiprep Binding Column and centrifuged at 4 000xg for 5 minutes. The flow-through liquid was discarded and centrifuged again at 14 000xg for 2 minutes without additional wash solution to remove excess ethanol. The GenElute Maxiprep Binding Column was transferred to a fresh 50ml collection tube. One millilitre of elution solution (10mM Tris-HCl, 1mM EDTA pH8.0) was added to the column and centrifuged at 4 000xg for 5 minutes. The results were observed by mixing 2µl of the solution with 5µl of loading buffer and the total volume was run on a 2% agarose gel using 1kb marker.

3.2.10 Ethanol precipitation

Two and a half volumes absolute ethanol and 1/10 volume of 3M NaAc were routinely used and the mixture was put on ice for 30 minutes. The solution was spun at 14 000xg for 15 minutes and the supernatant was carefully removed. Then 50µl of room temperature 70% of Ethanol (EtOH) was added and spun for 5 minutes; the supernatant was very carefully removed. The pellet was dried in the Speed vaccum for 5 minutes and then resuspended in 50-200µl TE (10mM Tris, pH8.0, 1mM EDTA).

3.2.11 Digestion of DNA with restriction enzymes

Two hundred microlitres of insert-containing plasmid DNA from 3.2.10, 25µl of SuRE/Cut Buffer H, 5µl EcoR1 (Roche) and 20µl of water was added into an Eppendorf tube and incubated at 37ºC for 2 hours. The results were

observed by mixing 2µl of the solution with 5µl of loading buffer and the total volume was run on a 2% agarose gel using 1kb marker.

3.2.12 DNA concentration determination

Five microlitres of DNA was added to 95µl of TE and the dilution was added to a 100µl cuvette. A Pharmacia Genequant spectrophotometer was used at 260 and 280nm to determine the concentration and purity of the DNA.

3.2.13 LightCycler experiments

The Roche Lightcycler was used as platform for the new assay in combination with the FastStart DNA Master PLUS SYBR Green reaction mix. Reactions of 20µl were composed of 4µl mastermix, 2µl Taq polymerase enzyme, 5µl of primer mix (final concentration 200 µM each) 5 microlitre DNA and 4µl water. The following profile was used throughout: 10 minutes at 95°C followed by 45 cycles consisting of 10 seconds at 95°C, 10 seconds at 60°C and 15 seconds at 72°C. Fluorescence measurements in channel F1 were taken at the end of each elongation step at 72°C. The amplicons were then denatured at 95°C for 10 seconds, annealed at 60°C for 1 minute and heated at 0.1°C per second to 95°C to determine their melting temperatures. During the melting step, fluorescence data was captured continuously in channel F1. Every experiment contained a no-template control without DNA to look for contamination and also to get a background profile.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Construction of an internal control using pBR322

A competitive internal control, using the same primers as the Roche Amplicor HIV-1 DNA assay, but amplifying a different amplicon, was designed. The reasoning behind it was to amplify a short length (109bp, 167bp HIV SK primers included) of pBR322, specially selected for having a high (Adenine + Thymine) A+T content so that it would have a low melting point. The amplicon generated from integrated HIV by the two SK primers is 157 bp in size and was calculated to have a Tm of 85°C, while the pBR322-derived fragment should have a Tm of 75-78°C depending on which program is used for the calculation. Hybrid primers were designed to prime on pBR322, besides having priming sites for the two Roche primers, SK145 and SKCC1B. The result of amplifying pBR322 DNA using primers PS1 and PS2 is shown in **Figure 4.1.**

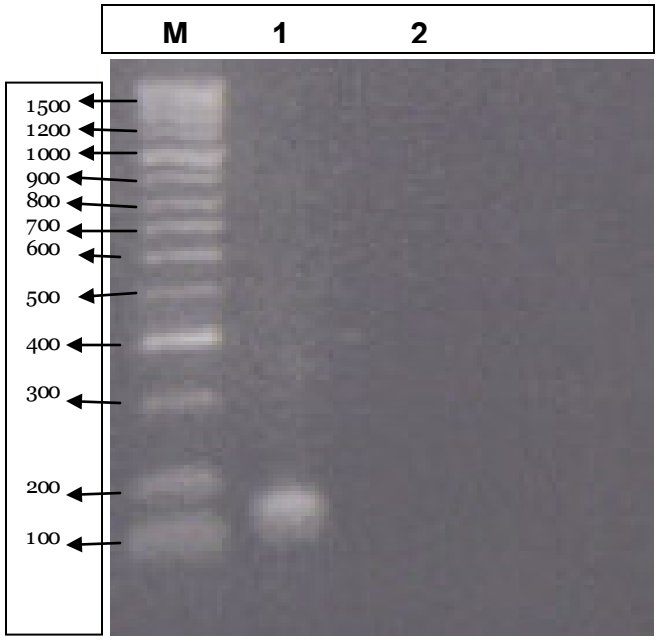

Figure 4.1 Electrophoretic analysis of pBR322 amplification

Lane M is the molecular marker (Promega 100 bp ladder),
lane 1 indicates the amplified fragment.

A band of the correct size (between 100 and 200bp) was visible in lane 1, while the no template control in lane 2 was empty. This PCR product was subsequently cloned into the Promega pGEM-T Easy vector which has overhanging thymine (Ts) to enable accommodation of the amplicon having adenine (A) overhangs. The ligated mixture was used to transform E. coli by electroporation, and a number of colonies were picked up for analysis from Ampicillin plates. These were stabbed onto a new Ampicillin plate and used to inoculate small cultures which served as templates for direct amplification of inserts from the plasmids. The resulting PCR products were separated on an agarose gel **(Figure 4.2).**

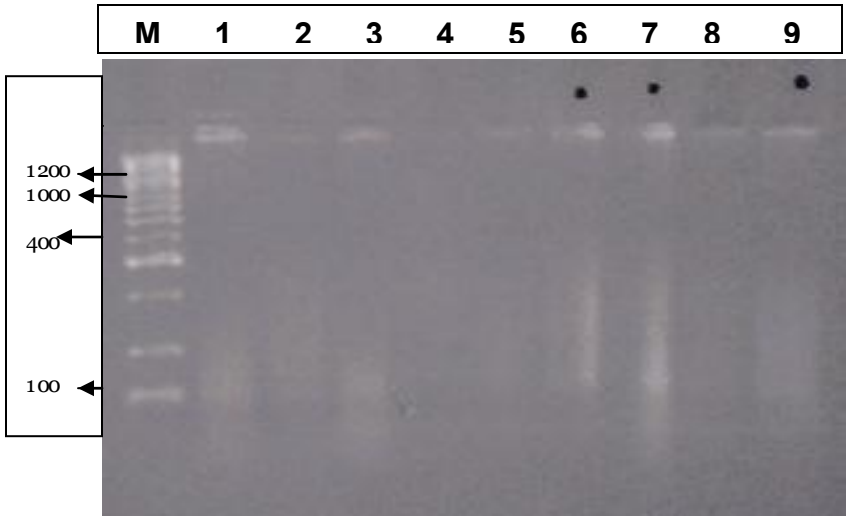

Figure 4.2 Agarose gel electrophoresis of amplicons

From crude extracts of plasmid-containing colonies 1, 6, 7 and 9 were chosen for further analysis.

Plasmid DNA was purified from the rest of the cultures of the colonies chosen for further analysis. Since the insertion site of the PCR fragments in pGEM-T Easy is flanked closely by EcoR1 restriction sites, the plasmids were digested by EcoR1 and analyzed by agarose gel electrophoresis (result not shown). One of the clones showing the correctly-sized fragment was selected and inoculated into 100ml medium for isolating a large amount of plasmid DNA. In turn, this DNA was digested with EcoR1 and analyzed by gel electrophoresis **(Figure 4.3).**

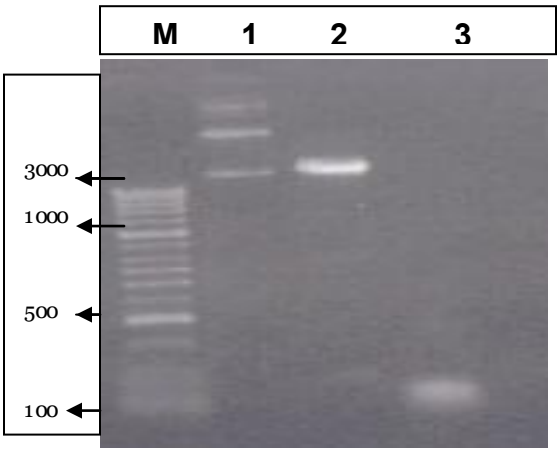

Figure 4.3 Agarose gel electrophoresis of EcoR1 digested plasmid DNA

Lane 1 supercoiled plasmid; lane 2, digested plasmid and lane 3,
the original PCR fragment.

The EcoR1-digested plasmid produced an insert of between 100 and 200bp, while the original fragment was somewhat smaller. The size difference can be due to a slight size difference (about 10bp due to the EcoR1 sites), as well as the effect of salt from the restriction buffer. The migration of small DNA fragments is easily perturbed by minor differences in salt and/or size.

The plasmid containing the insert was now deemed ready for testing as an internal control. It was thought better to have the insert removed by EcoR1 digestion to change it from supercoiled plasmid to linear DNA, an easier target for PCR.

4.2 Testing the internal control

The concentration of the digested plasmid was determined by UV spectrophotometry, from which the copy number was calculated. This was deemed necessary to establish the amplification characteristics of the construct. A dilution series was made and analyzed on the LightCycler,

the results of which are shown in **Figure 4.4.** From this it is clear that the lower limit of detection is in the region of 1 000 target copies/ml, which works out at 20 copies per reaction.

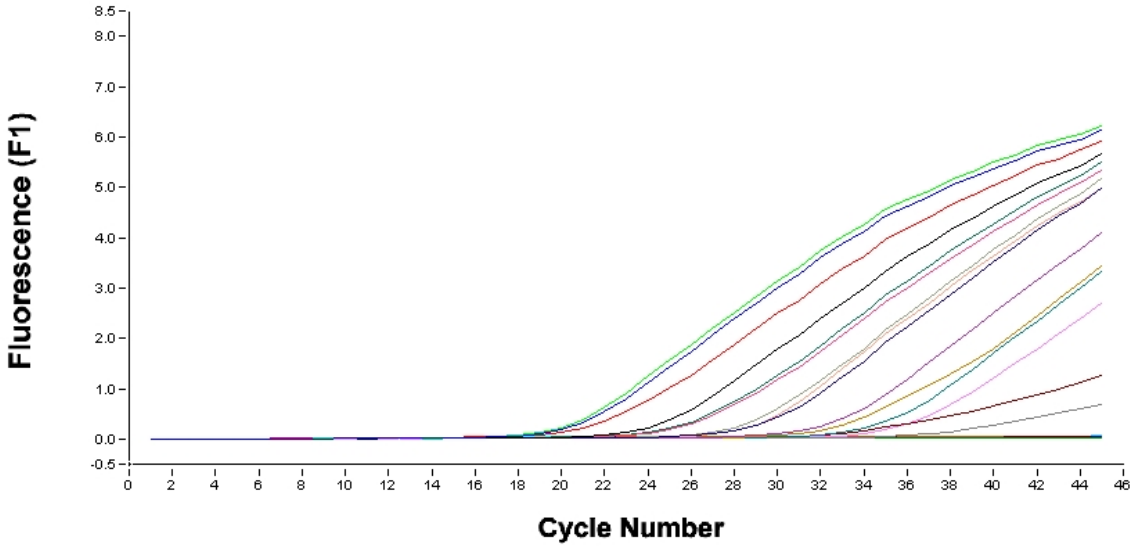

Figure 4.4 Determination of the detection sensitivity of the internal control reaction.

A 10-fold dilution series ranging from 1 million to10 copies/ml were run in triplicate.

Table 4.1. Crossing points (Cp) of the dilution series

Copy number	10^6	10^5	10^4	10^3	10^2	10^1
Cp	19,7 ± 0,8	23,1 ± 0,9	27,0 ± 0,6	31 ± 1.3	35*	>45

*Only 2 of the 3 lines came up

The standard curve constructed from the dilution series had a slope of -3,6 and an efficiency of 92%. It can be seen in **Table 4.1** that the crossing point differences between successive 10-fold dilutions are close to 4, where it should ideally be 3,33. As the same primers are used for the internal control and HIV-1 DNA detection and since the amplicon sizes are similar, these figures can be applied to the primary reaction as well. This

assumption implies that the lowest copy number detectable by this method is 20 copies of integrated HIV-1 DNA per reaction, which does not bode well for its intended application.

Strand separation, annealing, and slow denaturation while continuously following the fluorescence resulted in the set of melting curves shown in **Figure 4.5.** The melting point of amplicon (Tm) turned out to be 81 ±1˚C, somewhat higher than expected. It should be noted that the formulas used for predicting the Tm of DNA molecules assume "ideal" conditions, where the salt concentration, including magnesium, is exactly known. Unfortunately, the exact compositions of commercial Taq polymerase buffers are not given and may contain components different from the classical buffers, which may influence Tm values.

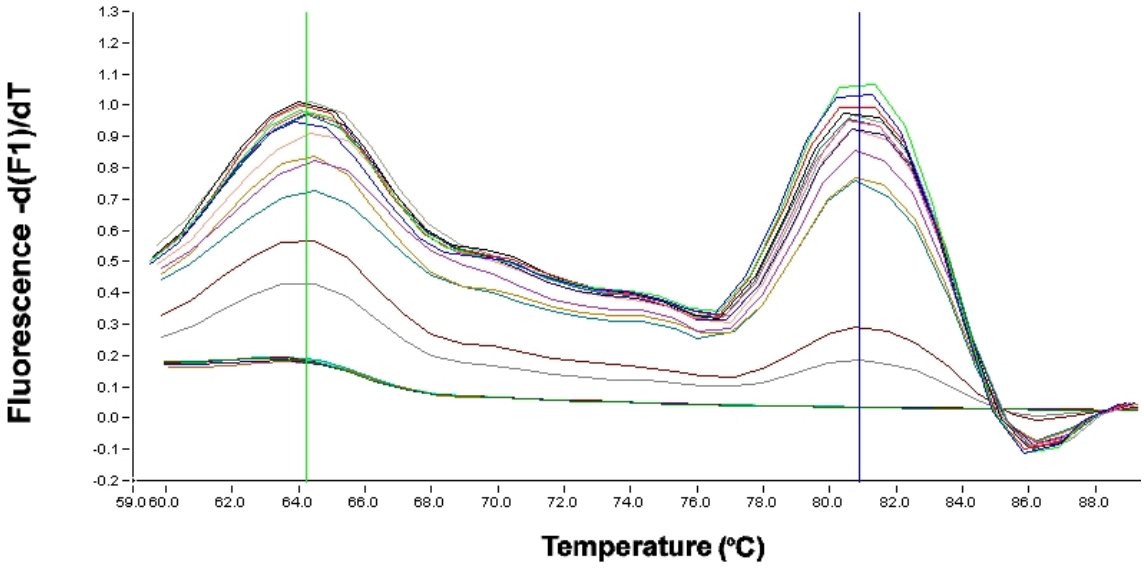

Figure 4.5 Melting curves of the amplicons generated in Figure 4.4.

Apart from the peak at 81˚C, another is seen at 64˚C, even in the no template controls (the lines at the bottom). Surprisingly, in the amplification plot **(Figure 4.4),** no amplification is seen in the no template controls. The peak is most probably caused by primer dimers or other primer-primer interactions, and they are not seen in the amplification plots

since the data capture is done at 72˚C, where the structures are melted completely. These secondary reactions are most probably responsible for the relatively poor efficiency of the reaction, since a sizable portion of the primers are sequestered in this primer-primer interaction. In an effort to reduce the effect of this side-reaction, the annealing temperature was varied between 55 and 65˚C, and the primer concentration between 100 and 200μM using the 1000 copies/ml samples as target (results not shown). The optimal conditions turned out to be annealing temperature (Ta) of 60 degrees and primer concentration of 200μM as it gave a good trade-off between primer dimer formation and sensitivity. These conditions were used for the rest of the work.

4.3 Addition of positive control

An artificial positive control was created by reamplification of remaining material from a Cobas Amplicor-tested positive sample. No attempt was made to determine its concentration by spectrophotometry, as too much handling of this amplicon may cause contamination. A dilution series was made to find a suitably diluted sample to use as realistic positive control. It was then added to reactions containing internal control to see the effect of having both targets in the same reaction. The amount of internal control was chosen as 500 copies/ml, halfway between the lowest value where all 3 came up (1 000 copies/ml) and the next value where none came up (100 copies/ml). It can be seen from **Figure 4.6** that a high copy number sample (blue line) had a low crossing point, but that all the others, including the no template control, crossed the threshold at cycle 33, since they all contain the same amount of internal control. Also, note that the internal control crossed the threshold at the expected range, between cycle 31 and 35 **(Table 4.1),** showing that it is not shifted by competition with the positive control.

The melting curves of the amplicons are shown in **Figure 4.7.** The Tm of the positive control, predicted to be 85˚C, turned out to be 84˚C, not too far from the expected value. That brings it close to the Tm of the internal control, but far enough to distinguish them reliably. The high concentration (blue line) made a single peak at 84˚C, while an intermediate concentration (green line) showed a double peak.

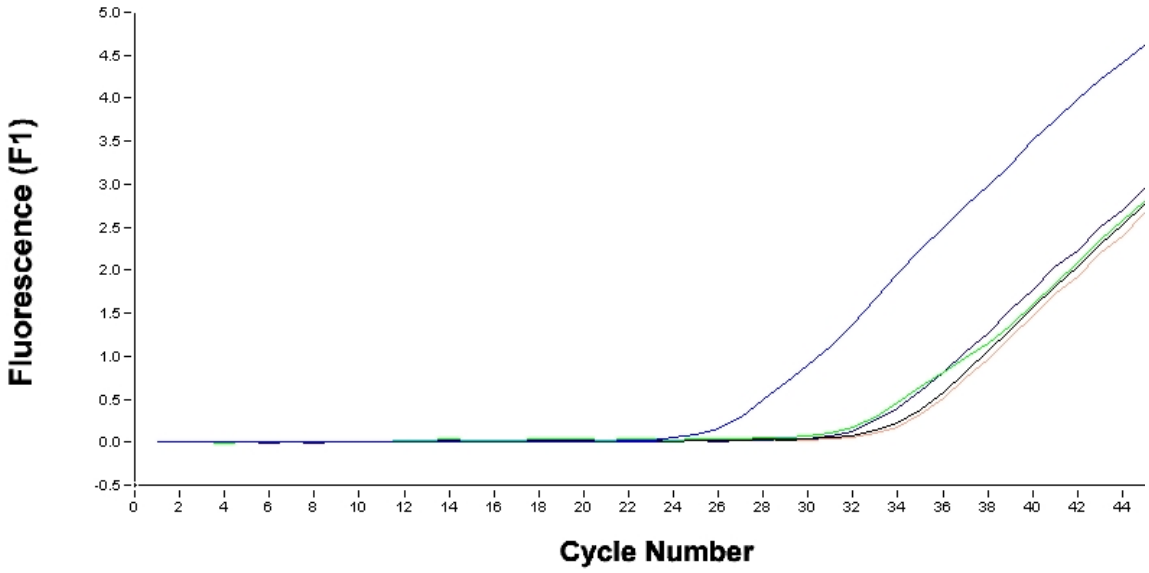

Figure 4.6 Amplification of samples containing internal control and various amounts of positive control.

All the others had only the single internal control peak at 80-81˚C. This means that the internal control is strong enough to come up in all samples, but weak enough to be suppressed by the presence of a high copy number of primary target. Unfortunately, the copy number of the artificial positive control could not be established accurately without running the risk of contaminating the laboratory with the same amplicon that is the target for the rest of the study.

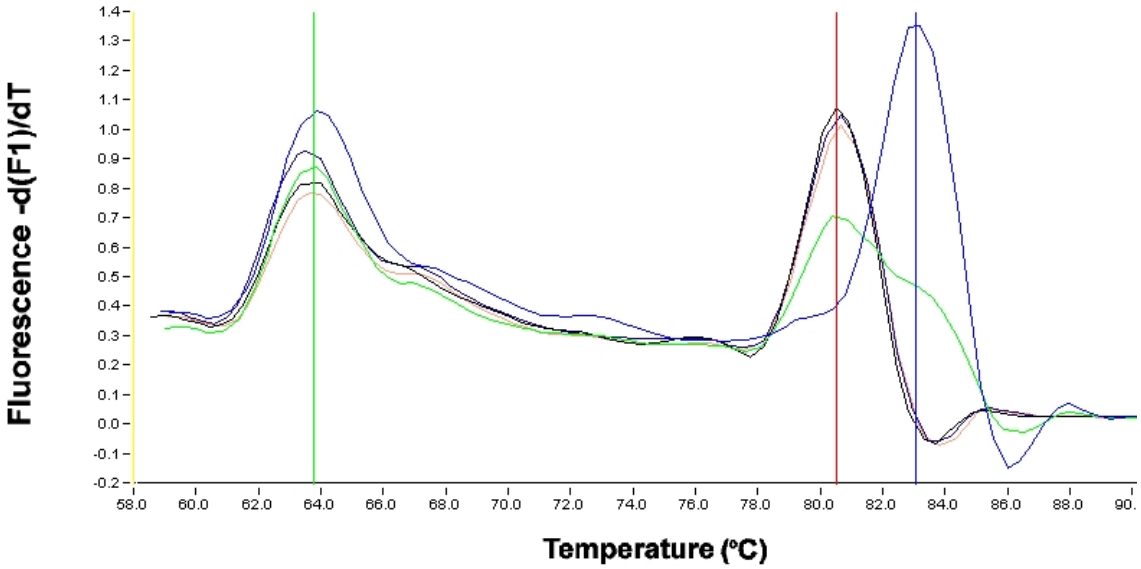

Figure 4.7 Melting curves of the samples in figure 4.6.

4.4. Evaluation using patient samples

The final test of the method was to retest a number of samples that were tested before in the laboratories of PathCare in Cape Town. DNA was isolated from 50 patient samples and 5µl of each were tested in the assay described here, containing 500 copies/ml of internal control. An example of the results is shown in **Figure 4.9.** Six of the 30 samples tested in that run were found positive by the Roche Amplicor HIV-1 DNA method, but only 3 came up with the in-house assay, the first line to cross the threshold being the positive control. It is known that the proviral load can be very low in adult patients, while babies may have a spectrum of loads, from very low to very high. An ideal method should be sensitive enough to identify the lowest level possible while retaining a large dynamic range so as not to be saturated by high proviral loads. The melting curves of the samples in **Figure 4.8** are shown in **Figure 4.9.**

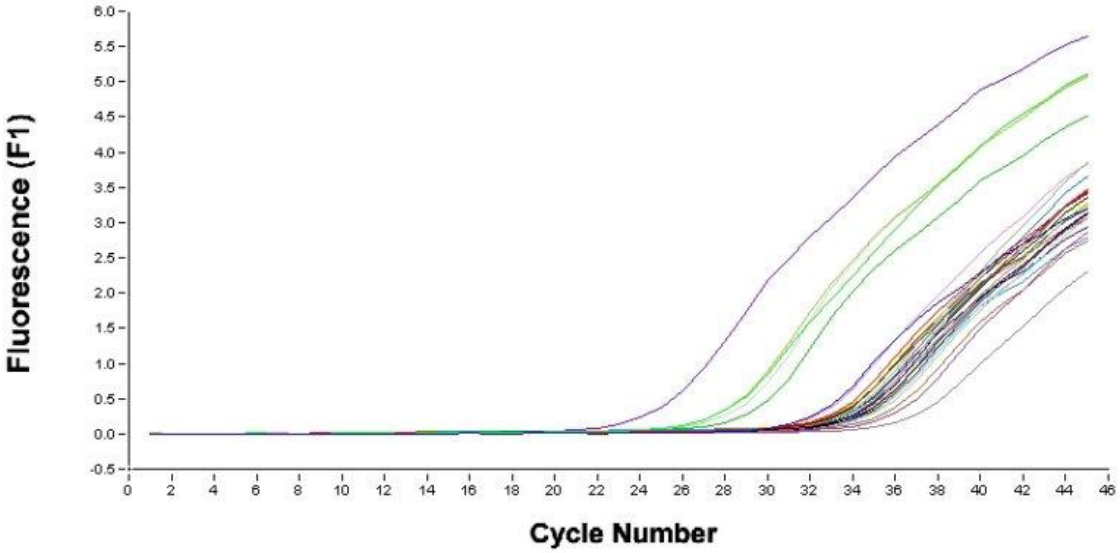

Figure 4.8 Amplification curves of 30 patients' samples.

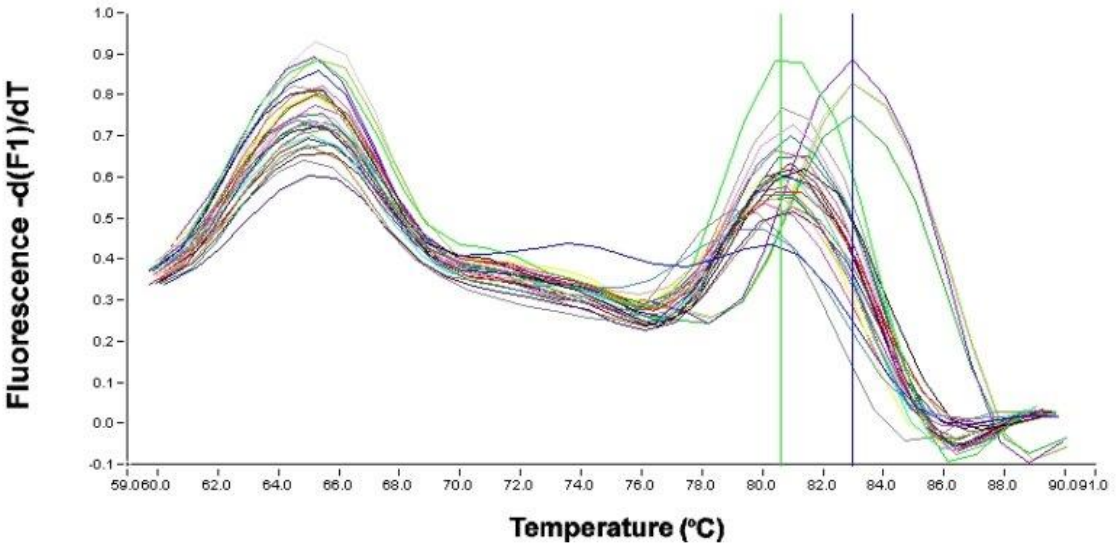

Figure 4.9 Melting curves of the samples shown in figure 4.8.

No information was known about the patients in this group as the samples have been anonymised to protect patient identity, which made it impossible to stratify patients into sex or age groups. It is possible that the samples that were positive in the in-house assay were from babies and

the others from adults. In this case, the method could potentially be useful for early detection of mother-to-child transfer of HIV-1, although it would take a brave person to use a method that is not ultrasensitive for this purpose. The unfortunate conclusion from this evaluation of real-life samples is that the assay described in this thesis will miss roughly half of the positives identified by the Roche method in general use.

The lack of sensitivity can be blamed on a number of factors:
Firstly, while the Roche method uses 50µl of extract, the in-house method uses only 5, giving it a built-in tenfold disadvantage. This becomes especially important in cases where very low levels of target are expected. PCR can potentially detect a single copy of a DNA target and the chances are better for a target molecule to be present in a larger volume. Unfortunately, it is impractical to scale up the in-house assay as the maximum practical volume that can be used in the LightCycler capillary is 25µl. Scaling up will also increase the cost as most of the running cost of real-time PCR is in the reagents used.

Secondly, the assay can probably be split into 2 capillaries per patient, one for HIV-1 DNA and one for the internal control. This will eliminate competition between the two reactions and could help push down the limit of detection. On the other hand, this will double the cost of the assay as well as increase the complexity, two things that we wanted to avoid.

Thirdly, SYBR Green assays rely purely on detection of the amplicon by amplification and subsequent identification by melting of the amplicon. The Roche method relies on detection of the amplicon by an amplicon-specific capture probe followed by ELISA detection of the bound amplicon. The assay thus relies on two amplification steps, namely, amplification of the HIV-1 DNA target, followed by signal amplification in the ELISA step.

Together, these steps give the assay incredible sensitivity, but also a very low saturation level, meaning that it cannot be used quantitatively.

The assay described in this thesis is thus not suitable for diagnostic use and another approach should used to get better sensitivity. Most in-house assays described in the literature use some form of probe detection, either Taqman, molecular beacons, or scorpions. This seems to be the most promising avenue to explore for an alternative assay. Meanwhile, the Roche assay with its batching requirement and labour intensity will remain the mainstay of HIV-1 molecular diagnostics.

CHAPTER 5

REFERENCES

Abbas, A. K., & Lichtman AH., 2000. Congenital and Acquired Immunodeficiencies. Cellular and molecular Immunology. China: Saunders. 463-475.

Adler, M. W., 1993. HIV infection. ABC of AIDS. Peckham, C. & Giaquinto, C., (Eds.). Tavistock Square, London: BMJ. 58.

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

Arrive, E., Newell, M., Ekouevi, D.K., Chaix, M., Thiebaut, R., *et al*., 2007. Prevalence of resistance to nevirapine in mothers and children after single-dose exposure to prevent vertical transmission of HIV-1; a meta-analysis. *International Journal of Epidemiology*. Oct. 36(5):

Avert, 2008. South Africa HIV & AIDS Statistics. http://www.avert.org/ worldstats.htm**.** Viewed on 24/08/2009.

Babl, F. E., Regan, A. M., & Pelton, S. I., 1999. Abnormal body fat distribution in HIV-1 infected children on antiretrovirals. *Lancet*. Apr 10; 353 (9160): 1243-1244.

Benjamin, E., Coico, R., & Sunshine, G., 2000. Immunodeficiency and other disorders of the immune system. Immunology. Dr. S. Gottesman, (Ed). New York: A John Wiley & Sons, Inc. 361-364.

Borkowsky, W., Stanley, K., Douglas, S. D., Lee, S., Wiznia, A., Pelton., *et al.*, 2000. Immunologic response to combination nucleoside analogue plus protease inhibitor therapy in stable antiretroviral therapy-experienced human immunodeficiency virus infected children. *J Infect Dis*. Jul ; 182 (1): 96-103.

Bryson, Y., Luzuriaga K., Sullivan, J., & Wara, D., 1993. Proposed definitions for in utero versus intrapartum transmission of HIV-1. *N Engl Med.* 327: 1246- 1247.

Bukrinskaya, A., 2007. HIV-1 matrix protein: A mysterious regulator of the viral life cycle. Virus research. 124: 1-11.

Burns, D. N., Mofenson, L.M., 1999. Paediatric HIV-1 infection. *Lancet*. 354 (suppl 2): S1-S6.

Butler, K. M., Husson, R. N., Balis, F.M., Brouwers, P., Eddy, J., el-Amin, D., *et al.,* 1991. Dideoxyinosine in children with symptomatic human immunodeficiency virus infection. *N Engl J Med*. Jan 17; 324 (3): 137-144.

Clapham, P. R. & Weiss, R. A., 1997. Spoilt choice of receptors, *Nature* 388: 230-231.

Chinen, J. & Shearer, W. T., 2002. Molecular virology and immunology of HIV infection. *Journal Allergy Clinical Immunology. Volume 110, Issue 2, August 2002*. 189-198.

Clavel, F., Guetard, D., Brun-Vezinet, F., Chamaret, S., Rey, M. A., Santos-Ferreira, M. O., *et al.,* 1986. Isolation of a new human retrovirus from West African patients with AIDS. *Science*; 233: 343-6.

Coffin, J. M., 1995. HIV Population Dynamics in vivo: Implications for genetic variation, pathogenesis and therapy. *Science* 267: 483-489.

Connor, E. M., Sperling, R. S., Gelber, R., Kiselev, P., Scott, G., O'Sullivan, M. J., *et al.,* 1994. Reduction of maternal-infant transmission of human immunodeficiency virus type 1 with zidovudine treatment. Paediatric AIDS Clinical Trials Group Protocol 076 Study Group. *N Engl J Med*. Nov; 99(6): 1173-1180.

Coovadia, A., Marais, B., Abrams, E., *et al.,* 2006. Virologic response to NNRTI treatment among women who took single-dose nevirapine 18-36 months earlier. *13th Conference on Retroviruses and Opportunistic Infections.* Feb.

De Cock, K., Fouler, M. G., Mercier, E., *et al.,* 2000. Prevention of mother-to-child transmission in resource-poor countries: Translating research into policy and practice. *JAMA* 2000; 283: 1175-82.

De Martino, M., Tovo, P. A., Balducci, M., Galli, L., Gabiano, C., Rezza, G., & Pezzotti, P., 2000. Reduction in mortality with availability of antiretroviral therapy for children with perinatal HIV-1 infection. Italian register for HIV in children and the Italian National AIDS Registry. *JAMA.* Jul 12; 284(2): 190-197.

Doranz, B. J., Rucker, J., Yi, Y., Smyth, R. J., Samson, M., Peiper, S. C., *et al.*, 1996. A Dual-tropic primary HIV I isolate that uses fusion and the b-

chemokine receptors CKR-5, CKR-3 as fusion co-factors. *Cell* 85: 1149 – 1158.

Douek, D. C., Koup, R.A., McFarland, R. D., Sullivan, J.L., & Luzuriaga, K., 2000. Effect of HIV on thymic function before and after antiretroviral therapy in children. *J Infect Dis*. Apr; 181 (4): 1479-1482.

Dunn, D. T., Brandt, C.D., Krivine, A., Cassol, S. A., Roques, P., Borkowsky, W., *et al.,* 1995. The sensitivity of HIV-1 DNA polymerase chain reaction in the neonatal period and the relative contributions of intra-uterine and intra-partum transmission. *AIDS*. Sep;9 (9): F7-11.

Dunn, D. T., Newell, M. L., Ades, A. E., & Peckham, C. S., 1992. Risk of human immunodeficiency virus type 1 transmission through breastfeeding. *Lancet* 340; 385-588.

England, K., Thorne, C., & Newell, M., 2006. Vertically acquired paediatric co-infection with HIV and hepatitis C virus. *Lancet Infect Dis*:6: 83-90.

Englund, J. A., Baker, C. J., Raskino, C., McKinney, R. E., Petrie, B., Fowler, M. G., *et al.,* 1997. Zidovudine, didanosine or both as the initial treatment for symptomatic HIV-infected children. AIDS Clinical Trials Group (ACTG) study 152 Team. *N Engl J Med*. Jun 12; 336 (24): 1704-1712.

Feng, F., Broder, C. C., Kennedy, P. E., Berger, E. A., 1996. HIV I entry co-factor: functional CDNA cloning of seven-transmembrane, G protein-coupled receptors. *Science* 272: 872-877.

Giles, R. E., Perry, K. R., & Parry, J. V., 1999. Simple/rapid test devices for anti-HIV screening: Do they come up to the mark? *J Med Virol*, September: 59: 1: 104-109.

Giaquinto, C., Rampon, O., & De Rossi, A., 2006. Antiretroviral Therapy for prevention of mother-to-child HIV transmission. *Clinical drug invest* 2006: 26 (II): 611-627.

Glass, A. J., 2006. Diagnosis and monitoring of HIV infection. Department of medical Virology, University of Pretoria, Tshwane Academic Division, NHLS. *SA Fam Pract*: 48(8): 46a-c.

Hahn, B., Shaw, G., Cock, K., & Sharp, P., 2000. *AIDS as a Zoonosis: Scientific and Public Health Implications.* 287: 607 -614.

Harms, G., Schulze, K., Moneta, I., Baryomunsi, C., Mbezi, P., & Poggensee, 2005. Mother-to-child transmission of HIV and its prevention:

awareness and knowledge in Uganda and Tanzania. *Journal of Social Aspects of HIV/AIDS,* 2(2): 258-266.

Hauri, A. M., Armstrong, G. L., & Hutin, Y. J. F., 2004. The global burden of disease attributable to contaminated injections given in healthcare settings. *International Journal of STD & AIDS,* 15: pp. 7-16.

Holmes, W. & Kwarteng, T, 2001. Parent to child transmission of HIV: policy considerations in the Asia-Pacific region. *Journal of Clinical Virology,* Volume 22, Issue 3, October: 315-324.

Jaquet, D., Levine, M., Ortega-Rodriguez, E., Faye, A., Polak, M., Vilmer, E., & Levy-Marchal, C., 2000. Clinical and metabolic presentation of lipostrophic syndrome in HIV-infected children. *AIDS*. Sep 29; 14(14); 2123-2128.

Jeena, P. M., Coovadia, H. M., & Bhagwanjee, S., 1996. Prospective controlled study of the outcome of human immune-deficiency virus antibody positive children admitted to an intensive care unit. *Cri Care Med:* 24: 963-967.

Jourdain, G., Ngo-Giang-Huong, N., Le Coeur, S., Bowonwatanuwong, C., *et al.,* 2004. Intrapartum Exposure to Nevirapine and Subsequent Maternal Responses to Nevirapine-Based Antiretroviral Therapy. *NEJM*. 15 July, 351(3).

Kahn, P., 2003. Do clades matter for HIV vaccines? IAVIReport, 7: 1-6.

Keren, D. F. & Warren, J. S., 1992. Diagnostic immunology. Section Immunopathologic processes. Immunodeficiency. Warren, J. S. (Ed.). Baltimore, Hong Kong, London. 112-114.

Kiwanuka, N., Gray, R., Serwadda, D., *et al*., 2004. The incidence of HIV-1 associated with injections and transfusions in prospective cohort, Rakai, Uganda. *AIDS* 18(2): 342-344.

Kline, M. W., Dunkle, L. M., Church, J. A., Goldsmith, J. C., Harris, A. T., Federici, M. E., *et al.,* 1995. A phase I/II evaluation of stavudine (d4T) in children with human immunodeficiency virus infection. *Paediatrics*. Aug; 96(2Pt 1): 247-252.

Kline, M. W., Van Dyke, R. B., Lindsey, J. C., Gwynne, M., Culnane, M., Diaz, C., *et al.,* 1999. Combination therapy with stavudine (d4T) plus didanosine (ddI) in children with human immunodeficiency virus infection.

The pediatric AIDS Clinical Trials Group 327 Team. *Pediatrics.* May; 103 (5); e62.

Kuby, J., 1997. *Immunology.* New York: W.H. Freeman and Company.

Kuiken, C., Foley, B., Guzman, E., & Korber, B., 1999. The Evolution of HIV. *Determinants of HIV-1 Protein Evolution.* The Johns Hopkins University Press: 432-465.

Lee, E.J., Kantor, R., Zijenah, L., *et al.,* 2005. Breast milk shedding of drug resistant HIV-1 subtype C in women exposed to single-dose nevirapine. *J Infect Dis* 192(7).

Liitsola, K., Holmstrom, P., Laukkanen, T., Brummer-Korvenkontio, H., Leinikki, P., & Salminen, O. M., 2000. Analysis of HIV-1 genetic subtypes in Finland reveals good correlation between molecular and epidemiological Data. *Scand Journal Infect Dis*. 32: 475-480.

Lockman, S., Shapiro, R.L., Smeaton, L.M., Wester, C., Thior, I., *et al.,* 2007. Response to antiretroviral therapy after a single, peripartum dose of Nevirapine. *NEJM*. January, 356(2).

Machuca, R., Bogh, M., Salminen, M., Gerstoft, J., Kvinesdal, B., Pedersen, C., *et al.,* 2001. HIV-1 subtypes in Denmark. *Scand Journal Infect Dis*. 33: 697-701.

Madhi, S. A., Schoub, B., Simmank, K., Blackburn, N., & Klugman, K. P., 2000. Increased burden of respiratory-associated severe lower respiratory tract infections in children infected with human immunodeficiency virus type 1. *J Pediatr:* 137: 78-84.

Choisy, M., Woelk, C. H., W., Guegan, J., & Robertson, D., 2004. Comparative study of adaptive molecular evolution in different Human Immunodeficiency Virus groups and subtypes. *Journal of virology*, Feb 2004: Volume 78, No. 4. 1962-1970.

Marlink, R., Kanki, P., Thior, I., Travers, K., Eisen, G., Siby, T., et al., 1994. Reduced rate of disease development after HIV-2 infection as compared to HIV-1. Science; 265: 1587-90.

McCutchan, F., 1999. The Evolution of HIV. *Global Diversity in HIV.* The University Press: 41-72.

McKinney, R. E., Jr., Johnson, G. M., Stanley, K., Yong, F. H., Keller, A., O'Donnell, K. J., *et al.,* 1998. A randomized study of combined zidovudine-

lamivudine versus didanosine monotherapy in children with symptomatic therapy-naive HIV-1 infection. The Pediatric AIDS Clinical Trials Group Protocol 300 study Team. *J Pediatr*. Oct; 133 (4): 500-508.

McKinney, R. E., Jr., Maha, M. A., Connor, E. M., Feinberg, J., Scott, G. B., Wulfsohn, M., *et al.,* 1991. A multicenter trial of oral zidovudine in children with advanced human immunodeficiency virus disease. The protocol 043 study Group. *N Engl J Med*. Apr 11; 324 (15): 1018-1025.

Michael, N. L., Herman, S. A., Kwok, S., Dreyer, K., Wang, J., Christopherson, C., *et al.,* 1999. Development of calibrated viral load standards for group M subtype of Human Immunodeficiency Virus type 1 and performance of an improved AMPLICOR HIV-1 MONITOR test which isolates diverse subtypes. *Journal of clinical microbiology, August.* 37 (8): 2557- 2563.

Miller, D., 1987. The virus and its spread. In: Miller, D., ed. *Living with AIDS & HIV.* Hong Kong: Macmillan press, LTD. 1-20.

Mirochnick, M., Fenton, T., Gagnier, P., Pav, J., Gwynne, M., Siminski, S., *et al.,* 1998. Pharmacokinetics of nevirapine in human immunodeficiency virus type 1-infected pregnant women and their neonates. Paediatric AIDS Clinical Trials Group Protocol 250 Team. *J Infect Dis*. Aug; 178 (2): 368-74.

Molecular cloning, vectors pBR322 DNA., 2009. http://www.fermentas.com/en/products/all/molecular-cloning/vector-phase/sd004. Viewed on 05/01/2010.

Moore, J.P., 1997. Co-receptors: implications for HIV pathogenesis and therapy. *Science* 276: 51-52.

Nachman, S. A., Stanley, K., Yogev, Pelton, S., Wiznia, A., Lee, S., *et al.,* 2000. Nucleoside analogs plus ritonavir in stable antiretroviral therapy-experienced HIV-infected children. A randomized controlled trial. Pediatric AIDS Clinical Trials Group 338 study Team. *JAMA.* Jan 26; 283 (4): 492-498.

National Department of Health, 2004. Antiretroviral Therapy in Adults. National Antiretroviral Therapy Guidelines 1st ed. http://doh.gov.za/docs/ factsheets: Viewed on 16/08/2006.

Nduati, R., John, G., Mbori-Ngacha, D., Richardson, B., Overbaugh, J., Mwatha, A., *et al.,* 2000. Effect of breastfeeding and formula feeding on

transmission of HIV-1, a randomized clinical trial, *J. Am. Med. Assoc*. 283: 1167-1174.

Newell, M. L., Loveday, C., Dunn, D., Kaye, S., Tedder, R., Peckham, C., *et al*., 1995. Use of polymerase chain reaction and quantitative antibody tests in children born to human immunodeficiency-1 infected mothers. *J Med Virol*, Dec, 47; 4: 330-335.

Newell, M. L., 1998. Mechanisms and timing of mother-to-child transmission of HIV-1 AIDS, 12: 831-837.

Newell, M. L., 2003. Antenatal and perinatal strategies to prevent mother-to-child transmission of HIV infection. *Transaction of the Royal Society of Tropical Medicine and Hygiene,* 97; 22-24.

Parren, P. W. H. J., Moore, J. P., Burton, D. R., Sattentaw, Q. J., 1999. The Neutralizing anti-body response to HIV I: Viral evasion and escape from humoral immunity. *AIDS* 13: S137 – S162.

Paediatric European Network for Treatment of AIDS, 1998. A randomized double blind trial of the addition of lamivudine or matching placebo to current nucleoside analogue reverse transcriptase inhibitor therapy in HIV-infected children. *AIDS*, 12: F151-F160.

Peter, A. C., 2006. Mother-to-child transmission of HIV in Botswana: An ethical perspective on mandatory testing. Developing world Bioethics ISSN 1471-8731 (print): 1471-8847 (online). Volume 6 Number 1: 1-12.

Powderly, W. G., Saag, M. S., Chapman, S., Yu, G., Quant, B., & Clendeninn, N.J., 1999. Predictors of optimal virological response to potent antiretroviral therapy. *AIDS.* October 1, 13; 14: 1873-1880.

Price, R. W. & Perry, S. W., 1994. Understanding the AIDS Dementia complex (ADC). HIV, AIDS and the brain. Research publications; Association for research in nervous and mental disease. Price, R. W., (Ed). New York: Raven Press. 7.

Ratner, L., Haseltine, W., Patarca, R., *et al.,* 1985. 'Complete nucleotide sequence of the AIDS Virus, HTLV-III'. *Nature* 313 (6000): 277-284.

Reddington, C., Cohen, J., Baldillo, A., Toye, M., Smith, D., Kneut, C., *et al.,* 2000. Adherence to medication regimens among children with human immunodeficiency virus infection. *Pediatr Infect Dis. J.* Dec; 19 (12): 1143-1153.

Relicio, K. & Holodniy, M., 2002. HIV-1 RNA and viral load. *Clinical laboratory Medicine.* 22: 593-610.

Rogers, M. F., Chin-Yih Ou, Rayfield, M., Thomas, P., Ellie, E., Schoenbaum, M. D., *et al.,* 1989. Use of the polymerase chain reaction for early detection of the sequences of human immunodeficiency virus in infants born to seropositive mothers. *The New England Journal of Medicine.* 320 (25): 1649-1654.

Roitt, I., Brostoff, J., & Male, D., 2001. Immunology. Secondary immunodeficiency. Male, D. (Ed). London, New York: Mosby. 317-321.

Roux, K. H. & Taylor, K. A., 2007. AIDS virus envelope spike structure. *Current opinion in Structure Biology.* 17: 1-9.

Schmid, G. P., Buve, A., Mugyenyi, P., *et al*., 2004. Transmission of HIV-1 infection in Sub-Saharan Africa and effect of elimination of unsafe injections. *Lancet* 363: 482-488.

Sigal, L. H. & Ron, Y., 1994. Immunology and inflammation. Basic mechanisms and clinical consequences. Immunodeficiency syndromes B. AIDS. Ron, Y. (Ed). New York, San Fransisco: McGraw Hill, inc. 445.

Simon, F., Mauclere, P., Roques, P., Loussert-Ajaka, I., Muller-Truwin, M. C., Saragosti, S., et al., 1998. Identification of a new human immunodeficiency virus type 1 distinct from group M and group O. *Nature Medicine*; 9: 1037-7.

South Africa Department of Health Guidelines for the management of HIV-infected children, 2005. http://www.doh.gov.za/docs/policy: Viewed on 25/08/2009.

South African Department of Health, 2005. Guidelines for management of HIV-infected children. http://www.doh.gov.za/docs/policy: Viewed on 3/09/2006.

Spencer, D., 2005. The clinical practice of HIV medicine. Goldstream Publishers. Johannesburg. pp. 4-30.

Structure and genome of HIV., 2009. http://en.wikipedia.org/wiki/Structure_and_genome_of_HIV. Viewed on 13/12/2009.

The viral load count., 2009. http://www.kingcountry.gov/About/metrokc.aspx. Viewed on 03/12/2009.

Thirsk, E. R., Kapongo, M. C., Jeena, P. M., Liebeschhuetz, S., York, D. F., Vega, G., *et al.,* 2003. HIV-exposed infants with acute respiratory failure secondary to acute lower respiratory infections managed with and without mechanical ventilation. *S Afri Med J,* August: 93: 8: 617-620.

Tozzi, A. E., Pezzotti, P., & Greco, D., 1990. Does breastfeeding delay progression to AIDS in HIV-infected children? *AIDS*: 1293-1294.

Turner, B. G. & Summers, M. F., 1999. Structural Biology of HIV. *Journal of Molecular Biology* 285: 1-32.

UNAIDS, 2008. Report on the global AIDS epidemic. http://data. unaids.org/pub/GlobalReport/2008/jc1510_2008_global_report_pp29_62_en. pdf, viewed on 4/09/2009.

UNAIDS, 2003. Factsheet. Sub-Saharan Africa. Genova. UNAIDS. Available at: http://www.unaids.org/Barcelona/presskit/factsheet/ FSssafrica_en.polf. Viewed on 23/05/2006.

UNAIDS, 2004. AIDS epidemic update. December. Genera: UNAIDS at: http://www.unaids.org/wad2004/report.html. Viewed on 13/05/2006.

Van Dyk, A., 2001. HIV/AIDS: origins and effects. HIV AIDS care and counseling. A multidisciplinary approach. Van Dyk, A. (Ed). Pinelands, CT: Hanli Venter, 7, 13-15: 29.

Van Rossum, A. M., Niesters, H. G., Geelen, S. P., Scherpbier, H. J., Hartwig, N. G., Weemaes, *et al.*, 2000. Clinical and virologic response to combination treatment with indinavir, zidovudine and lamivudine in children with human immunodeficiency virus-1 infection: A multicenter study in the Netherlands. On behalf of the Dutch study group for children with HIV-1 infections. *J Pediatr*. Jun; 136 (6): 780-788.

Viganò, A., Dally, L., Bricalli, D., Sala, N., Pirillo, M., Saresella, M., *et al.,* 1999. Clinical and immuno-virologic characterization of the efficacy of stavudine, lamivudine and indinavir in human immunodeficiency virus infection. *J Pediatr.* Dec; 135 (6): 675-682.

Viganò, A., Vella, S., Saresella, M., Vanzulli, A., Bricalli, D., Di Fabio, S., *et al.,* 2000. Early immune reconstitution after potent antiretroviral therapy in HIV-infected children correlates with the increase in thymus volume. *AIDS.* Feb 18; 14(3): 251-261.

Walles, S. L., Pitt, J., Colgrove, R., McIntosh, K., Chung, P. H., Colson, A., *et al.,* 2000. HIV-1 genotypic zidovudine drug resistance and the risk of maternal infant's transmission in the women and infants transmission study. The women and transmission study group. *AIDS.* Feb 18; 14(3): 263-71.

Watson, D. C. & Farley, J. J., 1999. Efficacy of and adherence to high active antiretroviral therapy in children infected with human immunodeficiency virus type 1. *Pediatr Infect Dis J.* Aug; 18 (8): 682-689.

Westby, M., Nakayama, G. R., Butler, S. L., & Blair, W. S., 2005. Cell-based and bio-chemical screening approaches for the discovery of novel HIV-1 inhibitors. Antiretroviral research. 67: 121-140.

Witten, G. Q. & Perelson, A. S., 2004. Modelling the cellular-level interaction between the immune system and HIV. *South African Journal of Science* 100, September/October 2004: 447-451.

Wiznia, A., Stanley, K., Krogstad, P., Johnson, G., McNamara, J., Moye, J., *et al.,* 2000. Combination nucleoside analog reverse transcriptase inhibitors plus nevirapine, nelfinavir or ritonavir in stable antiretroviral therapy-experienced HIV-1 infected children. Week 24 results of a randomized controlled trial-PACTG 377. Pediatric AIDS Clinical Trials Group 377 study Team. AIDS Res Hum Retroviruses. Aug 10; 16(12): 1113-1121.

Young, N. L., Shaffer, N., Chaowanachan, T., *et al.,* 1998. RNA and DNA PCR for early diagnosis of infants born to HIV-infected mothers, Thailand. *Int Conf AIDS*. 12: 794.

Zwi, K. J., Pettifor, J. M., Soderlund, N., Meyers, T., 2000. HIV infection and in-hospital mortality at an academic hospital in South Africa. *Arch Dis Child*: 83: 227-230.